IMPACT OF DIFFERENT METABOLIC UNCOUPLERS ON THE SPECIFIC DEGRADATION RATE OF TOLUENE IN A DIFFERENTIAL BIOFILTRATION REACTOR

A thesis

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Abstract:

In this work, a differential biofiltration reactor was used to explore the potential of metabolic uncouplers to improve pollutant (toluene) degradation rates. Metabolic uncouplers were reported to reduce the cell mass in activated sludge systems, but are untested in biofilters and the current work is the first to report the impact of different metabolic uncouplers in a biofilter. Initially soil was used as a biofilter bed and later experiments were conducted in pure cultures in a biofilm reactor.

A simple diffusion system was developed to generate the desired concentration of toluene to the system. Gas chromatography and a carbon dioxide analyzer were connected online to the reactor which improved the precision of the data collected and also the robustness of the measurements.

Preliminary experiments including effect of substrate concentration, different nutrients and temperature were done to optimize the conditions before starting the metabolic uncoupler screening studies in soil. Based on the results, inlet toluene concentration between 180 ppm and 250 ppm was used throughout the studies. Also it was found that the toluene degraders were nitrogen limited. Temperature studies showed that the elimination capacity (EC) increased with increasing temperature, from 34 ± 1.4 $g.m^{-3}.h^{-1}$ to 49.8 ± 2.6 $g.m^{-3}.h^{-1}$ for temperatures of 20 to 45 ^{o}C, respectively.

Nine potential metabolic uncouplers were screened in batch serum bottles. The nine uncouplers tested were dinitrophenol (dNP), p-nitrophenol (pNP), benzoic acid (BA), carbonylcyanide p-trifluoromethoxy phenylhydrazone (FCCP), carbonylcyanide m-chloromethoxy phenylhydrazone (CCCP), pentachlorophenol (PCP), malonic acid (MA), m-chlorophenol (mCP) and 2, 4, 6-trichlorophenol (TCP). Other than dNP and pNP (nitrogen containing uncouplers), seven other uncouplers were further tested in the differential biofilter reactor. Only PCP and TCP increased the toluene degradation rate significantly. PCP increased the toluene degradation rate by 35% at 140 µM, whereas 4051 µM TCP increased the rate by 18%. Though FCCP behaved as a classical uncoupler when compared with others, the EC increase was not significant.

Five toluene degraders were isolated from soil subjected to toluene and were identified using 16s rDNA/18s rDNA analysis. Out of five, two potential toluene degraders, *Stenotrophomonas*

maltophilia and *Pseudomonas putida* were used to develop a biofilm reactor. PCP, TCP and CCCP were tested in the biofilm reactors and found that PCP increased the surface elimination capacity (SEC) by 85% at 140 μM in *S. maltophilia* biofilm reactor and CCCP increased the SEC by 27% at 1 μM in *P. putida* biofilm reactor. Finally a simple model was developed to calculate the energy uncoupling coefficient for non-growth systems like ours to quantitatively represent the uncoupling mechanism.

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திருக்குறள்

(Classical Language Tamil)

தொட்டனைத் தூறும் மணற்கேணி மாந்தர்க்குக்
கற்றனைத் தூறும் அறிவு.

Thirukkural

As deep you dig the sand spring flows
As deep you learn the knowledge grows.

Chapter 1: Introduction

1.1 Biofiltration overview

Biological control of air pollution has many operational and cost advantages over the conventional physico-chemical methods in most of the chemical industries[1]. Biofiltration have been used for almost 100 years for waste treatment especially in treating highly concentrated effluents (Metcalf and Eddy, 2003). It is an air pollution control technology (APCT) frequently used for treating odour and volatile organic compounds (VOC's) from waste air streams. It is a cost-effective approach to volatile organic compound (e.g. toluene) removal for large air flows (> 1000 m^3h^{-1} and mostly low concentrations < 1000 ppm) (Devinny et al., 1999). In biofiltration, polluted air is blown[2] through a porous media, typically a mixture of compost, soil or wood chips that supports a population of microbes. Under optimum conditions, these microorganisms convert the absorbed biodegradable contaminants mostly into carbon dioxide salt and water (Deshusses and Johnson, 2000). Moreover, in biofiltration the microbial biomass is static/immobilized to the bedding material and the treated fluid is mobile/flows through the filter (Girard et al., 2009). The biological degradation process in a biofiltration can be written as follows,

$$\text{Organic Pollutant} + O_2 \xrightarrow{\text{Microbes}} CO_2 + \text{Salt} + H_2O + \text{Heat} + \text{Biomass} \qquad (1.1)$$

A suitable packing material should provide minimal pressure drop, minimal tendency for compaction, neutral pH (however, varies among different microbes), good water holding capacity, pore volume greater than 80%, particle diameter of greater than 4 mm and total organic matter content of more than 55% (mostly) (Oh and Choi, 2000). The parameters which are used to express the performance of the biofilters are pollutant loading capacity (L), elimination capacity (EC) and removal efficiency (RE). These are expressed in Eq. (1.2), (1.3) and (1.4) (Kennes and Veiga, 2001)[3],

[1] Petrochemical industry, paint industry, pharmaceutical industry, wastewater treatment, meat processing etc.,

[2] Diffuses through in case of landfills and waste ponds.

[3] Nomenclature is defined at the end.

$$L = \frac{C_{g,in} \times Q}{V} (g.m^{-3}.h^{-1}) \quad (1.2)$$

$$EC = \frac{(C_{g,in} - C_{g,out}) \times Q}{V} (g.m^{-3}.h^{-1}) \quad (1.3)$$

$$RE = \frac{(C_{g,in} - C_{g,out}) \times 100}{C_{g,in}} (\%) \quad (1.4)$$

1.2 History of biofiltration

Biofiltration is considered as one of the less energy utilizing technologies in treating air pollutants. Though it has been employed widely in odour treatment and VOC removal for the past 100 years in industrial scale, it has been naturally occurring in soil for millions of years. Germans were the first to get a patent for this technology during 1941 (Leson and Winer, 1991). Between the years 1960 and 1990, there was a huge development in the field of biofiltration. In 1963, biofilter was used effectively for treating odour from waste water treatment plants in California (Pomeroy, 1982). During 1977, the first soil biofilter was designed for organic waste gas removal in Germany (Bohn and Bohn, 1986). During 1987, it was discovered that odour removal through biofiltration was due to biodegradation and not by sorption. It was also found that the removal efficiency (RE) of soil bed biofilter filled with different media (Carlson and Leiser, 1966). Most of the biofiltration research was carried out in European countries until late 80's. In the past three decades, many biofilters were installed in and huge number of research articles pertaining to biofiltration were published in journals and conference proceedings (Lee et al., 2002; Leson and Winer, 1991; Oh and Choi, 2000). Fig. 1.1 compares the different APCT technologies available so far in treating the industrial air pollutants.

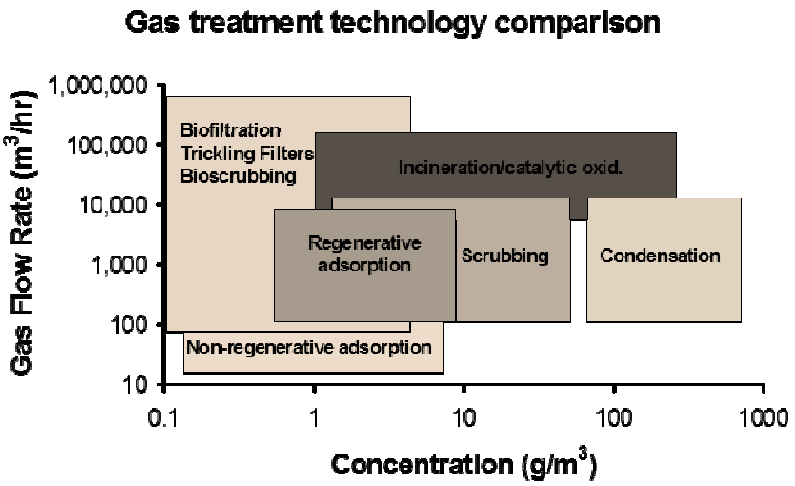


Figure 1.1: Comparison of different APCTs (Devinny et al., 1999). J.S. Copyright 2012 Reproduced with permission of TAYLOR & FRANCIS GROUP LLC-BOOKS in the format Journal via Copyright Clearance Center (Order detail ID: 62054904).

1.3 Biofiltration operating parameters

Biofiltration is a simple process, depends on many factors which are considered to be most critical in its operation. They include temperature, pH, pressure drop, moisture content, bed porosity, packing materials, air flow rate, nutrient requirement, oxygen requirement, inlet pollutant concentration, maintenance, residence time, microorganisms and acclimation time. These are all the most important physical, chemical and biological parameters influencing the biofiltration process and are described in detail in the following sections. Fig. 1.2 shows the operation of a typical biofilter used to treat a polluted air stream at an industrial scale.

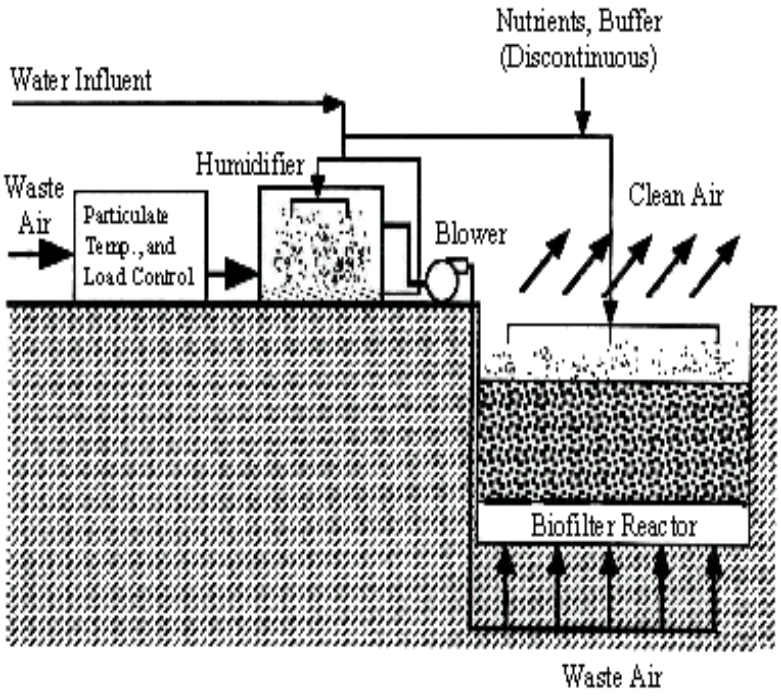


Figure 1.2: Schematic view of a conventional below ground open biofiltration system (Devinny et al., 1999). J.S. Copyright 2012 Reproduced with permission of TAYLOR & FRANCIS GROUP LLC-BOOKS in the format Journal via Copyright Clearance Center (Order detail ID: 62054904).

1.3.1 Temperature

Biofilters are normally operated at ambient temperature. Most researchers have reported no significant changes in the pollutant degradation rate with temperatures between 20 and 30 ^{o}C (Diks and Ottengraf, 1991). However, cooling is mostly needed to avoid microbial death above 40 ^{o}C (Leson and Winer, 1991) unless the microbes are thermophilic in nature. There are a few reports which suggests that changing the operating temperature would increase the removal efficiency considerably[4] (Sorial et al., 1994). The biodegradation reaction which takes place in a biofiltration system is exothermic, due to the consequence of the microbial activity. This also accounts for the change in the bed temperature (Delhomenie and Heitz, 2005). Moreover, it was proved that the quantity of energy released by the biological reaction can reach

[4] A temperature change from 11^{o} C to 15.5^{o} C increased the RE from 92% to 100%.

maximum of 50 kcal.h^{-1}, which means that the temperature gradients within the filter bed of the order of 2 to 4 ^{o}C and even may reach 10 ^{o}C sometimes for higher VOC inlet concentrations (Hwang et al., 2002). A study on toluene degradation rates at different operating temperatures showed that maximum toluene degradation rates were obtained at between 30 and 35 ^{o}C (Park et al., 2002). This optimum temperature was also recommended for the removal of benzene, toluene, ethylbenzene and xylene (BTEX) (Lee et al., 2002).

1.3.2 pH

Similar to temperature, pH is an important parameter to monitor and maintain in biofiltration. Beyond the optimum range of pH microbial activity is severely affected in biofiltration as most of the microbes in biofilters are neutrophilic in nature (Delhomenie and Heitz, 2005). The by-products of microbial degradation in a biofilter are mostly organic acids (e.g. acetic acid). Oxidation of halogenated organics and reduced sulphur compounds (e.g. H_2S) may produce inorganic acid by-products. Moreover, pollutants that have heteroatoms are also converted into acid products, which tend to reduce pH (Christen et al., 2002). Accumulation of these acids may reduce the pH of the bed media below the active pH range[5] for the microbial degradation. A drop in pH may also lead to excess carbon dioxide and intermediate production (Ottengraf and Vandenoever, 1983). In order to overcome this problem, buffering materials like calcium carbonate, limestone etc., are usually added to the bed (e.g. biofilters treating ammonia vapour). However, biofilters using acidophilic bacteria for degrading hydrogen sulphide may tolerate a lower pH (van Groenestijin and Hesselink, 1994). A study carried out on pH during BTEX degradation showed that maximum degradation was observed at pH between 7.5 and 8.0. However, for alkyl-benzene degradation, it was reported between 3.5 and 7.0 (Lee et al., 2002).

1.3.3 Pressure drop

Large pressure drop across the biofilter can result in air channelling in the bed. This will also increase the blower power requirement. Increase in the moisture and decrease in the bed pore size may also lead to an increase in pressure drop.

[5] pH between 6 and 9.

Accumulation of biomass may also contribute to the increase in pressure drop (Farmer et al., 1995). Overall biofilters dimensions also influence the pressure drop in biofilter bed. Usually, the biofilter bed volume ranges between 10 and 3000 m^3 (Delhomenie and Heitz, 2005). For a typical biofilter the pressure drop ranges between 1 and 10 hPa. Several methods have been developed to prevent filter bed clogging and thereby pressure drop due to excess biomass accumulation. These methods are in general helpful in nutrient control and the introduction of biomass predators (e.g. protozoa) in the biofilter bed on top of pressure drop control strategy (Delhoménie et al., 2003; Woertz et al., 2002).

1.3.4 Moisture content

Microbial activity is hugely dependent on the amount of moisture present in the biofilter bed. Moreover, reduced moisture content may also lead to cracking of biofilter bed (Kampbell et al., 1987). Biofilter researchers have already found the highest performance for treating BTEX in a typical biofilter at moisture content between 47-60% dry weight for compost (Ottengraf, 1987) and between 60-70% dry weight for peat (Beerli and Rotman, 1989) . Furthermore, humidity of the pollutant stream entering the biofilter should also be monitored periodically to prevent drying out of the bed (Wang and Govind, 1997). Usually around 95% relative humidity is maintained for the pollutant stream entering the biofilter and to achieve this. The pollutant stream can be prehumidified before entering the biofilter. Sometimes water can be sprayed on to the biofilter bed periodically in addition to the prehumidification. It was determined that, in a biofilter treating high concentration of pollutants, evaporation and stripping can cause water losses up to 70 g of water per day per kg filter bed (Delhomenie and Heitz, 2005). Though water is produced in biofiltration due to oxidation reaction, it is not sufficient enough to maintain the moisture content (e.g. in toluene oxidation only 4 mole of water is produced per mole of toluene and 9 moles of oxygen).

1.3.5 Bed porosity

In order to maintain an even flow rate of the pollutant gas and to decrease the pressure drop in a biofilter, adequate bed porosity is most essential[6]. A typical biofilter which uses soil as its bed medium should have the bed air filled porosity in the range of 35 to 40% (Leson and Winer, 1991). This is because majority of the porosity is associated with large interparticle pores in the soil and that is preferably air filled to minimize pressure drop. Generally the biofilter bed is mixed with packing materials in order to increase its porosity and to decrease the compaction (Bohn, 1992).

1.3.6 Packing materials

Choosing suitable packing materials for biofiltration operation is very important for the effective operation of a biofilters. Factors which need to be considered before selecting a good packing material include a) type of packing material b) packing porosity c) packing moisture capacity d) packing nutrient content and e) sorption characteristics of the packing surfaces. In addition, adsorption characteristics of the packing material and the target chemical should also be studied before selecting a proper packing material in biofiltration. Natural packing materials like soil, compost or peat are often used as packing material in biofiltration as they are inexpensive (Oh and Choi, 2000). However, these types of packing materials tend to settle and compact which in turn result in increased pressure drop and channelling. In order to improve degradation of hydrophobic VOCs which don't partition well into the aqueous phase and recalcitrant compounds with microorganisms, Granulated Activated Carbon (GAC) has been used as a packing material in compost biofilters. Mixtures of GAV and compost are reported to be effective for treating certain VOCs (Aizpuru et al., 2003). Inert materials such as ceramic or glass can able to maintain a rigid structure with large pores which minimize pressure drop build ups in a biofilter (Aizpuru et al., 2005).

[6] Bed porosity also impacts the residence time and surface area available for mass transfer.

1.3.7 Air flow-rate

One of the major advantages of using a biofilter is, it can handle higher inlet gas flow rates in the range of 100 to 100,000 $m^3.hr^{-1}$ when compared with other air pollution control technologies. When the flow rates are too high, the residence time becomes shorter which would lead to an incomplete biodegradation. Furthermore, if the flow rate is more, the water in the biofilter bed would get stripped by the flow which tends to desiccate the biofilter. A typical biofilter requires an airflow rate of 0.01 cfm per square foot of surface area (Leson and Winer, 1991).

1.3.8 Nutrient requirement

Aerobic microorganisms present in the biofilter media require nutrients such as nitrogen, phosphorus, potassium, sulphur and trace elements in addition to oxygen and carbon for their growth. Though the biofilter media[7] have the residual nutrients, extra nutrients are needed for the long-term performance of biofilters (Yang et al., 2002). Since nitrogen is the second most important element in the biomass next to carbon, addition of nitrogen to the biofilter media can increase the performance of a biofilter significantly (Morales et al., 1998). A study of a compost biofilter treating toluene proved that its performance strongly depended on the nitrogen supply and suggested that a stoichiometric mass ratio[8] of 3.8 assuming that bacteria contained 13% of their mass as nitrogen and 50% as carbon (Delhomenie et al., 2001). However, there are few microorganisms which can fix nitrogen from the atmosphere (e.g. green sulphur bacteria) (Chu and Alvarez-Cohen, 1998).

1.3.9 Oxygen requirement

Biofilters are driven by aerobic oxidation and hence require oxygen which is normally supplied with the pollutant stream. A minimum of 100 moles of oxygen per each mole of oxidizable gas should be supplied to those aerobic biofilters (Williams and Miller, 1992). This is because increase in the oxygen concentration will dilute the inlet pollutant stream and thereby increasing the biodegradability. In usual practise a

[7] Soil, compost, peat, wood bark etc.,

[8] reactive carbon/reactive nitrogen

supply of additional oxygen to the biofilter is provided using an air feed blower to the upstream of prehumidification.

1.3.10 Inlet pollutant concentration

Biofilters perform best when treating a pollutant concentration less than 1000 ppm. Higher inlet pollutant concentrations will lead to substrate inhibition which will inhibit the microbial activity. Moreover, higher inlet concentration will also lead to an insufficient oxygen availability (Ottengraf, 1987). Seed and Corsi (1994) found that 30 ppm of toluene had a removal efficiency of 99% but when the inlet concentration was doubled, the efficiency decreased to 82%. Moreover, studies suggest that at lower pollutant (toluene) concentration, the elimination capacity was observed to be lower when compared to a higher pollutant concentration, in a differential biofiltration reactor using compost as a bed media (Beuger and Gostomski, 2009).

1.3.11 Maintenance

Maintenance of a biofiltration system is required periodically and especially during the initiation process. Moreover, periodic sampling of the biofilter bed for the percentage of moisture and nutrient content is recommended (Leson and Winer, 1991). Extreme weather can also affect the performance of a biofilter. During heavy rainfall and snow, the biofilter should be monitored for excess water or snow more than twice a day in order to make sure there are no adverse gas flows. Addition of wood bark layer on the biofilter surface may prevent the compaction caused due to heavy rain.

1.3.12 Empty bed residence time (EBRT)

Both air flow rate and EBRT are parameters that have significant impact on biodegradation performance of a biofilter (Elmrini et al., 2004). Either an increase in the biofilter bed volume or decrease in pollutant gas flow-rate will increase the EBRT and thereby increasing the biodegradability. Increasing the EBRT will produce higher removal efficiencies. In order to improve the biofiltration performance, the EBRT should always be greater than the time needed for diffusion processes in case of low operating flow rates. Most of the research reports suggest that longer EBRT give rise

to better VOC removal efficiencies (Christen et al., 2002; Delhoménie et al., 2002; Martin Jr et al., 2002; Yoon and Park, 2002). However, to attain longer EBRT, larger filter bed volumes are required. EBRT value also depends on other operating parameters such as pollutant concentration, biodegradability level and the available bed volumes (Delhomenie and Heitz, 2005).

1.3.13 Microorganisms and acclimation time

Bed media used in most of the biofilters are natural packing materials like soil, peat, compost etc. They are the major source of microbial population. A major advantage in biofiltration is that the viability of the microorganisms is maintained for a longer period than any other treatment processes, although the system is not in function for a longer period. This is because of using natural materials as the filter bed. However, if an inert packing material is used in a biofilter then it needs a microbial exposure[9] before a biofilm develops, as microorganisms are considered as the catalysts for pollutant degradation in biofilters. Choice of microbes is usually done as per the composition of the pollutant. A single microbial population is enough to degrade certain pollutants and for certain group of pollutants, even a consortium of microorganisms is used (Nanda et al., 2012). An acclimation time required by the microorganism for handling a new substrate environment can take a few days to a few weeks in general (Li and Liu, 2006; Torkian et al., 2003). This lag phase can be shortened by introducing an inoculum[10] to the bed media. A typical biofilter usually contains 10^6 to 10^{10} cfu of bacteria and actinomycetes per gram of bed and fungi in the range of 10^3 to 10^6 cfu per gram of bed (Ottengraf, 1987). The degrading species present in a biofilter is usually between 1 and 15% of the total microbial population (Delhomenie et al., 2001; Pedersen et al., 1997). So far much of the biofiltration research has been focussed on bacteria; however, fungi have also been exploited (García-Peña et al., 2001; Spigno et al., 2003). Compost has been reported to use bacteria belonging to group *Proteobacteria, Actinobacteria, Bacteroidetes* and Firmicutes (Chung, 2007). Although restricted information is available on the microbial communities involved in biofiltration, new technologies such as denaturing

[9] activated sludge is usually added to the inert packing material used in biofilters.

[10] varies with the type of pollutant to be degraded in a biofilter.

gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and single strand confirmation polymorphism (SSCP) have allowed for a better understanding of microbial population dynamics in open and closed biofilter systems (Chung, 2007; Xie et al., 2009). Table 1.1 shows the list of microorganisms which were reported to degrade different VOCs.

Table 1.1: Identified VOC degrading microbes (Delhomenie and Heitz, 2005)

S. No	Pollutant	Microbes
1.	Benzene	*Pseudomonas* sp., *Alcaligenes xylosoxidans, Cladosporium sphaeraspermum, Exophiala lecanii-corni*, *Phanerochaete chrysosporium*
2.	Styrene	*C. sphaeraspermum, Exophiala lecanii-corni Tsukamurella, Pseudomonas, Sphingomonas, Xanthomonas Exophiala jeanselmei*
3.	Toluene	*Acetinobacter* sp., *Pseudomonas putida, Pseudomonas pseudoalcaligenes, Exophiala lecanii-corni, Scedosporium apiospermum, Corynebacterium jeikeium, C. nitrilophilus, Turicella oritidis, Pseudomonas mendocina, Sphingobacterium thalphophilum, Micrococcus lutens, Cladophalophoria* sp.
4.	Trichloroethane	*Pseudomonas putida*
5.	Xylene	*Pseudomonas pseudoalcaligenes*

1.4 Few VOCs treated through biofiltration

Control of volatile organic compound (VOC) emissions into the atmosphere from industrial facilities has become more critical following the amendment of 1990 Clean Air Act in United States (Aizpuru et al., 2001). Toluene, benzene, ethylbenzene and xylene are few examples of VOCs commonly used as solvents in the manufacture of paints, cosmetics, gasoline and adhesives. Though other air pollution control technologies like adsorption and incineration can be effective in treating the VOCs, they can generate unwanted by-products and may not be suitable for handling high flow pollutant stream with low concentrations of contaminants. The reliability of biofiltration for the treatment of VOCs has been proven in a very large number of reports as it is more suitable to treat low concentration and high volume of VOCs in a cost effective approach (Mpanias and Baltzis, 1998; Zilli et al., 1993). Moreover, biofilters are good at handling pollutants which are poorly soluble in water due to the higher superficial area available for mass transfer.

1.5 Few non-VOCs treated through biofiltration

Biofiltration is also used widely in treating complex odorous waste air containing hydrogen sulphide. The removal efficiencies for H_2S degradation is generally higher than that of VOC degradation although the concentrations of individual VOC species are lower (Iranpour et al., 2005). Biofilters tend to be used for applications with lower H_2S loadings due to the concerns of inhibition of H_2S removal and packing deterioration by sulphuric acid production over the long term. However, there are few successful reports for biofilters been operated at low pH and high H_2S concentrations (Nicolai and Janni, 2000; Yang and Allen, 1994). Ammonia is another highly odorous pollutant usually treated through biofilters in most of the food processing and petrochemical refining industries. Many researchers indicated that biofiltration technology is particularly effective in treating large air streams with low ammonia concentration (Baquerizo et al., 2005). Biofiltration is also a promising option for the control of methane emissions to atmosphere contained in biogas (Nikiema et al., 2007).

1.6 Choice of model pollutant in biofiltration research

Selecting a model pollutant for a biofiltration research is always important. Among the volatile organic compounds, toluene is one of the well-studied compounds in both laboratory-scale biofilters and industrial-scale biofilters. Moreover, toluene is one of the widely used solvents in the production of paints, gums, resins and rubber. It is also used widely as a reagent in the production of drugs, dyes and perfumes. In addition, toluene is highly volatile and is poorly soluble[11] in water. Furthermore, the American Conference of Government Industrial Hygienists has set the following threshold limit values (TLVs) for the concentration of this compound in air: a) the time weighed average (TWA) is 0.375 $g.m^{-3}$, b) the short time exposure level is 0.560 $g.m^{-3}$ and c) the olfactory threshold value is 8.8×10^{-3} $g.m^{-3}$ (Guelfo et al., 1987). Based on these reasons toluene has been used as a model pollutant in the current biofiltration research.

In addition to toluene, VOCs such as benzene, xylene and styrene may also be used as a model pollutant as few recent reports suggest that these pollutants are effectively degraded using biofiltration. Removal efficiencies higher than 68% were reported for xylene degradation in a typical lab scale biofilter at a pollutant loading rates lesser than 60 $g.m^{-3}.h^{-1}$ (Rene et al., 2009a). Studies carried out in a compost biofilter for treating xylene vapour has showed an EC of 73 $g.m^{-3}.h^{-1}$ with a removal efficiency of 91% (Torkian et al., 2003). Removal efficiencies higher than 90% were achieved for inlet benzene loading rates lesser than 40 $g.m^{-3}.h^{-1}$ in a laboratory scale biofiltration set up with compost as the filter bed (Rene et al., 2009b). Under steady state conditions, average removal efficiency of 84% at loading rates between 60 and 120 $g.m^{-3}.h^{-1}$ was achieved for styrene in a compost biofilter. However, maximum EC of 81 $g.m^{-3}.h^{-1}$ was obtained at a styrene loading rate of 120 $g.m^{-3}.h^{-1}$ (Bina et al., 2004).

1.7 Research objectives

Although biofiltration is a simple and environmental friendly technology, several challenges need to be overcome. Specifically, the degradation rate is low in traditional biofilters (in other words lower EC) contributing to the large size of a biofilter. Table

[11] Henry's law coefficient of 0.26 and water solubility ranges 515-627 $g.m^{-3}$.

1.2 compares the footprints of widely used APCTs, which clearly shows that there is a need to design a smaller footprint biofilter for handling higher volumetric flow rate with a higher pollutant degradation rate. This is one of the objectives of the current research work. The second objective is to explore the possible employability of the metabolic uncouplers in biofilters for increasing the pollutant degradation rate of the microbes present in the biofilter medium. Metabolic uncouplers were reported to decrease the yield in growth systems like activated sludge treatment and in non-growth systems like biofilters it is expected to increase the maintenance by making energy generation less efficient thereby increasing the substrate uptake rate. This will be a novel study in the field of biofiltration. Chapter 2 will discuss in detail about the metabolic uncouplers.

Table 1.2: Comparison of the size of different APCTs (Devinny et al., 1999; Menasveta et al., 2001; Theodore, 2008).

S. No	Common APCT	Average footprint (m^2)
1.	Open Biofilter	3000
2.	Closed Biofilter (similar to gas phase bioreactor)	8.0
3.	Adsorber (activated carbon)	30
4.	Cyclone	1.5
5.	Electrostatic precipitator	1.5
6.	Absorber	1.1
7.	Catalytic Oxidizer	0.3
8.	Incinerator	0.2

In order to fulfil the 2 main objectives mentioned above, the following specific sub objectives were included in this research work:

- Increase the robustness in measuring the inlet and outlet concentration of toluene by connecting a gas chromatography system and a carbon dioxide analyser online to the existing biofiltration reactor (with soil as the bed media) system developed by Beuger 2009.
- Investigate the effect of different metabolic uncouplers on the specific degradation rate of toluene in a biofiltration reactor (with soil as the bed medium) system.

- Determine the long term impact of uncouplers on EC and their stability.
- Isolate toluene degraders from soil and develop a biofilm reactor to carry out pure culture experiments.
- Investigate the effect of selected metabolic uncouplers (from soil studies) on the specific degradation rate of toluene in the pure culture biofilm reactor.

1.8 Nomenclature

$C_{g, in}$	inlet concentration of the pollutant gas	$g.m^{-3}$
$C_{g, out}$	outlet concentration of the pollutant gas	$g.m^{-3}$
Q	volumetric flow rate of the pollutant gas	$m^3.h^{-1}$
V	biofilter bed volume	m^3

1.9 References

Aizpuru, A., Dunat, B., Christen, P., Auria, R., García-Peña, I., Revah, S., 2005, Fungal biofiltration of toluene on ceramic rings. Journal of Environmental Engineering 131, 396-405.

Aizpuru, A., Malhautier, L., Roux, J., Fanlo, J., 2003, Biofiltration of a mixture of volatile organic compounds on granular activated carbon. Biotechnology and Bioengineering 83, 479-488.

Aizpuru, A., Malhautier, L., Roux, J.C., Fanlo, J.L., 2001, Biofiltration of a mixture of volatile organic emissions. Journal of the Air & Waste Management Association 51, 1662-1670.

Baquerizo, G., Maestre, J.P., Sakuma, T., Deshusses, M.A., Gamisans, X., Gabriel, D., Lafuente, J., 2005, A detailed model of a biofilter for ammonia removal: Model parameters analysis and model validation. Chemical Engineering Journal 113, 205-214.

Beerli, M., Rotman, A., 1989. Control of VOC emissions by use of peat biofilters in the flexography industry. In: Fisrt International Conference on Environmental Issues for Converters, Jacksonville, Florida, USA.

Beuger, A.L., Gostomski, P.A., 2009, Development of a biofilter with water content control for research purposes. Chemical Engineering Journal 151, 89-96.

Bina, B., Dehghanzadeh, R., Pourmoghadas, H., Kalantary, A., Torkian, A., 2004, Removal of styrene from waste gas stream using a biofilter. Journal of Research in Medical Sciences 9, 280-288.

Bohn, H., 1992, Consider Biofiltration for Decontaminating Gases. Chemical Engineering Progress 88, 34-40.

Bohn, H., Bohn, R., 1986, Gas scrubbing by bio-washers and bio-filters. Pollution Engineering 18, 34-35.

Carlson, D., Leiser, C., 1966, Soil beds for the control of sewage odors. Journal of Water Pollution Control Federation, 829-840.

Christen, P., Domenech, F., Michelena, G., Auria, R., Revah, S., 2002, Biofiltration of volatile ethanol using sugar cane bagasse inoculated with *Candida utilis*. Journal of Hazardous Materials 89, 253-265.

Chu, K.H., Alvarez-Cohen, L., 1998, Effect of nitrogen source on growth and trichloroethylene degradation by methane-oxidizing bacteria. Applied and Environmental Microbiology 64, 3451-3457.

Chung, Y.C., 2007, Evaluation of gas removal and bacterial community diversity in a biofilter developed to treat composting exhaust gases. Journal of Hazardous Materials 144, 377-385.

Delhoménie, M.C., Bibeau, L., Bredin, N., Roy, S., Broussau, S., Brzezinski, R., Kugelmass, J.L., Heitz, M., 2002, Biofiltration of air contaminated with toluene on a compost-based bed. Advances in Environmental Research 6, 239-254.

Delhoménie, M.C., Bibeau, L., Gendron, J., Brzezinski, R., Heitz, M., 2003, A study of clogging in a biofilter treating toluene vapors. Chemical Engineering Journal 94, 211-222.

Delhomenie, M.C., Bibeau, L., Roy, S., Brzezinski, R., Heitz, M., 2001, Influence of nitrogen on the degradation of toluene in a compost-based biofilter. Journal of Chemical Technology and Biotechnology 76, 997-1006.

Delhomenie, M.C., Heitz, M., 2005, Biofiltration of air: A review. Critical Reviews in Biotechnology 25, 53-72.

Deshusses, M.A., Johnson, C.T., 2000, Development and validation of a simple protocol to rapidly determine the performance of biofilters for VOC treatment. Environmental Science and Technology 34, 461-467.

Devinny, J.S., Deshusses, M.A., Webster, T.S., 1999, Biofiltration for air pollution control. CRC Press LLC, New York, USA.

Diks, R.M.M., Ottengraf, S.P.P., 1991, Verification Studies of a Simplified Model for the Removal of Dichloromethane from Waste Gases Using a Biological Trickling Filter .1. Bioprocess Engineering 6, 93-99.

Elmrini, H., Bredin, N., Shareefdeen, Z., Heitz, M., 2004, Biofiltration of xylene emissions: bioreactor response to variations in the pollutant inlet concentration and gas flow rate. Chemical Engineering Journal 100, 149-158.

Farmer, R.W., Chen, J.S., Kopchynski, D.M., Maler, W.J., 1995. Reactor switching:proposed biomass control strategy for the biofiltration process. In: 3rd International In Situ and On-Site Bioreclamation Symposium, San Diego, CA-USA, 243-248.

García-Peña, E., Hernández, S., Favela-Torres, E., Auria, R., Revah, S., 2001, Toluene biofiltration by the fungus *Scedosporium apiospermum* TB1. Biotechnology and Bioengineering 76, 61-69.

Girard, M.G.M., Nikiema, J.N.J., Brzezinski, R.B.R., Buelna, G.B.G., Heitz, M.H.M., 2009, A review of the environmental pollution originating from the piggery industry and of the available mitigation technologies: towards the simultaneous biofiltration of swine slurry and methane. Canadian Journal of Civil Engineering 36, 1946-1957.

Guelfo, G., Sordelli, D., Pozzoli, L., 1987, Valori limite di soglia. ACGIH. G Ig Ind 12, 1-122.

Hwang, S.C.J., Wu, S.J., Lee, C.M., 2002, Water transformation in the media of biofilters controlled by *Rhodococcus fascians* in treating an ethyl acetate-contaminated airstream. Journal of the Air & Waste Management Association 52, 511-520.

Iranpour, R., Cox, H.H.J., Deshusses, M.A., Schroeder, E.D., 2005, Literature review of air pollution control biofilters and biotrickling filters for odor and volatile organic compound removal. Environmental Progress 24, 254-267.

Kampbell, D.H., Wilson, J.T., Read, H.W., Stocksdale, T.T., 1987, Removal of Volatile Aliphatic-Hydrocarbons in a Soil Bioreactor. JAPCA Journal of Air and Waste Management 37, 1236-1240.

Kennes, C., Veiga, M.C., 2001, Bioreactors for waste gas treatment. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Lee, E.Y., Jun, Y.S., Cho, K.S., Ryu, H.W., 2002, Degradation characteristics of toluene, benzene, ethylbenzene, and xylene by *Stenotrophomonas maltophilia* T3-c. Journal of the Air & Waste Management Association 52, 400-406.

Leson, G., Winer, A.M., 1991, Biofiltration - an Innovative Air-Pollution Control Technology for Voc Emissions. Journal of the Air & Waste Management Association 41, 1045-1054.

Li, L., Liu, J.X., 2006, Removal of xylene from off-gas using a bioreactor containing bacteria and fungi. International Journal of Biodeterioration and Biodegradation 58, 60-64.

Martin Jr, R.W., Li, H., Mihelcic, J.R., Crittenden, J.C., Lueking, D.R., Hatch, C.R., Ball, P., 2002, Optimization of biofiltration for odor control: model calibration, validation, and applications. Water Environment Research, 17-27.

Menasveta, P., Panritdam, T., Sihanonth, P., Powtongsook, S., Chuntapa, B., Lee, P., 2001, Design and function of a closed, recirculating seawater system with denitrification for the culture of black tiger shrimp broodstock. Aquacultural Engineering 25, 35-49.

Metcalf, I., Eddy, H., 2003, Wastewater engineering: treatment and reuse. McGraw-Hill, New York, USA.

Morales, M., Revah, S., Auria, R., 1998, Start-up and the effect of gaseous ammonia additions on a biofilter for the elimination of toluene vapors. Biotechnology and Bioengineering 60, 483-491.

Mpanias, C.J., Baltzis, B.C., 1998, An experimental and modeling study on the removal of mono-chlorobenzene vapor in biotrickling filters. Biotechnology and Bioengineering 59, 328-343.

Nanda, S., Sarangi, P.K., Abraham, J., 2012, Microbial biofiltration technology for odour abatement: An introductory review. Journal of Soil Science and Environmental Management 3, 28-35.

Nicolai, R., Janni, K., 2000. Designing biofilters for livestock facilities. Second International Conference on Air Pollution from Agricultural Operations, Iowa, USA, 376-383.

Nikiema, J., Brzezinski, R., Heitz, M., 2007, Elimination of methane generated from landfills by biofiltration: a review. Reviews in Environmental Science and Bio/Technology 6, 261-284.

Oh, Y.S., Choi, S.C., 2000, Selection of suitable packing material for biofiltration of toluene, m- and p-xylene vapors. Journal of Microbiology 38, 31-35.

Ottengraf, S.P.P., 1987, Biological-Systems for Waste-Gas Elimination. Trends in Biotechnology 5, 132-136.

Ottengraf, S.P.P., Vandenoever, A.H.C., 1983, Kinetics of Organic-Compound Removal from Waste Gases with a Biological Filter. Biotechnology and Bioengineering 25, 3089-3102.

Park, D.W., Kim, S.S., Haam, S., Ahn, I.S., Kim, E.B., Kim, W.S., 2002, Biodegradation of toluene by a lab-scale biofilter inoculated with *Pseudomonas putida* DK-1. Environmental Technology 23, 309-318.

Pedersen, A.R., Moller, S., Molin, S., Arvin, E., 1997, Activity of toluene-degrading *Pseudomonas putida* in the early growth phase of a biofilm for waste gas treatment. Biotechnology and Bioengineering 54, 131-141.

Pomeroy, R.D., 1982, Biological Treatment of Odorous Air. Journal of Water Pollution Control 54, 1541-1545.

Rene, E.R., Lopez, M.E., Murthy, D.V.S., Swaminathan, T., 2009a, Removal of xylene in gas phase using compost ceramic ball biofilter. International Journal of Physical Sciences 4, 638-644.

Rene, E.R., Murthy, D.V.S., Swaminathan, T., 2009b, Treatment of Benzene Vapors from Contaminated Air Stream in a Laboratory-Scale Compost Biofilter. Macedonian Journal of Chemistry and Chemical Engineering 28, 119-123.

Sorial, G.A., Papadimas, S.P., Suidan, M.T., Speth, T.F., 1994, Competitive Adsorption of Vocs and Bom - Oxic and Anoxic Environments. Water Research 28, 1907-1919.

Spigno, G., Pagella, C., Daria Fumi, M., Molteni, R., Marco De Faveri, D., 2003, VOCs removal from waste gases: gas-phase bioreactor for the abatement of hexane by Aspergillus niger. Chemical Engineering Science 58, 739-746.

Theodore, L., 2008, Air pollution control equipment calculations. A John Wiley & Sons, INC., USA, New Jersey.

Torkian, A., Dehghanzadeh, R., Hakimjavadi, M., 2003, Biodegradation of aromatic hydrocarbons in a compost biofilter. Journal of Chemical Technology and Biotechnology 78, 795-801.

van Groenestijin, J.W., Hesselink, P.G.M., 1994, Biotechniques for Air Pollution Control. Biodegradation 4, 282-301.

Wang, Z., Govind, R., 1997, Biofiltration of isopentane in peat and compost packed beds. AIChE Journal 43, 1348-1356.

Williams, T.O., Miller, F.C., 1992, Odor Control - Biofilters and Facility Operations .2. Biocycle 33, 75-79.

Woertz, J., van Heiningen, W., van Eekert, M., Kraakman, N., Kinney, K., Van Groenestijn, J., 2002, Dynamic bioreactor operation: effects of packing material and mite predation on toluene removal from off-gas. Applied Microbiology and Biotechnology 58, 690-694.

Xie, B., Liang, S., Tang, Y., Mi, W., Xu, Y., 2009, Petrochemical wastewater odor treatment by biofiltration. Bioresource Technology 100, 2204-2209.

Yang, H., Minuth, B., Allen, D.G., 2002, Effects of nitrogen and oxygen on biofilter performance. Journal of the Air & Waste Management Association 52, 279-286.

Yang, Y., Allen, E.R., 1994, Biofiltration control of hydrogen sulfide 1. Design and operational parameters. Journal of the Air & Waste Management Association 44, 863-868.

Yoon, I.K., Park, C.H., 2002, Effects of gas flow rate, inlet concentration and temperature on biofiltration of volatile organic compounds in a peat-packed biofilter. Journal of Bioscience and Bioengineering 93, 165-169.

Zilli, M., Converti, A., Lodi, A., Delborghi, M., Ferraiolo, G., 1993, Phenol Removal from Waste Gases with a Biological Filter by *Pseudomonas-Putida*. Biotechnology and Bioengineering 41, 693-699.

Chapter 2: Metabolic Uncouplers

2.1 Introduction

Metabolic uncouplers are powerful chemicals, which can influence energy production in cells by uncoupling electron transport from oxidative phosphorylation reactions and thereby making ATP production less efficient from a substrate oxidation perspective. They were originally used to elucidate energy production in mitochondria (Brand et al., 2010) and more recently to reduce biomass yield in waste water treatment and disrupt biofilm formation (Hassani et al., 2011; Lou et al., 2007; Low and Chase, 1998). The term "metabolic uncoupler" comes from their ability to carry protons across cellular membranes in the protonated form, dissociate and move back across the membrane in a cyclical fashion. This transport of protons diminishes the protomotive force required for ATP production. Application of metabolic uncouplers in environmental research is rapidly increasing each year. However, more research was done in growth systems like activated sludge processes but least in trickle bed and non-growth systems like biofiltration. This chapter will explore in detail about the mechanism of chemical uncoupling and its application in activated sludge to decrease the biomass yield. In addition, the possibilities of employing potential metabolic uncouplers in biofilters will also be explored.

2.2 Metabolic Uncoupling in Microorganisms

Cells gain energy by breaking down molecules through catabolic pathways. During cell proliferation, this energy is used by the cells' anabolic pathways to create new cell mass. Cells are capable of regulating their metabolic reactions and the biosynthesis of their enzymes to achieve maximum efficiency and economy (Lehninger, 1975) and, under rapidly growing conditions, the biomass yield is directly proportional to the ATP produced (Brock and Madigan, 1991). This assumption of a coupling between anabolism and catabolism is contradicted by the observation that resting cell suspensions can utilize energy sources in the complete absence of growth and by the fact that the correlation between ATP and biomass formation is often very poor (Russell and Cook, 1995).

Some of these variations in growth efficiency or yield can be explained by maintenance energy (Russell and Cook, 1995). Though energy produced through catabolism can be used for growth, microbes utilize a portion of that energy to "maintain" the cells through

the requirement of protein turn-over, maintaining concentration gradients and hence termed as maintenance energy (Buchanan and Fulmer, 1928). The other endogenous processes which are considered to have significant effects on yield in activated sludge treatment are programmed cell death (PCD), microbial starvation, viral infection and predation. However, the current state of knowledge about some of these processes is very limited (Hao et al., 2010).

When the microbes are grown in the presence of metabolic uncouplers, energy uncoupling can be induced as the ATP synthesis is less efficient, thus requiring more substrate oxidation per ATP molecule produced, which effectively increases the maintenance coefficient. This uncoupling occurs in such a manner that the catabolism of the substrate can continue unaffected, while the anabolism is restricted due to lack of ATP (Diks and Ottengraf, 1991; Holubar et al., 1999; Kirchner et al., 1991; Liu and Tay, 2001; Low et al., 2000). The metabolic uncoupler interference is assumed to make less energy available for biomass yield in the case of growth systems like activated sludge, but not disrupt waste degradation (Yang et al., 2003). Since maintenance is a function that detracts from growth, the contribution of maintenance energy is more pronounced when there is low or nil growth. In other words, if the energy needed to maintain the cell increases, there will be less energy available for anabolism and hence less biomass yield (Liu and Tay, 2001; Low and Chase, 1998; Low et al., 2000). Studies using dinitrophenol as a metabolic uncoupler in glucose-limited culture of *K. aerogenes* showed an increase in the maintenance energy in both nitrogen- and carbon-limited aerobic chemostat cultures (Neijssel, 1977). Thus the typical nature of metabolic uncouplers makes them potentially useful in controlling excess microbial growth in growth systems like activated sludge treatment.

A wide group of natural compounds are known to be metabolic uncouplers of oxidative phosphorylation inside the cell which include compounds like ammonia, oxidants, detergents, heavy metals, organic solvents, fatty acids (Wojtczak and Schönfeld, 1993) (Skulachev, 1991), animal defensins (Lehrer et al., 1991) and plant thionins (Bohlmann and Apel, 1991). Though the uncoupling mechanism can occur naturally in all microorganisms during various biochemical syntheses, it is also possible to induce an uncoupling reaction with the help of synthetic chemical uncouplers.

2.3 Weak Acidic Metabolic Uncouplers

Most of the synthetic metabolic uncouplers are lipophilic weak acids which possess protonophoric activity (Zubay, 1998). Their key molecular feature is being able to pass across the membrane in a charged state by delocalising the charge across the molecule, usually involving a benzene ring structure. They are able to transport the protons across the membrane much faster than the proton pumps of the respiratory chain, with SF 6847 (Tyrphostin 9) uncoupling at a ratio of 1 molecule per 20 respiratory chains (Lewis et al., 1994). Weak acids like phenols, benzimidazoles, salicylic acids, cumarines, and aromatic amines etc., are known to induce uncoupling (Hanstein, 1976) (Terada, 1981).

According to the chemiosmotic theory, energy for ATP production in the form of the chemical potential of H^+ across a H^+ impermeable membrane is supplied by redox reactions. ATP is produced from ADP and P_i (inorganic phosphate) when H^+ enters the cell via H^+-ATPase. Using 2,4-dinitrophenol as an example (Fig 2.1), the anionic form of the weak acid metabolic uncoupler can trap the H^+ ions in the low pH environment of the external membrane-water interface and becomes a neutral compound. This neutral compound traverses the membrane to the cell interior and releases the H^+ ion due to the higher pH environment. Then the anionic form of the metabolic uncoupler returns to the original interface where it again traps an H^+ ion. This uncoupler cycle continues and decreases the H^+ gradient across the membrane which results in uncoupling (Terada, 1981, 1986) . The net effect of uncoupling is to shunt protons through the membrane and not through the F_0F_1 (coupling factors) ATPase. A similar uncoupling mechanism is followed by most of the common metabolic uncouplers (Fig 2.1).

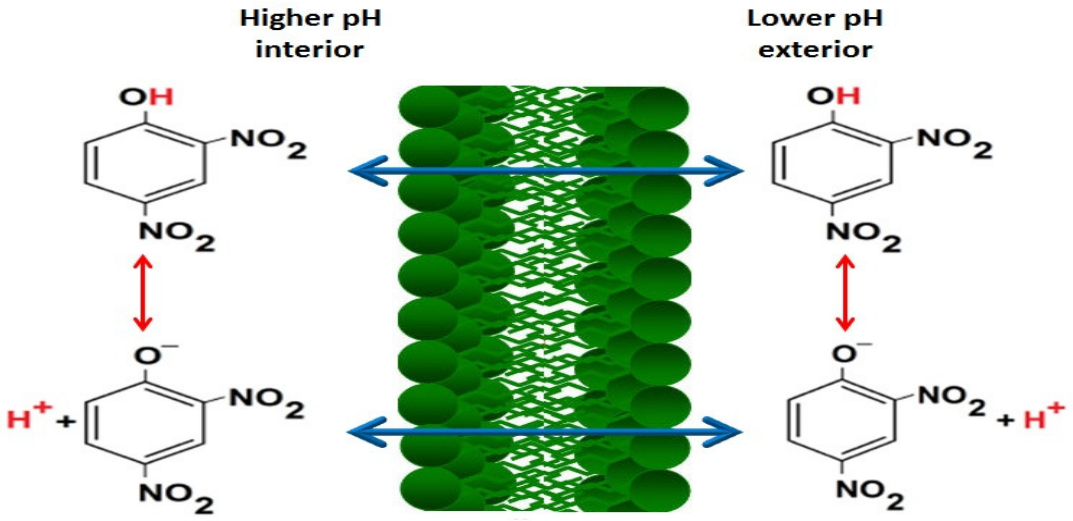


Figure 2.1: The mechanism for the 2,4-dinitrophenol uncoupling reaction. The net flux of H^+ ions due to uncoupling is from the lower pH exterior to higher pH interior. The net flux of DNP via membrane diffusion and protonation/deprotonation reactions is in the counter clockwise direction.

2.4 Common Metabolic Uncouplers

Among a large group of metabolic uncouplers reported in the literature, carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP), carbonylcyanide m-chloromethoxy-phenylhydrazone (CCCP), tetrachlorosalicylanilide (TSA), pentachlorophenol (PCP) and 2, 4-dinitropenol (DNP) are the most widely used metabolic uncouplers in research (Fig. 2.2).

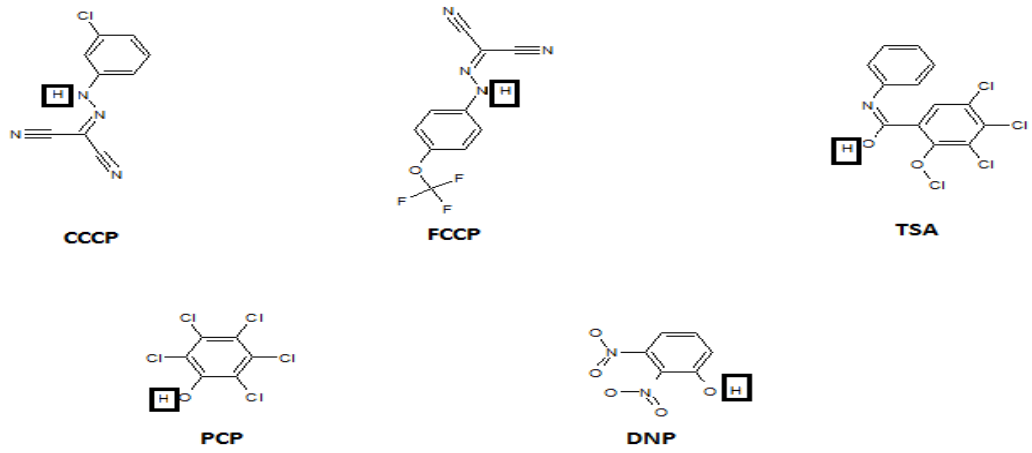


Figure 2.2: Protonophoric order of commonly used metabolic uncouplers FCCP>CCCP>TSA>PCP>DNP (Lewis et al., 1994). The ionisable H^+ is noted by a black box on each metabolic uncoupler.

2.5 Effect of pH and pK_a in uncoupling

The knowledge of pK_a or effective pH range of a metabolic uncoupler is considered essential for their selection (Hiraishi and Kawagishi, 2002; Salmond et al., 1984). In addition, knowledge about the internal pH, external pH and bulk pH of the cell is very important in understanding the protonophoric activity of the metabolic uncoupler inside and outside of a cell. Though both pH and pKa influence the uncoupling process, they are not the only parameters involved in the uncoupling mechanism. Solvation free energies of the anions and heterodimer formation during the protonophoric transport are also contributing to the uncoupling mechanism. However, less work has been done on understanding the intrinsic activity of the metabolic uncoupling process inside the cell (Spycher et al., 2005).

Bacteria and archaea adapted to an acidic environment maintain a cytoplasmic pH higher than that of the exterior, whereas the reverse is true for those growing in a high pH environment. To maintain cytoplasmic pH at the more neutral value, cells accept a substantial energy loss through inversion of $\Delta\Psi$ (electrical potential) in extreme acidic environment and expenditure of $\Delta\Psi$ in extreme basic environments. Over the range of microbes studied, it was found that the crossover point where cytoplasmic pH equals external pH lies between pH 7–8. However, not all species can actually grow at this external pH. The external pH that supports growth and the transmembrane pH difference

available for energy generation (ΔpH) vary in breadth but, in general, microorganisms can grow in wide ranges of environmental pH from pH 0 to above pH 13 (Nordstrom and Alpers, 1999; Nordstrom et al., 2000).

It was found that at pH 8.5 and higher concentrations of CCCP and FCCP, the uncoupling mechanism across the membrane failed compared to the uncoupling at pH 7 in marine bacteria (MacLeod et al., 1988). The difference in the metabolic activities at varying pH values was attributed to the pK_a values of 6.1 for CCCP and 5.7 for FCCP. Moreover, this failure was attributed to the increase in the pH difference between the external pH and the intracellular pH following the addition of the metabolic uncoupler (MacLeod et al., 1988). Therefore, as the pH was increased above the pKa of the protonophore, the amount of the protonated form able to enter the membrane became too low to maintain this cycle (McLaughlin and Dilger, 1980; Mitchell, 1966), which could have been the reason behind the failure of proton shuttling mechanism in case of CCCP and FCCP. Hence maintaining the external pH below the pKa of metabolic uncoupler and the internal pH above the pKa of metabolic uncoupler appears to be necessary for proton movement in and out of the membrane. However, more research is needed to prove this hypothesis.

In another study, the effects of chlorophenol and nitrophenol on activated sludge treatment were investigated (Yang et al., 2003). They showed that the metabolic uncouplers with lower pKa values were more effective in uncoupling energy metabolism, thereby favouring sludge reduction. The uncoupler m-chlorophenol with a pKa of 8.8 yielded 87% sludge reduction whereas p-chlorophenol with a pKa of 9.2 yielded only 58% sludge reduction. Similarly, o-nitrophenol with a pKa of 7.17 yielded 86% sludge reduction when compared to m-nitrophenol with a pKa of 8.28 which yielded only 65% sludge reduction. One possible explanation for these results is that the lower pK_a resulted in a weaker protomotive force resulting in a reduced biomass yield. Moreover, lower pKa values favour deprotonation of the phenolic hydroxyl group in chlorophenolic and nitrophenolic uncouplers inside the cell, thereby supporting the uncoupling cycle. At a weaker protomotive force, less sludge will be produced as there will be less ATP generation. Moreover, at a critical concentration of uncoupler for which the protomotive force is so low, there will be no ATP generation and hence no biomass yield (Yang et al., 2003). However, the bulk solution pH was not reported in this study, which might have clarified the uncoupling pattern. Although the theory seems to explain the results well, more testing

must be completed to definitively state uncouplers with a pK_a lower than the internal pH of the cell are more effective uncouplers.

Compounds like trinitrophenol, a very acidic phenol, can also induce uncoupling but its activities is weaker than those of protonophoric metabolic uncouplers due to its low pKa (Hanstein and Hatefi, 1974) (Hanstein and Kiehl, 1981). Hence, the pK_a of chemical uncouplers, the bulk solution pH and internal pH of different microbial cells should aid decisions of which chemical uncoupler may be best to pursue in research. Table 2.1 summarises the pK_a values of a range of metabolic uncouplers used in activated sludge treatment.

Table 2.1: The pK_a values of a range of metabolic uncouplers.

Metabolic Uncoupler	**pK_a**	**Reference**
benzoic acid	4.20	(Kwan and Voelker, 2003)
carbonylcyanide m-chloromethoxy-phenylhydrazone	6.09	(Bona et al., 1993)
carbonylcyanide p-trifluoromethoxy-phenylhydrazone	6.10	(Bona et al., 1993)
2,4-dinitrophenol	4.09	(Zhang et al., 2010)
m-chlorophenol	8.80	(Yang et al., 2003)
malonic acid	2.83	(Liu et al., 2010)
p-nitrophenol	7.15	(Zhang et al., 2010)
pentachlorophenol	4.70	(Kocherginsky, 2009)
2,4,6-trichlorophenol	7.50	(Poole and Cook, 2000)
3,3',4',5-tetrachlorosalicyanilide	7.60	(McLaughlin and Dilger, 1980)
trinitrophenol	0.40	(Hanstein and Hatefi, 1974)

2.6 Studies of different Metabolic Uncouplers in Growth System(s)

Yield of excess biomass during certain biological treatment requires costly disposal. Especially, in activated sludge processes the treatment of excess sludge may account for up to 65-70% of the total plant operation cost (Yang et al., 2003). Hence, reducing biomass yield is important in activated sludge processes or where unwanted biofilm formation can occur and the potential of uncouplers in these applications has been explored (Low and Chase, 1998; Okey and Stensel, 1993). These studies have been based on the idea that energy in cells used for maintenance takes precedence over growth. With metabolic uncouplers present, more energy is required for the maintenance of the cell and so less growth will occur. In the last decade, around twelve metabolic uncouplers have been tested in waste water treatment systems and shown to have an impact on the biomass yield (Hiraishi and Kawagishi, 2002; McLaughlin and Dilger, 1980; Ray and Peters, 2008; Wei et al., 2003; Yang et al., 2003; Zheng et al., 2008).

Yang et al. (2003) tested the effect of four metabolic uncouplers (p-chlorophenol, m-chlorophenol, m-nitrophenol and o-nitrophenol) in reducing biomass yield from an activated sludge process and reported that the biomass yield was reduced when increasing the metabolic uncoupler concentration from 0 to 0.15 mM. They concluded that among the four metabolic uncouplers tested, m-chlorophenol was the most effective, reducing the biomass yield by 87% at a concentration of 0.15 mM. It was reported that biomass yield was reduced by 49% when paranitrophenol (pNP) was added to the culture. However, when considering both the sludge reduction and COD removal efficiency, m-chlorophenol was the most effective metabolic uncoupler in this system. Paranitrophenol was also tested in a pure culture of *Pseudomonas putida*, in a chemostat, and it was found that pNP at 0.72 mM reduced the biomass yield by 62% with a simultaneous increase in the specific substrate uptake rate (Low and Chase, 1998). These studies again conclude that in presence of metabolic uncouplers, maintenance energy increases and for that reason the substrate uptake rate also increases.

TSA has been tested in a number of activated sludge systems. Growth yield was reduced by 78% with TSA at 2.3 µM with no significant effect on substrate removal efficiency (MacLeod et al., 1988). A similar study carried out by Aragon et al. (2009) to understand the effect of TSA and 2, 4-dinitrophenol (2,4-DNP) in reducing the excess biomass yield

in activated sludge reported that TSA reduced the biomass yield by 30% at 2.3 µM concentration but 2,4-DNP had no effect. Another study with TSA in an activated sludge system revealed that the excess biomass yield was reduced by 80% per day at a TSA dose of 1% (Hassani et al., 2011). Ye and Li (2005) reported that 0.43 mM of TSA decreased the biomass yield by 56% in an activated sludge process without affecting the substrate utilization rate.

The effect of 2, 4, 6-trichlorophenol (TCP) and malonic acid (MA) was investigated in an activated sludge system to reduce the sludge generation in a sequencing batch reactor (SBR) for treating organic waste water. TCP, at a concentration of 0.01 mM, reduced the sludge generation by 47% without influencing the chemical oxygen demand (COD) removal efficiency and sludge settleability. Whereas MA at a concentration of 0.05 mM, reduced the sludge generation by 30%. However it slightly affected the COD removal and seriously deteriorated the sludge settleability (Verduyn et al., 1992). Another study using 2,4,6 TCP as an uncoupler in activated sludge reported that TCP at a concentration of 1.2 mM reduced the sludge yield by 67% without affecting the sludge settleability (Tao et al., 2010). The feasibility of TCP as metabolic uncoupler to reduce sludge generation in the sequencing batch reactor for 90 days for the treatment of municipal wastewater showed that with 10 µM TCP, 47% sludge reduction was achieved. The group also reported that the COD removal efficiency and sludge settleability were not affected at 10 µM TCP (Zheng et al., 2008).

Studies were carried out using benzoic acid, CCCP and FCCP separately in a non-activated sludge system to understand their effect on biomass yield. Benzoic acid at a concentration of 10 mM altered the metabolic fluxes in yeasts and thus decreased the biomass yield. However, the percentage of reduction was not shown, nor the reasoning why this concentration was used in this study (Verduyn et al., 1992). Similarly, CCCP and FCCP at a concentration of 10 µM reduced the biomass yield in *E. coli* by 37% for FCCP and 22% for CCCP (MacLeod et al., 1988).

2,4- DNP was tested in an activated sludge treatment and showed a 21% decrease in the biomass yield with 93% COD removal at a concentration of 0.03 mM (Chen et al., 2008). Both 2, 4-DNP and pentachlorophenol (PCP) were used as model metabolic uncouplers to study the impact of chemical stress on microbiological metabolism (Ray and Peters,

2008). It was found that DNP, between 0.26 mM and 0.76 mM, and PCP, at 0.056 mM and 0.14 mM, caused decreases in biomass growth yield, but did not inhibit the substrate utilization rate. Table 2.2 summarizes the effective concentration of some of the metabolic uncouplers tested so far in growth systems.

Table 2.2: A list of common metabolic uncouplers and their effective concentration in growth systems and impact on yield.

Metabolic Uncoupler	**Effective conc. Reported (µM)**	**Decrease in growth yield (%)**	**Application**	**Reference**
carbonylcyanide m-chloromethoxy-phenylhydrazone	0.01	37	metabolic flux reduction in yeast	(Slonczewski et al., 2009)
carbonylcyanide p-trifluoromethoxy-phenylhydrazone	0.01	22	metabolic flux reduction in yeast	(Slonczewski et al., 2009)
2,4-dinitrophenol	760	21	Activated sludge	(Chen et al., 2008)
m-chlorophenol	160	87	Activated sludge	(Wang et al., 2010)
malonic acid	50	30	Activated sludge	(Büscher et al., 2009)
p-nitrophenol	860	62	Activated sludge	(Yang et al., 2003)
pentachlorophenol	140	80	Activated sludge	(Nicolaou et al., 2010)

2,4,6-trichlorophenol	10	67	Activated sludge	(Zheng et al., 2008)
3,3',4',5-tetrachlorosalicyanilide	2.28	80	Activated sludge	(Saini and Wood, 2008)

Though different metabolic uncouplers are effective in controlling the excess biomass yield in growth systems, their fate and residual toxicity following their application has not been explored. This raises a question about the usage of such toxic chemicals in growth systems which can affect the environment due to their uncontrolled release. In a recent study, the fate and residual toxicity of one of the metabolic uncouplers, TCP was tested in an activated sludge system treating municipal waste water. The study reported that residual TCP in the effluent ranged between 2 μM and 5 μM when the TCP in the feed was 10 μM. The study also concluded that although the concentration of the trace TCP was too low to affect the ecosystem, the long term accumulation of TCP may be an issue (Qiao et al., 2011). Similarly, the legal limit for DNP in surface water is 0.4 μM (Metcalf and Eddy, 2003) but the effective concentration of DNP reported (Chen et al., 2008; Zhang et al., 2010) in various activated sludge processes is well above this limit. Similarly, the international legal limit of chlorophenols in surface water is 0.003 nM (Girard et al., 2009), but the effective concentration of chlorophenols (PCP, TCP etc.,) reported in different activated sludge processes is higher than the stipulated level. So, although the addition of chemical uncouplers to activated sludge reactors is effective in reducing the excess biomass yield, questions still exist about the practical use of uncouplers in activated sludge and other systems discharging uncoupler-contaminated water to the environment. A more accurate ecological risk assessment methodology may provide some insight into the true benefits of uncouplers in activated sludge (Connell, 1999).

2.7 Metabolic Uncouplers in fixed film processes

Traditional fixed film, packed bed reactors for waste air and water treatment (trickle beds) require the control of biomass accumulation (Aly Hassan and Sorial, 2011; Pintar et al., 1997). Metabolic uncouplers could represent another tool in controlling biomass build-up,

however no reports on their use in this type of reactor system are available. Based on the studies conducted in activated sludge treatment, uncouplers should also be effective in controlling the excess biomass growth in trickle bed reactors or any fixed film processes to treat gaseous or aqueous pollutants. However, research is needed to prove this hypothesis.

Application of metabolic uncouplers in fixed film membrane bioreactors to prevent biofouling has been successful in recent years. FCCP and CCCP are used widely in reducing the membrane biofouling in membrane bioreactors (Xu and Liu, 2010). Another study investigated the effects of 2, 4-dinitrophenol on reduction of biofouling in a membrane bioreactor employed in waste water treatment. Nearly 65% biofouling inhibition was observed at 0.05 mM concentration of 2, 4-dinitrophenol. In addition, the 2,4-dinitrophenol had enhanced the biofilm detachment from nylon membrane in the membrane bioreactor (Xu and Liu, 2011). However, further research is needed in this field with other potential metabolic uncouplers previously tested in activated sludge processes.

2.8 Metabolic Uncouplers in non-growth system

Metabolic uncouplers show promise for sludge reduction but to our knowledge, no work has been performed on the effect of metabolic uncouplers in non-growth systems like biofiltration. These systems are driven mainly by maintenance energy, so the addition of the uncoupler should increase the substrate degradation rate. Moreover, in the case of an air treatment biofilter, the water released is minimal and the uncoupler could be recycled, as compared to activated sludge treatment, where it lost to the environment. However in biofilters, research will need to be done to understand how the microorganisms' maintenance metabolism will respond to the stress of the uncouplers both in short term and long term applications.

2.9 References

Aly Hassan, A., Sorial, G.A., 2011, Treatment of dynamic mixture of hexane and benzene vapors in a trickle bed air biofilter integrated with cyclic adsorption/desorption beds. Chemosphere 82, 521-528.

Bohlmann, H., Apel, K., 1991, Thionins. Annual Review of Plant Biology 42, 227-240.

Bona, M., Antalik, M., Gazova, Z., Kuchar, A., Dadak, V., Podhradský, D., 1993, Interaction of carbonyl cyanide 3-chlorophenylhydrazone with cytochrome c oxidase. General Physiology and Biophysics 12, 533-542.

Brand, M., Lou, P.H., Hansen, B., Olsen, P., Tullin, S., Murphy, M., 2010, Mitochondrial uncouplers with an extraordinary dynamic range. Biochemical Journal 407, 129-140.

Brock, T.D., and , Madigan, M.T., 1991, Biology of microorganisms, 6th Edition. Prentice-Hall, Englewood Cliffs, New Jersey, USA.

Buchanan, R.E., and , Fulmer, E.I., 1928, Physiology and biochemistry of bacteria. The Williams & Wilkins Co.,, Baltimore, USA.

Büscher, J.M., Czernik, D., Ewald, J.C., Sauer, U., Zamboni, N., 2009, Cross-platform comparison of methods for quantitative metabolomics of primary metabolism. Analytical Chemistry 81, 2135-2143.

Chen, G.W., Yu, H.Q., Xi, P.G., Xu, D.Q., 2008, Modeling the yield of activated sludge in the presence of 2, 4-dinitrophenol. Biochemical Engineering Journal 40, 150-156.

Connell, D.W., 1999, Introduction to ecotoxicology. Wiley-Blackwell, London, UK.

Diks, R., Ottengraf, S., 1991, Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter. Bioprocess and Biosystems Engineering 6, 131-140.

Girard, M.G.M., Nikiema, J.N.J., Brzezinski, R.B.R., Buelna, G.B.G., Heitz, M.H.M., 2009, A review of the environmental pollution originating from the piggery industry and of the available mitigation technologies: towards the simultaneous biofiltration of swine slurry and methane. Canadian Journal of Civil Engineering 36, 1946-1957.

Hanstein, W., Hatefi, Y., 1974, Trinitrophenol: a membrane-impermeable uncoupler of oxidative phosphorylation. Proceedings of the National Academy of Sciences 71, 288-292.

Hanstein, W.G., 1976, Uncoupling of oxidative phosphorylation. Biochimica et Biophysica Acta 456, 129-142.

Hanstein, W.G., Kiehl, R., 1981, Energy-dependent accumulation of the uncoupler picrate and proton flux in submitochondrial particles. Biochemical and Biophysical Research Communications 100, 1118-1125.

Hao, X.D., Wang, Q.L., Zhu, J.Y., Van Loosdrecht, M.C.M., 2010, Microbiological endogenous processes in biological wastewater treatment systems. Critical Reviews in Environmental Science and Technology 40, 239-265.

Hassani, A., Nejaei, A., Torabian, A., 2011, Excess sludge minimization in conventional activated sludge pilot plant by three chemical matters. International Journal of Environmental Research 5, 981-988.

Hiraishi, A., Kawagishi, T., 2002, Effects of chemical uncouplers on microbial biomass production, metabolic activity, and community structure in an activated sludge system. Microbes and Environments 17, 197-204.

Holubar, P., Andorfer, C., Braun, R., 1999, Effects of nitrogen limitation on biofilm formation in a hydrocarbon-degrading trickle-bed filter. Applied Microbiology and Biotechnology 51, 536-540.

Kirchner, K., Gossen, C., Rehm, H.J., 1991, Purification of exhaust air containing organic pollutants in a trickle-bed bioreactor. Applied Microbiology and Biotechnology 35, 396-400.

Kocherginsky, N., 2009, Acidic lipids, H+-ATPases, and mechanism of oxidative phosphorylation. Progress in Biophysics and Molecular Biology 99, 20-41.

Kwan, W.P., Voelker, B.M., 2003, Rates of hydroxyl radical generation and organic compound oxidation in mineral-catalyzed fenton-like systems. Environmental Science and Technology 37, 1150-1158.

Lehninger, A.L., 1975, Biochemistry, 2nd Edition. Worth Publishers, Inc., New York, USA.

Lehrer, R.I., Ganz, T., Selsted, M.E., 1991, Defensins: endogenous antibiotic peptides of animal cells. Cell 64, 229-230.

Lewis, K., Naroditskaya, V., Ferrante, A., Fokina, I., 1994, Bacterial resistance to uncouplers. Journal of Bioenergetics and Biomembranes 26, 639-646.

Liu, J., Takada, R., Karita, S., Watanabe, T., Honda, Y., 2010, Microwave-assisted pretreatment of recalcitrant softwood in aqueous glycerol. Bioresource Technology 101, 9355-9360.

Liu, Y., Tay, J.H., 2001, Strategy for minimization of excess sludge production from the activated sludge process. Biotechnology Advances 19, 97-107.

Lou, P.H., Hansen, B.S., Olsen, P.H., Tullin, S., Murphy, M.P., Brand, M.D., 2007, Mitochondrial uncouplers with an extraordinary dynamic range. The Biochemical Journal 407, 129-140.

Low, E.W., Chase, H.A., 1998, The use of chemical uncouplers for reducing biomass production during biodegradation. Water Science and Technology 37, 399-402.

Low, E.W., Chase, H.A., Milner, M.G., Curtis, T.P., 2000, Uncoupling of metabolism to reduce biomass production in the activated sludge process. Water Research 34, 3204-3212.

MacLeod, R., Wisse, G., Stejskal, F., 1988, Sensitivity of some marine bacteria, a moderate halophile, and Escherichia coli to uncouplers at alkaline pH. Journal of Bacteriology 170, 4330-4337.

McLaughlin, S., Dilger, J.P., 1980, Transport of protons across membranes by weak acids. Physiological Reviews 60, 825-863.

Metcalf, I., Eddy, H., 2003, Wastewater engineering: treatment and reuse. McGraw-Hill, Newyork, USA.

Mitchell, P., 1966, Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biological Reviews 41, 445-501.

Neijssel, O., 1977, The effect of 2, 4-dinitrophenol on the growth of *Klebsiella aerogenes* NCTC 418 in aerobic chemostat cultures. FEMS Microbiology Letters 1, 47-50.

Nicolaou, S.A., Gaida, S.M., Papoutsakis, E.T., 2010, A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation. Metabolic Engineering 12, 307-331.

Nordstrom, D.K., Alpers, C.N., 1999, Negative pH, efflorescent mineralogy, and consequences for environmental restoration at the iron mountain superfund site, california. Proceedings of the National Academy of Sciences 96, 3455-3462.

Nordstrom, D.K., Alpers, C.N., Ptacek, C.J., Blowes, D.W., 2000, Negative pH and extremely acidic mine waters from Iron Mountain, California. Environmental Science and Technology 34, 254-258.

Okey, R.W., Stensel, H.D., 1993, Uncouplers and activated sludge—the impact on synthesis and respiration. Toxicological & Environmental Chemistry 40, 235-254.

Pintar, A., Berčič, G., Levec, J., 1997, Catalytic liquid-phase oxidation of aqueous phenol solutions in a trickle-bed reactor. Chemical Engineering Science 52, 4143-4153.

Poole, R.K., Cook, G.M., 2000, Redundancy of aerobic respiratory chains in bacteria? Routes, reasons and regulation. Advances in Microbial Physiology 43, 165-224.

Qiao, J.L., Wang, L., Qian, Y.F., 2011, Fate and residual toxicity of a chemical uncoupler in sequencing batch reactor under metabolic uncoupling conditions. Environmental Engineering Science 29, 599-605.

Ray, S., Peters, C.A., 2008, Changes in microbiological metabolism under chemical stress. Chemosphere 71, 474-483.

Russell, J.B., and, Cook, G.M., 1995, Energetics of bacterial-growth - balance of anabolic and catabolic reactions. Microbiology Review 59, 48-62.

Saini, G., Wood, B.D., 2008, Metabolic uncoupling of *Shewanella oneidensis* MR-1, under the influence of excess substrate and 3, 3′, 4′, 5-tetrachlorosalicylanilide (TCS). Biotechnology and Bioengineering 99, 1352-1360.

Salmond, C.V., Kroll, R.G., Booth, I.R., 1984, The effect of food preservatives on pH homeostasis in *Escherichia coli*. Journal of General Microbiology 130, 2845-2850.

Skulachev, V.P., 1991, Fatty acid circuit as a physiological mechanism of uncoupling of oxidative phosphorylation. FEBS Letters 294, 158-162.

Slonczewski, J.L., Fujisawa, M., Dopson, M., Krulwich, T.A., 2009, Cytoplasmic pH measurement and homeostasis in bacteria and archaea. Advances in Microbial Physiology 55, 1-79, 317.

Spycher, S., Escher, B.I., Gasteiger, J., 2005, A quantitative structure-activity relationship model for the intrinsic activity of uncouplers of oxidative phosphorylation. Chemical Research in Toxicology 18, 1858-1867.

Tao, W., Chengquan, Y., Weimin, L., Haiwen, X., Jing, L., 2010, Effect of 2, 4, 5-trichlorophenol on A~ 2/O process sludge yields. Environmental Science and Management 35, 38-41.

Terada, H., 1981, The interaction of highly active uncouplers with mitochondria. Biochimica et Biophysica Acta 639, 225-242.

Terada, H., 1986, Determination of Log P_{oct} by high-performance liquid chromatography, and its application in the study of quantitative structure-activity relationships. Quantitative Structure-Activity Relationships 5, 81-88.

Verduyn, C., Postma, E., Scheffers, W.A., Van Dijken, J.P., 1992, Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8, 501-517.

Wang, J., Zhang, Z., Zheng, P., Li, C., 2010, The influence and mechanism of influent pH on anaerobic co-digestion of sewage sludge and printing and dyeing wastewater. Water SA 33, 467-472.

Wei, Y., Van Houten, R.T., Borger, A.R., Eikelboom, D.H., Fan, Y., 2003, Minimization of excess sludge production for biological wastewater treatment. Water Research 37, 4453-4467.

Wojtczak, L., Schönfeld, P., 1993, Effect of fatty acids on energy coupling processes in mitochondria. Biochimica et Biophysica Acta 1183, 41-57.

Xu, H., Liu, Y., 2011, Control and cleaning of membrane biofouling by energy uncoupling and cellular communication. Environmental Science and Technology 45, 595-601.

Yang, X.F., Xie, M.L., Liu, Y., 2003, Metabolic uncouplers reduce excess sludge production in an activated sludge process. Process Biochemistry 38, 1373-1377.

Zhang, H.M., Zhou, Q.H., Wang, Y.Q., 2010, Studies on the Interactions of 2, 4-dinitrophenol and 2, 4-dichlorphenol with trypsin. Journal of Fluorescence 20, 507-516.

Zheng, G., Li, M., Wang, L., Chen, Z., Qian, Y., Zhou, Q., 2008, Feasibility of 2, 4, 6-trichlorophenol and malonic acid as metabolic uncoupler for sludge reduction in the sequence batch reactor for treating organic wastewater. Applied Biochemistry and Biotechnology 144, 101-109.

Zubay, G.L., 1998, Biochemistry. WCB Publisher, Boston, MA, USA.

Chapter 3: Differential Biofiltration Reactor

3.1 Introduction

A differential biofilter reactor with water content control developed by Beuger and Gostomski (2009) has been modified in order to enable online monitoring of inlet and outlet toluene and carbon dioxide concentration (Beuger and Gostomski, 2009). Furthermore, the diffusion system used in the earlier research has also been modified in the current research. These modifications are intended to improve the research reactor for data collection and stability. This chapter will discuss in detail the experimental set-up of the existing differential biofilter reactor and various modifications carried out to it to improve the robustness of the reactor used in this current research.

3.2 Differential reactor

A differential reactor is normally used in catalytic studies for determining the rate of the reaction as a function of either concentration or partial pressure. The basic criterion for a reactor being differential is that the conversion of the reactants in the bed is extremely low as is the change in temperature and reactant concentration through the bed. As a result, the reactant concentration through the reactor is essentially constant and approximately equal to the inlet concentration (Fogler, 2004). A differential reactor is normally considered to be gradientless one and the reaction rate is considered spatially uniform within the bed (Anderson, 1976). Due to the low conversion achieved in the reactor, the heat release per unit volume will be small so that the reactor operates essentially in an isothermal manner. Though differential reactors gives good kinetic data, even small analytical errors usually lead to inaccurate rates (Tajbl et al., 1966). Differential reactors include batch reactor, continuous stirred tank reactors and plug flow reactors. In recent years, the concept of differential reactors has been employed in biofiltration because it is easy to control contaminant concentration and parameters like temperature, water content, nutrients etc., in a differential reactor, which is most important in biofiltration (Carberry, 1964)(Moser, 1988). Moreover, for employing metabolic uncouplers in a biofiltration system, a differential biofilter reactor would be best choice in terms of controlled addition and removal since most of metabolic uncouplers are not eco-friendly. In addition, the ability to control water content through the membrane in a differential biofiltration reactor makes it more suitable for uncoupler

studies. Based on these reasons, a differential biofilter reactor is used in the current research.

3.3 Matric potential

Water present in soil, compost and other porous media is mainly retained by the matric forces in pores. Matric potential is a measure of water availability to microorganisms which is fundamental in biofiltration. Hence knowledge about matric potential is most important in controlling the water content of a biofilter media (Papendick and Campbell, 1981). Matric potential is usually given by the following relationship (Eq. 3.1),

$$\boldsymbol{\Psi} = \frac{RT}{V_W} \ln a_W \quad (\text{J.m}^{-3}) \tag{3.1}$$

Generally, the matric potential is created by the effect of capillary forces of pores and particle surfaces adsorbing water. At a saturation point, matric potential becomes zero as all the pores are filled with liquid water. When suction or a gravitational force is applied to the saturated porous medium, water drains from the medium until equilibrium between matric potential and gravity is established at lower water content (Bohn and Bohn, 1999).

3.4 Configuration of the differential biofilter reactor

The differential biofilter reactor configuration is in contrast to an integral laboratory biofilter where most of the parameters change along the length of the reactor. The reactor converts the traditional plug flow biofilter into a CSTR by employing the internal recycle (Beuger and Gostomski, 2009) (Badilla et al., 2011). Soil (Park house Garden Supplies- Appendix D) is used as a biofilter bed media in this reactor for the current research purpose. Moreover, this reactor is also configured to control the water content through a hydrophilic membrane connecting the biofilter to a water reservoir under vacuum. This helps to set and maintain the matric potential of the biofilter. Furthermore, this configuration allows an easy method to introduce a metabolic uncoupler into the biofilter. In addition, the internal gas reservoir in this version gives good mixing, eliminating interparticle concentration, avoids temperature gradients and even eliminates bypass in the biofilter bed.

The water chamber is placed under vacuum by lowering the external water reservoir below the water chamber. The vacuum is formed because the free surface of external

reservoir is lower than the membrane and air cannot pass through the pores of the membrane. This technique is predominantly used in soil physics to measure and manipulate the water content of the soil. The most important criteria to be noted here is, since the hydrophilic membrane stops convective air flow, the gas reservoir is not under vacuum. Other than the slight pressure increase due to gas flow, the gas reservoir is operated at atmospheric pressure. The magnitude of the vacuum applied to the water side of the membrane can be varied by changing the height between the external water reservoir and the membrane. Because of the hydraulic link across the membrane, the vacuum controls the matric potential in the soil and thus the physical amount of water in the soil at equilibrium. This arrangement allows equilibrium of matric potential and the dissolved nutrients or metabolic uncouplers between the water chamber and the water in the soil. If the matric potential in the soil rises due to condensation or oxidative water production, excess water drains away from the soil into the water chamber. The same applies to the dissolved components, with movement between the soil and the water chamber driven by the concentration gradients. Fig. 3.1 shows configuration of the typical differential biofilter reactor used in the current research which was initially developed by Beuger and Gostomski (2009).

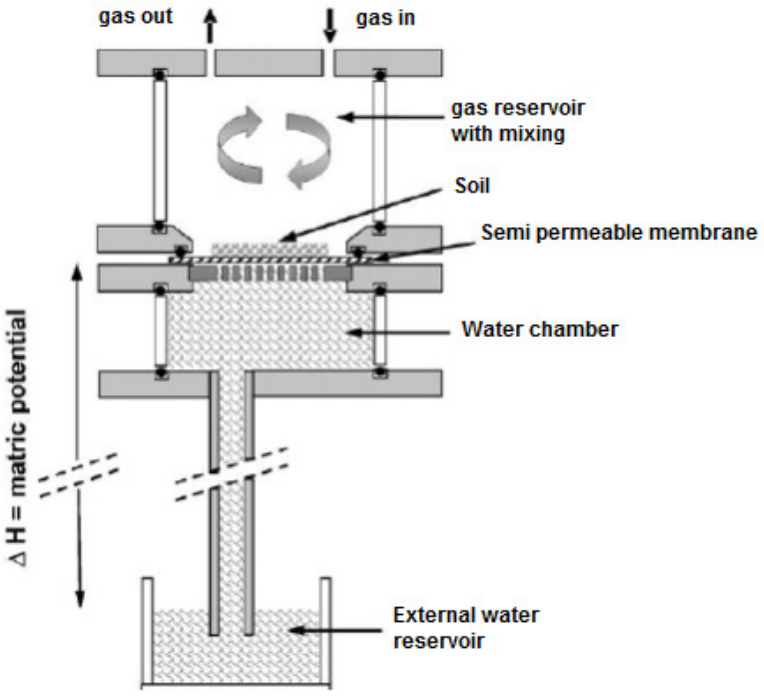


Figure 3.1: A cut section of differential biofilter reactor with water content control

3.5 Development of water retention apparatus

The water retention in soil affects the diffusion of gases and nutrients in and out of the soil and hence affecting the biological activity. The mobility of microorganisms within the soil may also be affected by the water potential of the soil matrix, hence affecting the ability of microbes to reach and degrade compounds. It is therefore important to be able to control the matric potential of a biofilter to eliminate the influence it can have on the system. A measure of ability of the bed material to hold water can be described by the water retention curve. The matric potential can be set by using a hanging column of water under the soil sample. The water in the soil sample reaches equilibrium with the water in the reservoir below. The height between the soil and the top of the reservoir gives a specific matric potential (Kirkham, 2005). It can be calculated from Eq.3.2,

$$Water\ Retention = \frac{m_{wet} - m_{dry}}{m_{dry}} \tag{3.2}$$

Among various empirical relationships used to estimate the relationship between water content and matric potential, the widely used one is Van Genuchten's model (Tuller and Or, 2005) which is shown in eq.3.3,

$$\Theta = \frac{\theta - \theta_r}{\theta_s - \theta_r} = \left[\frac{1}{1+(\alpha\psi_m)^n}\right]^m \text{(dimensionless)} \tag{3.3}$$

where, $m = 1 - \frac{1}{n}$

In the current research, Zap Cap filters (Fig. 3.2) was used to measure the water retention of the soil which is used in the differential biofilter reactor. The experiment was conducted at varying matric potentials. Zap Cap filters consisted of a 0.2 µm pore size hydrophilic cellulose filter, and hence could be used to hold the hanging water column.

Figure 3.2: Zap Cap filter (Sigma-Aldrich, 2012)

The Zap Cap filter was modified for use in for the water retention experiment. A small hole was drilled down the membrane where the suction tube is connected, by removing the cellulose membrane. A new cellulose membrane was cut to fit over the space and glued onto the Zap Cap. This allowed the air bubble to move out of chamber during suction. PVC clear single hose tube of 50 mm diameter was used to connect the bottom end of the Zap Cap filter with the water reservoir. At first, the tube was held above the Zap Cap and filled with 0.01 M $CaCl_2$ and the filter was tapped lightly to remove the air bubbles from the chamber. When all the air bubbles were removed, the attached tube was lowered into the beaker (containing 0.01 M $CaCl_2$), ensuring no air bubbles were present in it. The Zap Cap was then placed in a clamp on a stand and the height was adjusted so that the difference in height between the liquid level and the filter gave the required tension. The soil (mentioned earlier) is sieved using mesh no.6 (3.6 mm opening) sieve before being used in the experiment. A layer of 4 mm thick and 100 mm diameter (~ 8.65 g wet weight) was placed on top of the cellulose membrane. The matric potential was applied between -5 and -20 cm H_2O. The whole experiment was carried out in room temperature.

After equilibrating the soil for 7 days, the soil was removed and its water content was determined by oven drying at 105 °C for 24 hours. Fig. 3.3 shows the experimental setup of Zap Cap filter used to carry out the water retention experiment.

Figure 3.3: Photo of Zap Cap filter used to determine the water retention curve

The relationship between matric potential and gravimetric water content was determined for the experimental soil (Fig. 3.4 and 3.5). It was observed that there was a rapid decrease in the water content when the matric potential was decreased from -5 cm H_2O to -20 cm H_2O. Large matric potential changes (> -20 cm H_2O) were not investigated based on the earlier work carried out in studying the water retention curve for compost (Beuger and Gostomski, 2009) . Comparing the shapes of the curves in Fig. 3.4 and 3.5, it is clear that the garden soil used in the current research has the texture similar to silt loam. However, the shape of curves in Fig. 3.4 were not 100% comparable with the typical water retention curve shown in Fig. 3.5, and hence it was concluded that soils of different texture have a very different soil-water retention curves and absolute values are difficult to compare.

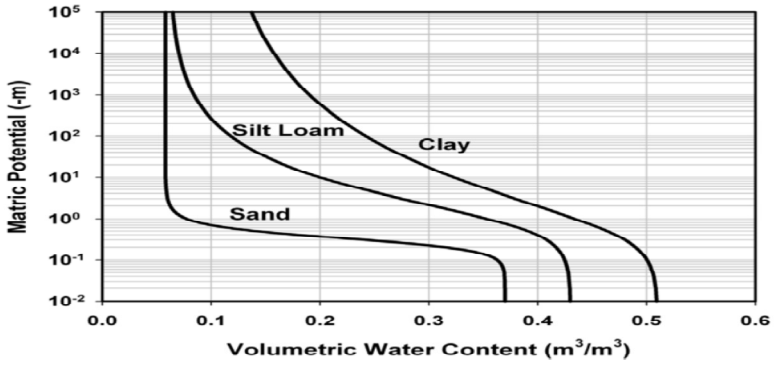


Figure 3.4: Typical soil water characteristic curves for soils of different texture (Tuller and Or, 2005).

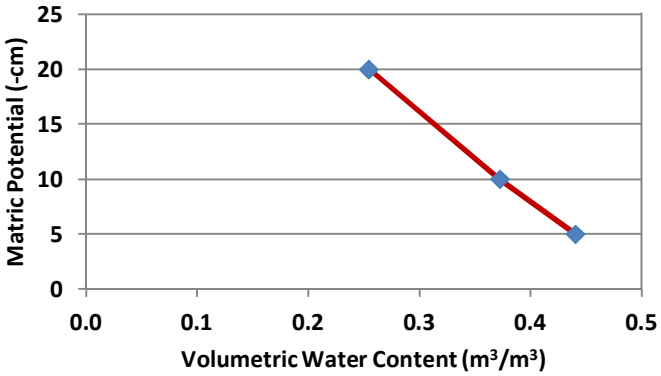


Figure 3.5: Water retention curve (wetting curve) for soil used in current study. The red solid line is the Van Genuchten model fit. The bulk density used is 0.64 g.cm^{-3}

The parameters used to fit the Van Genuchten model in Fig. 3.5 is shown in the Table 3.1. Excel Solver^{TM} and least square methods were used to determine the parameters on all 3 data points.

Table 3.1: Fitted parameters of the Van Genuchten model

Parameter	Value
α (cm^{-1})	0.049
n (dimensionless)	1.344
θ_s (cm^3.cm^{-3})	0.496
θ_r (cm^3.cm^{-3})	-0.513

3.6 Diffusion system

Generating a dilute concentration of volatile contaminant gas (e.g. toluene) in biofiltration research is one of the most critical steps. Therefore, it is desirable to have a highly precise dilute gas generation technique to minimize potential analytical errors. Several techniques are currently available for the preparation of dilute contaminant gas stream from pure contaminant liquid and they are generally classified as static and dynamic methods (Gautrois and Koppmann, 1999; Helmig et al., 2003; Possanzini et al., 2000; Rhoderick, 2008; Tumbiolo et al., 2005). Static methods are based on the introduction of known amounts of individual components in the form of gas or liquid into a vessel of known volume, whereas dynamic methods are based on a continuous flow of components into a chamber or a tube wherein the mixing occurs (Namiesnik, 1984). Though the static

methods have the advantage of ease of operation, for handling larger volume of volatile organic compounds it lacks reliability mainly due to the reaction of constituents on the container walls, especially at dilute concentration levels (Rhoderick and Miller, 1993). For these compounds, the diffusion technique, which is one of the widely used dynamic methods, provides more reliable dilute concentrations. By selecting a suitable diffusion system a low concentration of volatile organic compound with a wide concentration range is attainable. Especially, the capillary diffusion based method has often been used for the production of dilute volatile and semi-volatile organic compounds (Gautrois and Koppmann, 1999; Helmig et al., 2003; Possanzini et al., 2000).

Diffusion methods are based on the principle of dilution of the vapour of a liquid diffusing from a container through a capillary or directly from a capillary into a space through which a stream of diluting gas is passed. The theoretical background of diffusion methods was first reported by Altshuller and Cohen (1960). The driving force in the diffusion method is the concentration gradient up the tube. The reservoir which contains the liquid contaminant acts as a source governed by the temperature at which it is maintained. This temperature defines the vapour pressure above the contaminant liquid. It was also reported that an optimal internal diameter of a diffusion tube ranges between 0.2 and 2 cm for an effective diffusion (Altshuller and Cohen, 1960).

The diffusion coefficient depends on pressure and temperature of the diffusion system and can be expressed by the following relationship (eq.3.4) (Namiesnik, 1984),

$$D = D_{298} \left(\frac{T}{298}\right)^{n} \frac{1}{P} \quad (\mathrm{m^2.s^{-1}}) \tag{3.4}$$

The value of the constant n is usually assumed to be 2.00 but sometimes 1.75. Maintaining constant diffusion conditions, i.e., geometric dimensions of a diffusion system, temperature, pressure and flow rate of the diluting gas, a gaseous mixture containing a constant concentration of the diffusing component is obtained (Namiesnik, 1984).

The change in temperature will change the vapour pressure of toluene and by using Antoine's equation, vapour pressure of the toluene can be calculated (eq. 3.5).

$$\ln p_v = A - \frac{B}{T+C} \quad (\text{mm Hg}) \tag{3.5}$$

Table 3.2 shows the values of constants A, B and C for toluene which are gathered from different literature.

Table 3.2: Antoine coefficients for toluene at different temperature ranges

Temperature (K)	A	B	C	Reference
273.0-323.0	4.14	1377.58	-50.51	(Pitzer and Scott, 1943)
273.1-297.9	4.24	1426.45	-45.96	(Besley and Bottomley, 1974)
303.0-343.0	4.08	1346.38	-53.51	(Gaw and Swinton, 1968)
308.5-384.7	4.08	1343.94	-53.77	(McGarry, 1983)
420.0-580.0	4.54	1738.12	0.39	(Ambrose et al., 1975)

The output rate of toluene vapour can be controlled by the length and diameter of the diffusion flask (eq. 3.6) as well as the temperature and pressure of the diffusion system (eq. 3.4). In addition, the concentration of toluene vapour generated from the diffusion system can be controlled by varying the air flow rate (eq. 3.7).

$$q_d = \frac{D\,A_t}{L} \times ln\,\frac{P}{P-p_v}\ (\mathrm{cm^3.s^{-1}}) \tag{3.6}$$

$$C_{dif} = \frac{q_d}{F_g} \times 10^6\ (\mathrm{ppm}) \tag{3.7}$$

The design of the custom made glass diffusion flask (V = 10 mL) used in the current research is shown in Fig. 3.6a and the diffusion system reported earlier by Beuger and Gostomski (2009) is shown in Fig. 3.6b. Pure HPLC grade toluene was filled into the diffusion flask. Two different lengths (50 mm and 90 mm) for diffusion section of the flasks were used in the study; however the diameter of the diffusion section of the flask was kept constant at 3.5 mm. The diffusion flask was kept inside a 1000 mL reagent bottle (Fig.3.6a) and the bottle was sealed with a rubber stopper. This system is partly submerged (up to the neck of the reagent bottle) and clamped inside a temperature controlled water bath (GD100, Grant Instruments, Cambridge, England). The whole water bath was insulated using polyethylene foam (thickness 15 mm), leaving a little free space

at the top for allowing 1/8'' stainless steel tubing to pass inside and outside of the reagent bottle through the rubber stopper. Carbon dioxide free, dry air was passed continuously into the top of the reagent bottle at constant flow rate (25 mL.min^{-1}). The flow rate was maintained by a mass flow controller connected to a readout unit (M100B & Type 247D readout, MKS Instruments, Andover, MA, USA). The outlet toluene vapour stream was used to feed the differential biofiltration reactor.

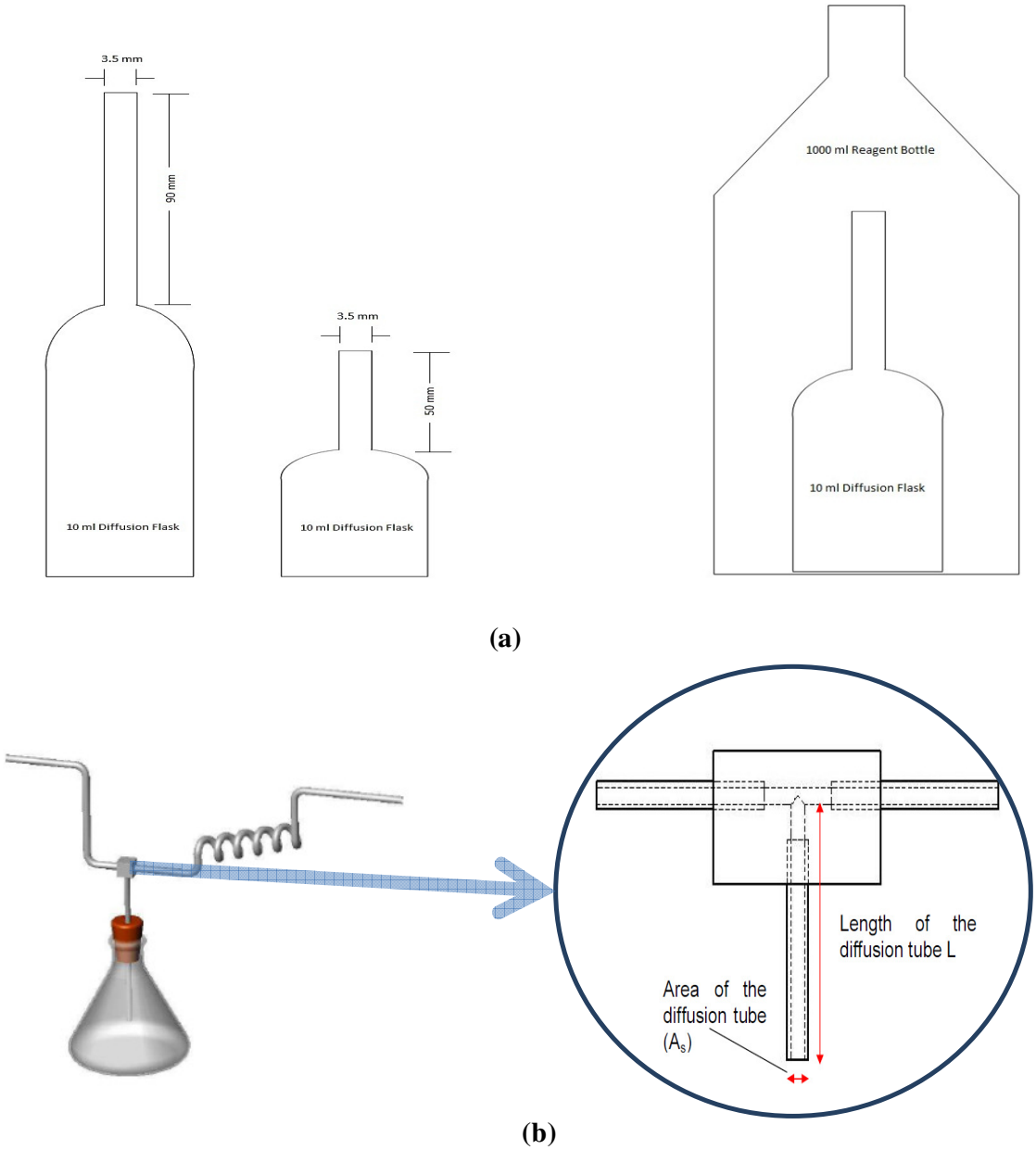


Figure 3.6: Schematic diagram of the custom made diffusion flask and diffusion system **(a)** current study **(b)** reported earlier by Beuger and Gostomski (2009).

The temperature range in which the diffusion system was operated ranged between 5 and 50 ^{o}C. The difference between the calculated and experimental concentration of toluene generated by this diffusion system is shown in Table 3.3. It can be seen from the table that the experimental values are in close agreements with their respective theoretical predictions. Two major disadvantages in using the earlier reported system (Fig. 3.6b) a) induced turbulence and b) chances of water getting into the diffusion system (fully submerged system and thereby diluting the concentration of toluene), were eliminated by using the current diffusion setup (Fig. 3.6a).

Table 3.3: Difference between experimental and calculated toluene concentration generated by the diffusion system at different temperatures and diffusion tube lengths.

Diffusion flask inner diameter (mm)	Diffusion tube length (mm)	T (K)	F_g (ml/min)	C_{diff} theoretical (ppm)	C_{diff} experimental (ppm)	Factor (experimental/ theoretical)
3.5	50	278	25	42.17	46.6 ± 0.45	1.1
3.5	50	283	25	58.95	61.92 ± 0.42	1.05
3.5	50	293	25	110.96	109.4 ± 1.63	0.98
3.5	50	298	25	149.70	145.6 ± 1.64	0.97
3.5	50	303	25	199.97	190.55 ± 1.04	0.95
3.5	50	308	25	264.70	246.97 ± 1.28	0.93
3.5	50	313	25	347.52	320.10 ± 1.86	0.92
3.5	50	318	25	452.92	407.40 ± 1.56	0.9
3.5	50	323	25	586.51	518.74 ± 2.63	0.88
3.5	90	303	25	111.09	97.8 ± 1.82	0.88
3.5	90	308	25	147.06	131.5 ± 1.10	0.89
3.5	90	313	25	193.07	180.1 ± 0.30	0.93

3.7 Experimental set-up

Four differential reactors are used in the current research. All the four reactors are based on a similar design reported by Beuger and Gostomski (2009) although the specific assembly is slightly different. These reactors are mainly constructed out of glass and operated continuously. The bottom water reservoir is made with a 50 mm long glass piece of OD 100 mm and thickness 5 mm. It is clamped between two stainless plates of diameter 180 mm and thickness 12 mm. The glass reservoir is sealed between the two

stainless plates by Viton O-rings of ID 91 mm and cross section 2.35 mm (Dotmar Engineering Plastics Ltd, Christchurch, New Zealand). The top of the plate holds an 80 mm diameter stainless steel perforated disk and on top of it a membrane (Mixed cellulose ester, pore size 0.45 µm, diameter 90 mm, Micro Analytix Private Ltd, Auckland, New Zealand) is placed. The Viton O-ring is kept at the top of the membrane to prevent leaks and glass-on-glass grinding. A large piece of glass (similar to the water reservoir) is clamped between the bottom plate and a top head stainless plate. Again a Viton O-ring is used in the top plate groove to give good sealing and to prevent leak. Three ports; and a 1/8'' liquid port, and a thermo-well, made out of 1/4'' tubing were welded in. All three ports are all fitted with 1/8'' stainless steel Swagelok fittings (Swagelok, Solon, OH, USA). Water reservoir bottom plate has two pieces of 1/8" stainless steel tubing welded in. One is used for removal of air bubbles under the membrane and the other one for connection to the water reservoir. The top part of this tube is connected to a small piece of 1/8'' Viton tubing with a Y-connector. The Y-connector has two pieces of Viton tubing, which are in contact with the bottom of the membrane support to be able to remove all entrapped air underneath. Four threaded stainless steel rods are used to assemble the reactor. The whole set up is fastened by tightening the nuts on the threaded rods. The reactor is designed in such a way; the head plate can be removed any time without disturbing the seal on the membrane. Fig. 3.7 shows the assembly of the experimental differential biofilter reactor.

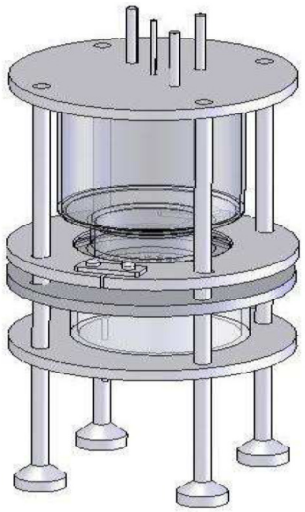


Figure 3.7: Differential biofilter reactor assembly

3.8 Process description

Fig. 3.8 shows the process flow diagram of the complete experimental set-up. The following sub sections will discuss about the operation of individual components shown in the process flow diagram. All stainless steel tubing used in this whole set up are 1/8th inch ID and are connected using Swagelok fittings.

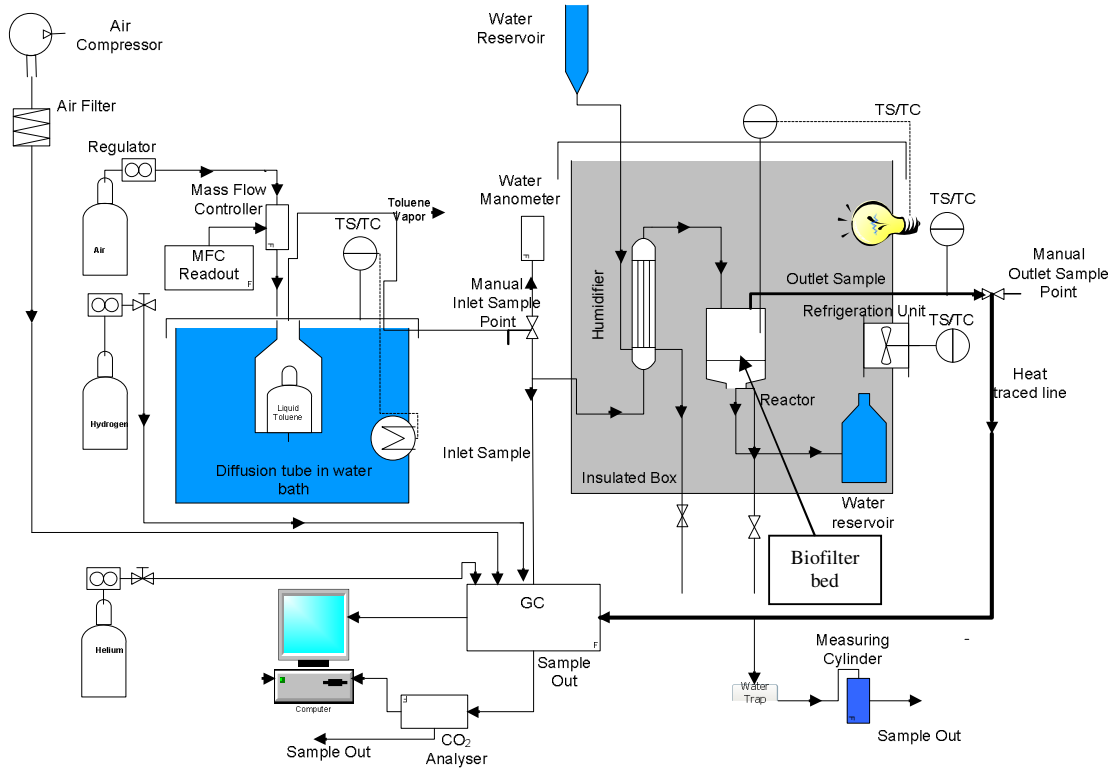


Figure 3.8: Experimental process flow diagram

3.8.1 Generation of toluene vapour

A dry air bottle (BOC, New Zealand) supplies air at a regulated pressure of 200 kPa to the mass flow controller. Using mass flow controller, the flow rate of the dry air passing can be fixed. The inlet flow rate is maintained at 25 mL min^{-1} throughout the studies. In addition, the size of the diffusion flask used in the diffusion system is also fixed (either the longer one or shorter one). The water bath temperature is the only variable parameter which can be changed to generate a desired concentration of toluene vapour for the experimentation. The toluene laden vapour flow past a manual inlet sample port and a

water manometer before it reaches the reactor. The manual inlet sample port is made of 1/8'' septum injector nut (Valco Instrument Co., Houston, TX, USA) attached to a Swagelok 1/4'' female branch tee to 1/8'' female NPT (SS-400-3TTF) and Swagelok bored through male connector (SS-200-1-2BT). The septum is GC septa (Blue 3/8'', Alltech Associates Ltd., Deerfield, IL). The water manometer is used to measure the inlet pressure and also helps to observe any blockage in the entire flow path (from diffusion system to Gas Chromatography outlet). Fig.3.9 shows all the four water baths with temperature controllers which also show a glimpse of the partly submerged diffusion system and mass flow controller attached to a readout unit.

Figure 3.9: Four water baths containing submerged diffusion system controlled by temperature controllers.

3.8.2 Humidification

The toluene laden air passes through a shell-in-tube humidifier (Perma Pure LLC, Toms River, NJ, USA) before it enters the reactor. The humidifier can handle an air flow rate up to 10 L min^{-1}. Water is supplied to the humidifier by a siphon method. A 50 ml glass burette with deionized water is placed approximately 30 cm above the humidifier and connected to the water inlet of the humidifier with Viton tubing. A short piece of the Viton tubing is connected to the water outlet of the humidifier and capped off. As the water in the humidifier is evaporated into the inlet stream, the water is replaced from the

burette. The water vapour is transferred between the liquid water and flowing gas stream inside the humidifier. This process is driven by the partial pressure of the water vapour on opposing sides of the Nafion membrane in the humidifier. No extra heating of the humidifier is required as the air achieves 100% relative humidity at the box temperature which is shared by the reactor. Fig.3.10 shows the schematic diagram of the humidifier used in current research.

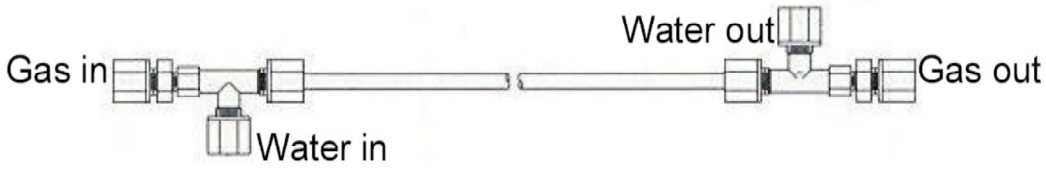


Figure 3.10: Schematic representation of Perma Pure Humidifier.

3.8.3 Reactor

Assembling and loading of the reactor is very important before the operation of the reactor. An improper assembling and loading may lead to leaking, breakage, biotic issues etc., In order to avoid contamination in the reactor before the addition of soil, all the reactor parts including the O-rings are soaked in 1% Virkon solution (antimicrobial solution) overnight. Following the soaking, they are autoclaved for 30 min at 121 ^{o}C prior to assembly. The reactor is assembled inside a laminar flow chamber to minimise contamination. Firstly, the bottom plates are fitted with threaded rods so that it can stand on legs. Inside the groove of the bottom plate, a Viton O-ring is placed to form a seal between the glass tubing and the metal plate. The support ring is lined up on the top of the glass so that the threaded rods can be put in place. The Y-piece is made to sit below the perforated membrane support disc. The nuts on the threaded rods are tightened to seal the water reservoir. The membrane is wetted out in deionised water and placed on top of the perforated support disc. In order to prevent contamination in both external and internal water reservoir, the water in the external reservoir is autoclaved at 121 ^{o}C for 45 min. The external reservoir is placed below the internal reservoir in order to prevent pressure build up under the membrane. The air in the internal reservoir is removed using 20 mL plastic syringe which leads to the filling up the vacuum of the internal reservoir with water. Unless there is any leak, the whole reservoir can be filled in less than 5 min. Any air that is trapped underneath the membrane can be removed by slightly tilting the reactor in the

direction of the air bubble. Once all the air bubbles are removed, the syringe used to suck the air from internal reservoir will fill up with water which is an indication of no further air inside the internal reservoir.

8.65 g of sieved soil (no.6 mesh) is loaded on the membrane using a stainless steel ring and slightly padded in order to get good contact between the soil and the membrane. The metal ring is removed and the glass gas reservoir is placed on top of the Viton O-ring in the metal plate placed above the membrane. The reactor is closed by fitting the top plate with Viton O-ring on the other end of the gas glass reservoir. Finally the whole reactor set up is made gas tight by screwing the nuts hand tight. A simple leak test can be done by closing either the inlet port or outlet port of the reactor and blowing air through one of the open ports.

Following the assembling and loading, the reactor, humidifier and the external water reservoir bottle (500 mL) are kept inside a wooden bench top box (560mm x 360mm x 300 mm) insulated with polyethylene foam (thickness 15 mm). The temperature inside the box is controlled by a temperature controller (LTR-5, LAE Electronics, Italy) using 60W incandescent bulb as a heating source. A cooling load is applied to the system by a refrigeration unit (Tropicool-XC3000A, 12V DC, Thermoelectric Refrigeration Ltd., New Zealand) when the room temperature approaches the reactor temperature. Tension is applied onto the soil by placing the external water reservoir below the membrane as explained in Section 3.5. The reservoir bottle is closed by a rubber stopper. Through a tee outside the wooden bench top box, the inlet sample line enters both the reactor inlet port and also the gas chromatography (GC) system (discussed in the following section). The outlet port of the reactor is connected with a tee. One end of the tee is connected to the top of the reservoir bottle and the other end is connected to the GC System (discussed in the following section) through a manual sample outlet port (similar to the manual sample inlet port). The outlet line is heat traced at 40 ^{o}C and insulated in order to prevent condensation. Using a split, a part of the outlet flow is connected to a water trap using a Tygon tube before it is dipped into a 100 mL measuring cylinder with water. This measuring cylinder with water is used to regulate the outlet pressure when the outlet sample is analysed by the GC. Whenever, the outlet sample is not analysed by GC, it will bubble through the measuring cylinder and whenever it is analysed by GC, there is no flow through the measuring cylinder set up. The water level in the measuring cylinder is always maintained

at a fixed height in order to maintain the pressure and flow of the reactor outlet constant. Fig. 3.11 shows the experimental setup of one of the four reactors used in the current research.

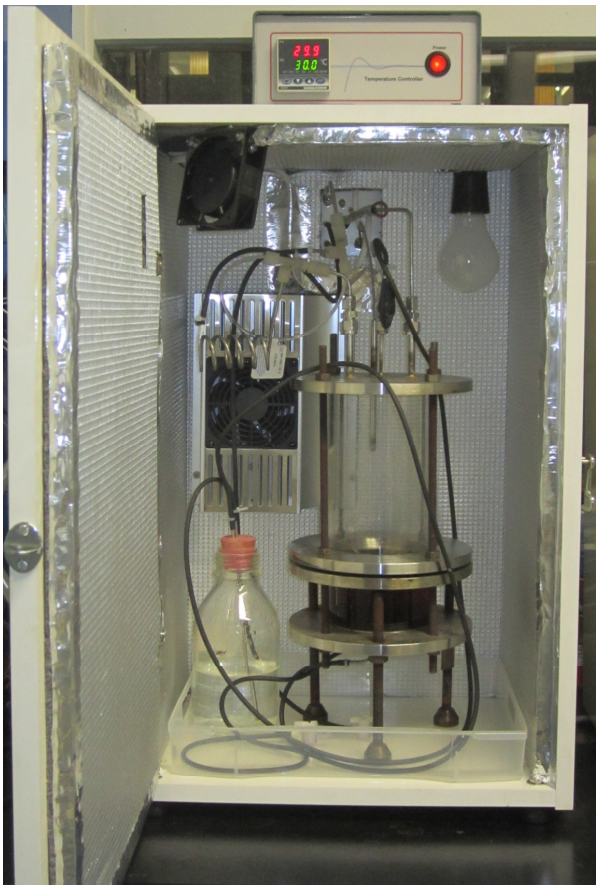
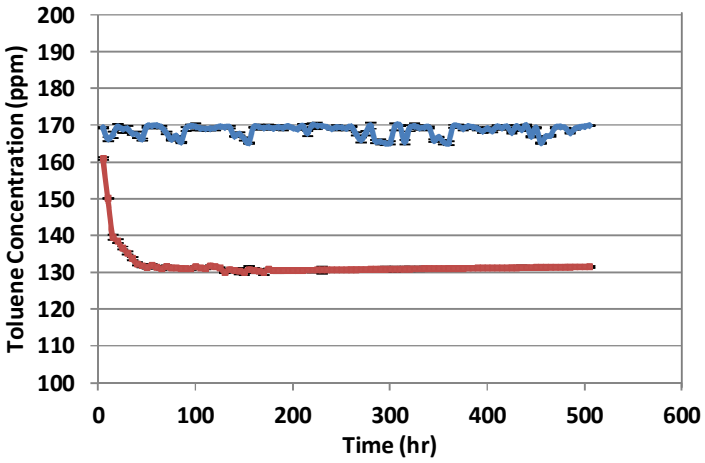
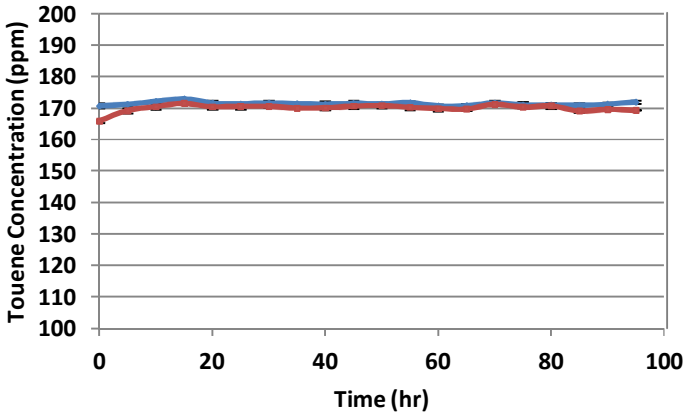


Figure 3.11: Experimental set-up of one of the four reactors.

Fig. 3.12(a) and 3.12(b) compares the performance of one of the four reactors without soil which was assembled with and without autoclaving. It is clearly evident that despite of initial toluene losses in both the experiments due to the interaction of toluene vapour with water in the internal reservoir prior to equilibrium, the performance of the autoclaved reactor is proved to be good as the toluene loss is negligible. This clearly shows that the reactor which was assembled without autoclaving should have allowed the contaminants to grow, which should have used toluene as a sole carbon source for their growth and survival.



(a)



(b)

Figure 3.12: (a) Performance of one of the four reactors which was assembled without autoclaving **(b)** Performance of one of the four reactors which was assembled following autoclaving. Blue line is inlet concentration and red line is outlet concentration. Error bars are the standard deviations.

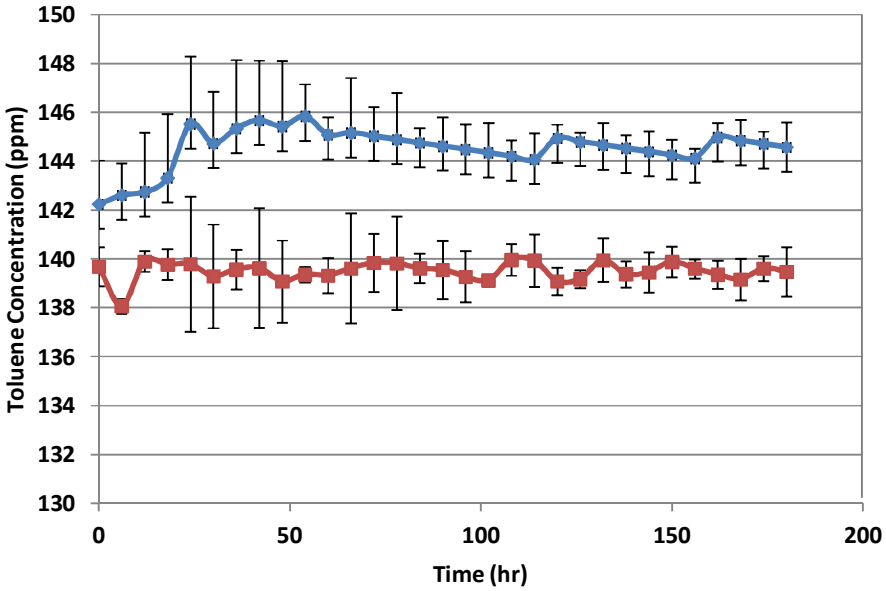
3.8.4 Online sample analysis

Continuous real time monitoring of inlet and outlet toluene concentration is achieved by connecting a gas chromatography (GC) system (SRI-8610C, SRI Instruments, CA, USA) online to the reactors (Appendix A). Integration of an online sample monitoring system with the reactor setup increased the precision and robustness of the measurement. The GC

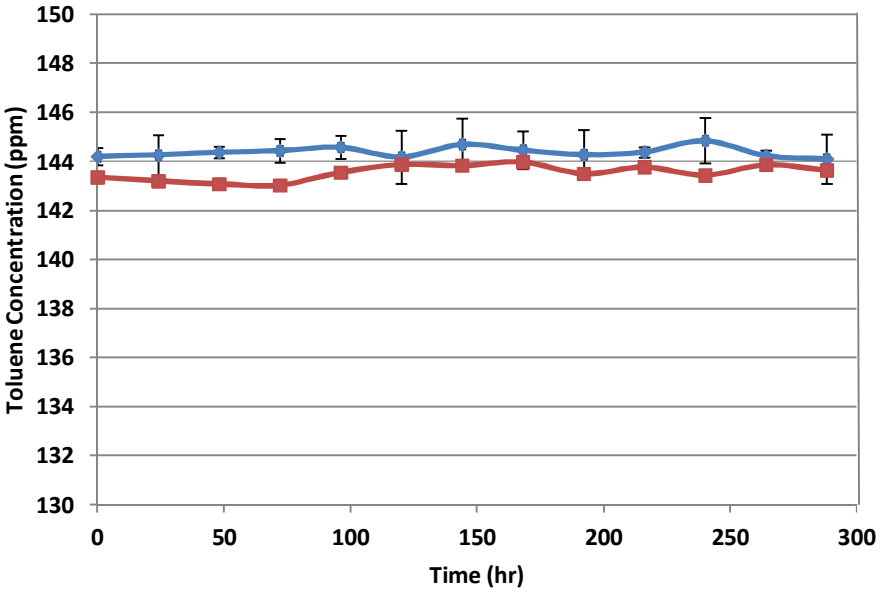
has ten sample inlet ports at the back in which eight are used for connecting the inlet and outlet lines of all four reactors. The GC uses a flame ionization detector, capillary column (1.5 mL) and 5 ml sample loop for detection and analysis of the sample. The initial flame ignition is done manually through a flame ignition switch present at the side panel of the GC. This step is followed whenever the GC is restarted. Helium is used as a carrier gas (5 psi and 10 mL min^{-1}), air (5 psi and 250 mL min^{-1}) and hydrogen (20 psi and 25 mL min^{-1}) for flame ignition. The temperature of column oven is maintained at 180 ^{o}C. Air for the GC is supplied from a compressor and hence it is filtered through oil and vapour removal filter (F64, Norgren Martonair Ltd, Staffordshire, England) before entering the GC. The air pressure is regulated at 30 psi by an in-built regulator in the filter.

In case of the compressor failure, air to GC is supplied from the dry air bottle (which supplies air to the diffusion system). This is done by an automatic pilot valve which is connected to the regulator of the filter. The other two gases are supplied to GC through individual gas bottles. The GC is programmed in such a way it can analyse the inlet concentration followed by outlet concentration of each reactor (out of 4) in every 8th hour in a 24 hours period. Hence three sets of inlet and outlet concentrations are measured in different times of the day for all four reactors. Each analysis is programmed for 1 hour and during that period the sample is injected 4 times at equal intervals following a first 20 min of flush with the sample to be tested. The 20 min flush time and 1 hr sample analysis time are calculated following series of trial and error. The GC is connected to a computer through USB cable and controlled by Peaksimple^{TM} software. Manual sample inlet and outlet ports are provided for all reactors as a backup, if the online sampling system is stopped for any reasons like GC maintenance, power failure, shut down period etc., Fig. 3.13 shows the online SRI GC in operation and Fig. 3.14a & 3.14b compares the inlet toluene concentration of one of the reactors when measured offline and online. It is clearly evident from Fig. 3.14a and Fig. 3.14b, the robustness of the measurement increased in online analysis when compared to offline. Standard deviation for inlet sample analysis decreased by 65% for online sampling when compared to the offline sampling. Similarly, for outlet sample analysis it decreased by 79% for online sampling when compared with the offline sampling. The major cause for this is assumed to be the human error associated with the offline measurement.

Figure 3.13: SRI-8610C connected online to reactor system



(a)



(b)

Figure 3.14: (a) Offline measurement of inlet and outlet toluene concentration in one of the four reactors **(b)** Online measurement of inlet and outlet toluene concentration in one of the four reactors. Closed blue diamonds (◆) are inlet concentrations and closed red squares are outlet concentrations (■). Error bars are the standard deviations.

3.8.5 Online carbon dioxide analysis

Carbon dioxide is one of the by-products of toluene degradation and it is measured from the outlet sample of all the four reactors. A carbon dioxide probe (GMP343, Vaisala Inc, CO, USA) works on the principle of infra-red (IR) absorption. It is connected to the outlet sample loop purge port of the GC (Fig. 3.15), so that whenever the GC is analysing the toluene concentration in the inlet and outlet of each reactors, the carbon dioxide probe also measures the concentration of carbon dioxide in the inlet and outlet stream of each reactors. In addition, the outlet samples are at 100% relative humidity as they are above 30 ^{o}C and hence the possibilities of condensation inside the carbon dioxide analyser can be avoided. The probe is connected to computer through a readout unit (MI70, Vaisala Inc, CO, USA) and the measured carbon dioxide concentration is monitored and recorded online using MI70 link software. The probe also measures the sample temperature which is also monitored and recorded online using MI70 link software. Fig. 3.15 shows the

online operation of carbon dioxide probe and readout connected to the outlet sample port of GC.

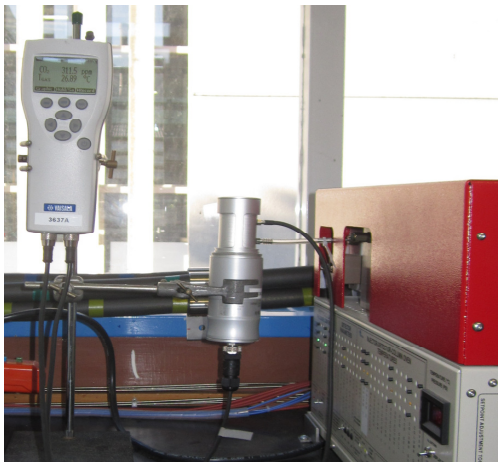


Figure 3.15: Carbon dioxide probe connected online with the outlet of GC and read out records the measured carbon dioxide.

Fig. 3.16 shows the online carbon dioxide measurement from the inlet and outlet sample of one of the four reactors[1]. It is observed from the figure that the inlet concentration of carbon dioxide is zero which consistent with the supplier specification for the dry air.

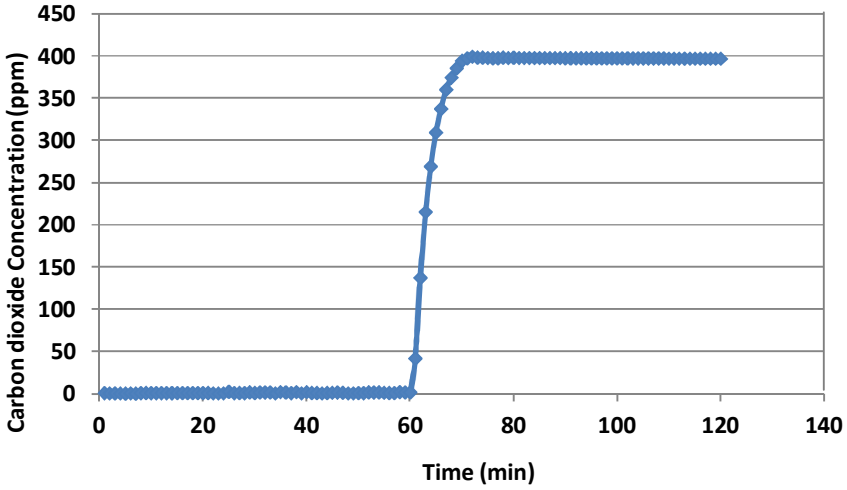


Figure 3.16: Online carbon dioxide analysis in the inlet and outlet streams of one of the four reactors.

[1] GC was programmed to measure inlet first and then outlet for 60 minutes each.

3.9 Conclusions

Introduction of new simple diffusion system (which is designed in the current research) improved the generation of toluene vapour which helped to generate the desired inlet toluene concentration for the biofiltration reactor system. The new diffusion system generated a near theoretical concentration of toluene vapour when compared to the old one (maximum experimental to theoretical ratio of 1.1 @ 5 ^{o}C and minimum of 0.88 @ 50 ^{o}C). Online sample analysis through GC improved the precision of the data collected and enhanced the robustness of the process. Overall, the quality of the experiment and data collection was improved due to the modifications carried out to the existing biofiltration system.

3.10 Nomenclature

A_t	diffusion tube cross sectional area	m^2
a_w	water activity	dimensionless
C_{dif}	concentration at the exit of the diffusion flask	ppm
D	diffusion coefficient at pressure P and temperature T	$m^2\ s^{-1}$
D_{298}	diffusion coefficient at 298 K and 1 atm	$m^2\ s^{-1}$
F_g	gas flow rate	$m^3\ s^{-1}$
L	length of the diffusion flask	m
m	constant	dimensionless
m_{dry}	mass of dry soil	kg
m_{wet}	mass of wet soil	kg
n	temperature coefficient (in diffusion equation)	dimensionless
n	empirical shape factor (matric potential equation)	dimensionless
p_v	vapour pressure of toluene	mm Hg
P	pressure in the diffusion system	mm Hg
q_d	diffusion rate	$m^3\ s^{-1}$
R	Universal gas constant	$J\ mol^{-1}\ K^{-1}$

T	Temperature	K
V_W	volume of water in soil	m^3
α	empirical shape factor	dimensionless
Θ	normalized water content at matric head h	dimensionless
θ	water content	$m^3\ m^{-3}$
θ_r	residual water content	$m^3\ m^{-3}$
θ_s	saturated water content	$m^3\ m^{-3}$
ψ_m	matric potential	$J\ m^{-3}$

3.11 References

Altshuller, A., Cohen, I., 1960, Application of diffusion cells to production of known concentration of gaseous hydrocarbons. Analytical chemistry 32, 802-810.

Ambrose, D., Lawrenson, I., Sprake, C., 1975, The vapour pressure of naphthalene. The Journal of Chemical Thermodynamics 7, 1173-1176.

Anderson, B., 1976, Experimental methods in catalytic research. Academic Press, San Diego, USA.

Badilla, D.B., Gostomski, P.A., Dalida, M.L.P., 2011, Influence of water content on biofiltration performance. ASEAN Journal of Chemical Engineering 2, 31-39.

Besley, L., Bottomley, G., 1974, Vapour pressure of toluence from 273.15 to 298.15 K. The Journal of Chemical Thermodynamics 6, 577-580.

Beuger, A.L., Gostomski, P.A., 2009, Development of a biofilter with water content control for research purposes. Chemical Engineering Journal 151, 89-96.

Bohn, H.L., Bohn, K.H., 1999, Moisture in biofilters. Environmental Progress 18, 156-161.

Carberry, J., 1964, Designing laboratory catalytic reactors. Industrial & Engineering Chemistry 56, 39-46.

Fogler, S.H., 2004, Elements of Chemical Reaction Engineering, 4th Edition. Prentice Hall, New Jersey, USA.

Gautrois, M., Koppmann, R., 1999, Diffusion technique for the production of gas standards for atmospheric measurements. Journal of Chromatography A 848, 239-249.

Gaw, W., Swinton, F., 1968, Thermodynamic properties of binary systems containing hexafluorobenzene. Part 4.—Excess Gibbs free energies of the three systems

hexafluorobenzene+ benzene, toluene, and p-xylene. Transactions of Faraday Society. 64, 2023-2034.

Helmig, D., Revermann, T., Pollmann, J., Kaltschmidt, O., Jiménez Hernández, A., Bocquet, F., David, D., 2003, Calibration system and analytical considerations for quantitative sesquiterpene measurements in air. Journal of Chromatography A 1002, 193-211.

Kirkham, M.B., 2005, Principles of soil and plant water relations. Academic Press, New York, USA.

McGarry, J., 1983, Correlation and prediction of the vapor pressures of pure liquids over large pressure ranges. Industrial & Engineering Chemistry Process Design and Development 22, 313-322.

Moser, A., 1988, Bioprocess technology: kinetics and reactors. Springer Verlag, New York, USA.

Namiesnik, J., 1984, Generation of standard gaseous mixtures. Journal of chromatography 300, 79-108.

Papendick, R., Campbell, G., 1981, Theory and measurement of water potential [in soil, organic materials, plants, seeds, and microorganisms. Soil Science Society of America, Madison, USA.

Pitzer, K.S., Scott, D.W., 1943, The thermodynamics and molecular structure of benzene and its methyl derivatives. Journal of the American Chemical Society 65, 803-829.

Possanzini, M., Di Palo, V., Brancaleoni, E., Frattoni, M., Ciccioli, P., 2000, Dynamic system for the calibration of semi-volatile carbonyl compounds in air. Journal of Chromatography A 883, 171-183.

Rhoderick, G.C., 2008, Differences between propane in nitrogen versus air matrix analyzed using gas chromatography with flame-ionization detection. Journal of Chromatography A 1187, 226-231.

Rhoderick, G.C., Miller, W.R., 1993, Development of hydrocarbon gas standards. Journal of Chromatography A 653, 71-81.

Tajbl, D., Simons, J., Carberry, J., 1966, Heterogeneous catalysis in continuous stirred tank reactor. Industrial & Engineering Chemistry Fundamentals 5, 171-175.

Tuller, M., Or, D., 2005, Water retention and characteristic curve, In: Hillel, D. (Ed.) Encyclopedia of soils in the environment. Academic Press, Oxford, UK.

Tumbiolo, S., Vincent, L., Gal, J.F., Maria, P.C., 2005, Thermogravimetric calibration of permeation tubes used for the preparation of gas standards for air pollution analysis. Analyst 130, 1369-1374.

Chapter 4: Effect of Substrate Concentration, Nutrients and Temperature on Removal

4.1 Introduction

Biofiltration involves a combination of different chemical and biological processes: absorption, diffusion, adsorption and degradation of the gaseous contaminant and desorption of the degradation products (Vergara-Fernández et al., 2007). Biofilter performance can be directly influenced by various operational parameters such as the filter bed characteristics, nutrient supply, contaminant concentrations, temperature and gas flow rates (Jorio et al., 2000a). Understanding the extent of impact of these parameters in a biofilter is very important both in industrial scale and lab scale biofilters. The differential biofiltration reactor system described in Chapter 3 can be effectively utilized in investigating the effect of these operational parameters on the pollutant (toluene) degradation rate. This chapter will discuss the studies with the lab scale differential biofiltration reactor to understand the impact of substrate/pollutant (toluene) concentration, nutrients and temperature on the removal rate.

4.2 Pollutant/Substrate concentration

The inlet pollutant concentration and the flow rate quantify the amount of pollutant to be degraded in a biofilter. Biofiltration is highly efficient for dilute (concentrations < 1000 ppm), easily biodegradable pollutant gases (e.g. benzene, toluene etc.). Conversely, biofiltration is less proficient in the treatment of highly concentrated emissions of moderately or poorly biodegradable pollutant gases (e.g. dimethyl sulphide, dichloroethane, etc.) (Kennes and Veiga, 2001; Kumar et al., 2008). There are a few reports on the effect of inlet pollutant concentration on the mass transfer rate which suggest that an increase in the inlet pollutant concentration improves the mass transfer rate of the pollutant to the water/biofilm (Jorio et al., 2000b; Wang et al., 2006). However, high concentrations of some recalcitrant pollutant gases may inhibit[1] the metabolic activity of the microbial consortium present in the biofilter bed (Madigan et al., 2009). Moreover, a high inlet concentration (below the inhibition level) in the air

[1] e.g. a EC of 35 $g.m^{-3}.h^{-1}$ was observed for high concentrations of alpha-pinene

stream increases the biomass production if nutrients are added, which potentially restricts air flow and creates channelling in the filter bed (du Plessis et al., 1998; Sorial et al., 1998). Knowledge about the effect of pollutant concentration is essential in describing the effect of that pollutant on the biodegradation rate. The biodegradation rate in a biofilm is usually described using a modified form of Monod's growth equation (Eq. 4.1) (Doran, 1995). When substrate inhibition affects the growth rate, Andrew's substrate inhibition equation (Andrews, 1968) is one option that can be used to model the growth rate (Eq. 4.2). However, for non-growth systems susceptible for substrate inhibition like biofilter, the growth rate (μ) and the maximum growth rate (μ_{max}) in Eq. 4.2 can be replaced with EC and EC_{max} (Eq. 4.3). The Eq. 4.3 does not have mass transfer as an explicit term. Hence, substrate concentration can influence EC directly in the traditional way through the K_s term or indirectly through a higher mass transfer rate causing more biofilm to be engaged in the biofilter bed.

$$\mu = \frac{\mu_{max} S}{K_S + S} \tag{4.1}$$

$$\mu - \frac{\mu_{max} S}{K_S + S + K_i S^2} \tag{4.2}$$

$$EC = \frac{EC_{max} S}{K_s + S + K_i S^2} \tag{4.3}$$

The substrate concentration is usually much higher than the K_s and K_i value for the majority of the biofilter operation and hence the biodegradation reaction is often zero order over a large range of substrate concentrations (Ottengraf and Vandenoever, 1983).

4.3 Nutrients

The bed material in a biofilter is typically soil, peat, bark, compost or other materials that contain a large variety of indigenous microorganisms (Alahari and Apte, 2004; Madigan et al., 2009). In addition to providing a physical support for the microorganisms, these materials also provide some amount of minor and trace nutrients. The presence of sufficient nutrients in the biofiltration medium is required for the maintenance of

microbial activity and the consequent degradation of pollutants. However, the continuous supply of supplemental nutrients may also lead to undesirable problems such as biomass overgrowth with eventual clogging (Delhoménie and Heitz, 2005).

Microorganisms present in the biofilter bed use the carbon present in the pollutant (e.g. VOCs) as a carbon source for cell material, as carbon is the most important building block in any cell (Kennes and Veiga, 2001). Biofilters which treat non-carbon containing pollutants (e.g. NH_3, H_2S etc.,) are mostly autotrophs and hence occasionally need to be supplemented with additional carbon sources. After carbon, nitrogen is the most essential nutrient for microbial growth. It makes up about 15% of the dry cell mass and is a major constituent of nucleic acids and proteins. The bulk of available nitrogen in nature is in inorganic form, with most microorganisms able to use ammonia, and some can also use nitrate. A large fraction of the nitrogen[2] used by microorganisms is recycled after organisms die and lyse (Madigan et al., 2009).

The effect of nitrogen concentration and its chemical form on biofilter performance has been often reported (Delhomenie et al., 2001; Song and Kinney, 2005). An increase in removal efficiency by 59% is reported in a biofilter treating hexane after the addition of potassium nitrate to the biofilter bed (Morgenroth et al., 1996). There are also reports on the application of gaseous ammonia as a nitrogen source to the biofilter bed increasing the EC by 10 times (Kibazohi et al., 2004; Morales et al., 1998).

Next to nitrogen, phosphorus, sulphur and potassium are considered essential for many intracellular processes in a microorganism (ATP production, disulphate bond formation, maintenance of cellular pH, etc.) (Alahari and Apte, 2004; du Plessis et al., 1998; Sorial et al., 1998). Addition of phosphorus increased the VOC removal rate by 70% in a compost biofilter (Morgenroth et al., 1996). However, there are no reports that sulphur or potassium addition increased the performance of a biofilter (Beuger and Gostomski, 2009). However, it is necessary to maintain a threshold concentration for all these macronutrients in a biofilter in order to maintain the normal microbial metabolism (Prado et al., 2002). In addition to the macronutrients, cells require micronutrients like vitamins, magnesium, iron, calcium, copper, zinc and molybdenum in the form of trace elements for maintaining various metabolic pathways. For this reason, micronutrients are

[2] Least amount is lost through volatilization

often added along with macronutrients to biofilters in trace levels (Delhoménie and Heitz, 2005).

Though both macro- and micronutrients are required in biofilter media for adequate biofilter performance, there is no consensus on the optimal concentration required for each of these nutrients. Routine top up of these nutrients is often needed to maintain good biofilter performance during a long run or higher inlet loads (Cherry and Thompson, 1997; Gribbins and Loehr, 1998; Morgenroth et al., 1996). Conversely, there are also few biofilters reported to be used for extended time without nutrient addition (Cárdenas-González et al., 1999; Devinny et al., 1999; Weckhuysen et al., 1993). Therefore, the concentration, frequency and type of nutrients needed for treating different gaseous pollutants with various biofilter bed media remains highly empirical.

4.4 Temperature

Maintaining an optimum temperature in biofiltration is very crucial as the microorganisms involved in the biodegradation reaction can show maximum activity only over certain temperature ranges. The pollutant degradation rate usually increases with a rise in biofilter bed temperature until an optimum is reached. On the other hand, for the majority of gases, the solubility of the gas in the aqueous phase decreases, which in turn makes the contaminant less readily available for microbes (Yoon and Park, 2002). Since the biodegradation taking place in a biofilter is an exothermic process, it will add heat to the biofilter bed, which will also contribute to the overall temperature in the biofiltration process. The temperature of the biofilter bed increases when the cells are most active, which usually happens in a temperature range of 30-40 ^{o}C for toluene degraders (Kiared et al., 1997). There are a few reports which recommend 40 ^{o}C as the optimum operating temperature for biofilters (Leson and Winer, 1991; Ottengraf and Vandenoever, 1983). However, based on the type of microorganisms involved in degrading the particular pollutant, their optimum temperature range will change for attaining maximum degradation rate.

Hence, the major objective of this chapter is to understand the effects of toluene (substrate) concentration, different nutrients and temperature on the toluene degradation rate. These studies will help further to select a threshold toluene concentration and working temperature to attain high EC. Moreover, it will also help to understand about

the limiting nutrients, so that the use of chemicals containing those limiting nutrient can be avoided.

4.5 Experimental setup and methods

Experiments for studying the effect of substrate concentration and the effect of nutrient addition used the setup of Reactor 1 whereas, experiments for studying the effect of temperature used the setup of Reactor 2 as described in section 3.7.

4.5.1 Substrate concentration studies

Reactor 1 was operated for 4 months at 30 ^{o}C (optimum temperature for most microbes). Inlet concentrations were varied until a steady EC was observed at each concentration. Toluene inlet concentration was varied between 46.6 ± 0.5 ppm and 649.6 ± 4.2 ppm by varying the water bath temperature (as described in section 3.8.1) between 5 ^{o}C and 55 ^{o}C for this study. A separate cooler was connected to the water bath in order to work below the room temperature for generating lower inlet toluene concentrations. The experiment was repeated in three cycles with first two cycles at increasing order of concentrations and the last cycle at mixed order of concentrations. The matric potential of the soil was kept constant at -10 cm H_2O for the whole experiment.

The following assumptions were made for fitting the data in Eq. 4.3,

1. Since the reactor used in the study was a differential reactor, there were no concentration gradients in the gas phase and in the interparticle space between the soil particles.
2. The biomass concentration and composition were constant.
3. There was no accumulation of toluene in the soil or in water phase, as the system is at steady state.
4. All environmental parameters are constant over all the steady states except for residual toluene concentration.

4.5.2 Nutrient addition

The external lower water reservoir which was connected hydraulically with the internal upper reservoir (the one below the membrane) was used for the addition of different

nutrient solutions (Table 4.1) to the reactor bed (soil). All the nutrient solutions[3] were autoclaved at 121 ^{o}C for 45 minutes before being used in the experiment. PBS washes were performed before swapping the nutrient solutions in the reactor.

Table 4.1: Different nutrients[4] added to the differential biofilter to determine the impact on elimination capacity.

Nutrient	Concentration (g.L^{-1})	Duration (days)
Nitrogen Source a) $NaNO_3$	4.00	5
Phosphate Source a) KH_2PO_4	0.24	43
b) $NaH_2PO_4.H_2O$	1.44	43
Sulphate, Magnesium, Ferrous Sources a) $MgSO_4.7H_2O$	0.2	7
b) $FeSO_4.7H_2O$	0.0008	7
Calcium Source a) $CaCl_2.2H_2O$	1.42	56
Tap water	NA	15

4.5.3 Temperature studies

The temperature of the insulated box containing the differential reactor was controlled between 20 ^{o}C and 50 ^{o}C. Temperatures near ambient and lower were obtained by

[3] Except tap water and calcium chloride all other nutrient solutions were prepared in 1X PBS (buffered at pH: 7.0) in order to eliminate the pH effect on microbial degradation.

[4] Due to time factor, nutrient study experiments were conducted with on salt for each nutrient.

adding a cooling load through a refrigeration unit (Tropicool-XC3000A, 12V DC, Thermoelectric Refrigeration Ltd., New Zealand) attached to the insulated box and a temperature controller (LTR-5, LAE Electronics, Italy) turning a 60W light bulb off and on. For studies conducted above the ambient temperature, the refrigeration unit was eliminated. The experiment was carried out by changing the differential reactor temperature in 5 ^{o}C intervals until a steady EC was observed.

4.6 Results and discussion

4.6.1 Substrate concentration effect

The effect of residual (outlet) toluene concentration on EC was studied by manipulating the inlet toluene concentrations to change the load. The experiments were conducted with an assumption that there would be no change in biomass in the soil layer unless nutrients were added. Before these experiments were started, the reactor (R1) was operated for 120 days, and any excess nutrients to stimulate growth were assumed to be exhausted. Three cycles of repeat experiments were conducted and the EC reached a maximum before substrate inhibition dominated (C_{max}) and above C_{max} it started to drop (Fig. 4.1-4.3).

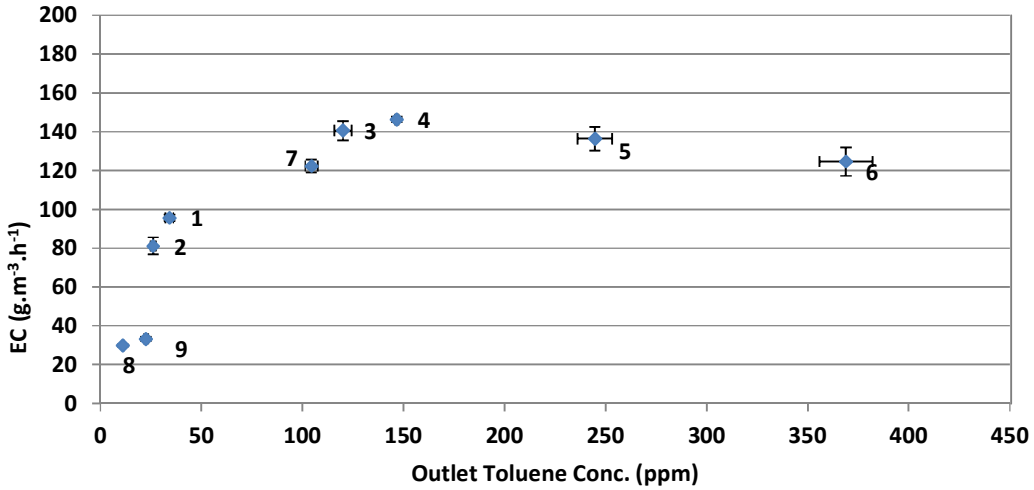


Figure 4.1: The relationship between the outlet (residual) toluene concentration on the EC. The numbers represent the order in which the curve was generated. This sample set is for cycle 1 experiment was obtained between day 120 and 188. Error bars are the standard deviations.

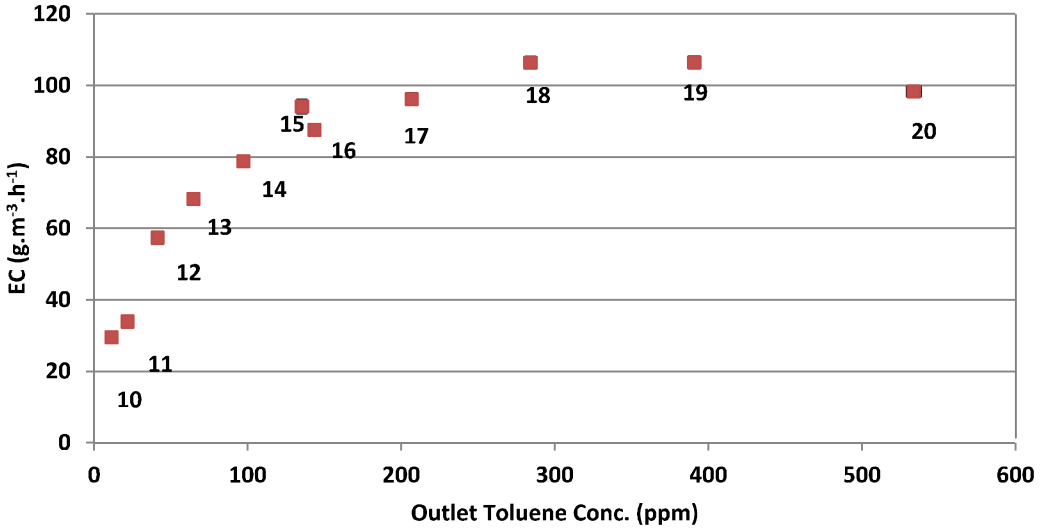


Figure 4.2: The relationship between the outlet (or residual) concentration on the EC. The numbers represent the order in which the curve was generated. This sample set is for cycle 2 experiment was obtained between day 189 and 222. Error bars are the standard deviations.

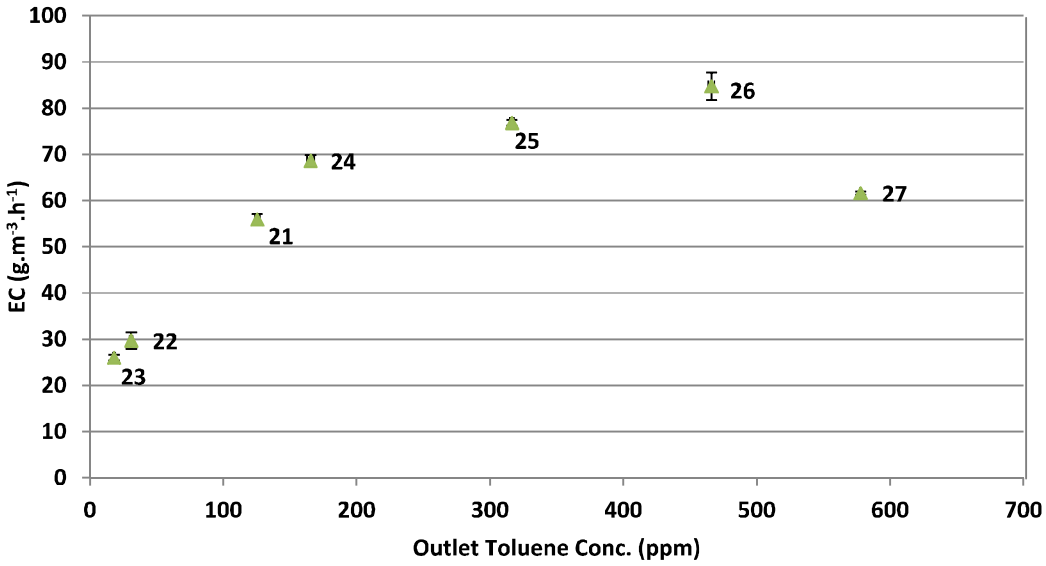


Figure 4.3: The relationship between the outlet (or residual) concentration on the EC. The numbers represent the order in which the curve was generated. This sample set is for cycle 3 experiment was obtained between day 223 and 245. Error bars are the standard deviations.

The first cycle results are shown in Fig. 4.1 with nine data points. The numbers corresponds to the order in which the experiment was done. The differential biofilter reactor was operated for nearly 4 months at a toluene residual concentration of 160 ppm and generated a lower steady EC of 38 $g.m^{-3}.h^{-1}$ until the start of this cycle (data not shown) when compared to the point 1. It is evident from the EC at point 1 that, the decrease in the toluene residual concentration decreased the substrate inhibition and only mass transfer limitation influenced EC. A similar trend was observed at point 2 as the toluene residual concentration (26.1 ppm) is further lowered at this point when compared to point 1. Following point 2, the toluene residual concentration was increased sequentially from point 3 through to point 6 (120-369 ppm). However, an increase in EC was observed only at points 3 and 4. At points 5 and 6, the EC started to decrease despite a higher residual toluene concentration. This response can either be attributed to substrate inhibition or oxygen limitation. However based on few earlier reports, oxygen limitation in a biofilter is unlikely to influence the EC at these loads (Deshusses et al., 1996; Shareefdeen et al., 1993) and hence the current response can be attributed to substrate inhibition. Therefore, it is clear that at point 4 maximum EC was measured and above which substrate inhibition started to dominate the EC more than the mass transfer limitation. Following point 6, the toluene residual concentration was decreased and a lower EC was observed. This trend was also observed at point 8, where the toluene residual concentration was lowest (11.1 ppm) in the whole cycle. Following point 8, the toluene residual concentration was increased (22.5 ppm) at point 9 and the EC also increased. Studies between points 7 and 9 confirmed once again that, substrate inhibition did not dominate the mass transfer limitation below C_{max} and EC also responded accordingly.

Following the first cycle of experiments, in order to understand further about the response of toluene degraders in the biofilter media (which was already subjected to varying substrate concentrations in cycle 1), a new cycle 2 (Fig. 4.2) was started with point 10 after day 188 at a residual toluene concentration of 11.3 ppm. The whole experiment was conducted with an increasing order of residual toluene concentration at a constant reactor temperature (30 ^{o}C). However at point 15, the experiment was conducted at a reactor temperature of 35 ^{o}C in order to have a one point comparison of EC at nearly the same toluene residual concentration but at different reactor temperature.

From point 10 to 14, the increase in residual toluene concentration increased the EC. At point 15, EC was higher than at point 16 though both the points are at nearly same residual toluene concentration. This is most likely because, at point 15 the biodegradation activity was increased due to an increase in the reactor temperature (35 ^{o}C) which was later decreased to 30 ^{o}C for points 16-20. From point 16 to point 18, EC increased with increased residual toluene concentration and at point 19, it reached the maximum value and hence the residual toluene concentration at that point was the maximum measured concentration (C_{max}) above which substrate inhibition started to influence EC. After point 19, EC started to drop and it further dropped at point 20 and this effect can be attributed to the dominance of substrate inhibition over mass transfer limitation.

Though cycle two followed a similar pattern to cycle one (mass transfer limitation dominated below C_{max} and substrate inhibition dominated after C_{max}), the curve generated from cycle two was at a lower EC compared with cycle one at similar residual toluene concentrations. Furthermore, the C_{max} also doubled in cycle 2 when compared to cycle 1. This difference could be possibly attributed to that less of the most active and efficient toluene degraders originally present in the biofilter media were still present in the biofilm. Moreover, it was believed that the other toluene degraders (sub population left following cycle 1) which were more resistant to toluene with lower specific degradation might have played a role in toluene degradation in cycle 2. The other possibility is nutrients could have leaked from those damaged, non-toluene tolerant cells or most effective toluene degraders into the environment. Probably less active toluene degraders (which survived the substrate inhibition in cycle 1) scavenged these nutrients and a small amount of growth occurred in the cycle 2 experimentation phase. However, this scavenging mechanism is not easy to validate until the initial and final biomass for each type of toluene degrader were measured in a natural biofilter medium, which is difficult to do in the diverse microbial consortium.

The second cycle raised a question about the repeatability of these studies (in which active toluene degraders are lost) in order to further explore the substrate effect on group of toluene degraders present in soil. Cycle three (Fig. 4.3) was conducted to further validate the hypothesis proposed from cycle two experiments. However, this time the experiment was conducted in random order of residual toluene concentration from

points 21 – 24 and increasing order of residual concentration from points 24 to 27 rather than an increasing or decreasing order like before (cycle one and two).

The soil which was subjected to cycle one and two was used again in this cycle. The EC observed at point 21 of cycle three was lower than the EC observed at point 20 of cycle two, which was consistent (in terms of loss in the microbial activity following high toluene load at the end of earlier cycle)with other two cycles as the residual toluene concentration at point 20 is higher than point 21. Points 22 and 23 with lower residual toluene concentration responded in similar pattern (lower EC) like in cycle 1 and 2. In addition points 24 and 25 with higher residual toluene concentration than point 21 also responded in similar pattern (higher EC) like in cycle 1 and 2. At point 26, the residual toluene concentration attained the maximum measured concentration (C_{max}) above which substrate inhibition started to influence EC more than the mass transfer limitation.

Similar to cycle one and two, a further increase in the residual toluene concentration after C_{max} decreased the EC. Hence, it can be concluded that the mass transfer limitation dominated before C_{max} and substrate inhibition dominated after C_{max}. This response was observed consistently in all three cycles. However, the curve generated from cycle three showed a further reduction in the EC at a nearly similar residual toluene concentration when compared with cycle two and further with cycle one. Overall by comparing the three curves generated by three cycles of experiments, EC dropped consistently (Fig. 4.1-4.3). The explanation provided for similar response in cycle two can also be applicable for cycle three for understanding this pattern. However, since the substrate inhibition started to dominate after C_{max}, it was hard to understand whether the whole biofilm was completely utilized (nil mass transfer limitation) at C_{max}. These experiments further implies that there were probably multiple toluene degraders involved in toluene degradation present in the biofilter bed (soil) which was used for all three cycles. Also each toluene degrader has its own C_{max} and also differing specific toluene degradation rate.

Another possible explanation for the reduced EC value between the cycles may be attributed to the depletion in nutrients with respect to the time. However, all the experiments were started after observing a steady EC for at least 2 weeks. Hence the loss of nutrients during the course of the experiments might not be significant.

The last possible explanation for the reduced EC may be the reduced oxygen level due to the thick biofilm formation, possibly due to the polysaccharide production by the microbes on the soil and hence the formation of anaerobic zones reducing the biodegradation efficiency. This may happen because of clogging of biofilter bed by excess biomass. However, additional experiments need to be done to understand the reason behind reduced biodegradation rate between the cycles. The experimental values for each cycle were fit to Eq. 4.3 (Table 4.2, Fig. 4.4).

Overall from the three cycles, it was found that the biodegradation efficiency was diminished between the cycles following the substrate inhibition in each cycle. Hence it can be concluded that running any experiments in a fresh soil at C_{max} of cycle 1 or in the flat region as shown in the Fig 4.4 is advisable to achieve maximum biodegradation. It was evident from the figure that variation of EC versus the residual toluene concentration showed an increase in EC with increasing residual toluene concentration to a certain value called C_{max}. Also it can be hypothesised that below C_{max}, mass transfer limitation dominated the substrate inhibition and vice versa after C_{max}. At C_{max}, entire active toluene degraders are assumed to be involved in the biodegradation kinetics. As the residual toluene concentration was increased above C_{max}, the EC started to decrease and this response can be referred as a substrate inhibition regime. Hence these parameters will further help to model a real biofilter in order to achieve optimal performance.

Table 4.2: Parameters used to fit modified Andrew's substrate inhibition model.

Parameter	Cycle 1	Cycle 2	Cycle 3
EC_{max}	238.8 $g.m^{-3}.h^{-1}$	145.5 $g.m^{-3}.h^{-1}$	113.9 $g.m^{-3}.h^{-1}$
K_S	0.2 $g.m^{-3}$ (53 ppm)	0.3 $g.m^{-3}$ (79 ppm)	0.3 $g.m^{-3}$ (79 ppm)
K_i	0.6 $g.m^{-3}$ (158 ppm)	0.2 $g.m^{-3}$ (53 ppm)	0.2 $g.m^{-3}$ (53 ppm)
C_{max}	0.9 $g.m^{-3}$ (245 ppm)	1.1 $g.m^{-3}$ (285 ppm)	1.7 $g.m^{-3}$ (466 ppm)

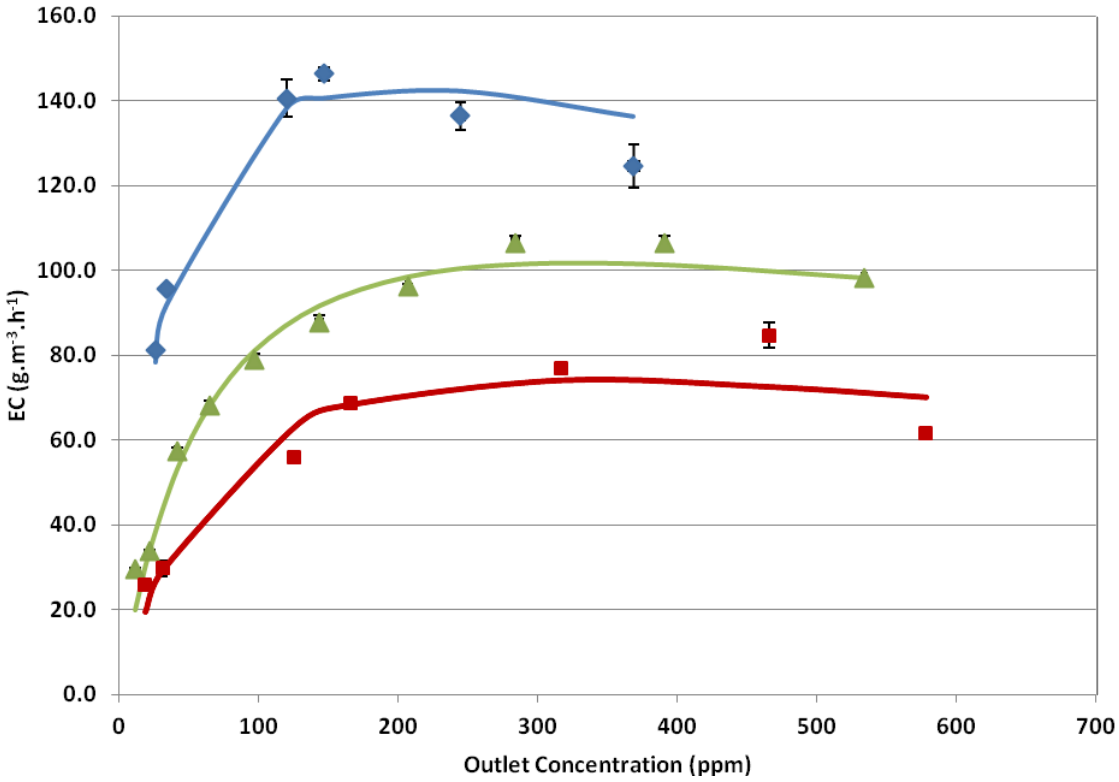


Figure 4.4: Fitting the substrate inhibition model (Eq. 4.3) for all three cycles. Cycle one is the blue closed diamonds (◆), Cycle two is the closed green triangles (▲) and Cycle three is the closed red squares (■). Error bars are the standard deviations.

4.6.2 Nutrient effect

Before starting the nutrient studies in the differential biofilter reactor, the reactor was operated for 7 days as an acclimation period for toluene degraders present in the biofilter media (soil). Moreover, the reactor was started with tap water[5] initially. A steady EC was observed after the 7th day with tap water. In the current study, tap water was considered as a control and the corresponding EC was considered as control EC. In order to avoid possible contamination in the liquid phase, all the nutrient solutions

[5] Potable water supplied by Christchurch city council for houses and workplaces.

(including tap water) used in this experiment were autoclaved before introducing into the reactor. In addition, other than the tap water and calcium chloride, all other nutrients were buffered and the pH was adjusted between 6.8 and 7.2 prior to introducing them into the reactor. Fig. 4.5 shows the effect of different nutrient additions on the EC of the differential biofilter reactor.

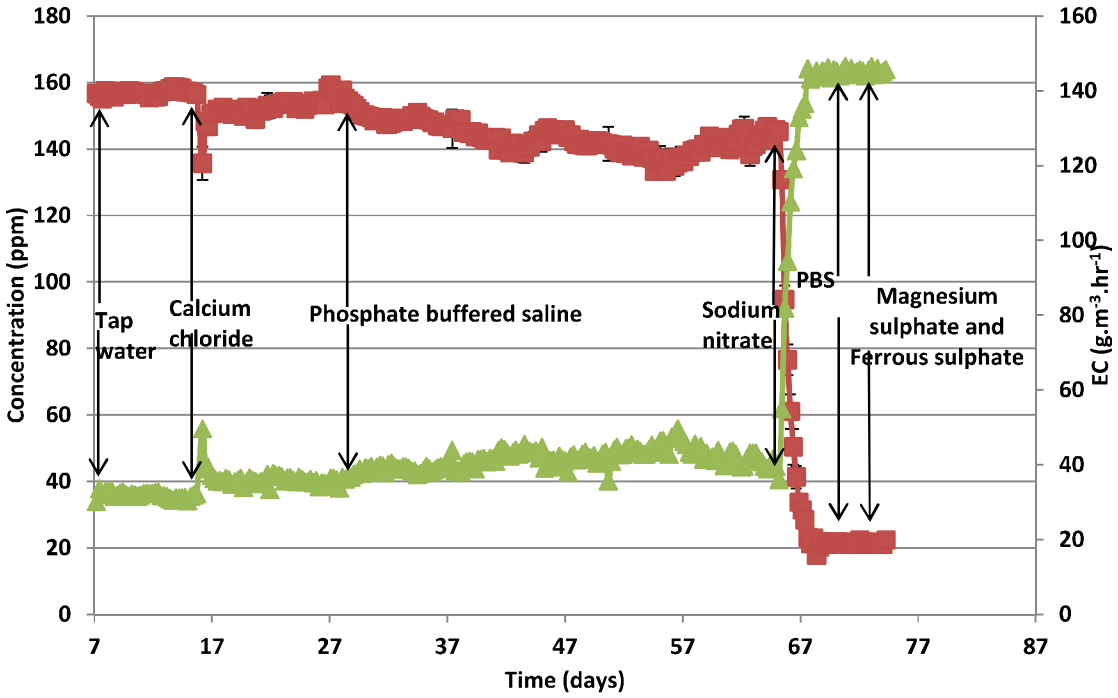


Figure 4.5: Elimination capacity, closed green triangles (△), and residual toluene concentration, closed red squares (■) for nutrient addition studies at different time intervals. Error bars are the standard deviations.

Following the steady state EC (31.9 ± 0.8 $g.m^{-3}.h^{-1}$) with tap water, at day 16, 0.01 M calcium chloride (Houba et al., 2000) was added after removing the tap water which slightly increased the EC. The steady state EC observed following the addition of calcium chloride was 35.9 ± 1 $g.m^{-3}.h^{-1}$. Since this marginal increase in EC was not considered significant (Acuña et al., 2002; Beuger and Gostomski, 2009; Jorio et al., 2000a; Morgenroth et al., 1996; Weckhuysen et al., 1993), the toluene degraders present in the soil were not calcium-limited. After achieving steady state EC at day 29,

phosphate buffered saline (PBS) was added by removing the calcium chloride from the reactor. Similar to calcium chloride, it slightly increased the EC but not significantly. However, this slight increase was observed after 10 days (day 39) of PBS addition. The steady state EC after PBS addition was observed as 41.5 ± 2.7 $g.m^{-3}.h^{-1}$. Since this marginal increase in EC was not considered significant, it was considered that similar to calcium chloride, PBS had nearly nil influence on EC. Following this experiment, it was decided to prepare all experimental solutions in PBS and also the pH of all test solutions were adjusted to 7.0 before loading into the reactor. Moreover, PBS washes were performed whenever a new test solution was loaded and removed[6] from the reactor. This was done to eliminate the pH effect on the microbial degradation.

After the day 66, PBS was replaced by 0.05 M sodium nitrate which increased the EC 11 fold after 12 days. The steady state EC observed following the addition of sodium nitrate was 145 ± 0.9 $g.m^{-3}.h^{-1}$. This response when compared with other tested nutrients proved that nitrogen was the substrate limiting the growth of toluene degraders present in the biofilter bed. Biomass increase in the soil was observed through visual inspection (white layer of microbial growth was seen on the soil surface). Nitrogen limitation was observed in other related research work conducted in different biofilters for treating different volatile organic compounds (VOCs) (Acuña et al., 2002; Beuger and Gostomski, 2009; Jorio et al., 2000a; Morgenroth et al., 1996; Weckhuysen et al., 1993).

After achieving a new steady state EC, the sodium nitrate solution was replaced with PBS water to get a new control EC value on day 69. No significant change in the EC was observed following this change. This response clearly proved that EC increase during the addition of sodium nitrate was only due to biomass growth. On 74^{th} day, PBS was replaced with magnesium sulphate and ferrous sulphate solution and no further change in EC was observed. This proved that the toluene degraders are neither Mg/Fe nor sulphate limited. It was also clear from these studies that the current experimental setup gave an easy and controlled environment for nutrient addition and removal in addition to control the water content of the biofilter bed. Since all the nutrient solutions were autoclaved before use and both the internal and external water reservoirs were

[6] The pH of tested solutions upon removal from the reactor ranged between 6.5-6.8.

sealed, the possibilities of microbial growth in the nutrient solution was minimised. Hence it was relatively easy to study the nutrient effect in the current experimental setup. The data from Fig. 4.5 is summarized in Fig. 4.6.

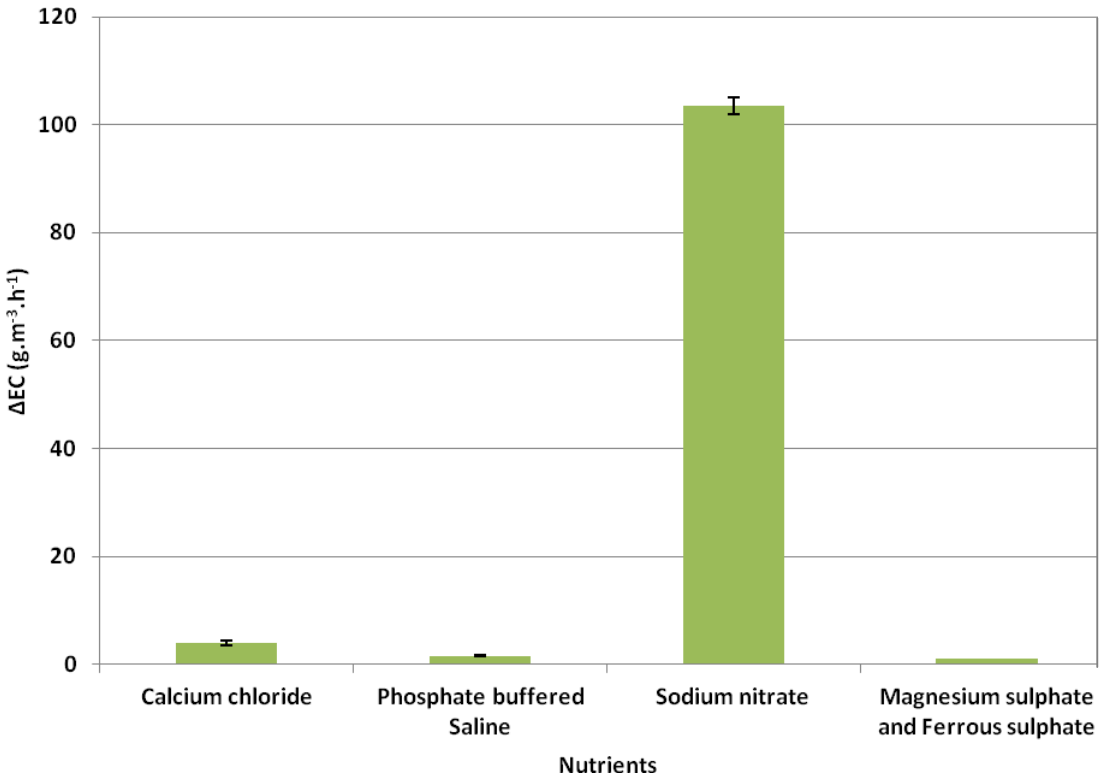


Figure 4.6: Overall results of nutrient effect on the EC of soil in the differential biofilter reactor. Error bars are the standard deviations.

4.6.3 Temperature effect

The operating temperature of the differential biofilter reactor was increased stepwise from 20 ^{o}C to 50 ^{o}C during the experiment. Increasing the temperature of the reactor increased the EC to a maximum of 49.8 ± 2.6 g.m^{-3}.h^{-1} at 45 ^{o}C (Fig. 4.7). However, the EC started to drop steeply above 45 ^{o}C. The average residual toluene concentration at this point was observed as 240±3 ppm. In addition it was also observed that the increase in EC was gradual between 20 ^{o}C and 45 ^{o}C but after that EC started to drop steeply. However, a similar study reported that maximum specific toluene degradation rate was observed at 30 ^{o}C in a biofilter (Lee et al., 2002). Hence from the current study it can be concluded that a highest intensity of the metabolic microbial activity in soil was seen at

45 ^{o}C which was 18% lower than the optimal temperature reported earlier by Beuger and Gostomski (2009) in compost. Moreover in soil, it was also possible that the group of toluene degraders present had a wide range of overlapping optimal temperatures.

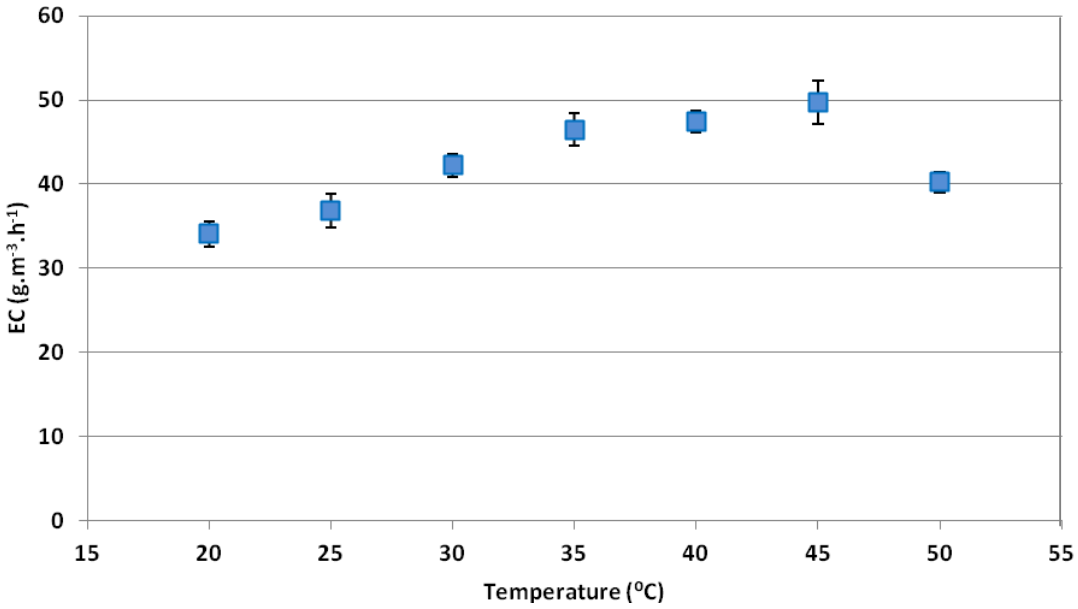


Figure 4.7: Effect of temperature on elimination capacity. Error bars are the standard deviations.

It was also observed from all the experiments which were discussed in this chapter that the initial (before the start of individual experiments) steady state EC value ranged between 30 and 100 $g.m^{-3}.h^{-1}$ (data not shown) between the runs (though soil from same source was used). A possible reason for this response is the difference in the packing density and biofilm loading which might have contributed to the variation in biodegradation.

4.7 Conclusions

The differential biofilter reactor used in the studies showed a high degree of flexibility in manipulating environmental parameters, such as substrate concentration, nutrients and operating temperature while controlling water content. From the substrate concentration studies, it was demonstrated that below C_{max}, mass transfer limitation influenced the EC and above C_{max}, substrate inhibition was the dominant influence. At

C_{max}, entire active toluene degraders are assumed to be involved in the biodegradation kinetics. As the residual toluene concentration was increased above C_{max}, the EC started to decrease and this response can be referred as a substrate inhibition regime. Furthermore, it was understood that conducting similar repeat substrate concentration studies in the same biofilter media (soil) will lose active toluene degraders. This is due to substrate inhibition during earlier experimentation cycles. Studies carried out using different nutrients clearly proved that the toluene degraders present in the soil were nitrogen limited. This was evident from the 5 fold increase in the EC under the influence of nitrogen source but the other nutrients did not show a significant increase in EC. Temperature studies showed that the EC of differential biofilter reactor increased with increasing temperature, from 34 ± 1.4 $g.m^{-3}.h^{-1}$ to 49.8 ± 2.6 $g.m^{-3}.h^{-1}$ for temperatures of 20 to 45 ^{o}C, respectively. This increase in EC was due to an increase in the activity of the toluene degraders present in the biofilter bed.

4.8 Nomenclature

EC	elimination capacity	$g.m^{-3}.h^{-1}$
EC_{max}	maximum elimination capacity	$g.m^{-3}.h^{-1}$
S	substrate concentration	$g.m^{-3}$
K_i	inhibition constant	$g.m^{-3}$
K_s	toluene half saturation constant	$g.m^{-3}$
C_{max}	maximum measured substrate concentration	$g.m^{-3}$
μ	specific growth rate	hr^{-1}
μ_{max}	maximum specific growth rate	hr^{-1}

4.9 References

Acuña, M.E., Villanueva, C., Cardenas, B., Christen, P., Revah, S., 2002, The effect of nutrient concentration on biofilm formation on peat and gas phase toluene biodegradation under biofiltration conditions. Process Biochemistry 38, 7-13.

Alahari, A., Apte, S.K., 2004, A novel potassium deficiency-induced stimulon in *Anabaena torulosa*. Journal of Biosciences 29, 153-161.

Andrews, J.F., 1968, A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates. Biotechnology and Bioengineering 10, 707-723.

Beuger, A.L., Gostomski, P.A., 2009, The Impact of Environmental Parameters on Toluene Degradation Using a Laboratory-scale Reactor with Internal Recycle. International Journal of Chemical Engineering 2(2-3), 87-95.

Cárdenas-González, B., Ergas, S.J., Switzenbaum, M.S., Phillibert, N., 1999, Evaluation of full-scale biofilter media performance. Environmental Progress 18, 205-211.

Cherry, R.S., Thompson, D.N., 1997, Shift from growth to nutrient-limited maintenance kinetics during biofilter acclimation. Biotechnology and Bioengineering 56, 330-339.

Delhomenie, M.C., Bibeau, L., Roy, S., Brzezinski, R., Heitz, M., 2001, Influence of nitrogen on the degradation of toluene in a compost-based biofilter. Journal of Chemical Technology and Biotechnology 76, 997-1006.

Delhoménie, M.C., Heitz, M., 2005, Biofiltration of air: a review. Critical Reviews in Biotechnology 25, 53-72.

Deshusses, M.A., Hamer, G., Dunn, I.J., 1996, Transient-state behavior of a biofilter removing mixtures of vapors of MEK and MIBK from air. Biotechnology and Bioengineering 49, 587-598.

Devinny, J.S., Deshusses, M.A., Webster, T.S., 1999, Biofiltration for air pollution control. CRC Press LLC, New York, USA.

Doran, P.M., 1995, Bioprocess engineering principles, Vol 1. Academic Press, New York, USA.

Du Plessis, C.A., Kinney, K.A., Schroeder, E.D., Chang, D.P.Y., Scow, K.M., 1998, Denitrification and nitric oxide reduction in an aerobic toluene-treating biofilter. Biotechnology and Bioengineering 58, 408-415.

Gribbins, M.J., Loehr, R.C., 1998, Effect of media nitrogen concentration on biofilter performance. Journal of the Air & Waste Management Association 48, 216-226.

Houba, V., Temminghoff, E., Gaikhorst, G., Van Vark, W., 2000, Soil analysis procedures using 0.01 M calcium chloride as extraction reagent. Communications in Soil Science & Plant Analysis 31, 1299-1396.

Jorio, H., Bibeau, L., Heitz, M., 2000a, Biofiltration of air contaminated by styrene: effect of nitrogen supply, gas flow rate, and inlet concentration. Environmental Science and Technology 34, 1764-1771.

Jorio, H., Bibeau, L., Viel, G., Heitz, M., 2000b, Effects of gas flow rate and inlet concentration on xylene vapors biofiltration performance. Chemical Engineering Journal 76, 209-221.

Kennes, C., Veiga, M.C., 2001, Bioreactors for waste gas treatment, Vol 4. Kluwer Academic Publishers, Faridabad, India.

Kiared, K., Fundenberger, B., Brzezinski, R., Viel, G., Heitz, M., 1997, Biofiltration of air polluted with toluene under steady-state conditions: experimental observations. Industrial & Engineering Chemistry Research 36, 4719-4725.

Kibazohi, O., Yun, S.I., Anderson, W.A., 2004, Removal of hexane in biofilters packed with perlite and a peat–perlite mixture. World Journal of Microbiology and Biotechnology 20, 337-343.

Kumar, A., Dewulf, J., Van Langenhove, H., 2008, Membrane-based biological waste gas treatment. Chemical Engineering Journal 136, 82-91.

Lee, E.Y., Jun, Y.S., Cho, K.S., Ryu, H.W., 2002, Degradation characteristics of toluene, benzene, ethylbenzene, and xylene by *Stenotrophomonas maltophilia T3-c*. Journal of the Air & Waste Management Association 52, 400-406.

Leson, G., Winer, A.M., 1991, Biofiltration - an Innovative Air-Pollution Control Technology for Voc Emissions. Journal of the Air & Waste Management Association 41, 1045-1054.

Madigan, M.T., Martinko, J.M., Dunlap, P.V., Clark, D.P., 2009, Brock biology of microorganisms, Vol 2. Pearson/Benjamin Cummings, London, UK.

Morales, M., Revah, S., Auria, R., 1998, Start-up and the effect of gaseous ammonia additions on a biofilter for the elimination of toluene vapors. Biotechnology and Bioengineering 60, 483-491.

Morgenroth, E., Schroeder, E.D., Chang, D.P.Y., Scow, K.M., 1996, Nutrient limitation in a compost biofilter degrading hexane. Journal of the Air & Waste Management Association 46, 300-308.

Ottengraf, S.P.P., Vandenoever, A.H.C., 1983, Kinetics of Organic-Compound Removal from Waste Gases with a Biological Filter. Biotechnology and Bioengineering 25, 3089-3102.

Prado, Ó., Mendoza, J., Veiga, M., Kennes, C., 2002, Optimization of nutrient supply in a downflow gas-phase biofilter packed with an inert carrier. Applied Microbiology and Biotechnology 59, 567-573.

Shareefdeen, Z., Baltzis, B.C., Oh, Y.S., Bartha, R., 1993, Biofiltration of methanol vapor. Biotechnology and Bioengineering 41, 512-524.

Song, J.H., Kinney, K.A., 2005, Microbial response and elimination capacity in biofilters subjected to high toluene loadings. Applied Microbiology and Biotechnology 68, 554-559.

Sorial, G.A., Smith, F.L., Suidan, M.T., Pandit, A., Biswas, P., Brenner, R.C., 1998, Evaluation of trickle-bed air biofilter performance for styrene removal. Water Research 32, 1593-1603.

Vergara-Fernández, A., Lara Molina, L., Pulido, N.A., Aroca, G., 2007, Effects of gas flow rate, inlet concentration and temperature on the biofiltration of toluene vapors. Journal of Environmental Management 84, 115-122.

Wang, J., Wu, C., Chen, J., Zhang, H., 2006, Denitrification removal of nitric oxide in a rotating drum biofilter. Chemical Engineering Journal 121, 45-49.

Weckhuysen, B., Vriens, L., Verachtert, H., 1993, The effect of nutrient supplementation on the biofiltration removal of butanal in contaminated air. Applied Microbiology and Biotechnology 39, 395-399.

Yoon, I.K., Park, C.H., 2002, Effects of gas flow rate, inlet concentration and temperature on biofiltration of volatile organic compounds in a peat-packed biofilter. Journal of Bioscience and Bioengineering 93, 165-169.

Chapter 5: Metabolic Uncoupler Screening Studies

5.1 Introduction

One of the biggest challenges in a traditional biofilter is to overcome the low volumetric degradation rate, which often makes the footprint excessive (Devinny et al., 1999). The volumetric degradation rate or elimination capacity (EC) is directly influenced by the specific biodegradation rate of the microorganism involved in degrading the pollutants. Application of metabolic uncouplers in biofiltration could improve the biodegradation rate of microorganisms, which in turn could increase the EC. The addition of metabolic uncouplers to the growth system decreases the biomass growth (Hiraishi and Kawagishi, 2002; Low et al., 2000; Torkian et al., 2003) whereas in energy-excess, non-growth systems like biofiltration, it is expected to increase the specific substrate uptake rate since the substrate requirement for maintenance energy should increase, which in turn should increase the EC. This chapter will discuss the screening studies investigating seven potential metabolic uncouplers (at different concentrations) on the specific biodegradation rate of air-borne toluene in both serum bottles and a differential biofilter reactor with soil as the bed medium.

5.2 Experimental setup and methods

The experiments for studying the effect of metabolic uncouplers in batch mode used serum bottles and a Varian-3800 gas chromatography system (Agilent Technologies, USA). Experiments to study the effect of seven potential metabolic uncouplers in continuous mode used the differential biofilter reactors (R2, R3 and R4) setup with online toluene and carbon dioxide monitoring system as described in Chapter 3. A phosphate buffered saline (PBS) solution (Uquillas et al., 2011) was used to prepare the required concentration of metabolic uncouplers used in both batch and continuous screening studies. The solution pH was adjusted to 7.0 and then autoclaved. Since most of these metabolic uncouplers were slow to dissolve in water at room temperature, autoclaving the solution at 121 ^{o}C helped to dissolve the metabolic uncouplers and as well in preparing an abiotic solution for the screening experiments.

5.2.1 Screening of metabolic uncouplers in batch mode

Nine metabolic uncouplers (Table 5.1) were chosen for the initial short-term screening tests their ability to increase the toluene degradation rate by soil microorganisms. Approximately 8.65 g wet weight of soil (Park House Garden Supplies, Christchurch, New Zealand) was placed on a Whatman filter paper (500 mm dia., Grade 1) in a funnel over a flask. A 0.01 M calcium chloride[1] solution was then used to make up 100 mL of metabolic uncoupler solutions at the concentration reported in the literature for activated sludge studies. However, based on earlier experiments, the concentration of dinitrophenol, carbonylcyanide m-chloromethoxy-phenylhydrazone (CCCP) and carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP) were decreased to 10% of the literature values for this screening study. The metabolic uncoupler solution was poured over the soil and then the soil sample was squeezed to remove any excess solution. The wet soil[2] treated with metabolic uncoupler was then transferred into a 125 mL serum bottle. Approximately 0.5 µL of HPLC (High Performance Liquid Chromatography) grade toluene was then injected into the serum bottle to generate approximately 700 ppm of toluene vapour in the head space sealed with a bung and a cap. Four control serum bottles were also used in this study with no soil, abiotic soil, soil without any metabolic uncoupler and soil with 2 mL of toluene degraders (mixed culture). The toluene degraders used in the control study were previously isolated from soil using similar method described in section 6.2.1 and mixed together to form the mixed culture. Each condition was tested in duplicate. All the serum bottles were incubated at 30 ^{o}C for 60 hours (Fig. 5.1). Periodically, toluene samples from the serum bottles were analysed by gas chromatography system to observe the degradation.

[1] Used in-order to maintain hardness/compactness of the soil.

[2] 20% (wet weight)water content was measured in the wet soil.

Figure 5.1: Batch mode, serum bottle experimental set-up (inside a 30 ^{o}C incubator).

Table 5.1: Metabolic uncouplers[3] used in batch mode serum bottle tests.

Metabolic Uncoupler	Effective conc. reported (µM)	Concentration tested (µM)	Solubility[4] (mM)	pKa
benzoic acid (BA)	10000	10000	23.8	4.20
carbonylcyanide m-chloromethoxy-phenylhydrazone (CCCP)	10	1	NA	6.09
carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP)	10	1	NA	6.10
2,4-dinitrophenol (DNP)	760	76	7.6	4.09
m-chlorophenol (mCP)	160	160	22.6	8.80
malonic acid (MA)	96	96	701.5	2.83
p-nitrophenol (pNP)	860	860	107.8	7.15
Pentachlorophenol (PCP)	142	142	0.15	4.70
2,4,6-trichlorophenol (TCP)	4051	4051	4.05	7.50

[3] References for effective concentration and pKa are discussed in Table 2.2.

[4] Yalkowsky, S.H., He, Y., 2003. Handbook of aqueous solubility data. CRC.

5.2.2 Screening of metabolic uncouplers in continuous mode

Following the batch screening studies, seven metabolic uncouplers[5] were used for further screening studies in the continuous differential biofilter reactors (Table 5.2). Based on the earlier studies carried out on nutrient limitation (section 4.6.2), preference was given to the non-nitrogen containing metabolic uncouplers for testing in continuous mode. Initially, all the biofilter reactors ran without metabolic uncouplers until a steady toluene degradation rate was observed following the introduction of soil inside the reactors. Following each metabolic uncoupler concentration tested, fresh PBS was used to remove the residual metabolic uncoupler in the soil until a steady toluene degradation rate was observed. The average inlet toluene concentration used in all three biofilter reactors was approximately 180 ppm. Each metabolic uncoupler experiment was carried out for more than 30 days at all uncoupler concentrations following the initial steady state EC (before the addition of metabolic uncoupler) in each of the three differential biofilter reactors. Following experimentation, the tested liquid samples (PCP and TCP) were sent for analysis at Hill Laboratories, New Zealand for PCP and TCP analysis.

Table 5.2: Metabolic uncouplers used in continuous mode screening test.

Metabolic Uncoupler	**Concentrations tested** (μM)
benzoic acid (BA)	5000, 10000 and 15000
pentachlorophenol (PCP)	70 and 140
2,4,6 trichlorophenol (TCP)	4051
malonic acid (MA)	25, 50 and 100
carbonylcyanide p-chloromethoxy phenylhydrazone (CCCP)	0.01, 0.001 and 0.002
carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP)	0.01
m-chlorophenol (mCP)	16, 160 and 1600

[5] Residual metabolic uncouplers were removed through multiple PBS washes at the end of each experiments.

5.3 Results and discussion

5.3.1 Batch mode screening test

The effect of nine metabolic uncouplers on toluene degradation in serum bottle tests is shown in Fig. 5.2. It was observed that in 60 hr period, pentachlorophenol, benzoic acid, p-nitrophenol, 2, 4, 6 trichlorophenol and m-chlorophenol increased the toluene degradation rate by 40% compared to the control soil with toluene degraders and 200% compared to the control soil without toluene degraders. CCCP had a better toluene degradation rate when compared with the control soil without toluene degraders. The addition of the uncouplers 2,4 dinitrophenol, malonic acid and FCCP decreased the toluene degradation rate when compared with the control soil with and without toluene degraders. On the basis of batch screening test results, pentachlorophenol, benzoic acid, 2, 4, 6 trichlorophenol, m-chlorophenol and CCCP were selected for further screening studies in a continuous biofilter reactor system. Later, it was decided to test both malonic acid and FCCP also in the continuous biofilter reactor system to understand their degradation dynamics.

It was possible that the soils in the short-term serum bottle tests were not growth limited during the 60 hr test period as there may have been residual nutrients in the soil which the microbe might have utilized. A similar response (higher EC) was also observed in the continuous biofiltration reactor during initial acclimation time (data not shown). Hence the toluene degradation might have been mostly due to growth (plus uncoupling) and not due to maintenance requirements enhanced by uncouplers. Hence seven out of nine metabolic uncouplers were selected for further screening studies in continuous biofilter reactor system. However, p-nitrophenol and 2, 4 dinitrophenol were not selected for further studies in the continuous biofilter reactor system as both of them contained nitrogen and previous work (section 4.6.2) demonstrated the toluene degraders in the soil were nitrogen limited after the acclimation period.

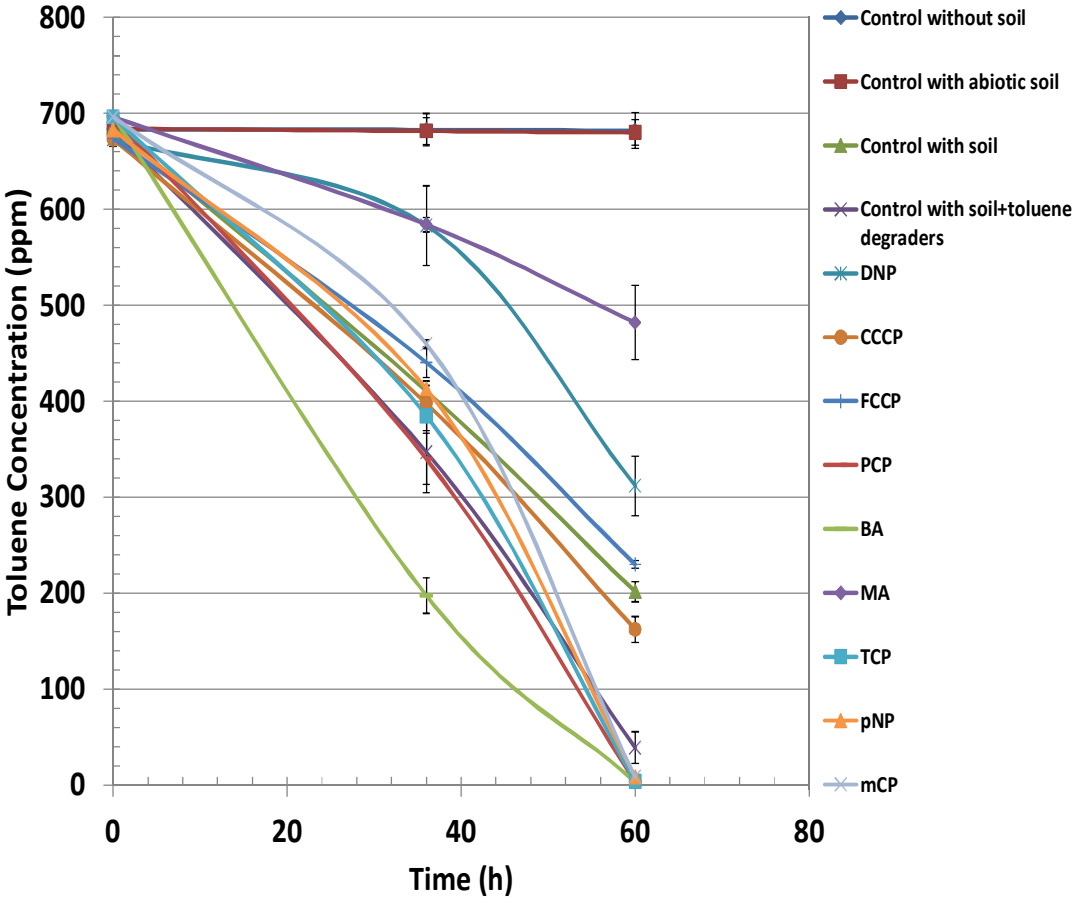


Figure 5.2: Effect of different metabolic uncouplers on toluene degradation rate in batch serum bottle tests with soil. Individual error bars are the standard deviation between the duplicates.

5.3.2 Continuous mode screening test

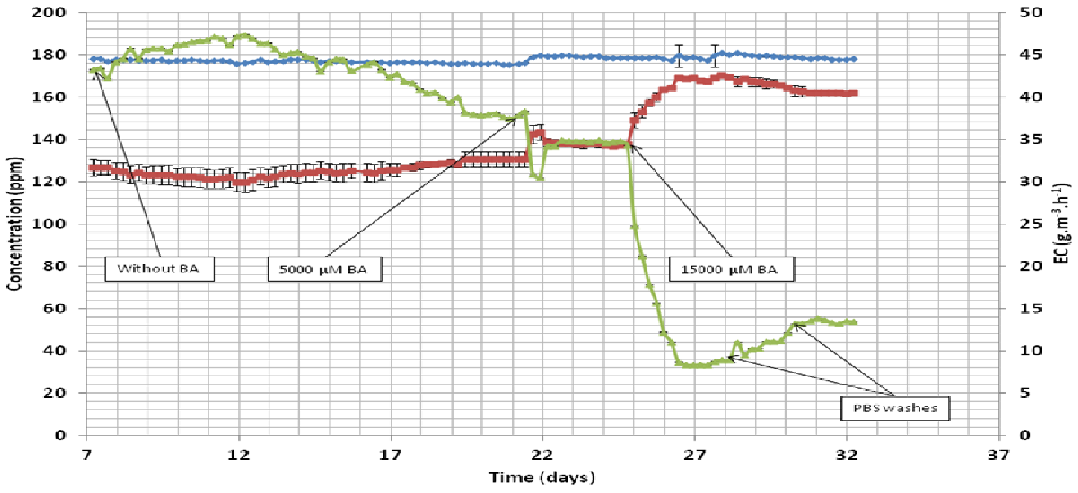
Following the batch mode screening experiments and identifying the limiting nutrient (section 4.6.2), seven potential (non-nitrogen containing) metabolic uncouplers were selected for further screening studies in continuous mode. In this investigation, different concentrations (Table 5.2) of the seven metabolic uncouplers were tested and the effect of individual uncoupler on the EC is discussed in detail in the following sections.

5.3.2.1 Effect of benzoic acid (BA)

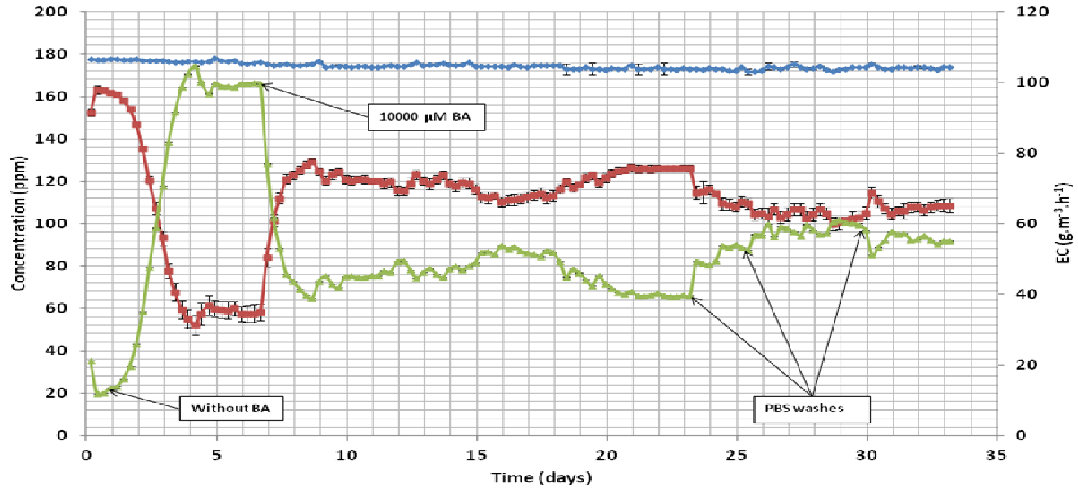
Reactor 3 was used for this study and was run initially for 22 days with fresh soil and PBS which generated a steady state EC of 38.9 $g.m^{-3}.h^{-1}$ (Fig. 5.3a). Following the

steady state response, PBS was replaced with 5000 µM benzoic acid buffered at pH 7 on the 22nd day. Following steady state EC, it was observed that addition of 5000 µM benzoic acid decreased the EC by 10.8% with reference to the initial EC. On 25th day, 5000 µM benzoic acid solution was replaced with a 15,000 µM benzoic acid solution in order to understand the effect of a higher benzoic acid concentration. However, the EC dropped significantly (9.7 $g.m^{-3}.h^{-1}$) following the addition of 15,000 µM benzoic acid. Following this response, the 15,000 µM benzoic acid solution was removed from the reactor on 28th day through multiple PBS washes. Despite the PBS washes, the EC recovered to 36% of the initial EC (Fig. 5.3a). This response was not consistent with an uncoupler response as per the chemiosmotic theory. The experiment was repeated with a fresh soil and PBS. After a steady state EC of 99 $g.m^{-3}.h^{-1}$, a 10,000 µM benzoic acid solution was added to the reactor replacing PBS. However, a similar trend compared to the earlier experiment was observed. The EC decreased by 60% when compared to the initial EC and after the PBS wash, it recovered only by 10% (Fig. 5.3b).

The benzoic acid concentration reported to be effective in influencing the metabolic fluxes of yeast was 10,000 µM (Verduyn et al., 1992). However, the concentrations tested in the non-growth differential biofiltration system ranged between 5000 µM and 15000 µM. But none of the concentrations tested in the system increased the EC consistent with a metabolic uncoupler rather it decreased the EC. This suggested that benzoic acid might have killed the toluene degraders which are mostly bacteria. Hence the response of benzoic acid in a eukaryotic growth system is dissimilar to a prokaryotic non-growth system. However, reducing the concentration of benzoic acid further below may lead to the possible degradation of benzoic acid by toluene degraders (Muthukumar et al., 2009; Wright, 1993). Hence, experiments at lower benzoic acid concentrations were not performed in the current study. Therefore, benzoic acid was considered ill-suited as a potential metabolic uncoupler for enhancing the toluene degradation rate in non-growth biofilter reactor system. The response of benzoic acid was found different in batch and continuous systems. This is because in batch mode benzoic acid was added in non-steady state condition (steady state in continuous mode), which means that though the soil contained toluene degraders; it might also contain non-toluene degraders which might have degraded the benzene.



(a)



(b)

Figure 5.3: Effect of benzoic acid on toluene degradation rate in a differential biofilter reactor with soil. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. **a)** Cycle 1 experiment with 5000 µM and 15,000 µM benzoic acid **b)** Cycle 2 experiment with 10,000 µM benzoic acid. Error bars are the standard deviation between the multiple sample injections in GC. (Note: Different soil was used in Cycle 1 and 2).

5.3.2.2 Effect of pentachlorophenol (PCP)

Reactor 2 was used for this study and was run initially for 29 days with fresh soil and PBS, which generated an initial steady state EC of 71.4 $g.m^{-3}.h^{-1}$ (Fig 5.4). PBS was replaced with a 70 µM PCP solution on the 29th day. The PCP a slowly increased the EC and a steady EC of 84 $g.m^{-3}.h^{-1}$ was observed after the 33rd day. Following this, a 140 µM PCP solution replaced the 70 µM PCP solution on the 34th day. It was observed that 140 µM PCP increased the EC by 35% when compared with the initial EC. A steady EC of 110 $g.m^{-3}.h^{-1}$ was observed after 50 days (Fig. 5.4). Due to the solubility limit of PCP (which is ~150 µM) studies at higher concentrations were not performed. Since PCP was not easily degradable by the soil microbes (Mikesell and Boyd, 1988) and that the system was nitrogen-limited, it was concluded that the increase in EC was not directly associated with growth on PCP. A PBS wash to remove the PCP from the reactor produced an EC of 109 $g.m^{-3}.h^{-1}$ and it did not drop back to the initial EC value. This response was not as expected for uncoupling, as the ATP production efficiency should have returned to the initial level, thereby dropping the EC to its original level.

At least two possibilities existed to explain these results in addition to some level of metabolic uncoupling:

i) the PCP killed microorganisms not associated with toluene degradation thereby freeing up nitrogen for the toluene degraders to grow, thus permanently increasing the EC;

ii) the PCP was not completely removed by the wash step due its hydrophobic property leaving it entrained in the lipid layer of the biomass and adsorbed to the soil;

Following the 140 µM PCP studies, the liquid was sent for PCP analysis (including the PBS washes). The results showed that only 18.4% of PCP did not end up in the removed liquid and subsequent PBS washes (Appendix C). This 18% loss of PCP may be attributed to the PBS wash not removing all PCP from the system. It also confirms that PCP was not significantly degraded by the toluene degraders. If uncoupling was happening, it is expected to be reversible with a return to the initial EC upon removal (Brand et al., 2010; Lou et al., 2007). These results imply that growth by the toluene degraders on nitrogen released by other organism probably increased the EC and PCP

did not uncouple the metabolism. However, additional experiments analysing the PCP fraction in the soil and with pure cultures of toluene degraders (biofilm) will help further clarify this response.

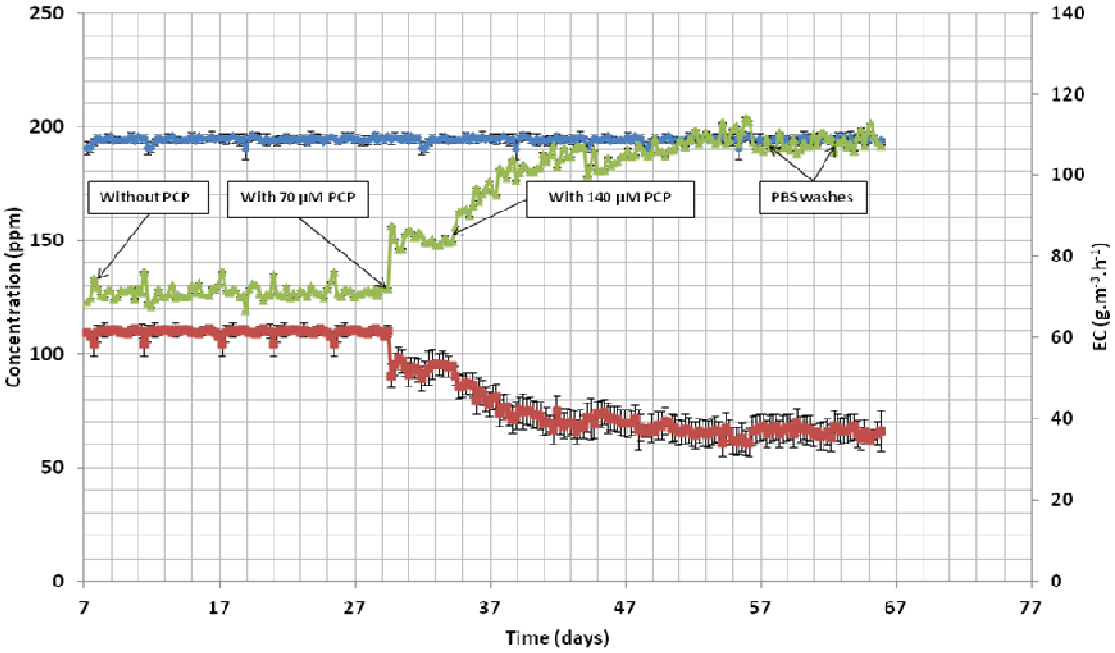


Figure 5.4: Effect of pentachlorophenol on toluene degradation rate in a differential biofilter reactor with soil. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. Error bars are the standard deviation between the multiple sample injections in GC.

5.3.2.3 Effect of 2, 4, 6 trichlorophenol (TCP)

Reactor 3 was used for this study and was run initially for 13 days until it reached a steady EC of 73 $g.m^{-3}.h^{-1}$ (Fig. 5.5). Based on the earlier experiment done with PCP, it was decided to use a higher concentration (4051 µM) of the similar but more soluble TCP to observe the EC change. At 4051 µM of TCP (which is its solubility limit), the EC increased by 18% when compared with the initial EC. However, similar to PCP, after removing the TCP from the reactor and washing with PBS, the EC did not return to the initial EC and dropped only by 2% when compared with the maximum EC generated by 4051 µM TCP (Fig. 5.5). The possible explanations for this response are

the same as those for PCP. Similar to PCP studies, TCP was also sent for analysis (after multiple PBS washes). The results showed that 68% of the TCP was missing from the liquid (Appendix C). This particular result raised a question about the possibilities of TCP degradation (either by toluene degraders or by any other microbe present in the soil or in combination of both), as the increased solubility should have aided its removal by washing. In addition, growth on nitrogen released by non-toluene degraders is still a possibility for the increased EC. Similar to PCP, it was decided to test TCP in a pure culture of toluene degrader (biofilm) in our differential biofilter reactor system in order to further understand the potential uncoupling mechanism of TCP clearly.

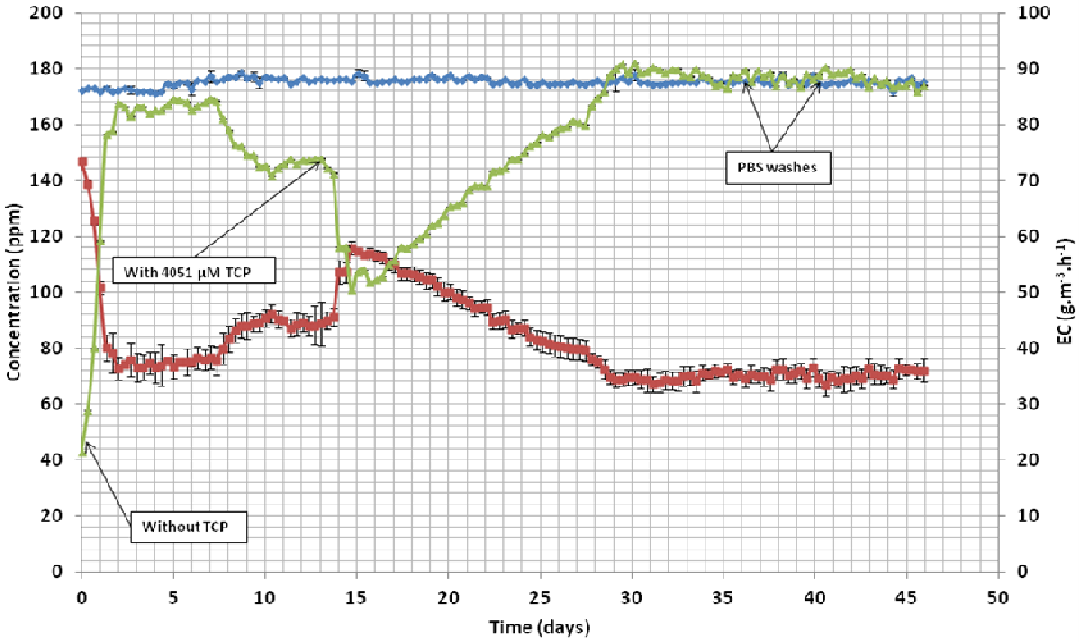


Figure 5.5: Effect of 2, 4, 6-trichlorophenol on toluene degradation rate in differential biofilter reactor with soil. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. Error bars are the standard deviation between the multiple sample injections in GC.

5.3.2.4 Effect of malonic acid (MA)

Reactor 2 was used for this study. It was run for nearly 35 days with fresh soil and PBS to get a steady state EC of 109 $g.m^{-3}.h^{-1}$ (Fig 5.6). Three different concentrations of

malonic acid were tested in this system with increasing concentration. Increasing concentrations of malonic acid decreased the EC. Initial addition of 25 µM malonic acid buffered at pH 7 on 36th day decreased the EC by 5.8%. Following the steady state EC, 25 µM malonic acid was replaced by 50 µM malonic acid on 40th day which nearly had zero influence on EC and hence on 46th day, 50 µM malonic acid was replaced with 100 µM malonic acid. The EC was decreased by another 11% (Fig. 5.6). The response of malonic acid was similar to the response of benzoic acid. Hence, the possible explanations for this response are the same as those for benzoic acid. Similar to benzoic acid studies, lower concentrations of malonic acid were not studied in the system, due to the potential malonic acid degradation (Ariya et al., 2002). Hence malonic acid was considered not suitable as a metabolic uncoupler in enhancing the toluene biodegradation from this system.

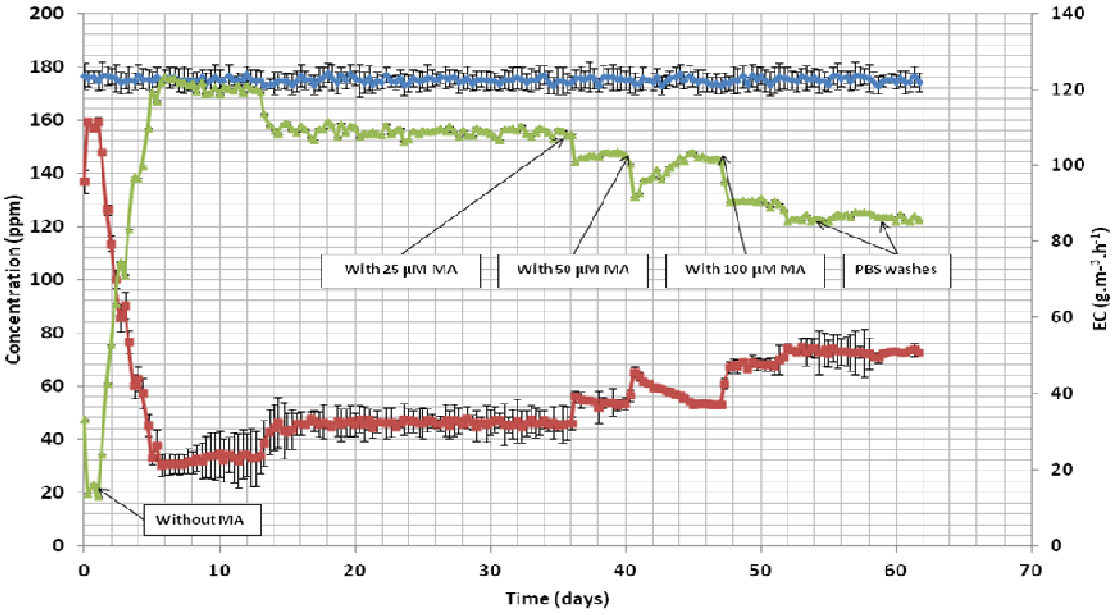
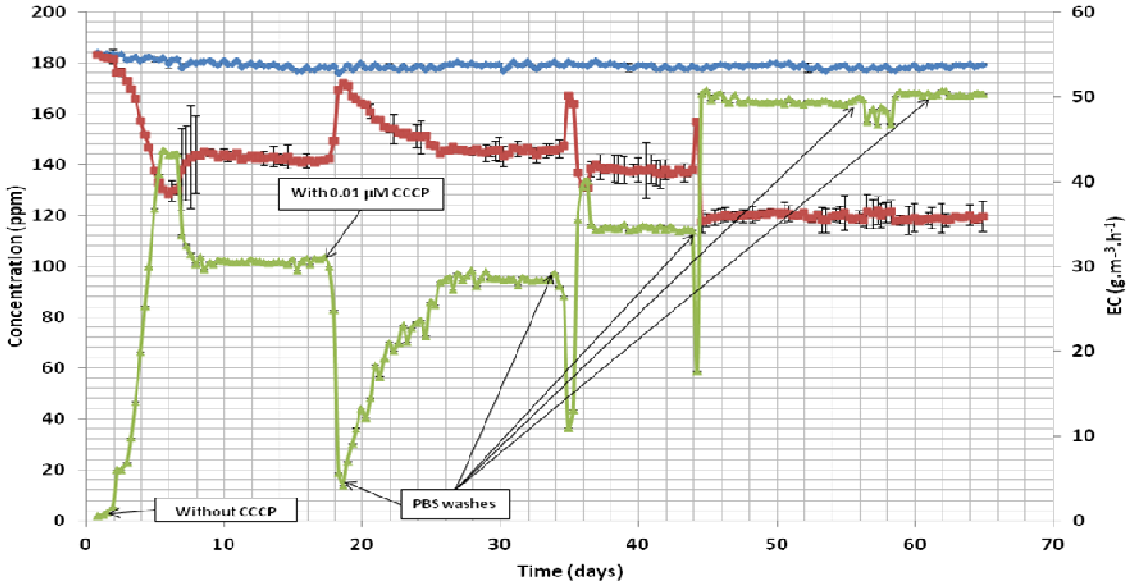


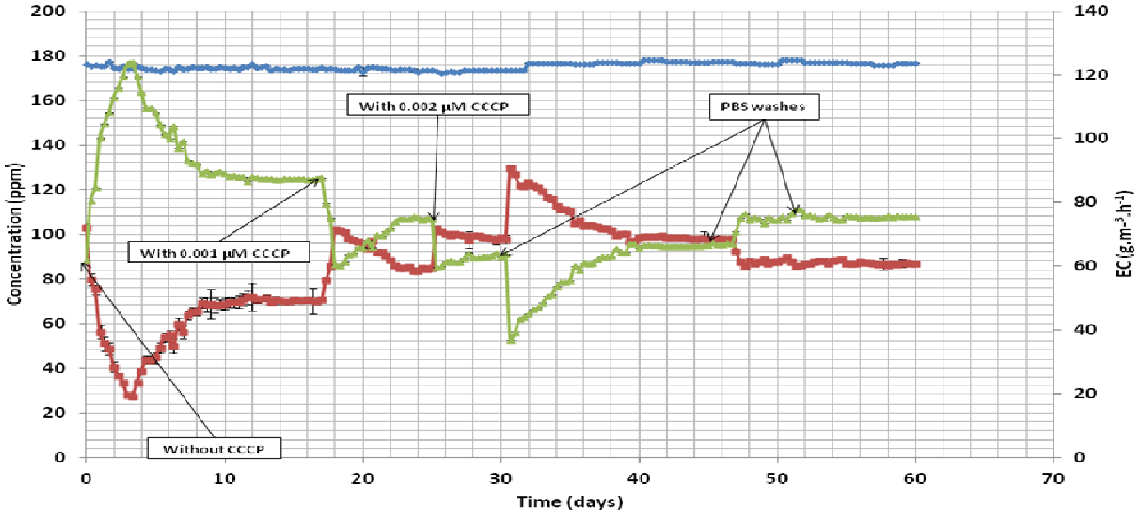
Figure 5.6: Effect of malonic acid on toluene degradation rate in differential biofilter reactor with soil. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. Error bars are the standard deviation between the multiple sample injections in GC.

5.3.2.5 Effect of carbonylcyanide p-chloromethoxy phenylhydrazone (CCCP)

Reactor 4 was used for this study. Two cycles of experiments were done with three different concentrations of CCCP. Following a steady state EC (31 $g.m^{-3}.h^{-1}$) with soil and PBS after 17 days, a 0.01 µM CCCP solution was added to the system replacing PBS and the EC was reduced by 87%. In order to avoid the complete loss of active toluene degraders, before attaining a steady EC, the 0.01 µM CCCP solution was washed from the system on the 19th day. A series of PBS washes was performed and it was observed that following every PBS wash, the EC increased. However, increase in the EC was not linear with each PBS wash. The maximum EC observed following series of PBS wash was 50 $g.m^{-3}.h^{-1}$. After this, further PBS washes did not influence the EC (Fig. 5.7a). A possible speculative reason for this response may be due to the slow diffusion rate of this compound into soil when compared with other metabolic uncouplers which took multiple washes to remove it from soil. However, the diffusion rate of CCCP is unknown. In addition it was observed that reason for the sharp decrease in EC following the addition of CCCP may be that the concentration used in the study was intolerable to the active toluene degraders present in the soil. The experiment was repeated with a fresh soil but at a lower CCCP concentration (0.001 µM). The CCCP addition dropped the EC by 15%. On the 25th day, 0.001 µM CCCP was replaced with a 0.002 µM CCCP solution. Following this change, the EC further dropped by 16% with reference to the earlier one. However, series of PBS washes to remove the 0.002 µM CCCP increased the EC closer to initial EC (Fig. 5.7b). The explanation provided for the similar response in cycle 1 can be again valid for this cycle. But, PBS washes did not increase the EC above the initial EC in cycle two when compared with cycle one. There are two possible speculative explanations for this response of CCCP a) the concentration of CCCP used was inhibiting/killing the toluene degraders and other microbes in soil. When it was removed the slow release of nitrogen had allowed the toluene degraders to bounce back to a higher level than original b) the concentration were inhibitorier (near C_{max}) than killing concentration. Hence CCCP was considered not suitable for similar studies like ours. However, conducting similar experiments in pure culture of toluene degrader (biofilm) in our differential biofilter reactor system will further help to understand the potential uncoupling mechanism of CCCP as the issue of diffusion rate would be nullified in biofilm studies to an extent.



(a)



(b)

Figure 5.7: Effect of carbonylcyanide p-chloromethoxy phenylhydrazone on toluene degradation rate in a differential biofilter reactor with soil. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. **a)** Cycle 1 experiment with 0.01 µM carbonylcyanide p-chloromethoxy phenylhydrazone **b)** Cycle 2 experiment with 0.001 and 0.002 µM carbonylcyanide p-chloromethoxy phenylhydrazone. Error bars are the standard deviation between the multiple sample injections in GC.

5.3.2.6 Effect of carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP)

Reactor 4 was used for this study. Since FCCP belongs to the same family as CCCP, it was decided to test FCCP only at one concentration to understand its effect on the EC. Following the steady state EC of 25 $g.m^{-3}.h^{-1}$ after 13 days, 0.01 µM FCCP was added to the system by replacing the PBS. The EC increased to 30 $g.m^{-3}.h^{-1}$ which was 20% higher than the initial EC. Following the removal of FCCP and subsequent PBS washes, the EC dropped to 27 $g.m^{-3}.h^{-1}$ (Fig. 5.8). Experiments at higher FCCP concentrations were not performed due to solubility limits. A further lower concentration of FCCP was not tested in the system under the assumption that a lower concentration either would little influence on the EC. When comparing the response of CCCP with FCCP, they behaved differently though both belong to the same family of compounds and have similar pKa values. In particular when compared to CCCP, FCCP behaved as a classic uncoupler in increasing the EC. But to be a 100% classic uncoupler, the response is expected to be reversible with a return to the initial EC upon removal, which was not the case here. Conversely, the diffusion rate of FCCP in soil is unknown. Based on the results from CCCP and FCCP studies, the diffusion rate of FCCP may be assumed higher than that of CCCP in soil which can be further correlated to their different response. Though FCCP did not increase the EC significantly in our soil differential biofiltration system, conducting similar experiments in pure culture of toluene degrader (biofilm) in our differential biofilter reactor system will further help to understand the uncoupling mechanism of FCCP clearly.

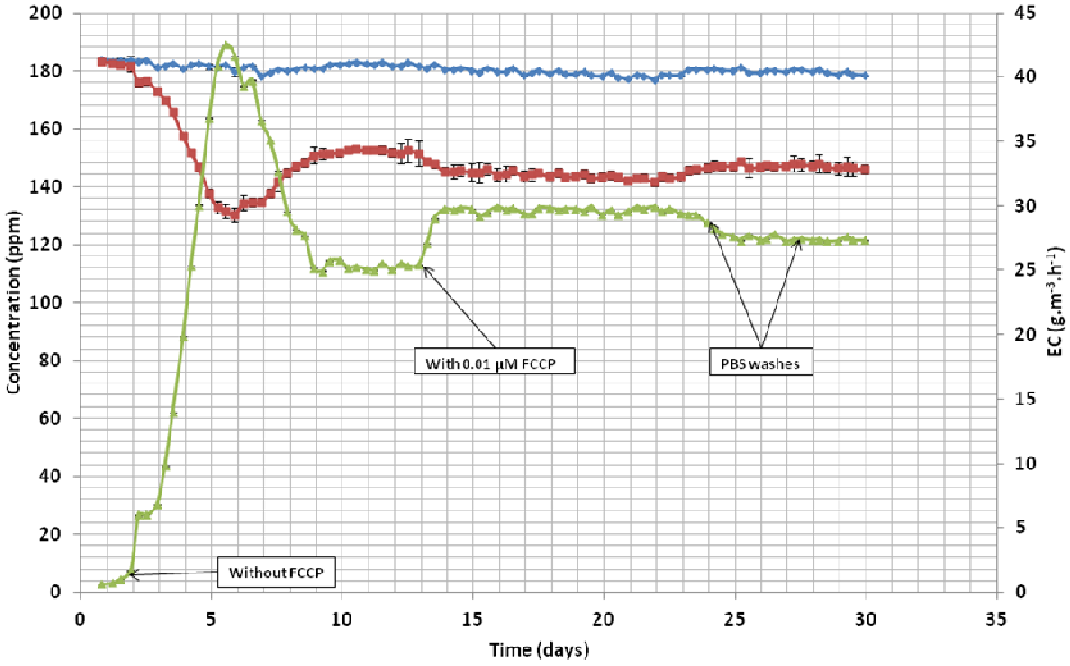


Figure 5.8: Effect of carbonyl cyanide p-trifluoromethoxy-phenylhydrazone on toluene degradation rate in a differential biofilter reactor with soil. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. Error bars are the standard deviation between the multiple sample injections in GC.

5.3.2.7 Effect of m-chlorophenol (mCP)

Reactor 4 was used for this experiment. Initially it was thought that mCP would be similar to PCP and TCP and hence the experiment was started at a higher concentration of 1600 µM after the initial steady EC value of 24 $g.m^{-3}.h^{-1}$ on the 9^{th} day. But the addition of mCP dropped the EC by 73%. However, this drop in EC was not similar to CCCP as the decreased EC remained constant in the current case. Hence it was decided to wash with PBS after removing the mCP and to use the same soil to test the lower concentrations of mCP in order to understand its influence on EC. After the removal of mCP and series of PBS washes, the EC recovered to 37% of the initial EC. Following this, experiments were conducted at lower mCP concentrations (16 µM and 160 µM). None of the concentrations increased the EC value above the initial EC and not even above the earlier EC. Moreover, a series of PBS wash following the removal of 160 µM

mCP did not show any significant change in the EC (Fig. 5.9). It is clear from this experiment that the initial concentration tested was toxic to the active toluene degraders and further reduced concentration tested was slightly inhibitory to the toluene degraders present in the soil. Hence it was concluded that mCP was not a potential metabolic uncoupler for enhancing the toluene degradation.

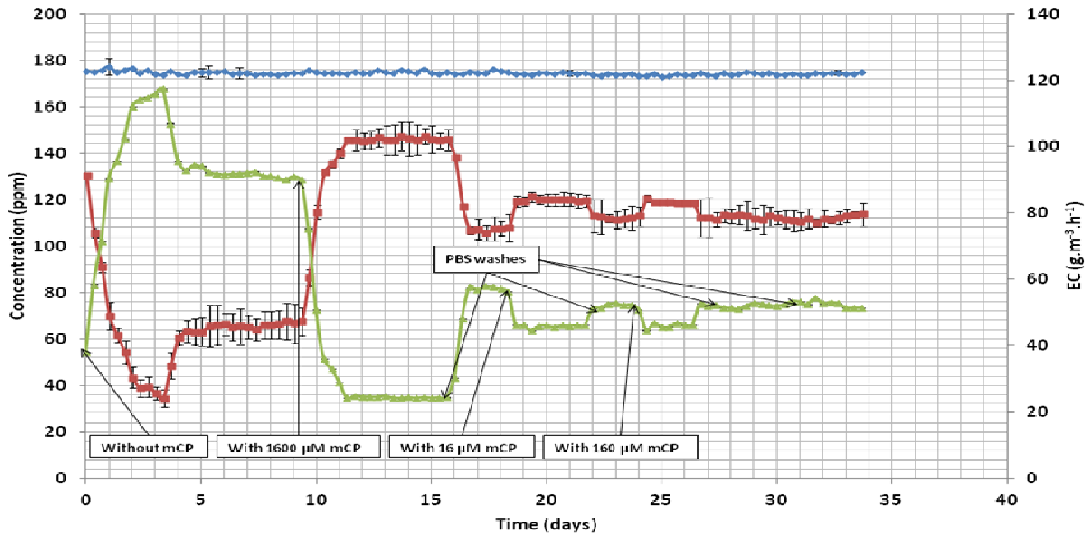


Figure 5.9: Effect of m-chlorophenol on toluene degradation rate in differential biofilter reactor with soil. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. Error bars are the standard deviation between the multiple sample injections in GC.

5.3.2.8 Overall summary

The overall effect of all seven metabolic uncouplers used in the current study is summarised and compared in a graphical representation in Fig. 5.10. It is clearly evident that the initial steady EC were ranged between 40 and 105 $g.m^{-3}.h^{-1}$ for the soil samples tested. In theory, the toluene degradation rate was expected to rise in presence of a metabolic uncoupler due to less efficient ATP production and when the metabolic uncoupler was removed decrease to the initial EC value. However none of the metabolic uncouplers used in this study followed this pattern. Though PCP, TCP and FCCP increased the EC, none of these responded reversibly following PBS washes. However, compared to PCP and TCP, the FCCP approached a classical uncoupler response by

showing signs of reversibility following PBS washes. But it did not increase the EC significantly like PCP and TCP. Benzoic acid, malonic acid and m-chlorophenol inhibited the biodegradation activity of the toluene degraders at all the concentrations tested. Though CCCP and FCCP have similar physiochemical properties their responses were totally opposite. It was also observed from the results that, on top of uncoupling, PCP, TCP and FCCP might have killed the non toluene degraders in the soil whereas other metabolic uncouplers tested might have killed the potential toluene degraders at the concentration tested. However, this hypothesis is hard to prove in mixed culture systems like soil. A possible explanation for the difference in the response of metabolic uncouplers in batch mode and continuous mode is the batch mode experiments were conducted at unsteady state conditions. Hence by testing all seven metabolic uncouplers in a pure culture system similar to the current study will further help to understand their uncoupling mechanism clearly.

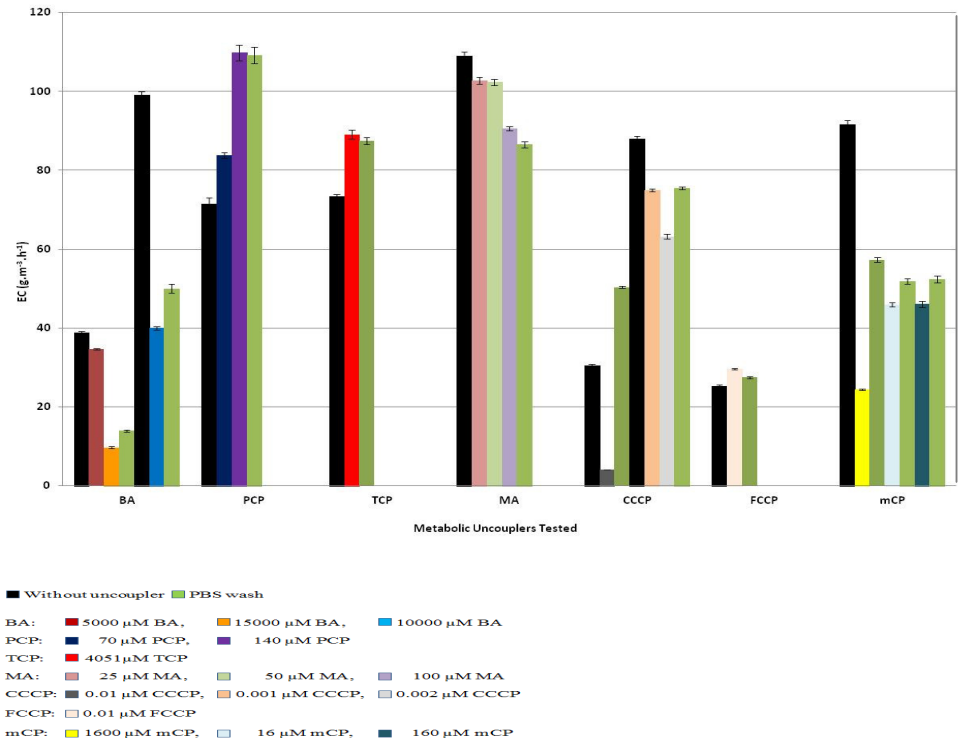


Figure 5.10: Overall results showing the effect of seven metabolic uncouplers on toluene EC in a continuous biofilter reactor with soil. Error bars are standard deviations. Bars under each metabolic uncoupler represent the order in which the experiments are carried out.

Another observation made from these experiments is, though same source of soil was used, a huge variability in the initial steady state EC was observed. A possible reason for this response is the difference in the packing density and biofilm loading which might have contributed to the variation in biodegradation. An additional observation made from the responses of seven metabolic uncouplers was, their different pKa values showed nearly nil influence on the biodegradation. Especially the pKa of CCCP and FCCP are same, however their responses were observed different. This clearly shows that the role of pKa was not significant. Though all the metabolic uncoupler solutions were buffered at pH 7 and used in the experiment, the pKa of these chemicals were expected to play a role in uncoupling mechanism (Hiraishi and Kawagishi, 2002). But it was clear from the results that none responded according to theory. Especially by comparing the pKa values of two uncouplers PCP (pKa: 4.7) and TCP (pKa: 7.5), which increased the EC significantly, it is very clear that pKa value did not influence the biodegradation. However, without knowing the internal pH and local external pH of the toluene degraders, it is hard to conclude the pKa effect on biodegradation and uncoupling mechanism in experimental systems like ours.

5.4 Conclusions

It was observed from the initial screening studies in serum bottle that in a 60 hr period, pentachlorophenol, benzoic acid, p-nitrophenol, 2, 4, 6 trichlorophenol and m-chlorophenol increased the toluene degradation rate by 40% compared to the control soil with toluene degraders and 200% compared to the control soil without toluene degraders. The rest of the uncouplers did not work as efficient as those one reported above. Hence the batch mode serum bottle studies helped to select the potential uncouplers in a short time for the further screening studies in continuous mode. From the screening studies conducted in continuous reactor, it was observed that the increase was less than 50%. Moreover, only PCP and TCP increased the EC significantly when compared with FCCP. None of these three metabolic uncouplers behaved reversibly as a classical uncoupler though FCCP showed closer signs of reversibility following PBS washes. In addition the metabolic uncoupler solutions (PCP and TCP) assayed following the experimentation showed decreased concentration when compared with the initial concentration tested. This may be either due to the lower solubility of these chemical which might have caused some residual amount of these metabolic uncouplers to stay in the soil even after multiple

PBS washes. Other metabolic uncouplers tested did not increase the EC and were inhibited the EC. However among these, the response of CCCP was totally different when compared to other 6 uncouplers tested. The EC increased for CCCP tested soil following PBS washes which was higher than the initial EC. Other than the possibilities of lower diffusivity of CCCP (which is not known), the reason for this response is unclear. Overall the response of metabolic uncouplers in growth mode (batch) and maintenance mode (continuous) was clearly distinguished. Since, the major intention of the current research is to increase the maintenance requirement of the toluene degraders in continuous mode and thereby to increase the specific substrate degradation rate, conducting similar studies in pure cultures of toluene degraders in pure culture biofilm reactors will help further to understand the exact biology of the effect of all these metabolic uncouplers.

5.5 References

Ariya, P.A., Nepotchatykh, O., Ignatova, O., Amyot, M., 2002, Microbiological degradation of atmospheric organic compounds. Geophysical Research Letters 29, 2077-2081.

Devinny, J.S., Deshusses, M.A., Webster, T.S., 1999, Biofiltration for air pollution control. CRC Press LLC, New York, USA.

Hiraishi, A., Kawagishi, T., 2002. Effects of chemical uncouplers on microbial biomass production, metabolic activity, and community structure in an activated sludge system. Microbes and Environments 17, 197-204.

Hiraishi, A., Kawagishi, T., 2002, Effects of chemical uncouplers on microbial biomass production, metabolic activity, and community structure in an activated sludge system. Microbes and Environments 17, 197-204.

Lou, P.H., Hansen, B.S., Olsen, P.H., Tullin, S., Murphy, M.P., Brand, M.D., 2007, Mitochondrial uncouplers with an extraordinary dynamic range. The Biochemical Journal 407, 129-140.

Low, E.W., Chase, H.A., Milner, M.G., Curtis, T.P., 2000, Uncoupling of metabolism to reduce biomass production in the activated sludge process. Water Research 34, 3204-3212.

Mikesell, M.D., Boyd, S.A., 1988, Enhancement of pentachlorophenol degradation in soil through induced anaerobiosis and bioaugmentation with anaerobic sewage sludge. Environmental Science and Technology 22, 1411-1414.

Muthukumar, K., Bharath, C., Pugalenthi, V., Velan, M., 2009, Biodegradation kinetics of benzoic and anthranilic acids by *Micrococcus sp*. Journal of Scientific and Industrial Research 68, 900-903.

Torkian, A., Dehghanzadeh, R., Hakimjavadi, M., 2003, Biodegradation of aromatic hydrocarbons in a compost biofilter. Journal of Chemical Technology and Biotechnology 78, 795-801.

Uquillas, J.A., Kishore, V., Akkus, O., 2011, Effects of phosphate-buffered saline concentration and incubation time on the mechanical and structural properties of electrochemically aligned collagen threads. Biomedical Materials 6, DOI: 10.1088/1748-6041/6/3/035008.

Verduyn, C., Postma, E., Scheffers, W.A., Van Dijken, J.P., 1992. Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8, 501-517.

Wright, J., 1993, Fungal degradation of benzoic acid and related compounds. World Journal of Microbiology and Biotechnology 9, 9-16.

Yalkowsky, S.H., He, Y., 2003, Handbook of aqueous solubility data. CRC, Danvers, USA.

Chapter 6: Isolation, Characterization and Preservation of Toluene Degraders from Soil

6.1 Introduction

The biodegradation effectiveness of a biofilter hugely depends on the microbial population present in the biofilter bed (Jeong et al., 2006). Bacteria and fungi are the most dominant groups of microorganisms contributing to the biodegradation of organic pollutants in a biofilter. However, bacteria are more advantageous than fungi due to their rapid growth rate and degradation rate (Adebusoye et al., 2007; Okamoto et al., 2003; Wang et al., 2008). The biodegradation mechanisms taking place in a biofilter are normally aerobic and are considered energetically favourable (Leson and Winer, 1991). Hence it is essential to understand the biodegradation capacity of different microbial species present in natural environments like soil, water, etc., In addition, it is also important to find an organism which can adapt to the non-natural system which uses the pollutant as the sole energy source. Moreover, to develop a pure biofilm reactor for treating particular gaseous pollutants (e.g. toluene), these studies are prerequisite. This chapter will discuss the isolation of toluene degraders from soil and will also discuss the primary and secondary characterization of different isolated toluene degraders along with the methods used in preserving the isolated strains.

6.2 Experimental methods

6.2.1 Isolation of toluene degraders

Reactor 3 was setup with 8.65 g (wet weight) of soil as described earlier in chapter 3. An inlet toluene concentration of 180 ppm (average) was maintained in the reactor. At steady state an outlet concentration of 132 ppm (average), a steady EC of 40 $g.m^{-3}.h^{-1}$ was observed after 30 days. Following this, the reactor was dismantled in a sterile environment and the soil was removed. A 1 g sample of this soil was used for a serial dilution. A standard serial dilution procedure (MacLowry et al., 1970) was followed and a 1 ml sample from 10^{-5} dilution was used to inoculate the agar plates using the spread plate method (Buck and Cleverdon, 1960). A control agar plate without any sample was also used in the experiment. A minimal salt medium (Shen et al., 1998) with 1.5% agar was used to prepare all the agar plates. Toluene was used as a sole carbon and energy

source for growth with the MS medium. Plates were incubated at 30 ^{o}C in a 5 litre glass desiccators containing a 100 mL beaker with 1% liquid toluene in Vacuum Pump Oil (vpo)[1] (Evans et al., 1991) with an approximate head space toluene concentration of 430 ppm. The plates were observed every day for growth and a fresh 1% liquid toluene in vpo was replaced daily.

6.2.2 Identification of the toluene degraders

Isolated toluene degraders were individually subjected to Gram staining technique (Hucker and Conn, 1923) as a primary identification step and were observed both in phase contrast and scanning electron microscopy. As a secondary identification technique, all the isolated toluene degraders were individually sub-cultured in Luria Bertani (LB) agar plates (Clermont et al., 2000) and then the over-night cultures were sent to Eco Gene Ltd (Auckland, New Zealand) for 16s rDNA and 18s rDNA analysis.

6.2.2 Preservation of the toluene degraders

Following the secondary identification of the isolated toluene degraders, they were sub-cultured in duplicates. One set of grown plates were stored in -4 ^{o}C for short term preservation and the other set of grown plates[2] were used to prepare 100 mL liquid cultures in LB media. 5 mL of the overnight culture was added to a 5 mL of 40% glycerol in a 15 mL sterile screw cap centrifuge tube and stored in both -20 ^{o}C and -80 ^{o}C deep freezers for long term preservation.

6.3 Results and discussion

6.3.1 Isolation

Colonies were observed in plates 1, 4 and 5 after 8 days whereas in plates 2 and 3 colonies were seen after 13 days. This difference can be attributed to the different toluene degrading metabolisms involved in different species, growth rates, induction pathways and possibly the initial concentration in the serial dilution. In addition the response of microorganisms in a synthetic medium is always different when compared

[1] Densities of toluene and vpo are nearly same (0.86 g.cm^{-3} for toluene and 0.85 g.cm^{-3} for vpo)

[2] Single colony was picked for inoculation.

to a natural media like soil, compost etc. (Pirbazari et al., 1990). However, the control plate displayed no growth and hence the contamination issue was eliminated though it is not most important in the current scenario. Figure 6.1 shows the colonies in five agar plates along with the control agar plate subjected to toluene as a sole carbon and energy source. Based on visual identification, a total of six different colonies were picked and sub-cultured (purified) further in LB agar plates. The streak plate technique was adopted and the experiment was repeated to obtain pure individual isolates of the toluene degraders.

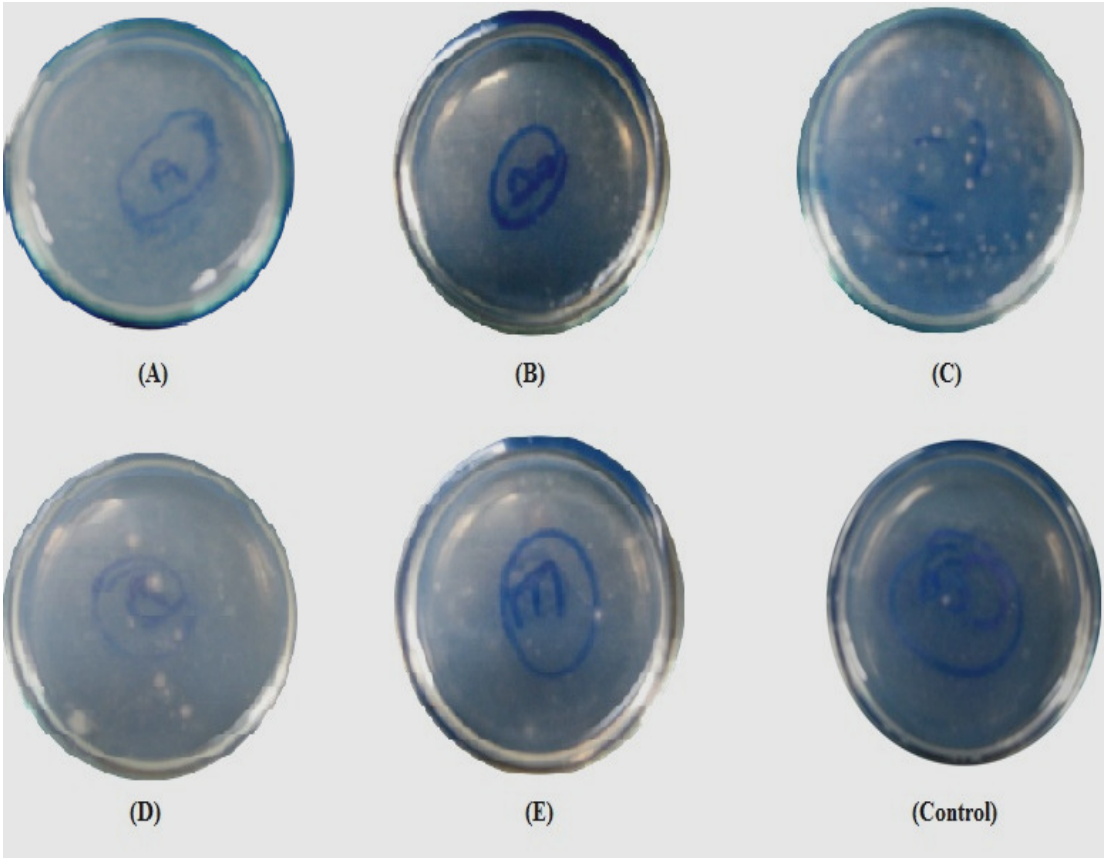
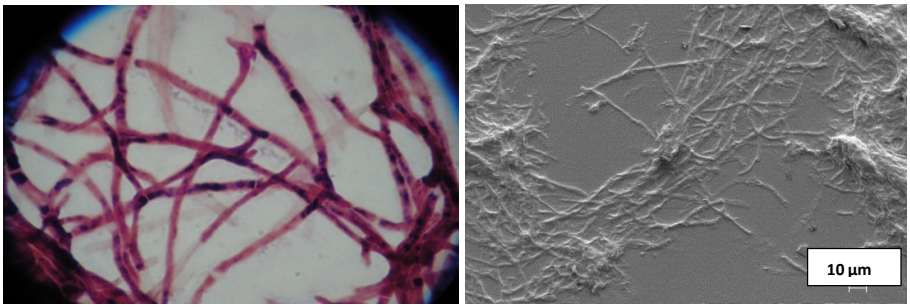


Figure 6.1: Growth of isolated toluene degraders on MS agar plates (A-E) along with control in desiccator equilibrated with toluene-vpo mixture containing 1% toluene. The plates were photographed after 2 weeks of incubation.

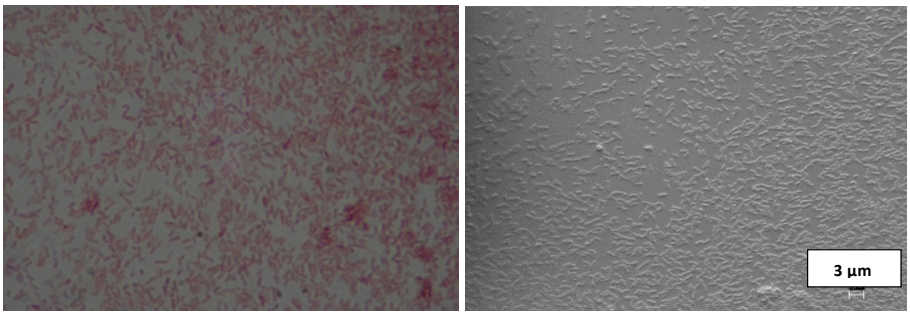
6.3.2 Primary identification

Four out of the five isolated toluene degraders were found to be rod shaped organisms in which three were Gram negative and one was Gram positive. The fifth isolate was found

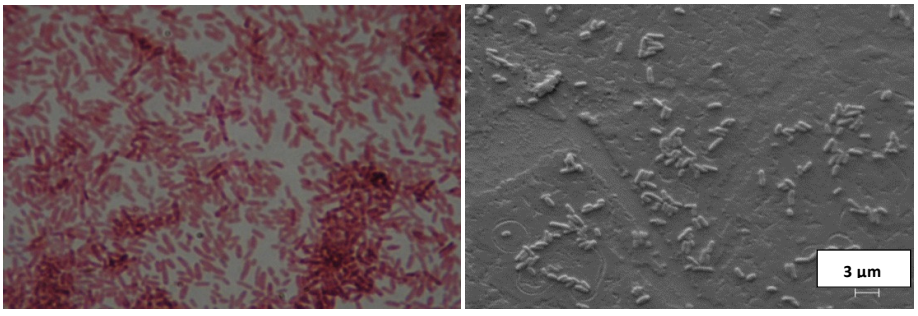
to be a fungus based on the morphology. The shapes (morphology) of all five isolates were further studied through scanning electron microscopy (SEM) (Appendix E) and compared to the results obtained from phase contrast microscopy following the Gram staining experiment. Figure 6.2 compares both the phase contrast and SEM results of 5 toluene degrading strains which were subjected to primary identification technique. The phase contrast images clearly shows whether the isolated organism is gram positive or negative and the SEM images clearly shows the size & shape of the isolated organisms.



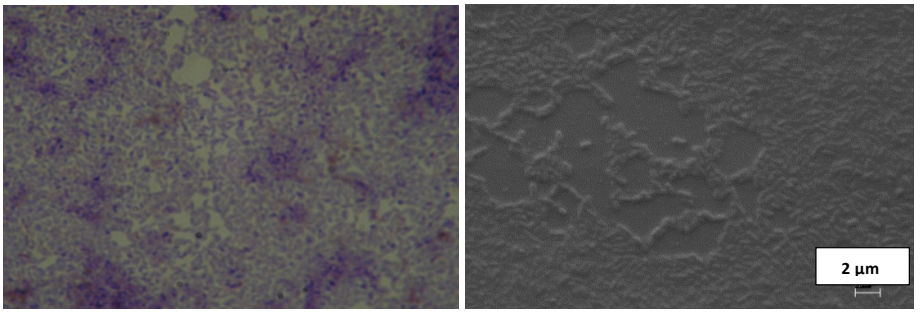
(A_P) **(A_S)**



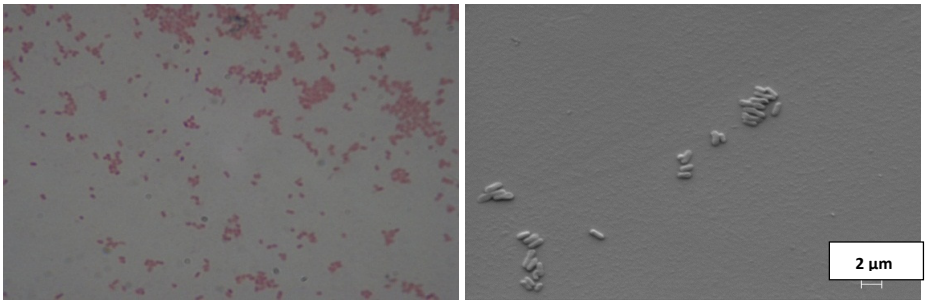
(B_P) **(B_S)**



(C_P) **(C_S)**



(D_P) **(D_S)**



(E_P) **(E_S)**

Figure 6.2: A_P-E_P are the phase microscopy images (100X) of five different toluene degraders subjected to gram staining. A_S-E_S are the corresponding SEM images. (A_S: 500X; B_S to E_S: 1000X) of those five isolates.

6.3.3 Secondary identification

Following the primary identification, the five isolates were subjected to taxonomical identification through amplification and sequencing of the 18s rDNA for the fungus and 16s rDNA for the four bacterial isolates. The sequences were compared with the database of known 18s rDNA and 16s rDNA sequences through blast search for identification (Fig. 6.3) (Appendix B).

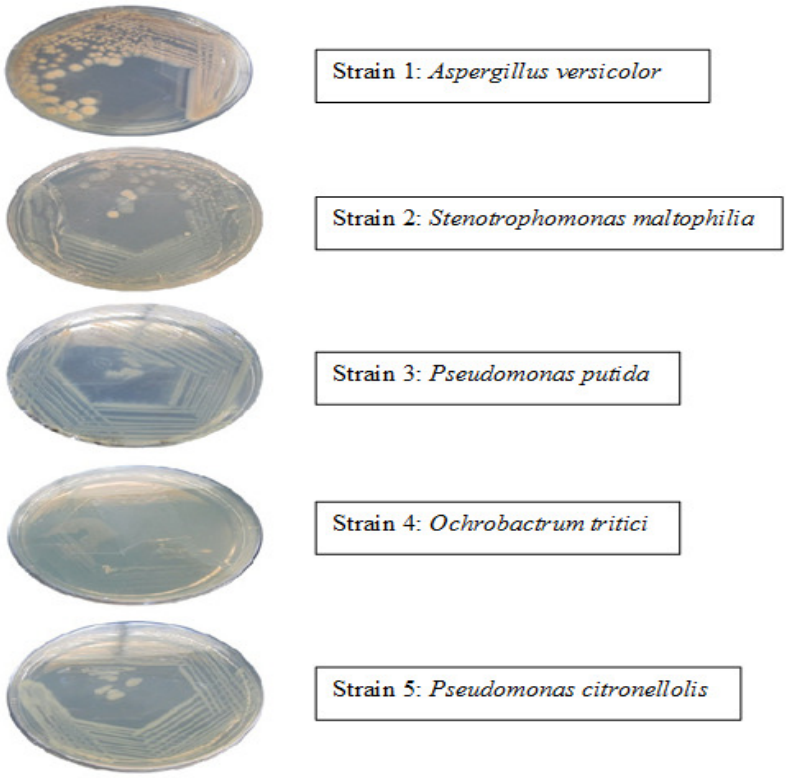


Figure 6.3: Five different toluene degrader strains identified using 18s rDNA and 16s rDNA sequencing studies by Eco Gene Ltd (Auckland, NZ).

Out of these five identified toluene degraders, three were already reported in literature and the other two were not reported elsewhere as potential toluene degraders. However, only *Pseudomonas putida* was studied extensively and reported to follow the toluene degradation (TOD) catabolic pathway to degrade toluene (Del Castillo and Ramos, 2007; Lee et al., 1995; Marqués and Ramos, 2006) and the pathways used by the other isolated strains to degrade toluene are unknown. In the TOD pathway, toluene is first oxidized to cis-toluene dihydrodiol through the action of toluene dioxygenase (TDO). Cis–toluene dihydrodiol is dehydrogenated to form 3-methyl catechol which is cleaved at the ortho position and then converted in three steps to form acetaldehyde and pyruvate before entering the tri-carboxylic acid (TCA) cycle (Fig. 6.4). Table 6.1 summarises the maximum toluene biodegradation percentage reported for the three potential toluene degraders isolated along with the other two isolated (non-reported) ones.

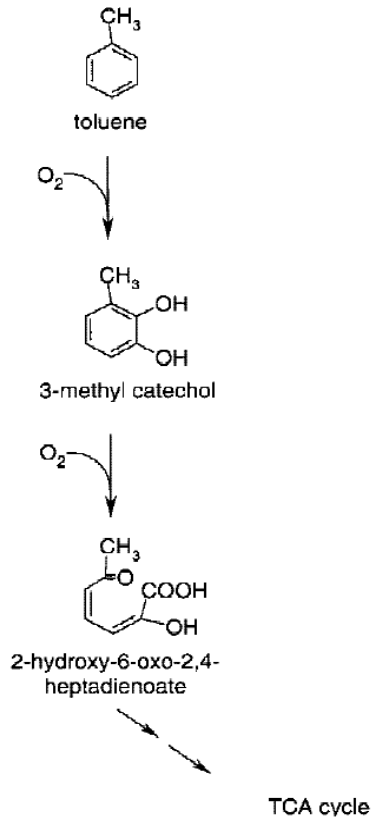


Figure 6.4: TOD pathway followed by *Pseudomonas putida* during toluene degradation (Reardon et al., 2000).

Table 6.1: Reported maximum toluene degradation for the isolated strains

Toluene degrader	**Maximum toluene biodegradation percentage reported (%) (RE)**	**Reference**
Stenotrophomonas maltophilia	83	(Lee et al., 2002)
Pseudomonas putida	95	(Men and Cheng, 2011)
Aspergillus versicolor	97	(Prenafeta-Boldú et al., 2012)
Ochrobactrum tritici	Unknown	-
Pseudomonas citronellolis	Unknown	-

The strains *Ochrobactrum tritici* and *Pseudomonas citronellolis* are the first to be isolated from New Zealand soil and hence both the strains were deposited in the NZ culture collection maintained by International Collection of Microorganisms from Plants (ICMP), New Zealand. ICMP accession number of 19448 and 19447 were given to these two strains *Ochrobactrum tritici* and *Pseudomonas citronellolis*. Later as per the request[3] from ICMP the other three isolated toluene degraders *Stenotrophomonas maltophilia*, *Pseudomonas putida* and *Aspergillus versicolor* were also deposited in ICMP with accession numbers 19446, 19449 and 19445. In addition to the deposition, all the five isolates were preserved in -4 ^{o}C for short term application and in -20 ^{o}C, -80 ^{o}C deep freezers for long term application.

6.4 Conclusions

Though toluene degraders are commonly found in soil and many people have already isolated and characterized them from soil, most of them are outside New Zealand. Since there are lots of time consuming procedures in purchasing pure cultures from microbial culture collections in and outside New Zealand, instead of purchasing the pure cultures, they were isolated from soil. The soil used was exposed to the outlet concentration of 132 ppm of toluene for nearly 30 days in the biofiltration reactor. Primary identification of the isolated toluene degraders through Gram staining and SEM analysis gave an insight into the morphology of the isolated species. Secondary identification using 16s rDNA and 18s rDNA amplification and sequencing studies helped to identify the taxonomy of five potential toluene degrading stains. Only *Pseudomonas putida, Stenotrophomonas maltophilia* and *Aspergillus versicolor* were reported as a toluene degrader in the literature and the other two isolates *Ochrobactrum tritici* and *Pseudomonas citronellolis* are novel toluene degraders. In addition, these newly reported toluene degraders were deposited in NZ culture collections maintained by ICMP, NZ. Following secondary identifications all the five toluene degraders were preserved under -4 ^{o}C, -20 ^{o}C and -80 ^{o}C for future experimentation to develop a pure biofilm reactor for toluene degradation.

[3] Our research group is the first to report these strains as potential toluene degraders in New Zealand.

6.5 References

Adebusoye, S.A., Picardal, F.W., Ilori, M.O., Amund, O.O., Fuqua, C., Grindle, N., 2007, Aerobic degradation of di-and trichlorobenzenes by two bacteria isolated from polluted tropical soils. Chemosphere 66, 1939-1946.

Buck, J.D., Cleverdon, R.C., 1960, The spread plate as a method for the enumeration of marine bacteria. Limnology and Oceanography 5, 78-80.

Clermont, O., Bonacorsi, S., Bingen, E., 2000, Rapid and simple determination of the *Escherichia coli* phylogenetic group. Applied and Environmental Microbiology 66, 4555-4558.

Del Castillo, T., Ramos, J.L., 2007, Simultaneous catabolite repression between glucose and toluene metabolism in *Pseudomonas putida* is channeled through different signaling pathways. Journal of Bacteriology 189, 6602-6610.

Evans, P., Mang, D., Kim, K.S., Young, L., 1991, Anaerobic degradation of toluene by a denitrifying bacterium. Applied and Environmental Microbiology 57, 1139-1145.

Hucker, G., Conn, H., 1923, Methods of Gram staining. Technical Bulletin of the New York State Agricultural Experimental Station 23, 1-23.

Jeong, E., Hirai, M., Shoda, M., 2006, Removal of p-Xylene with *Pseudomonas sp*. NBM21 in biofilter. Journal of Bioscience and Bioengineering 102, 281-287.

Lee, E.Y., Jun, Y.S., Cho, K.S., Ryu, H.W., 2002, Degradation characteristics of toluene, benzene, ethylbenzene, and xylene by *Stenotrophomonas maltophilia* T3-c. Journal of the Air & Waste Management Association 52, 400-406.

Lee, J.Y., Jung, K.H., Choi, S.H., Kim, H.S., 1995, Combination of the TOD and the TOL pathways in redesigning a metabolic route of *Pseudomonas putida* for the mineralization of a benzene, toluene, and p-xylene mixture. Applied and Environmental Microbiology 61, 2211-2217.

Leson, G., Winer, A.M., 1991, Biofiltration - an innovative air-pollution control technology for VOC emissions. Journal of the Air & Waste Management Association 41, 1045-1054.

MacLowry, J.D., Jaqua, M.J., Selepak, S.T., 1970, Detailed methodology and implementation of a semiautomated serial dilution microtechnique for antimicrobial susceptibility testing. Applied Microbiology 20, 46-53.

Marqués, S., Ramos, J.L., 2006, Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. Molecular Microbiology 9, 923-929.

Men, J., Cheng, F., 2011, Biodegradation and growth characteristics of a toluene-degrading strain. African Journal of Biotechnology 10, 13299-13306.

Okamoto, K., Izawa, M., Yanase, H., 2003, Isolation and application of a styrene-degrading strain of *Pseudomonas putida* to biofiltration. Journal of Bioscience and Bioengineering 95, 633-636.

Pirbazari, M., Voice, T.C., Weber Jr, W.J., 1990, Evaluation of biofilm development on various natural and synthetic media. Hazardous Waste and Hazardous Materials 7, 239-250.

Prenafeta-Boldú, F.X., Guivernau, M., Gallastegui, G., Viñas, M., Hoog, G.S., Elías, A., 2012, Fungal/bacterial interactions during the biodegradation of TEX hydrocarbons (toluene, ethylbenzene and p-xylene) in gas biofilters operated under xerophilic conditions. FEMS Microbiology Ecology. DOI: 10.1111/j.1574-6941.2012.01344.x.

Reardon, K.F., Mosteller, D.C., Bull Rogers, J.D., 2000, Biodegradation kinetics of benzene, toluene, and phenol as single and mixed substrates for *Pseudomonas putida* F 1. Biotechnology and Bioengineering 69, 385-400.

Shen, Y., Stehmeier, L.G., Voordouw, G., 1998, Identification of hydrocarbon-degrading bacteria in soil by reverse sample genome probing. Applied and Environmental Microbiology 64, 637-645.

Wang, C., XI, J.Y., HU, H.Y., WEN, X.H., 2008, Biodegradation of gaseous chlorobenzene by white-rot fungus *Phanerochaete chrysosporium*. Biomedical and Environmental Sciences 21, 474-478.

Chapter 7: Development of Biofilm Reactor for Metabolic Uncoupler Studies

7.1 Introduction

The major advantage of the current differential biofiltration reactor is its simple and effective configuration. Unlike most biofilm reactors, which operate with saturated biofilms (Ergas and McGrath, 1997; Kumar et al., 2008a; Kumar et al., 2008b; Kumar et al., 2009), this reactor system is suitable to examine unsaturated biofilms with less mass transfer resistance between the pollutant stream, biofilm, as the contaminant can transfer directly from the gas phase to the biofilm, with only water and dissolved species (uncouplers, etc.) exchanging across the membrane. Moreover, in regards to practical operation, excess biomass can be more easily controlled with direct access to the biofilm for physical or chemical treatment. Furthermore, addition and removal of metabolic uncouplers in the current reactor system is very easy through external and internal reservoirs while still maintaining constant water content.

Applications of pure cultures in biofiltration have not been well explored due to the practical difficulties in maintaining the aseptic environment throughout the operation. So far, only a few studies have reported the successful use of metabolic uncouplers in pure cultures (Low and Chase, 1998; Neijssel, 1977; Saini and Wood, 2008; Verduyn et al., 1992). However, these reports are based on growth systems and not in non-growth systems like biofiltration. Moreover, they were used only to control the excess biomass growth and not in increasing the biodegradation rate. Conducting metabolic uncoupler studies with a biofilm (pure culture) will further elucidate their impact on the specific substrate degradation rate. Moreover, it is preferable to use the pure cultures as a biofilm instead of soil or compost for testing the metabolic uncouplers in the differential biofiltration reactor because of the ease in calculating the change in the maintenance energy.

7.2 Biofilms

The term biofilm can simply and broadly be defined as communities of microorganisms that are attached to a surface (Geesey et al., 1977). Biofilms can either be a single microbe or multiple microbial species and can form on a variety of surfaces through extracellular

polymeric substances (EPS). Approximately 97% of the biofilm matrix contains water, which is bound to the capsules of microbial cells (Singh et al., 2006). Water binding capacity and mobility of the biofilm limits the diffusion process occurring inside the biofilm. In addition to EPS, absorbed nutrients, proteins, cell lysis products, particulate material and detritus from the immediate surrounding environment can also be present in a biofilm (Hans-Cur, 1995). Biofilms can be broadly classified into two types: saturated biofilms and unsaturated biofilms. A saturated biofilm is in an environment that has only liquid and solid phase. Whereas, the unsaturated biofilm has an extra phase in the form of gas, which is normally the main phase (Holden, 2001). Usually the mass transport in a biofilm is influenced by the biofilm structure, which again depends on the local availability of substrates. Solute transport in a biofilm is driven by convective transport within pores and water channels and also through diffusion in the denser aggregates. Thus the biofilm matrix shows a high degree of microheterogeneity due to the numerous microenvironments that co-exist within it (Horn and Morgenroth, 2006).

7.2.1 Biofilms in gas phase bioreactors

The potential of biofilm communities for air pollution control technologies (APCTs) was realized during 1980's. However, it is only during the past few decades that biofilm reactors have become a focus of interest for researchers in the field of air pollution control (Paul et al., 2005). The major biofilm reactors are categorized according to the principle they employ, such as the up-flow sludge blanket (USB), biofilm fluidised bed (BFB), expanded granular sludge blanket (EGSB), biofilm airlift suspension (BAS) and internal circulation (IC) methods (Nicolella et al., 2000). Biofilters and biotrickling filters are the most important among APCTs using membrane bioreactors with biofilms for treating waste gases. ECs of up to 397 $g.m^{-3}.h^{-1}$ have been reported for a toluene biodegradation in biofilters using this technology (Jacobs et al., 2003).

This chapter will discuss in detail the development of a biofilm reactor using the selected toluene degraders isolated from soil (section 6.3.1). In addition, it will also discuss extended metabolic uncoupler studies in the biofilm reactor using selected uncouplers from the soil screening studies (section 5.3.2) to understand their influence on the substrate degradation rate. Moreover, the energy uncoupling coefficients in the presence of all tested metabolic uncouplers are estimated.

7.3 Experimental methods

7.3.1 Selection of toluene degraders

Out of the five isolated and identified toluene degraders (section 6.3.3), two isolates *Pseudomonas putida* and *Stenotrophomonas maltophilia* were used as pure biofilms in the differential biofiltration reactor system. The reason behind the selection of these two isolates was based on the reported higher toluene degradation rates of these two bacterial species (Lee et al., 2002; Men and Cheng, 2011). Though another isolate *Aspergillus versicolor* was reported to have higher toluene degradation rate (Prenafeta-Boldú et al., 2012) than the selected two isolates, it was not selected as handling a fungus in our reactor system was considered harder than handling a bacteria due to the risk of membrane clogging in the short term. This is because of the size of fungus and also due to the possibilities of utilizing fungus spores as a host by certain bacterial species (Cruz and Ishii, 2011) which may lead to contamination issues. The other two isolates *Ochrobactrum tritici* and *Pseudomonas citronellolis* were not selected as there was no literature reporting the toluene degradation rates of these two isolates.

The selected two isolates were subjected to growth kinetics study in the presence and absence of toluene. Luria Bertani (LB) medium (Sigma Aldrich, USA) (Appendix B) was used as a growth medium for studies carried out in the absence of toluene. Whereas, minimal salt (MS) medium was used (Sigma Aldrich, USA) (section 6.2.1) with toluene (90 ppm) as the sole carbon source. A UV/Vis spectrophotometer (Shimadzu 1500, Kyoto, Japan) was used for optical density (OD) measurements at 600 nm.

7.3.2 Selection of metabolic uncouplers

Based on the screening studies conducted in soil (section 5.3.2), three potential metabolic uncouplers were selected for further studies in the biofilm reactor: pentachlorophenol (PCP), 2,4,6-trichlorophenol (TCP) and carbonylcyanide m-chloromethoxy-phenylhydrazone (CCCP). However, though carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP) responded as a metabolic uncoupler in the soil studies, it was not selected for further studies with the biofilms due to its insignificant influence on the EC. All three uncouplers were buffered with PBS (pH: 7.0) and autoclaved before using in the

reactor. Table 7.1 shows the concentrations of the three selected metabolic uncouplers tested in the biofilm reactor.

Table 7.1: Metabolic uncouplers tested in the biofilm reactor.

Metabolic Uncoupler	Concentration(s) tested (μM)
pentachlorophenol (PCP)	140 (maximum solubility)
2,4,6 trichlorophenol (TCP)	4.05×10^3 (maximum solubility)
carbonylcyanide p-chloromethoxy phenylhydrazone (CCCP)	0.001

7.3.3 Biofilm reactor loading

Reactors 1, 3 and 4 were used for the biofilm experiments. The procedure for setting up the reactors was similar to the method described in Ch. 3. However instead of soil, a biofilm was added to the membrane. Late logarithmic phase cultures[1] of *Pseudomonas putida* and *Stenotrophomonas maltophilia* were used for forming the respective biofilms. During initial experiments, 20 mL of the culture was used and later it was increased to 50 mL to increase the biofilm density. In addition during initial studies with 20 mL cultures, a nitrogen source (0.05 M sodium nitrate) was added in the liquid reservoir of the reactor to enhance further growth on the membrane, which was later not used for 50 mL cultures. The nitrogen source was replaced by a phosphate buffered saline (PBS) solution during the later studies. By placing the cells directly onto the membrane (0.0043 m^2 working surface area), the lag phase and biofilm establishment phases were shortened. The excess growth medium was drained away by the applying a negative matric potential. The wet cell weight (WCW) and dry cell weight (DCW) of the inoculum were measured using standard procedures (Doran, 1995). In addition the weight of the fresh membrane was also measured before adding the biofilm to it (which was used later in calculating the WCB/DCW of the biofilm post-experiment). The complete loading procedure was performed in a sterile environment to avoid contamination issues in the biofilm

[1] Grown under non-toluene environment in LB shake flasks (100 rpm) overnight at 30 ^{o}C.

development. Inlet toluene concentration of 180 ppm, 225 ppm and 250 ppm was used for reactors 4, 3 and 1 respectively throughout the experimentation.

7.3.4 Surface elimination capacity (SEC)

In order to estimate the toluene removal rates in a biofilm reactor, EC was replaced by the surface elimination capacity (SEC) which normalised the degradation by the total biofilm area rather than the biofilm volume. The SEC was calculated from Eq.7.1.

$$SEC = \frac{(C_{in}-C_{out})Q}{A_m} \ (g.m^{-2}.h^{-1}) \quad (7.1)$$

7.3.5 Scanning electron microscopy (SEM) and confocal microscopy studies

After each metabolic uncoupler study with a biofilm ended, the biofilm was removed carefully along with the membrane and a portion of it was subjected to SEM and confocal analysis. A Leica S440 (Wetzlar, Germany) SEM and a Leica TCS SP5 confocal microscope (Wetzlar, Germany) (Appendix E) were used to observe the samples at standard magnifications. Polaron 5000 sputter coater was used as conducting carbon paint in SEM analysis (Richards and Turner, 1984). The fluorescent dye acridine orange (120 µM) was used to stain the sample used for confocal analysis (Møller et al., 1996).

7.4 Results and discussion

7.4.1 Growth kinetics studies

Growth curves of *Pseudomonas putida* and *Stenotrophomonas maltophilia* grown in the LB medium (without toluene) were generated (Fig. 7.1). A maximum specific growth rate of 0.073 h^{-1} with a doubling time of 9.47 h was observed for *P. putida* and for *S. maltophilia* a maximum specific growth rate of 0.074 h^{-1} with a doubling time of 9.41 h was observed. This study was conducted to identify the late growth phase of each of these 2 isolates in order to load them in the biofilm reactor. Though the doubling time of *S. maltophilia* is comparable with the literature value of 8 h doubling time (Emerson and Moyer, 1997), the observed doubling time for *P. putida* was well higher than the reported literature value of 2 h doubling time (Kurbatov et al., 2005). This may be because of different *P. putida* sub-strain used in the current study.

Initial attempts to prepare bioreactor inoculums from the isolates with toluene as the sole carbon/energy source failed. No growth was observed even after 15 days in the flasks which were inoculated with these two species. Hence 1 mL of LB medium was added to both the flasks on 15th day which stimulated the growth of these two species. However, no optical density (OD) measurements were carried out for this experiment and only the visual inspection of the turbidity was done. It was decided to stop the experiment and the cultures were stored at -4 ^{o}C until they were used in the biofilm reactor.

For the initial experiments with metabolic uncouplers in the biofilm reactor, the isolates which were grown and maintained under a non-toluene environment were used by supplementing with a nitrogen source (0.05 M sodium nitrate) in the liquid reservoir of the biofilm reactor. However, in later stages only those isolates grown under a toluene environment in MS media were used without supplementing with nitrogen source.

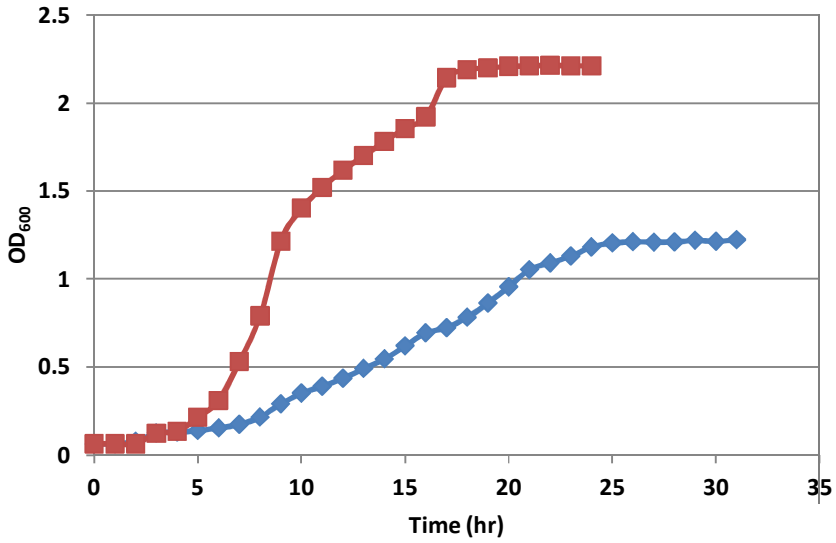


Figure 7.1: Optical density with time of the toluene degraders *P. putida* (■) and *S. maltophilia* (♦) on LB medium.

7.4.2 Studies on 2, 4, 6-trichlorophenol (TCP)

The reactor was started with an initial dry cell weight (DCW) of 18.2 ± 0.1 mg.m^{-2} of *P. putida* with 1X PBS. After 2 days, a nitrogen source (0.05 M sodium nitrate in PBS) was added to the system by replacing the PBS which increased the SEC (Fig. 7.2a). However,

this increase was related to the growth of *P. putida* in presence of the nitrogen source. After 15 days, the nitrogen source was removed from the reactor and replaced with 1X PBS (0.05 M PBS) (Appendix B). After a steady SEC of 0.20 $g.m^{-2}.h^{-1}$on day 18, TCP (in PBS at pH 7.0) at a concentration of 4051 µM replaced the PBS. The SEC started to drop steeply and when the SEC was 0.08 $g.m^{-2}.h^{-1}$, the TCP solution was removed from the system and two PBS washes were performed. However, the SEC did not improve and stayed at 0.07 $g.m^{-2}$.h (Fig. 7.2a). TCP at 4051 µM had a negative, irreversible effect on the toluene SEC for *P. putida.* This response is inconsistent with the simple metabolic uncoupling model (Ch. 2.2). By comparing this response with the TCP response in the soil studies (Fig. 5.5), two possible hypothesis can be made;

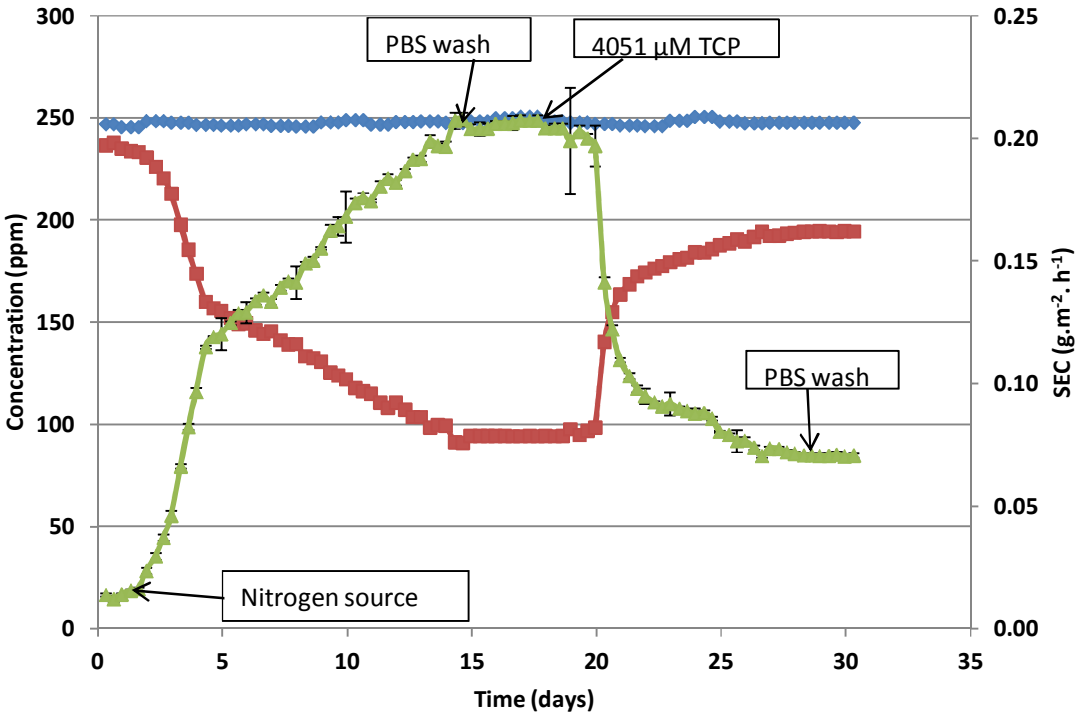
1. The increase in the soil EC by 18% following the addition of 4051 µM of TCP in soil studies compared to the permanent decrease in SEC in this experiment was because toluene degraders other than *P. putida* might have played a dominant role in toluene degradation in soil studies.
2. *P. putida* does not respond the same to TCP in pure culture as it does in a mixed culture (Der Yang and Humphrey, 2004).
3. The TCP concentration experienced by the cells might have been different in pure culture due to partitioning differences (soil sorption, etc.) leading to cell lysis.

In addition, there may be some unknown mechanism such as programmed cell death (PCD) (Kroemer et al., 1995) microbial starvation etc., which might have contributed to the different response for TCP in soil and pure biofilm studies. However, based on the *P. putida* biofilm studies, it is clear than TCP is not a potential uncoupler for *P. putida*. Following this study, the biofilm was removed and the DCW was measured and found to be 33.7 $mg.m^{-2}$. This increase in the biomass was accounted for the growth stimulated initially with nitrogen source.

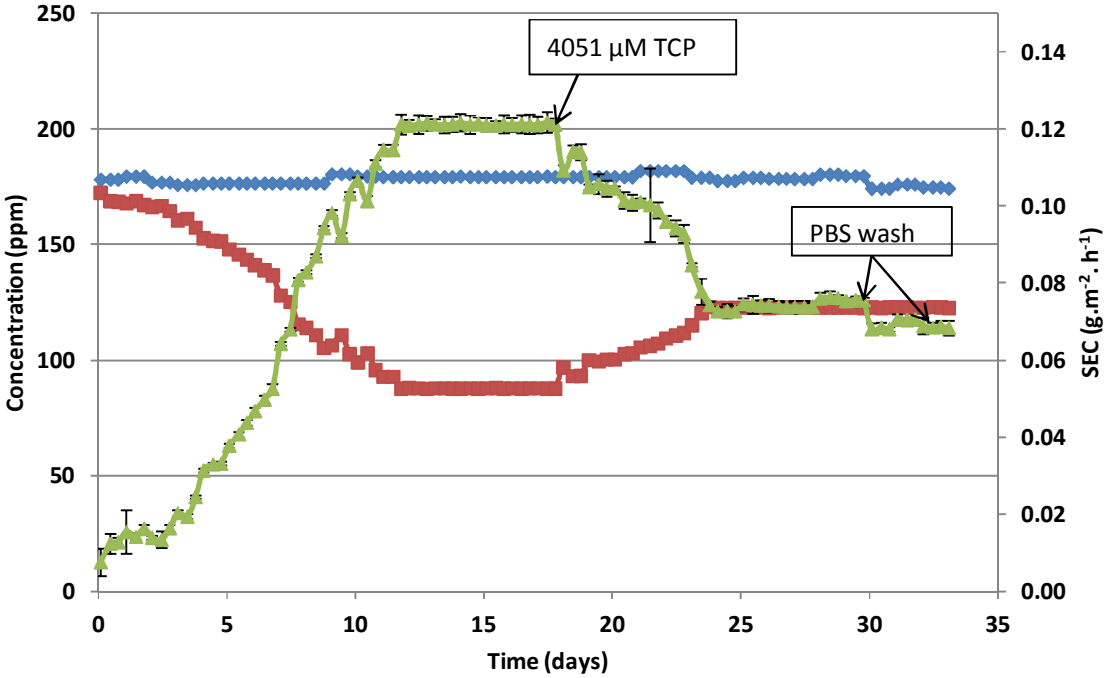
The experiment was repeated with a fresh biofilm of *S. maltophilia* with initial DCW of 13.9 $mg.m^{-2}$. However, this time no nitrogen source was supplied to the system as the inoculum used in the study was grown with toluene and moreover 50 mL of culture was used this time for generating the biofilm when compared with 20 mL culture which was used in the earlier study. After 11 days, a steady SEC of 0.12 $g.m^{-2}.h^{-1}$ was observed (Fig. 7.2b) and following this 4051 µM TCP was added to the reactor by replacing the PBS. A similar response when compared to the earlier studies in *P. putida* was observed. SEC

dropped steadily and reached a new steady state value of 0.08 $g.m^{-2}.h^{-1}$. PBS replaced the TCP on the 28th day. However, no recovery in SEC was observed. The hypothesis which was explained earlier for the similar response of TCP in *P. putida* is valid for *S. maltophilia* also.

Hence based on these similar results, TCP was not considered further as a potential metabolic uncoupler for studies in either *P. putida* or *S. maltophilia*. Following this study, the *S. maltophilia* biofilm was removed and the DCW was measured and found to be 49.2 $mg.m^{-2}$. This 350% increase in the biomass may be due to the secretion of stress induced extra polysaccharides (EPS) on the biofilm. However, without running a suitable control biofilm reactor in parallel with the biofilm reactor with TCP, this hypothesis cannot be validated. Moreover, no further study was conducted to prove this as TCP was not found to be a potential uncoupler. Another interesting observation made from these results is, though *P. putida* is found in most environmental samples grown up on toluene, they may not be the dominant degraders but just the easy growers on artificial media (Pirbazari et al., 1990).Table 7.2 summarizes the initial and final DCW measured for *P. putida* and *S. maltophilia* biofilms subjected to TCP.



(a)



(b)

Figure 7.2: Effect of TCP on toluene degradation rate in biofilm reactor. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. **(a)** Biofilm of *Pseudomonas putida* **(b)** Biofilm of *Stenotrophomonas maltophilia*. Error bars are standard deviations.

Table 7.2: Comparison of initial and final DCW under TCP influence for two pure culture biofilms.

Metabolic Uncoupler	Biofilm	Inoculum Volume (mL)	Supplemental nitrogen addition	Initial DCW ($mg.m^{-2}$)	Final DCW ($mg.m^{-2}$)
TCP	*P. putida*	20	Yes	18.2	33.7
	S. maltophilia	50	No	13.9	49.2

7.4.3 Studies on carbonylcyanide p-chloromethoxy phenylhydrazone (CCCP)

The reactor was started with an initial DCW of 18.9 $mg.m^{-2}$ of *P. putida* with 1X PBS. Addition of nitrogen source increased the SEC and after 8 days when a significant SEC was reached (0.16 $g.m^{-2}.h^{-1}$), the nitrogen source was removed from the reactor and PBS was replaced (Fig. 7.3). Once a steady SEC of 0.16 $g.m^{-2}.h^{-1}$ was observed, PBS was replaced with 0.001 µM CCCP. Following the addition of CCCP, the SEC increased 19% to a steady value of 0.19 $g.m^{-2}.h^{-1}$. On the 16^{th} day, CCCP was replaced with PBS and the SEC started to drop slowly and reached a steady SEC of 0.07 $g.m^{-2}.h^{-1}$ after 17 days. This decrease in SEC was found to be 56% lower than that of the initial SEC.

Though the response of CCCP in the current biofilm study with a reversible increase in SEC follows the simple uncoupler model, the reason for large drop in the SEC (below the initial SEC) following the removal of the CCCP is unclear. A possible explanation for this response is the active biofilm which was subjected to a stress following the addition of CCCP might have lost its stress induced activity following the removal of CCCP and hence the SEC might have decreased.

The overall response of CCCP in *P. putida* biofilm was completely opposite to CCCP studies in soil (Fig. 5.7). In soil studies, CCCP addition decreased the EC and the subsequent PBS wash improved the EC beyond the initial EC. However in the current biofilm study the response was totally opposite (Fig. 7.3). This suggests the possibilities of the earlier hypothesis (Ch 5.4) of lower diffusivity value of CCCP as a factor of influence in soil studies is true, because the thickness of soil (4000 µm) was larger than that of the biofilm (130 µm).

Following this study, the biofilm was removed and the DCW was measured and found to be 30.7 $mg.m^{-2}$. This 62% increase in the biomass was accounted for by the initial growth stimulated by nitrogen source on top of possible secretion of stress induced polysaccharides by the biofilm. Though CCCP showed some interesting results when compared to TCP, experiments to measure the loss of CCCP during the experiment were not done as no proper CCCP assay has been reported. A CCCP assay following the experiment might have given an idea about its influence on *P. putida* in enhancing the SEC in addition to the metabolic uncoupling. However, drop in the SEC following the

removal of CCCP indicated that the increase in SEC after the addition of CCCP was not due to the growth.

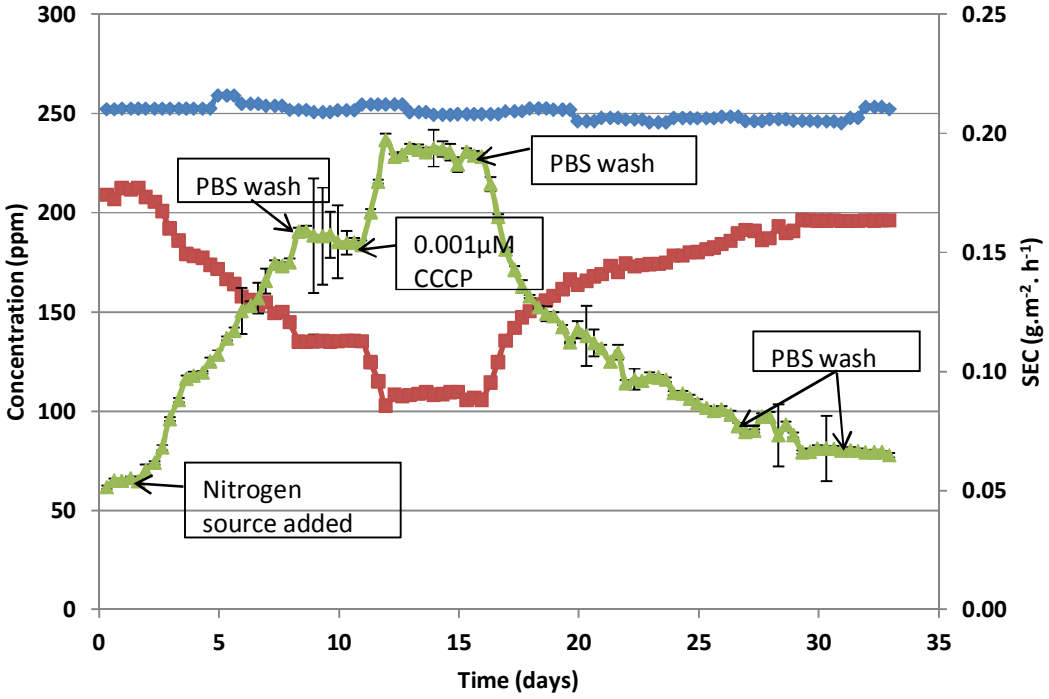


Figure 7.3: Effect of CCCP on toluene degradation rate in a *P. putida* biofilm reactor. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are SEC (Cycle 1 experiment). Error bars are standard deviations.

The experiment was replicated with *P. putida* at a higher initial DCW of 70.3 mg.m^{-2} of with 1X PBS. However, this time no nitrogen source was supplied to the system, as the inoculum used in the study was grown under a toluene environment. After a steady SEC of 0.15 g.m^{-2}.h^{-1} (Fig. 7.4), PBS was replaced with 0.001 µM CCCP on the 15th day. Following the addition of CCCP, the SEC dropped steeply. Though this response was not the same in regards to the initial dynamics as before, 10 days after CCCP addition, a steady higher SEC of 0.19 g.m^{-2}.h^{-1} was observed. This increase in SEC was 27% higher than that of the initial SEC.

Multiple PBS washes lowered the SEC but it remained above its initial value. The SEC did not collapse after the PBS wash like in Fig. 7.3. Following this study, the biofilm was

removed and the DCW was measured and it increased by 67% to 117.3 mg.m^{-2}. The same explanation provided for the increase in biomass for the earlier experiment is valid for this repeat experiment as well. Though the results of this repeat experiment are not exactly the same with the previous one, in both experiments CCCP increased the steady state SEC and after PBS washes the SEC dropped. These similar responses indicate that CCCP has uncoupler potential, but further experiments are required, especially to determine if the inoculation history (growth on toluene or complex carbon/energy) explains the response post-PBS wash.

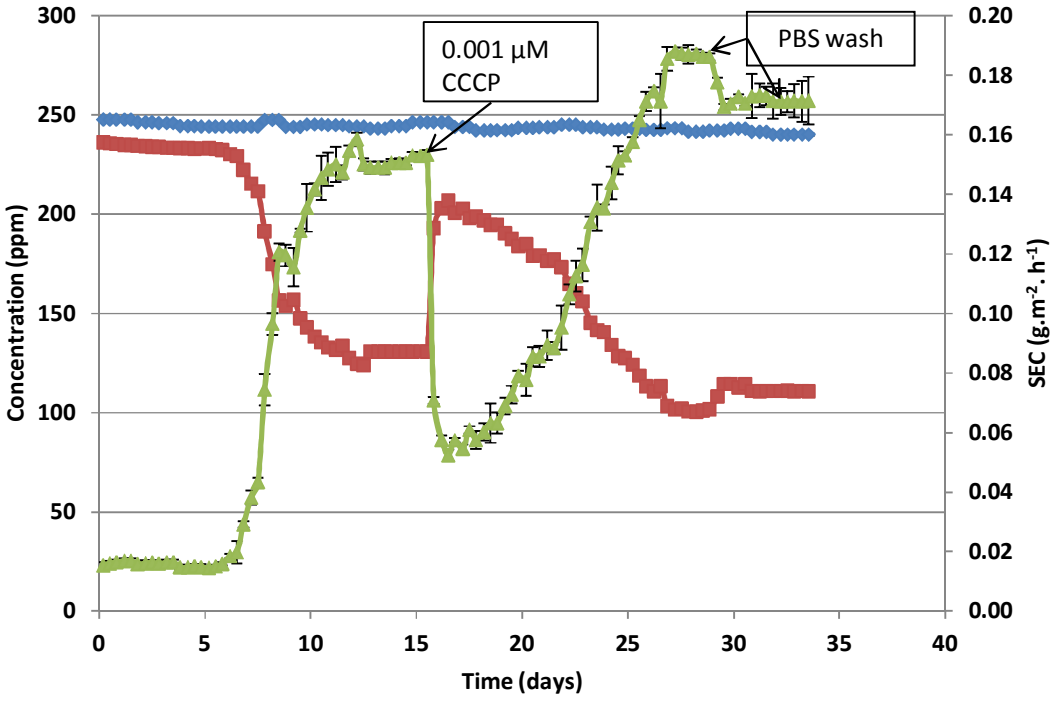
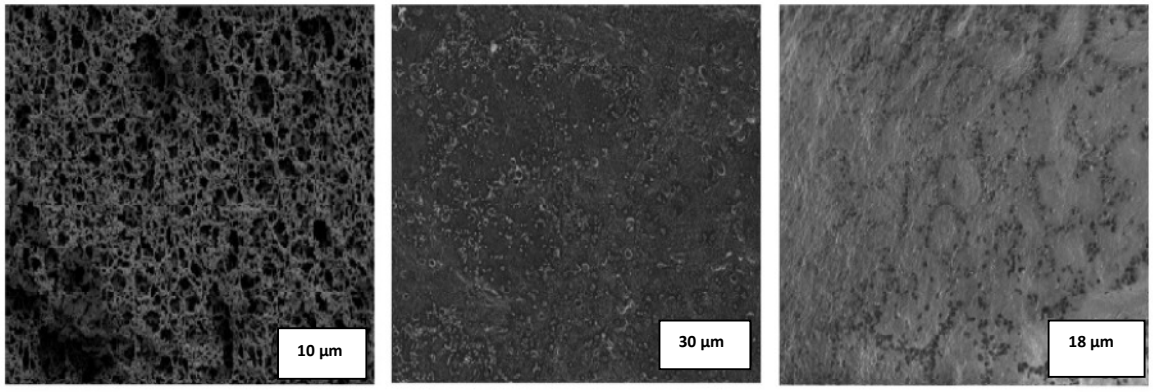


Figure 7.4: Effect of CCCP on toluene degradation rate in a *P. putida* biofilm reactor. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are SEC (Cycle 2 experiment). Error bars are standard deviations.

The biofilms used in these two experiments were observed under SEM and confocal microscopy to further understand the structure of the biofilms and to observe the possible production of stress induced EPS (due to CCCP addition). A fresh membrane (Mixed Cellulose Ester, A045A090C, Advantec MFS, USA) was used as a control for SEM analysis (Fig. 7.5a) and a membrane with fresh *P. putida* was used as a control for confocal

analysis (Fig. 7.6a and 7.6b). SEM results showed that the biofilm surface was rough with air interfaces/cavities (Fig. 7.5b and 7.5c). Earlier biofilm work also reported similar biofilm surface characteristics and claimed that rougher the interface, the larger is the area exposed for gas phase. In addition it was reported that more cavities enhanced substrate and oxygen transfer through the biofilm (Holden et al., 1997; Studer and Rudolf von Rohr, 2008). In addition to biofilm structure analysis in SEM, the biofilm thickness was also measured and found that the thickness varied between 100 µm and 160 µm (average). This variation was due to the difference in the volume of culture used between the cycles (20 mL for earlier with final SEC of 0.07 $g.m^{-2}.h^{-1}$ and 50 mL for later with final SEC of 0.17 $g.m^{-2}.h^{-1}$). This thickness matched the reported biofilm thickness of 71 to 239 µm by Vayenas *et al.* (2002) and 100 µm by England *et al.* (2005).

Confocal microscopy showed clearly the growth of *P. putida* when compared with the control biofilm (where cell density is nearly zero) (Fig. 7.6c and 7.6d). In addition it also showed the clumps of secreted EPS on top of growth. These analyses proved the hypothesis of the increase in the DCW was not only due to growth but also due to the production of stress induced EPS. Conducting EPS studies will help to understand further about this stress induced EPS in detail.



(a) **(b)** **(c)**

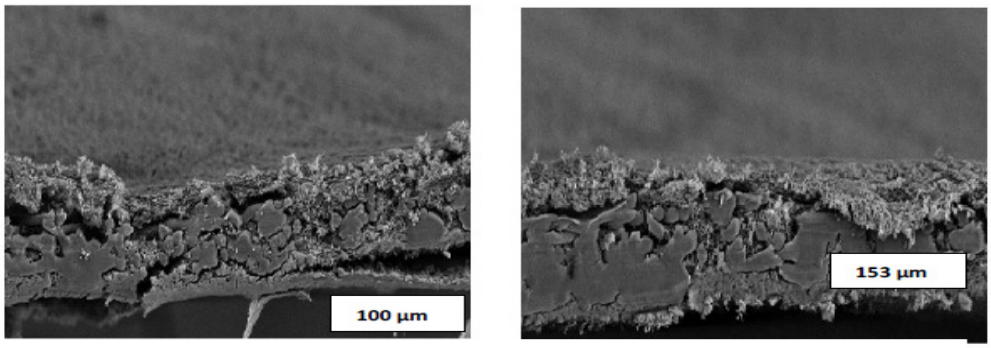


Figure 7.5: SEM pictures of biofilm subjected to CCCP studies **(a)** Control membrane **(b)** Top surface of the biofilm of *P. putida* (Cycle 1) **(c)** Top surface of the biofilm of *P. putida* (Cycle 2) **(d)** Side of the biofilm of *P. putida* (Cycle 1) **(e)** Side of the biofilm of *P. putida* (Cycle 2).

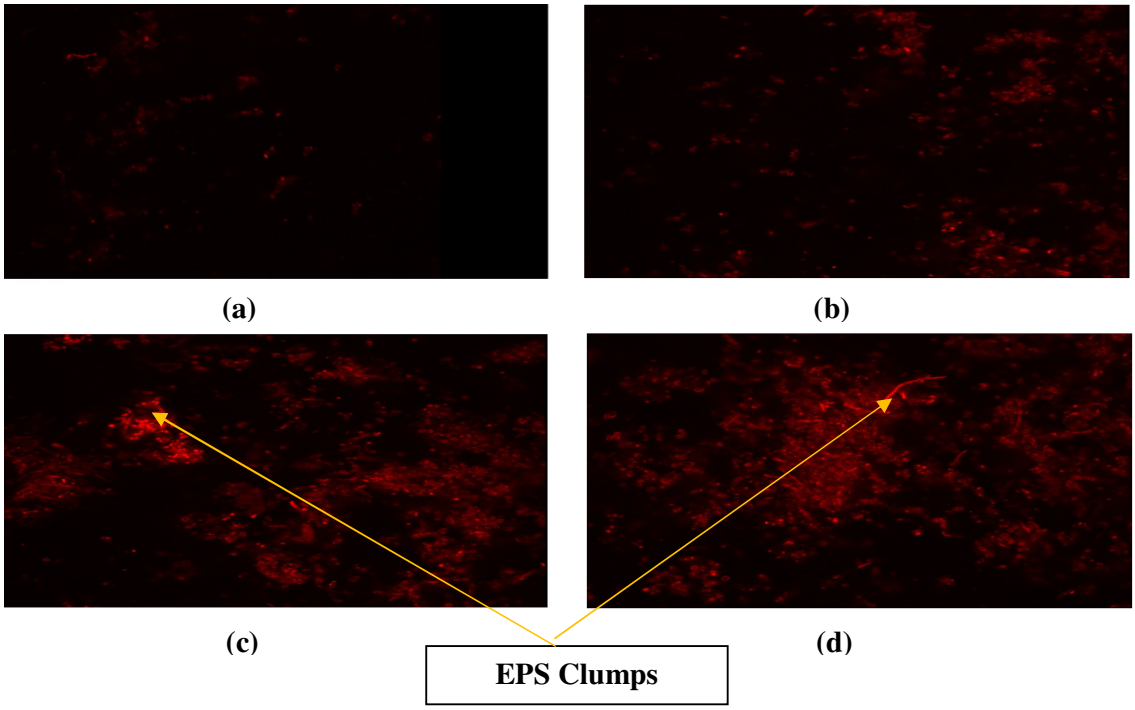


Figure 7.6: Confocal pictures (500X magnification) of biofilm subjected to CCCP studies **(a)** Control-Fresh biofilm of *P. putida* (18.9 $mg.m^{-2}$ DCW) (Cycle 1) **(b)** Control-Fresh biofilm of *P. putida* (70.3 $mg.m^{-2}$ DCW) (Cycle 2) **(c)** *P. putida* biofilm subjected to CCCP (30.7 $mg.m^{-2}$ DCW) (Cycle 1) **(d)** *P. putida* biofilm subjected to CCCP (117.3 $mg.m^{-2}$ DCW) (Cycle 2).

The influence of CCCP on a *S. maltophilia* biofilm, was tested in the biofilm reactor. The reactor was started with an initial DCW of 7.2 $mg.m^{-2}$ of *S. maltophilia* with PBS. A nitrogen source was added to increase the SEC and after 10 days a significant SEC was reached (0.15 $g.m^{-2}.h^{-1}$), the nitrogen source was removed from the reactor and replaced with PBS (Fig. 7.7). The switch to PBS did not influence the SEC and the steady state SEC remained at 0.15 $g.m^{-2}.h^{-1}$. On 13^{th} day of the run, PBS was replaced with 0.001 µM CCCP, which had no effect on the EC. After 10 days, CCCP was removed and PBS was replaced, and the SEC did not change. A slight drop in SEC was observed following the removal of CCCP which later came back to the earlier SEC value (Fig. 7.7). Explanation for this nil response of CCCP for *S. maltophilia* include the concentration tested might not be high enough for *S. maltophilia* to initiate uncoupling mechanism.

Following this study, the biofilm was removed and the DCW was measured and found to be 24.7 $mg.m^{-2}$. This increase in the biomass was attributed to growth stimulated initially with the nitrogen source in addition to secreted EPS. However, no further SEM or confocal studies were done as 0.001 µM CCCP was not a potential uncoupler for *S. maltophilia*. Based on soil studies (section 5.3.2.5), CCCP concentrations higher than 0.001 µM were not conducted but arguably higher concentrations might worth testing on *S. maltophilia*. Table 7.3 summarizes the initial and final DCW measured for *P. putida* and *S. maltophilia* biofilms subjected to CCCP.

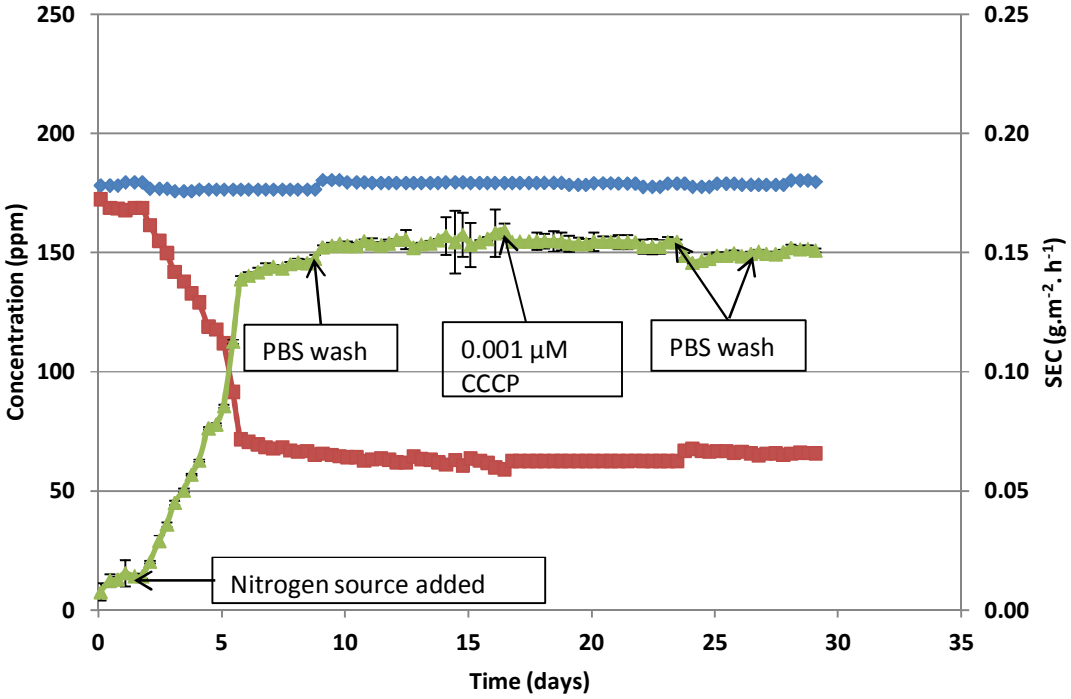


Figure 7.7: Effect of CCCP on toluene degradation rate in *S. maltophilia* biofilm reactor. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. Error bars are standard deviations.

Table 7.3: Comparison of initial and final DCW under CCCP influence

Metabolic Uncoupler	Biofilm	Inoculum Volume (mL)	Supplemental nitrogen addition	Initial DCW ($mg.m^{-2}$)	Final DCW ($mg.m^{-2}$)
CCCP	*P. putida* (Cycle 1)	20	Yes	18.9	30.7
	P. putida (Cycle 2)	50	No	70.3	117.3
	S. maltophilia	20	Yes	7.2	24.7

7.4.4 Studies on pentachlorophenol (PCP)

The reactor was started with an initial DCW of 18.2 $mg.m^{-2}$ of *P. putida* with 1X PBS. After 7 days, a nitrogen source (0.05 M sodium nitrate) was added to the system by replacing the PBS which increased the SEC. After 18 days, the nitrogen source was removed from the reactor and replaced with 1X PBS. After a steady SEC of 0.20 $g.m^{-2}.h^{-1}$ was achieved, PCP at a concentration of 140 µM was added to the system by replacing the PBS. The SEC started to drop steeply and reached a steady SEC of 0.09 $g.m^{-2}.h^{-1}$ after 47 days. When compared with the initial steady state SEC, this new SEC was 55% lower. Following this, PCP was removed from the system and PBS was replaced. However, the SEC did not improve and stayed constant (Fig. 7.8) indicating PCP was not a viable uncoupler for *P. putida* at this concentration. By comparing this response with the PCP response in the soil studies (Fig. 5.4), two possible explanations were proposed.

1. The increase in EC by 35% following the addition of 140 µM in soil studies was because toluene degraders that could be uncoupled by PCP played a key role in toluene degradation in soil studies.
2. It is very clear from the PCP biofilm studies that in presence of PCP the SEC dropped but at the same PCP concentration in the soil studies, EC increased. Therefore, PCP might have killed the other toluene degraders/non-toluene degraders and the nitrogen released by the dead cells was utilised by those other active toluene degraders which tolerated the PCP in the soil to grow.

Following this study, the biofilm was removed and the DCW was 50.1 $mg.m^{-2}$. This increase in the biomass was accounted for by the growth stimulated initially with nitrogen source on top of possible secretion of EPS.

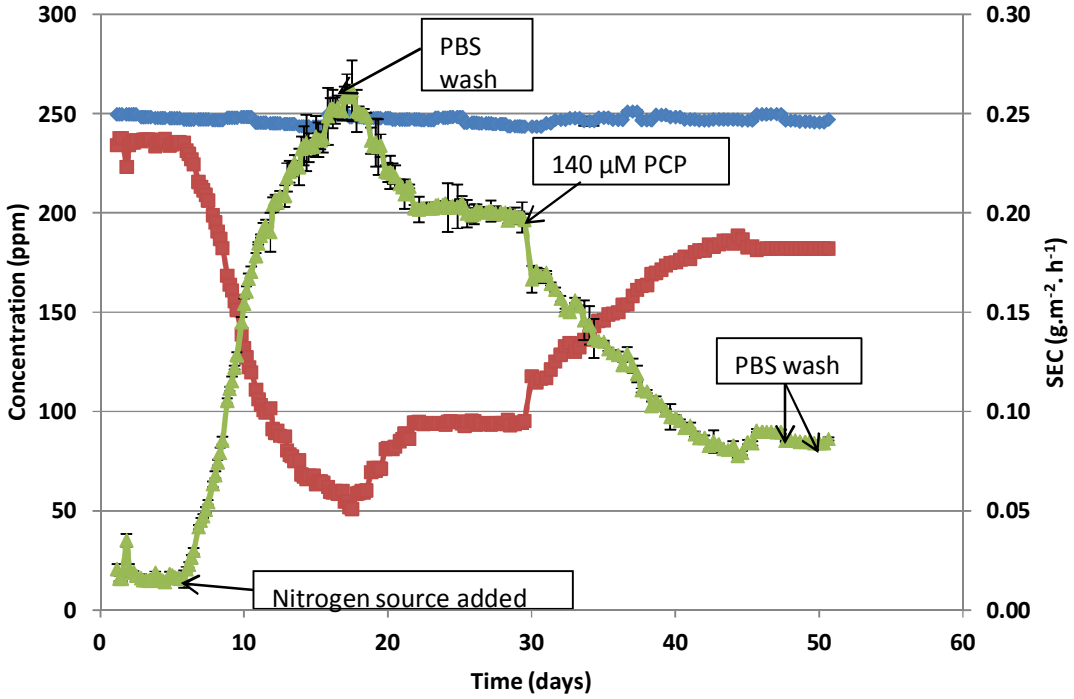


Figure 7.8: Effect of PCP on toluene degradation rate in *P. putida* biofilm reactor. Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. Error bars are standard deviations.

The influence of PCP on *S. maltophilia* was tested in the biofilm reactor. The reactor had an initial DCW of 5.4 $mg.m^{-2}$ of *S. maltophilia* with 1X PBS. Addition of the nitrogen source increased the SEC and after 10 days when a significant SEC was reached (0.16 $g.m^{-2}.h^{-1}$), the nitrogen source was removed from the reactor and PBS was replaced (Fig. 7.9). A steady SEC of 0.13 $g.m^{-2}.h^{-1}$ was observed after 29 days. Following this, a 140 µM PCP solution was added to the reactor replacing the PBS. The SEC dropped initially following the addition of PCP and it reached a maximum steady SEC of 0.24 $g.m^{-2}.h^{-1}$. When compared with the earlier steady state SEC, this new SEC was 85% higher. Following this, PCP was removed and PBS wash was performed which dropped the SEC back to the initial steady state SEC. This response was a classical uncoupler response. This response of PCP was similar with its response in soil studies. PCP behaved in a similar fashion by increasing the removal rate in both soil studies and in biofilm studies. However, the PBS wash in soil

studies did not reverse the PCP uncoupler effect in soil (Fig. 5.4), but it did in the *S. maltophilia* biofilm study (Fig. 7.9).

To test the response, 140 µM PCP was again added to the system which responded in a similar fashion and increased the SEC. However this time the steady state SEC was 17% lower than before. PBS washes after removing the PCP from the reactor did not return the SEC to the initial steady state SEC.

Overall by comparing the effect of PCP in soil and two pure culture biofilms, there are three possible explanations for the behaviour of PCP in soil,

1) PCP addition might have killed the non-toluene degraders in the soil and the toluene degraders might have grown in addition to the metabolic uncoupling by PCP and generated a higher EC.
2) Removal of PCP might not have reduced the active metabolic activity of the toluene degraders and hence EC did not come back to the initial steady state EC.
3) Removal of PCP might have reduced the activity of the toluene degrader which was active in presence of PCP and the other non-active toluene degraders might have contributed to the EC after the removal of PCP.

However, all three hypothesis needs to be validated in soil. Based on the PCP assay at the end of the PCP studies in soil it was clear that PCP did not induce any growth of active toluene degraders in soil as the PCP loss was only 18% (Tab. 5.3). Similarly in biofilm studies, the PCP loss was observed as 7%. No solid explanation can be provided for this slight PCP loss in the biofilm study. However, running the experiment for more months (say 6 months) will help clarify this issue. Based on two cycles of PCP experiments in *S. maltophilia* and one cycle of PCP experiment in *P. putida* biofilm, it is clearly evident that *S. maltophilia* generated higher SEC and hence it can be concluded that the organism actively involved in toluene degradation in soil screening studies may be *S. maltophilia* and not *P. putida*. In addition, compared to soil and biofilm studies, the effect of PCP was found positive in both cases and hence it can be concluded that PCP can be a potential metabolic uncoupler for toluene degradation.

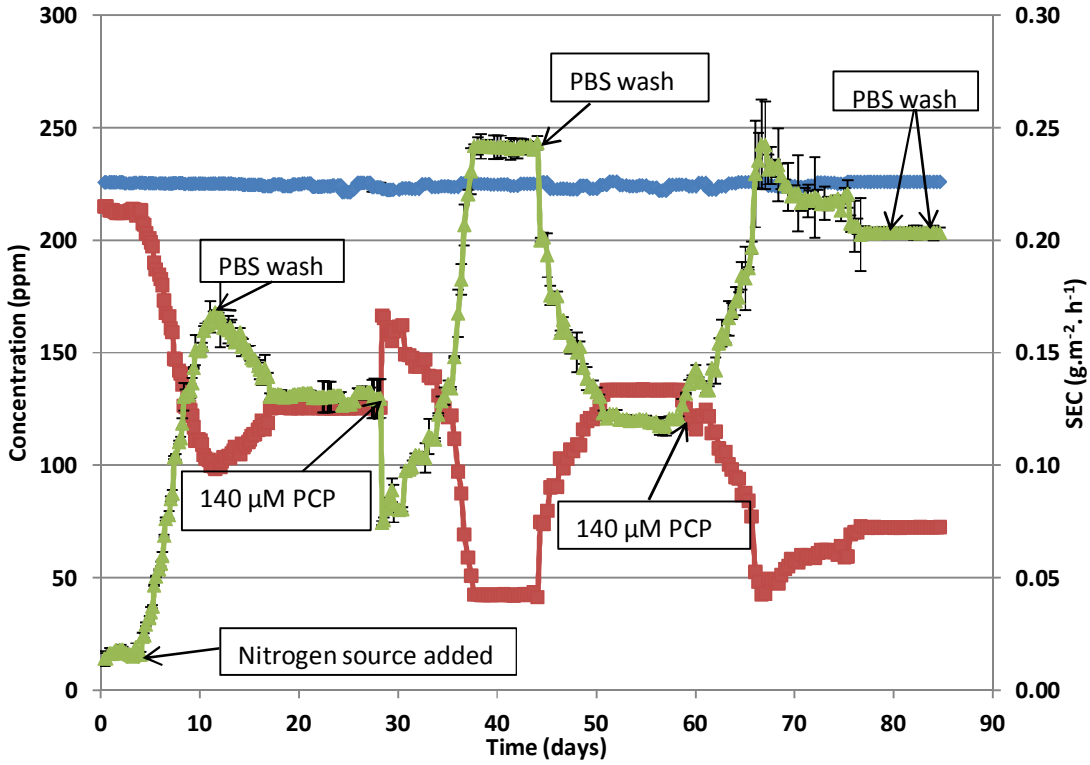
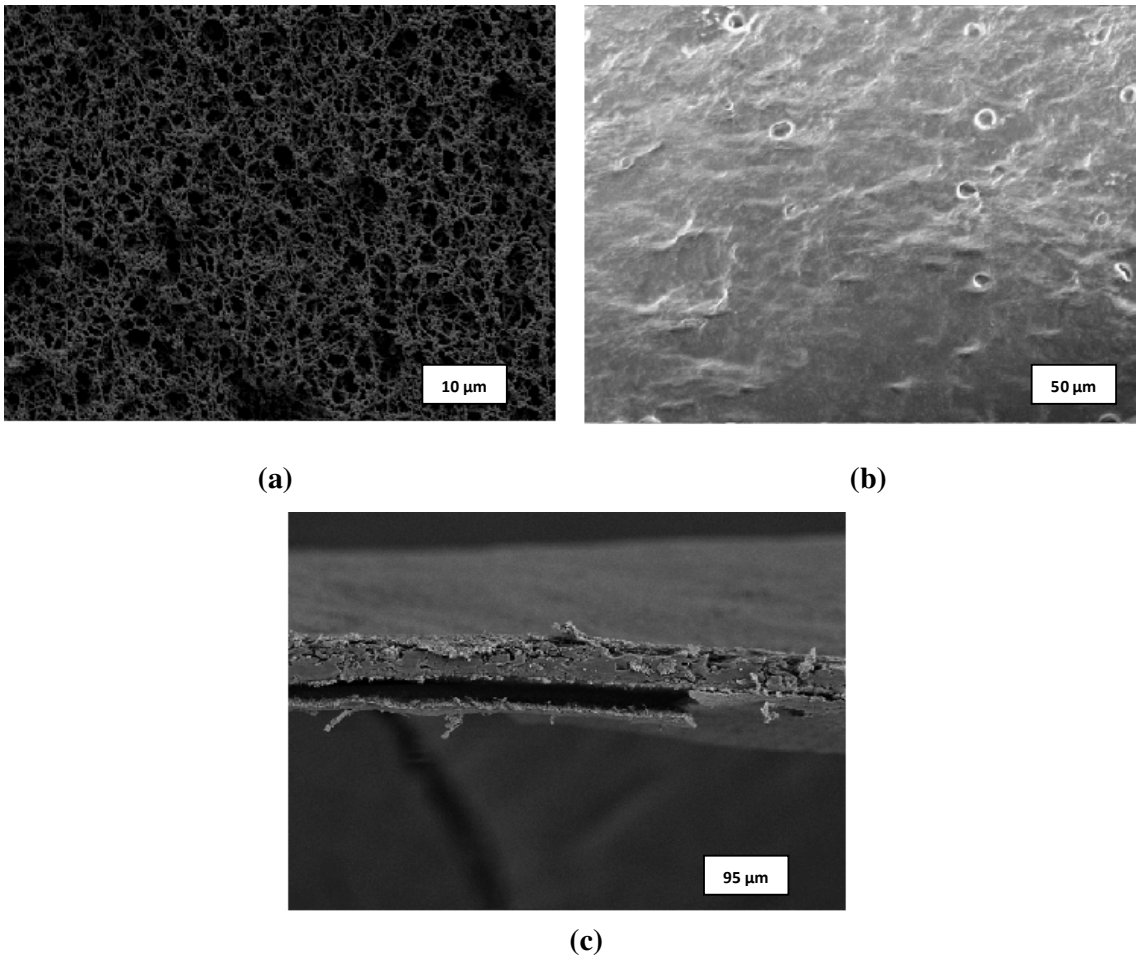


Figure 7.9: Effect of PCP on toluene degradation rate in *S. maltophilia* biofilm reactor (Cycle 1). Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations and closed green triangles (▲) are EC. Error bars are standard deviations.

Following this study, the biofilm was removed and the DCW was measured and found to be 30.8 $mg.m^{-2}$. This 470% increase in the biomass was attributed to nitrogen-induced growth on top of possible secretion of EPS. In order to study the biofilm further, the biofilm was subjected to SEM and confocal analysis. SEM results (Fig. 7.10) matched the observations made earlier with biofilm subjected to CCCP and hence the explanations provided earlier support the current observations also. However, the air interface observed (through SEM at similar magnification) in the biofilm subjected to PCP analysis was at least 2 to 3 times larger than that of the biofilm subjected to CCCP (Figs. 7.5b, 7.5c Vs Fig. 7.10b). This might have caused the higher SEC observed in the biofilm subjected to PCP (0.24 $g.m^{-2}.h^{-1}$ vs. 0.19 $g.m^{-2}.h^{-1}$). As described earlier, the larger the air interface cavity, the larger the mass transfer rate. The biofilm thickness was also analysed and was 70-95 µM (average) which was thinner than the biofilms subjected to CCCP. Table 7.4

summarizes the initial and final DCW measured for *P. putida* and *S. maltophilia* biofilms subjected to PCP.



(a) **(b)**

(c)

Figure 7.10: SEM pictures of S. *maltophilia* biofilm subjected to PCP studies **(a)** Control membrane **(b)** Top surface of the biofilm **(c)** Side of the biofilm.

Table 7.4: Comparison of initial and final DCW under PCP influence

Metabolic Uncoupler	Biofilm	Inoculum volume (mL)	Supplemental nitrogen addition	Initial DCW ($mg.m^{-2}$)	Final DCW ($mg.m^{-2}$)
PCP	*P. putida*	20	Yes	18.2	50.1
	S. maltophilia	20	Yes	5.4	30.8

Studies carried out with the *S. maltophilia* biofilm subjected to confocal microscopy showed clearly an increase in cell density (biomass and EPS) when compared with the control biofilm (Fig. 7.11a and 7.11b). Similar to our earlier studies, these analyses again proved our hypothesis of increase in the DCW was not only due to growth but also due to the production of stress-induced EPS.

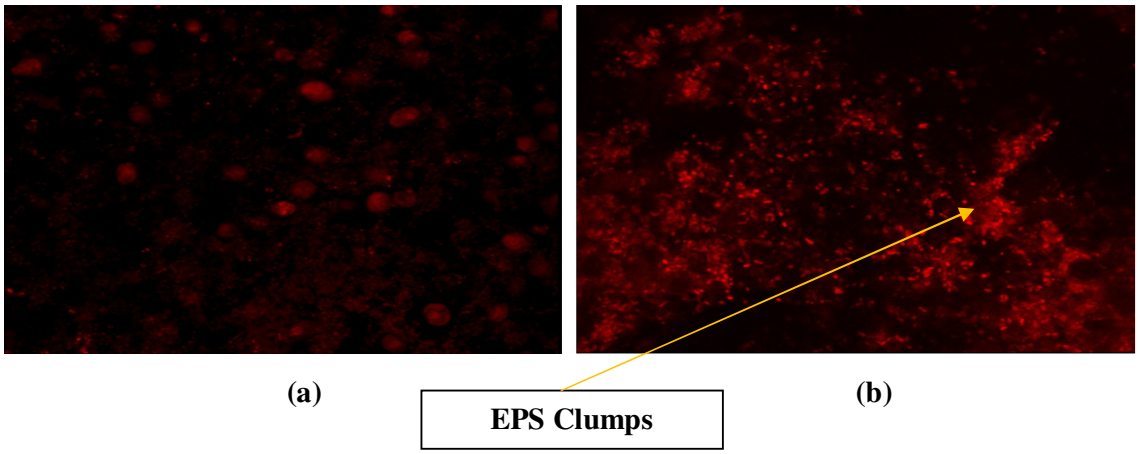


Figure 7.11: Confocal microscopy (500X magnification) of *S. maltophilia* biofilm subjected to PCP studies **(a)** Control/fresh biofilm of *S. maltophilia* (5.4 $mg.m^{-2}$ DCW) **(b)** *S. maltophilia* biofilm subjected to PCCP (30.8 $mg.m^{-2}$ DCW).

7.4.5 Overall interpretation

Overall by comparing the effects of TCP, CCCP and PCP on the biofilms of *P. putida* and *S. maltophilia* (Tab. 7.5), it is very evident that CCCP had the largest positive influence on the SEC in *P. putida* and PCP in *S. maltophilia*. As the effects of TCP on both these two biofilms did not support metabolic uncoupling, it was not selected as a best uncoupler. By comparing the percentage increase in SECs among CCCP and PCP, it is also very evident that PCP showed a higher percentage increase of 85% when compared with CCCP. Hence PCP was selected as the best uncoupler among the three tested uncouplers.

In order to test PCP again for its consistency, a new experiment was started with higher initial *S. maltophilia* DCW of 0.3 g with 1X PBS. However, this time no nitrogen source was supplied to the system, as the species used in the study was grown with toluene and 50 mL of culture was used for generating the biofilm when compared to 20 mL in the earlier studies. In addition to the regular inlet and outlet toluene measurements through online GC, this time carbon dioxide was also measured continuously with an online carbon dioxide

monitoring system. After a steady SEC of 0.12 $g.m^{-2}.h^{-1}$, PBS was replaced with 140 µM PCP on the 16th day (Fig. 7.12). Similar to the earlier PCP responses, this time the SEC started to increase gradually and reached a steady value of 0.15 $g.m^{-2}.h^{-1}$. It was also observed that the SEC rise was only 25% when compared to the earlier 85% increase. The reason behind this decreased effect was unknown and further experiments are required to understand this. The carbon dioxide values were also increased with the SEC. However, as reported earlier (section 3.8.6), the measured carbon dioxide did not equal the theoretical carbon dioxide to be produced during the toluene degradation with ~50% missing. A possible explanation for the missing carbon is the formation of polysaccharides by the biofilm. However, separate research needs to be conducted to investigate the missing carbon and also to validate this hypothesis. The steady SEC observed in the current experiment remained constant for more than 45 days (Fig. 7.12), which clearly showed that PCP influence was active for longer than a month.

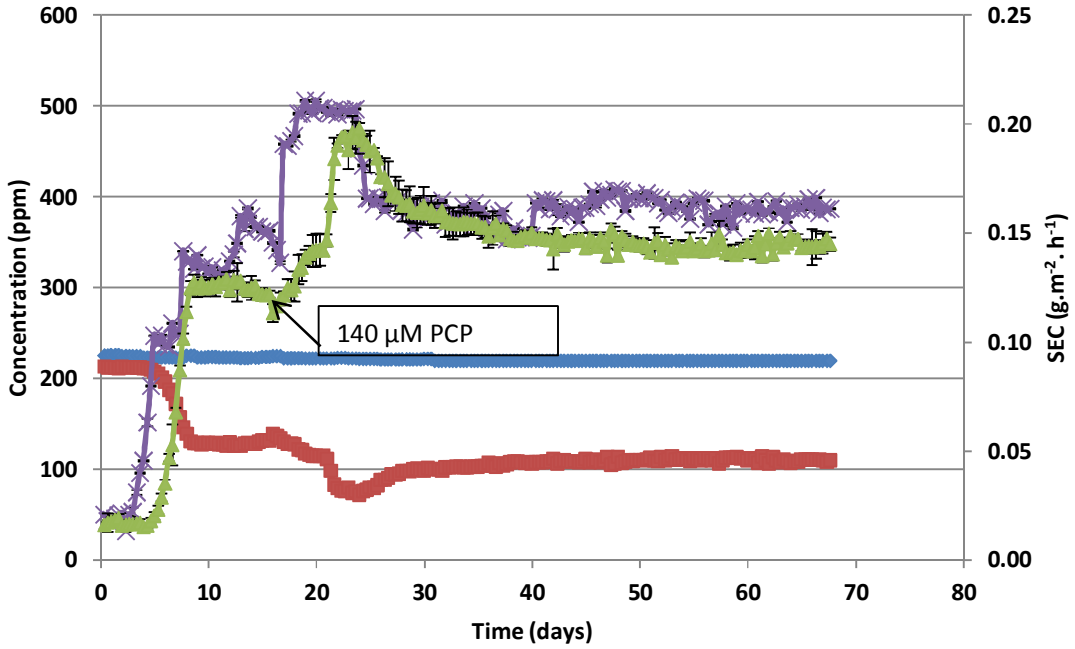


Figure 7.12: Effect of PCP on toluene degradation rate in *S. maltophilia* biofilm reactor (Cycle 2). Closed blue diamonds (◆) are inlet toluene concentrations, closed red squares (■) are outlet toluene concentrations, violet crosses (X) are carbon dioxide concentrations and closed green triangles (▲) are SEC. Error bars are standard deviations.

Table 7.5: Summary of steady effects of TCP, CCCP and PCP on *P. putida* and *S. maltophilia* biofilms.

Metabolic Uncoupler	Biofilm	Pre-exposure SEC ($g.m^{-2}.h^{-1}$)	Post-exposure SEC ($g.m^{-2}.h^{-1}$)
TCP	*a) P. putida*	0.20	0.08
	b) S. maltophilia	0.12	0.08
CCCP	*a) P. putida*	0.16 (Cycle 1) 0.15 (Cycle 2)	0.19 (Cycle 1) 0.19 (Cycle 2)
	b) S. maltophilia	0.15	0.15
PCP	*a) P. putida*	0.20	0.09
	b) S. maltophilia	0.13 (Cycle 1) 0.12 (Cycle 2)	0.24 (Cycle 1) 0.15 (Cycle 2)

7.4.6 Modelling the SEC in presence of uncouplers

Experimental results showed that in presence of PCP, the SEC of *S. maltophilia* biofilm increased and in presence of CCCP, the SEC of *P. putida* increased. In addition both PCP and CCCP did not induce growth and hence the increase in the SEC was possibly due to the energy uncoupling mechanism. Developing a simple model (without a time factor) to calculate and understand the degree of uncoupling in a non-growth systems will help further to describe the uncoupling mechanism quantitatively.

Generally, the degree of energy uncoupling in presence of an uncoupler in a growth system can be expressed by the energy uncoupling coefficient (Liu et al., 1998):

$$E_u = \frac{(Y_{max})_c - (Y_{max})_u}{(Y_{max})_c} \quad (7.2)$$

For our non-growth biofilter system, Eq. 7.2 can be modified in terms of SEC and can be rewritten as,

$$E_u = \frac{(SEC_{max})_u - (SEC_{max})_c}{(SEC_{max})_c} \quad (7.3)$$

Positive E_u value will represent uncoupling and the negative E_u value will represent no uncoupling or the inhibition by the uncoupler. Eq. 7.3 is only applicable if no growth is associated in the uncoupling mechanism. When the uncoupler had nil effect on the system then E_u is zero.

Table 7.6 shows the calculated E_u values for TCP, CCCP and PCP tested on both *P. putida* and *S. maltophilia*. It is very clearly evident from the E_u values from Table 7.6 that consistent metabolic uncoupling response was observed in *P. putida* under the influence of 0.001 µM CCCP and in *S. maltophilia* under the influence of 140 µM PCP. However, based on the highest E_u found for PCP in *S. maltophilia*, it is understood that PCP is the best uncoupler among the three tested. These quantitative results will easily help to understand the difference between uncoupling and inhibition among different metabolic uncouplers at desired concentrations in various microbial biofilms used in non-growth systems.

Table 7.6: Calculated energy uncoupling coefficient

Metabolic uncoupler	**Concentration tested (µM)**	**Biofilm tested**	**E_u**
TCP	4051	a) *P. putida* b) *S. maltophilia*	-0.60 -0.33
CCCP	0.001	a) *P. putida* b) *S. maltophilia*	+0.19 and +0.27 0
PCP	140	a) *P. putida* b) *S. maltophilia*	-0.55 +0.85, +0.54 and +0.25

Overall, by comparing the effects of TCP, CCCP and PCP in soil and biofilm studies, it is evident that the response of mixed culture in the soil was totally different with the pure culture in the biofilm. In addition, the increase in the biomass was clearly observed in biofilm a study which was suspected in soil studies but unable to be measured due to the complexities involved. Though the addition of nitrogen triggered the growth of biomass, the biomass increase observed in biofilm studies which were not subjected to nitrogen addition (Figs. 7.2b, 7.4, 7.12) might be due to the formation of EPS. Two major possible disadvantages in long run due to the formation of these extra polysaccharides and/or stress induced EPS is the increase in the thickness of biofilm without any increase in activity but only increase in mass transfer resistance and increased pressure drop (in real biofilters). Hence it is very clear that conducting metabolic uncoupler screening studies in a pure culture biofilm system will help to understand the metabolic uncoupling concept better than a mixed culture system (like soil). The earlier uncoupler study carried out in pure culture growth systems by Low and Chase et al. (1998) and by Neijssel et al. (1977) also well supports this statement.

7.5 Conclusions

The biofilm reactor was used to study *S. maltophilia* and *P. putida* in the presence of three metabolic uncouplers TCP, CCCP and PCP at the concentrations reported effective in the soil screening studies. Results showed that PCP increased the SEC by maximum of 85% in *S. maltophilia* biofilm and CCCP increased the SEC by maximum of 27% in *P. putida* biofilm. However, TCP did not increase the SEC either in *S. maltophilia* or *P. putida* biofilms. The CCCP and PCP exposed biofilms were further studied by SEM and confocal analysis. SEM analysis showed that the biofilm subjected to PCP had larger and wider air interface cavities when compared with the biofilm subjected to CCCP. This implies the possibility of higher mass transfer in biofilms exposed to PCP than the CCCP influenced biofilm. In addition to biofilm surface analysis, SEM was also used to observe the thickness of the biofilms. Confocal results clearly showed that on top of growth, stress induced EPS formation contributed to the increased DCW at the end of each uncoupler studies. In order to understand the long term effectiveness of uncoupling, a separate biofilm study using *S. maltophilia* and the best uncoupler PCP was carried out and found that the SEC remained constant even after 70 days. Finally a simple model was developed

to calculate the energy uncoupling coefficient for non-growth systems like ours to quantitatively represent the uncoupling mechanism.

7.6 Nomenclature

A_m	Area of the membrane covered by the biofilm	m^2
C_{in}	Inlet concentration	$g.m^{-3}$
C_{out}	Outlet concentration	$g.m^{-3}$
E_u	Energy uncoupling coefficient	No unit
Q	Gas flow rate	$m^3.h^{-1}$
SEC	Surface elimination capacity	$g.m^{-2}.h^{-1}$
$(SEC_{max})_c$	Maximum observed surface elimination capacity	$g.m^{-2}.h^{-1}$
$(SEC_{max})_u$	Maximum observed surface elimination capacity in the presence of the uncoupler	$g.m^{-2}.h^{-1}$
$(Y_{max})_c$	Maximum observed yield	$g.g^{-1}$
$(Y_{max})_u$	Maximum observed yield in presence of uncoupler	$g.g^{-1}$

7.7 References

Cruz, A., Ishii, T., 2011, Arbuscular mycorrhizal fungus spores host bacteria and their biofilm efficient in nutrient biodynamics and soil-borne plant pathogen suppression. Biology Open 1, 52-57.

Der Yang, R., Humphrey, A.E., 1975, Dynamic and steady state studies of phenol biodegradation in pure and mixed cultures. Biotechnology and Bioengineering 17, 1211-1235.

Doran, P.M., 1995, Bioprocess engineering principles, Vol 1. Academic Press.

Emerson, D., Moyer, C., 1997, Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. Applied and Environmental Microbiology 63, 4784-4792.

Ergas, S.J., McGrath, M.S., 1997, Membrane bioreactor for control of volatile organic compound emissions. Journal of Environmental Engineering (ASCE) 123, 593-598.

Geesey, G., Richardson, W., Yeomans, H., Irvin, R., Costerton, J., 1977, Microscopic examination of natural sessile bacterial populations from an alpine stream. Canadian Journal of Microbiology 23, 1733-1736.

Hans-Cur, F., 1995, Sorption sites in biofilms. Water Science and Technology 32, 27-33.

Holden, P.A., 2001, Biofilms in unsaturated environments. Methods in enzymology 337, 125-143.

Holden, P.A., Hunt, J.R., Firestone, M.K., 1997, Toluene diffusion and reaction in unsaturated *Pseudomonas putida* biofilms. Biotechnology and Bioengineering 56, 656-670.

Horn, H., Morgenroth, E., 2006, Transport of oxygen, sodium chloride, and sodium nitrate in biofilms. Chemical Engineering Science 61, 1347-1356.

Jacobs, P., De Bo, I., Demeestere, K., Verstraete, W., Van Langenhove, H., 2003, Toluene removal from waste air using a flat composite membrane bioreactor. Biotechnology and Bioengineering 85, 68-77.

Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J., Mignotte, B., 1995, The biochemistry of programmed cell death. The FASEB Journal 9, 1277-1287

Kumar, A., Dewulf, J., Luvsanjamba, M., Van Langenhove, H., 2008a, Continuous operation of membrane bioreactor treating toluene vapors by *Burkholderia vietnamiensis* G4. Chemical Engineering Journal 140, 193-200.

Kumar, A., Dewulf, J., Van Langenhove, H., 2008b, Membrane-based biological waste gas treatment. Chemical Engineering Journal 136, 82-91.

Kumar, A., Dewulf, J., Vercruyssen, A., Langenhove, H.V., 2009, Performance of a composite membrane bioreactor treating toluene vapors: Inocula selection, reactor performance and behavior under transient conditions. Bioresource Technology 100, 2381-2387.

Kurbatov, L., Albrecht, D., Herrmann, H., Petruschka, L., 2005, Analysis of the proteome of Pseudomonas putida KT2440 grown on different sources of carbon and energy. Environmental Microbiology 8, 466-478.

Lee, E.Y., Jun, Y.S., Cho, K.S., Ryu, H.W., 2002, Degradation characteristics of toluene, benzene, ethylbenzene, and xylene by *Stenotrophomonas maltophilia* T3-c. Journal of the Air & Waste Management Association 52, 400-406.

Liu, Y., Chen, G.H., Paul, E., 1998, Effect of the S_0/X_0 ratio on energy uncoupling in substrate-sufficient batch culture of activated sludge. Water Research 32, 2883-2888.

Low, E.W., Chase, H.A., 1998, The use of chemical uncouplers for reducing biomass production during biodegradation. Water Science and Technology 37, 399-402.

Men, J., Cheng, F., 2011, Biodegradation and growth characteristics of a toluene-degrading strain. African Journal of Biotechnology 10, 13299-13306.

Møller, S., Pedersen, A.R., Poulsen, L.K., Arvin, E., Molin, S., 1996, Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative in situ hybridization and scanning confocal laser microscopy. Applied and Environmental Microbiology 62, 4632-4640.

Neijssel, O., 1977, The effect of 2, 4-dinitrophenol on the growth of *Klebsiella aerogenes* NCTC 418 in aerobic chemostat cultures. FEMS Microbiology Letters 1, 47-50.

Nicolella, C., van Loosdrecht, M., Heijnen, S.J., 2000, Particle-based biofilm reactor technology. Trends in Biotechnology 18, 312-320.

Paul, D., Pandey, G., Pandey, J., Jain, R.K., 2005, Accessing microbial diversity for bioremediation and environmental restoration. Trends in Biotechnology 23, 135-142.

Pirbazari, M., Voice, T.C., Weber Jr, W.J., 1990, Evaluation of biofilm development on various natural and synthetic media. Hazardous Wastes and Hazardous Materials 7, 239-250.

Prenafeta-Boldú, F.X., Guivernau, M., Gallastegui, G., Viñas, M., Hoog, G.S., Elías, A., 2012, Fungal/bacterial interactions during the biodegradation of TEX hydrocarbons (toluene, ethylbenzene and p-xylene) in gas biofilters operated under xerophilic conditions. FEMS Microbiology Ecology. DOI: 10.1111/j.1574-6941.2012.01344.x

Richards, S., Turner, R., 1984, A comparative study of techniques for the examination of biofilms by scanning electron microscopy. Water Research 18, 767-773.

Saini, G., Wood, B.D., 2008, Metabolic uncoupling of *Shewanella oneidensis* MR-1, under the influence of excess substrate and 3, 3′, 4′, 5-tetrachlorosalicylanilide (TCS). Biotechnology and Bioengineering 99, 1352-1360.

Singh, R., Paul, D., Jain, R.K., 2006, Biofilms: implications in bioremediation. Trends in Microbiology 14, 389-397.

Studer, M., Rudolf von Rohr, P., 2008, Novel membrane bioreactor: Able to cope with fluctuating loads, poorly water soluble VOCs, and biomass accumulation. Biotechnology and Bioengineering 99, 38-48.

Van Langenhove, H., De Bo, I., Jacobs, P., Demeestere, K., Dewulf, J., 2004, A membrane bioreactor for the removal of dimethyl sulphide and toluene from waste air. Water

science and technology: A Journal of the International Association on Water Pollution Research 50, 215-229.

Verduyn, C., Postma, E., Scheffers, W.A., Van Dijken, J.P., 1992, Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8, 501-517.

Chapter 8: Recommendations and Future Work

8.1 Summary

In this work, a differential biofiltration reactor was used to explore the potential of metabolic uncouplers to improve pollutant (toluene) degradation rates. Metabolic uncouplers were reported to reduce the cell yield in activated sludge systems, but were untested in biofilters. The current work is the first to report the impact of different metabolic uncouplers in a biofilter. Initially soil was used as a biofilter bed and later experiments were conducted in pure cultures in a biofilm reactor.

Generating a desired concentration of toluene for our differential biofiltration reactor was considered prerequisite before conducting any experiment and hence a simple diffusion system was developed to generate the desired concentration of toluene to the system (section 3.6). In addition, inclusion of mass flow controllers in the system helped to prevent the flow rate fluctuations (±3 $mL.min^{-1}$ in simple rotameter set-up) and facilitated in generating the desired toluene concentration nearly matching the theoretical calculations (Tab. 3.3). Inclusion of online gas chromatography and a carbon dioxide analyzer improved the precision and quality of the data collected. However, the amount of carbon dioxide measured did not match the calculated value which indicated that toluene carbon was being converted to something other than carbon dioxide. Only one fourth of the predicted theoretical carbon dioxide was measured in the outlet stream of the reactor at an average inlet toluene concentration of 200 ppm.

Preliminary experiments (section 4.6.1 to 4.6.3) including the effect of substrate concentration, different nutrients and temperature were done to optimize the conditions before starting the metabolic uncoupler screening studies in soil. Based on the results, inlet toluene concentrations between 180 ppm and 250 ppm at a constant flow rate of 25 $mL.min^{-1}$ were used throughout the studies. Moreover, it was found that substrate inhibition dominated above the toluene concentrations above approximately 200 ppm (outlet concentration). In addition, the results showed that the soil subjected to higher toluene concentration decreased the EC between 5% and 29% in repeat experiments. This may be due to substrate inhibition or due to the loss of active toluene degraders. The Nutrient studies showed that the toluene degraders present in the soil were nitrogen limited. Other macronutrients tested like phosphate, sulphate, magnesium, calcium and iron did not

increase the toluene degradation rate. Temperature studies showed that the EC increased with increasing temperature, from 34 ± 1.4 $g.m^{-3}.h^{-1}$ to 49.8 ± 2.6 $g.m^{-3}.h^{-1}$ for temperatures of 20 to 45 ^{o}C, respectively. However above 45 ^{o}C the majority of the toluene degraders which were active upto 45 ^{o}C were greatly inhibited and hence the EC dropped.

Based on published work in activated sludge treatment, nine potential metabolic uncouplers were selected for initial screening studies in batch serum bottles with toluene as the sole carbon source (section 5.2.1). The nine uncouplers tested were dinitrophenol (dNP), p-nitrophenol (pNP), benzoic acid, carbonylcyanide p-trifluoromethoxy phenylhydrazone (FCCP), carbonylcyanide m-chloromethoxy phenylhydrazone (CCCP), pentachlorophenol (PCP), malonic acid, m-chlorophenol and 2, 4, 6-trichlorophenol. Other than dNP and pNP (nitrogen containing uncouplers), seven uncouplers were selected for further testing in the differential biofilter reactor though the initial serum bottle screening studies were not 100% conclusive. Results showed that only PCP and TCP increased the toluene degradation rate significantly. PCP increased the toluene degradation rate by 35% at 140 µM, whereas 4051 µM TCP increased the rate by 18%. PCP and TCP assays were conducted following the screening studies with only 18% PCP and 68% TCP lost. Though FCCP behaved as a classical uncoupler when compared with others, the EC increase was not significant. Results also showed that possible lower diffusivity of CCCP in soil made it incompatible for soil studies. Other uncouplers decreased the EC in soil (A detailed summary is shown in Fig. 5.10). Hence PCP, TCP and CCCP were selected for further studies in a pure culture (toluene degraders) biofilm reactor system.

Five toluene degraders were isolated from soil subjected to toluene and were identified using 16s rDNA/18s rDNA analysis (section 6.3.3). The five isolates were *Stenotrophomonas maltophilia*, *Pseudomonas putida*, *Aspergillus versicolor*, *Ochrobactrum tritici* and *Pseudomonas citronellolis*. The five isolates were deposited in the New Zealand culture collection maintained by ICMP and this project was the first to report these five isolates as toluene degraders in New Zealand. Moreover, this is the first report in New Zealand for the two isolates *Ochrobactrum tritici* and *Pseudomonas citronellolis* in the ICMP culture collections.

Out of five isolates, two toluene degraders, *Stenotrophomonas maltophilia* and *Pseudomonas putida* were used to inoculate the reactor to create a biofilm (section 7.3.1).

PCP, TCP and CCCP were tested in the biofilm reactors and found that 140 µM PCP increased the surface elimination capacity (SEC) by 85% at in *S. maltophilia* and 0.001 µM CCCP increased the SEC by 27% at in *P. putida*. Further SEM analysis of the biofilm subjected to PCP and CCCP showed larger air interface cavities in the PCP exposed biofilms when compared with the biofilms subjected to CCCP. This implies the possibilities of that higher mass transfer in PCP tested biofilm than that of CCCP influenced biofilm. In addition to biofilm surface analysis, SEM was also used to observe the thickness of the biofilms and found a range of thickness varying between 100-160 µm in *P. putida* and 70-95 µm for *S. maltophilia*. Confocal results clearly showed that on top of growth, stress induced proteins which contributed to the increased DCW at the end of each uncoupler studies. In order to understand the long term effectiveness of uncoupling, another biofilm study using *S. maltophilia* and the best uncoupler PCP at 140 µM was carried out and the SEC increased by 36% and remained stable for 45 days. Finally a simple modeling study (without a time factor) was also performed to determine the energy uncoupling coefficient for the metabolic uncouplers used in biofilm reactor. This model equation helped us to represent the uncoupling mechanism quantitatively. Overall, it was found that the effects of TCP, CCCP and PCP in soil and biofilm studies were totally different. This difference is due to the presence of mixed culture in soil when compared to the pure culture in biofilm reactor. Though the increase in the biomass was evident (visibly) in soil studies, it was not quantified due to the complexities involved in quantifying the biomass increase in mixed culture systems like soil. However, the increase in the biomass was clearly observed in biofilm studies. Though the addition of nitrogen might have triggered the growth of biomass, the biomass increase observed in biofilm studies which were not subjected to nitrogen addition might be due to the possible formation of extra polysaccharides and/or stress induced protein. One of the major possible disadvantages in handling these extra polysaccharides and/or stress induced protein is the increase in mass transfer resistance due to the increase in the thickness of biofilm without any increase in activity. This mass transfer limitation can be measured by using the change in the pressure between the inlet flow rate and outlet flow rate (using an inert gas) in the reactor. However, this issue can be more easily handled in a pure culture biofilm studies than in soil studies. Hence it is very clear that conducting metabolic uncoupler screening studies in a pure culture biofilm system is more valid than mixed culture systems like soil. Moreover, similar studies conducted earlier by few researchers in pure culture growth systems also supports

this statement (Low et al., 2000; Neijssel, 1977). Though these metabolic uncouplers have been widely used in activated sludge, their residual toxicity was not studied well yet. However, using these metabolic uncouplers in closed systems like the one in the current research, the issues associated with environmental release can be avoided. Hence in terms of environmental issues concerned, using metabolic uncouplers in a closed system with lots of precautions is better than using them in open systems.

The major overall outcome of the current research is, metabolic uncouplers can be used effectively in biofiltration to increase the pollutant degradation rate. However, screening experiments have to be done for different types of pollutants to choose an appropriate metabolic uncoupler and appropriate pure culture.

8.2 Online carbon dioxide analysis

8.2.1 Recommendations

The inclusion of online carbon dioxide monitoring system helped to highlight the issue of other toluene degradation products. However, it was observed that at an average inlet toluene concentration of 200 ppm, the system generated approximately 50% theoretical concentration of carbon dioxide and found missing the remaining 50%. Hence it was hard to close the close carbon balance in the current research. This may be due to the possible utilization of carbon source for the production of polysaccharides by the toluene degraders present in the biofilter bed. Other possible carbon end points include soluble microbial metabolites, internal storage polymers and volatile substances like carbon monoxide. Measuring and understanding about all these missing carbon compounds further in a mixed culture systems like soil is hard due to the complexities involved in measuring the biomass. Hence it is recommended to study the missing carbon in pure culture biofilm system in order to avoid the complex biomass and/or other products estimation procedures.

8.2.2 Future work

The fate of the transformed pollutants is poorly understood in biofiltration. A fraction of carbon entering the system remains unaccounted for (Li and Moe, 2005). The biofilms in oxidative microbial processes in the waste treatment industry degrade waste organic compounds to carbon dioxide, biomass and other metabolites. A major portion is released

as carbon dioxide but carbon balance closure in these system remains debatable. An assumption in biofiltration that does not involve continuous/periodic nutrient addition is that at steady state there is no net biomass growth and the organism are in maintenance mode. However the carbon dioxide recovery is never 100 % and a fraction of carbon is either accumulating or exiting the system in some form. Some studies have attempted to close the carbon balance, yet most report 10% - 50% of the carbon as missing (Deshusses, 1997; Furer and Deshusses, 2000; Grove et al., 2009; Hassan and Sorial, 2009; Song and Kinney, 2000; Weber and Hartmans, 1996). With the alarming issue of global warming and industrial emissions, accounting of carbon from these biological systems will be paramount. Hence understanding the carbon flux and closure of carbon balance is very important to accurately predict and model a biofilters performance in the future. Hence, in addition to the online carbon dioxide analyser, in-corporation of an online mass spectrometer and a liquid total organic/inorganic carbon (TO/IC) analyser to the existing system will help to track and trace the missing carbon. This is the proposed research idea to investigate in the near future.

8.3 Metabolic uncouplers

8.3.1 Recommendations

The metabolic uncouplers used in the soil studies were selected based on their earlier reports on activated sludge treatment, as no other reports have yet shown their application in non-growth systems like ours. Hence, the concentration reported earlier was taken as a base for conducting the screening studies. Since the concentration reported was for a growth system, using the same concentration might not be a appropriate in non-growth systems where the system is in maintenance mode. So testing the metabolic uncouplers in lower concentrations with reference to the earlier reported concentration will further help to understand uncoupling pattern and there by the substrate degradation rate in non-growth systems. In addition, conducting similar screening studies in pure culture biofilm reactors at varied metabolic uncoupler concentrations will also help to understand their effect on individual species of toluene degraders. Since the pKa of the uncoupler and internal pH of the cell are important criteria in the uncoupling mechanism, while selecting the pure cultures of toluene degraders for metabolic uncoupler studies, a pre-understanding about these two important parameters would be handy. However the effect of pKa was not clearly

seen during the experimentation of different uncouplers in both soil and pure culture biofilm in the current study. The other important aspect with respect to the metabolic uncoupler is its solubility limit. Most of the uncouplers tested were heat solubilised to shorten to solubilisation time in room temperature. During heat solubilisation (especially at 121 ^{o}C), the possibilities of losing the activity of metabolic uncoupler cannot be completely ruled out. Though this concept was not proved, selecting metabolic uncouplers with high solubility range in water may prevent the possibilities of loss in the uncoupling activity. Moreover, it will also help to work with metabolic uncouplers in different concentration ranges.

8.3.2 Future work

FCCP behaved as a classical uncoupler when compared to others in soil studies. However, it was not considered for further studies in biofilm reactor based on the fact that it didn't improve the EC significantly in soil studies. Hence testing this metabolic uncoupler at different concentrations in soil may help further to understand about this potential uncoupler. This future work is currently under progress. Another work with respect to the long term stability studies of pentachlorophenol in both soil and biofilm reactor needs to be done which will help to understand its metabolic activity with respect to time. This work is also currently under progress. In addition to those seven metabolic uncouplers tested in soil studies, other reported potential uncouplers like tetrachlorosalicylanilide (TSA), dinitrophenol (DNP), 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile (SF 6847) and p-nitrophenol (pNP) (Lewis et al., 1994) may also be tested despite that they all are nitrogen containing compound. However, most of the toluene degraders are nitrogen limited which is a major pitfall in testing these compounds. One of the short term future goals is to develop an easy assay procedure for each of these metabolic uncouplers which are prerequisite to test the uncoupler before and after the analysis.

From both soil and biofilm studies, it was found that after the removal of metabolic uncouplers from the reactor, the EC/SEC did not drop back to the initial steady state value. This response is contradictory to the theoretical concept of metabolic uncoupling. Hence a separate research is needed to identify the issues behind this contradictory response of metabolic uncouplers.

Finally, the possibilities of employing these metabolic uncouplers in trickle bed reactor systems with controlled environment similar to our current system to regulate the microbial growth without hindering the substrate degradation rate is the long term future plan.

8.4 Microbiology

8.4.1 Recommendations

The two toluene isolates used in the biofilm reactor studies were *Pseudomonas putida* and *Stenotrophomonas maltophilia.* It was observed that the pure cultures of both these organisms which were grown in toluene environment showed shorter acclimation time in the biofilm reactor rather than those grown in non-toluene environment (section 7.4.1). In addition, introduction of higher initial biomass in the biofilm reactor improved the acclimation time of these organisms in the biofilm reactor. Hence addition of nitrogen to the biofilm reactor can be avoided by this way.

Maintaining a sterile environment is very hard while using pure culture for experiment like the current one. Though the reactor and tubings were sterilized and the preparation of biofilm was carried out in a sterile environment, one cannot rule out the possibilities of contamination in the biofilm which was subjected for toluene degradation. The chances of population collapse are huge in systems like the current one and hence conducting these kinds of experiments in a complete sterile environment may reduce this huge problem. At least introducing a 0.2 μm filter in the inlet side and outlet side of the reactor may decrease the population collapse to some extent.

In terms of isolation cost, it is cheaper to isolate the toluene degraders from the natural sources like soil. However, cost involved in identifying the isolated strain is huge (especially 16srRNA sequencing). Hence instead of isolation and screening, the appropriate microorganism may be procured from microbial culture collection centers. Procurement cost is always lower than the earlier cost.

8.4.2 Future work

Out of five toluene degraders isolated from soil subjected to toluene, only two were tested in the biofilm reactor. The other three isolates *Aspergillus versicolor*, *Ochrobactrum tritici* and *Pseudomonas citronellolis* could also be tested in the biofilm reactor with the selected

metabolic uncouplers to understand their uncoupling mechanisms. Online biofilm thickness monitoring device will be incorporated with the biofilm reactor to measure the biofilm thickness continuously. Other than the five isolated toluene degraders, other reported potential toluene degraders like *Burkholderia vietnamiensis G4* (Kumar et al., 2008), *Acinetobacter calcoaceticus* (Chalmers et al., 1991), *Acinetobacter Radioresistens* (Abdel-El-Haleem, 2004), *Ralstonia pickettii* (Tao et al., 2004) and *Burkholderia cepacia* (Mars et al., 1996) could be tested in the biofilm reactor. In addition confocal microscopy will be used in very effective manner to observe and study the stress induced metabolites on the surface of the biofilm using appropriate staining procedures. Finally, preliminary studies like substrate effect, temperature effect and nutrient effect will be studied in each biofilm reactors in similar way they were studied in soil. These studies will help to optimize the process parameters for the biofilm reactors.

8.5 General future work

An important issue with traditional biofilter is its huge size (3000 m^2 per m^3 of pollutants treated in an hour) (Theodore, 2008). Use of membrane reactors could solve this size issue to a great extent. Membrane reactors have been previously studied for biological gas treatment. As reviewed by Kumar *et al* (2008) the major advantages of membrane reactors over traditional packed beds are oxygen supply is received equally by the whole membrane resulting in more degradation and less clogging, higher inlet loading rate with greater elimination capacity, smaller foot-print and lower maintenance cost. A smaller foot print, plug flow biofiltration reactor needs to be developed from the existing differential biofiltration reactor system with biofilm on the gas side of the membrane. This reactor will be similar to a shell and tube heat exchanger. The biofilm will be developed in the gas-side of the membrane inside the tube side rather than shell side (Fig. 8.1). It will also eliminate the membrane mass transfer resistance, as the contaminant will transfer directly from the gas phase to the biofilm, with only water and dissolved species exchanging across the membrane. In regards to practical operation, excess biomass will be more easily controlled with direct access to the biofilm for physical or chemical treatment. This will allow both non-growth and growth systems to be easily tested in the same apparatus. However, with the biofilm directly exposed to the contaminated gas, the build-up of recalcitrant compounds such as dust or fats/oils or the displacement of the preferred microbial community by

contaminants or predators is more likely compared to traditional membrane reactors. However, standard biofilters and trickle beds are subject to the same problems.

Preliminary design calculations have given satisfactory results which will help us to develop a plug flow reactor efficiently for treating higher volumes of pollutants per unit volume of reactor. This novel work will pave the way for developing a cost effective biofilter reactor occupying less area and involving less manpower in treating higher volume of pollutants and higher specific productivity. Moreover, this reactor will also help to introduce the metabolic uncouplers in a controlled way/environment similar to the existing differential biofilter reactor system. This idea is one of the future ideas for our current research group, and hence it has to be first proved in a lab scale before scaling it up.

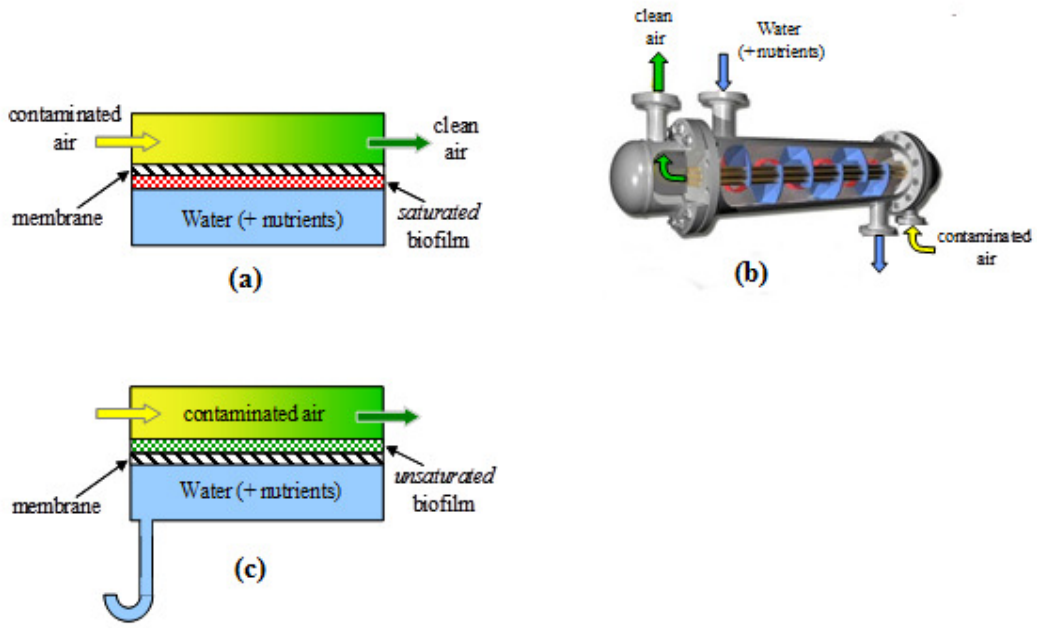


Figure 8.1 – A comparison of the biofilm location in a membrane reactor (a) – saturated biofilm on the water-side; (b) – traditional shell and tube configuration of a membrane bioreactor; (c) – an unsaturated biofilm on the gas-side of the membrane.

Additional work could include testing different substrates (pollutants) in the biofiltration reactor set-up. In particular methane gas is a possible replacement to toluene for similar studies as it is a greenhouse gas and posing a great threat to the increased global warming.

Similar to toluene degraders, methane degrading organisms (Chu and Alvarez-Cohen, 1998; Lontoh and Semrau, 1998) may be used in our biofilm reactor in presence and absence of metabolic uncouplers to study the degradation rate.

Finally a modelling study to understand and calculate the maintenance energy during the action of metabolic uncouplers in a pure culture biofilm reactor needs to be done. This study is one of the ways to quantitatively explain the metabolic uncoupling in pure biofilm under non-growth conditions.

8.6 References

Abdel-El-Haleem, D., 2004, Acinetobacter: environmental and biotechnological applications. African Journal of Biotechnology 2, 71-74.

Chalmers, R.M., Keen, J.N., Fewson, C.A., 1991, Comparison of benzyl alcohol dehydrogenases and benzaldehyde dehydrogenases from the benzyl alcohol and mandelate pathways in *Acinetobacter calcoaceticus* and from the TOL-plasmid-encoded toluene pathway in *Pseudomonas putida*. N-terminal amino acid sequences, amino acid compositions and immunological cross-reactions. Biochemical Journal 273, 99-107.

Chu, K.H., Alvarez-Cohen, L., 1998, Effect of nitrogen source on growth and trichloroethylene degradation by methane-oxidizing bacteria. Applied and Environmental Microbiology 64, 3451-3457.

Deshusses, M.A., 1997, Transient behavior of biofilters: Start-up, carbon balances, and interactions between pollutants. Journal of Environmental Engineering (ASCE) 123, 563-568.

Furer, C., Deshusses, M. 2000. Biodegradation in biofilters: Did the microbe inhale the VOC? In proceeding of the A&WMA 93rd annual meeting (Salt Lake City, Utah),1-13.

Grove, J.A., Zhang, H.X., Anderson, W.A., Moo-Young, M., 2009, Estimation of Carbon Recovery and Biomass Yield in the Biofiltration of Octane. Environmental Engineering and Science. 26, 1497-1502.

Hassan, A.A., Sorial, G., 2009, Biological treatment of benzene in a controlled trickle bed air biofilter. Chemosphere 75, 1315-1321.

Kumar, A., Dewulf, J., Luvsanjamba, M., Van Langenhove, H., 2008, Continuous operation of membrane bioreactor treating toluene vapors by *Burkholderia vietnamiensis* G4. Chemical Engineering Journal 140, 193-200.

Lewis, K., Naroditskaya, V., Ferrante, A., Fokina, I., 1994, Bacterial resistance to uncouplers. Journal of Bioenergetics and Biomembranes 26, 639-646.

Li, C., Moe, W.M., 2005, Activated carbon load equalization of discontinuously generated acetone and toluene mixtures treated by biofiltration. Environmental Science and Technology 39, 2349-2356.

Lontoh, S., Semrau, J.D., 1998, Methane and Trichloroethylene Degradation by *Methylosinus trichosporium* OB3b Expressing Particulate Methane Monooxygenase. Applied and Environmental Microbiology 64, 1106-1114.

Low, E.W., Chase, H.A., Milner, M.G., Curtis, T.P., 2000, Uncoupling of metabolism to reduce biomass production in the activated sludge process. Water Research 34, 3204-3212.

Mars, A.E., Houwing, J., Dolfing, J., Janssen, D.B., 1996, Degradation of toluene and trichloroethylene by *Burkholderia cepacia* G4 in growth-limited fed-batch culture. Applied and Environmental Microbiology 62, 886-891.

Neijssel, O., 1977, The effect of 2, 4-dinitrophenol on the growth of *Klebsiella aerogenes* NCTC 418 in aerobic chemostat cultures. FEMS Microbiology Letters 1, 47-50.

Song, J.H., Kinney, K.A., 2000, Effect of vapor-phase bioreactor operation on biomass accumulation, distribution, and activity: Linking biofilm properties to bioreactor performance. Biotechnology and Bioengineering. 68, 508-516.

Tao, Y., Fishman, A., Bentley, W.E., Wood, T.K., 2004, Oxidation of benzene to phenol, catechol, and 1, 2, 3-trihydroxybenzene by toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 and toluene 3-monooxygenase of *Ralstonia pickettii* PKO1. Applied and Environmental Microbiology 70, 3814-3820.

Theodore, L., 2008, Air pollution control equipment calculations. A John Wiley & Sons, INC., New Jersey, USA.

Weber, F.J., Hartmans, S., 1996, Prevention of clogging in a biological trickle-bed reactor removing toluene from contaminated air. Biotechnology and Bioengineering. 50, 91-97.

Appendix A

A.1 GC calibration

Continuous real time monitoring of inlet and outlet toluene concentration is achieved by connecting a gas chromatography (GC) system (SRI-8610C, SRI Instruments, CA, USA) online to the reactors. GC uses a flame ionization detector, capillary column (1.5 mL) and 5 ml sample loop for detection and analysis of the sample. Helium is used as a carrier gas (5 psi and 10 mL min^{-1}), air (5 psi and 250 mL min^{-1}) and hydrogen (20 psi and 25 mL min^{-1}) for flame ignition. The temperature of column oven is maintained at 180 ^{o}C. Air for the GC is supplied from a compressor and hence it is filtered through oil and vapour removal filter (F64, Norgren Martonair Ltd, Staffordshire, England) before entering the GC. The air pressure is regulated at 30 psi by an in-built regulator in the filter.

A calibration curve was generated using a known volume of liquid toluene in a known volume of air in a Tedlar bag. Table A.1 shows the different concentrations of toluene used in the calibration experiment and Fig. A.1 shows the generated calibration curve. This calibration curve was used to correct all toluene concentrations throughout the experiments.

Table A.1: GC calibration curve data

Concentration (ppm)	**Liquid toluene (µL)**	**Air (mL)**	**Average peak area**
233.04	1	1000	3985.60±90.49
116.52	1	2000	2221.84±294.91
77.68	1	3000	1516.79±109.98
58.26	1	4000	1040.64±126.51
46.61	1	5000	630.89±114.68

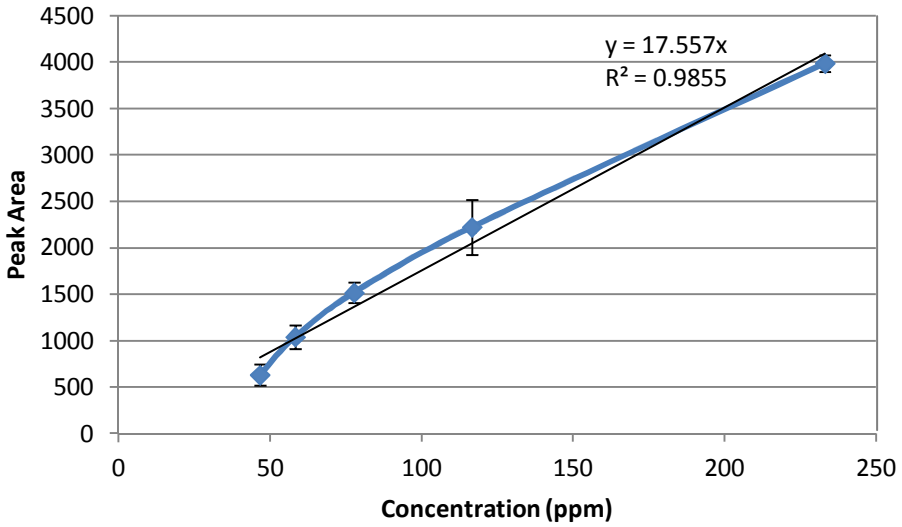


Figure A.1: GC calibration curve. The equation is based on a linear fit through the origin. Error bars are the standard deviations.

A.2 Humidifier

The water consumption rate of humidifier was monitored over time in order to check and confirm the 100% relative humidity of air. To calculate the theoretical water consumption rate, modified Clausius Clayperon equation was used (Eq. A.1).

$$\ln e_S = 53.67957 - \frac{6743.769}{T} - 4.8451.\ln T \qquad \text{(A.1)}$$

Theoretical water consumption rate (Eq. A.2) can be calculated with gas flow rate and from equations A.1 and A.2.

$$c = \frac{n.M_W}{V} = \frac{e_S}{RT} \qquad \text{(A.2)}$$

$$Consumption = c.F_g.t \qquad \text{(A.3)}$$

Water consumption rate was monitored continuously for 7 days and found that water was missing at an average rate of 0.93 mL/day at a consumption rate of 1.8 mL/day. A possible explanation for this response is liquid water might have slowly diffused through the Nafion membrane and formed a film of water inside the tube. The built up water might have slowly been dragged by the air flow into the reactor. However, the excess water will drain through

the membrane and the volume of excess water is too small and hence its influence in the results will have negligible influence any way.

A.3 Reactor leak testing

After assembling the reactors and before the start of experiments, leak testing was conducted to ensure the set-up is leak free. All possible sources of leaks were sprayed with soap solution. Any appearances of bubble were indicated as a leak. Any detected leaks were fixed before the experiments were initiated.

A.4 Online carbon dioxide analysis

The inclusion of online carbon dioxide monitoring system helped to highlight the issue of other toluene degradation products. However, during initial phase of experiments (soil studies), the data generated by the online carbon dioxide analyzer was not reliable due to two important issues: 1) Inlet toluene analysis of all the 4 reactors greatly affected the carbon dioxide measurement, as each inlet was measured three times a day for 1 hr per cycle totaling 3 hr in a 24 hr period and hence 12 hr for all 4 reactors. One disturbance at every 8 hours for an individual reactor greatly influenced the carbon dioxide measurement. After resetting the online GC program to measure inlet toluene once a day, the carbon dioxide measurements improved but not completely reliable. Hence, the inlet toluene analysis was totally removed from the online GC program and was measured only periodically through manual step change option in GC. This improvement solved the problem in the online carbon dioxide measurement to a great extent and hence it was used effectively during the final PCP studies in the biofilm reactor. 2) Variation in the temperature of the sample coming out from GC affected the online carbon dioxide analysis. This was because; the inlet of the online carbon dioxide analyzer was connected to the outlet of the GC by half meter $1/8^{th}$ inch Tygon tubing. Since this tube was not insulated and not heat traced, condensation was observed in the tube which caused carbon dioxide fluctuations. This issue was solved by shortening the Tygon tube length, insulating the tube and by placing the insulated carbon dioxide probe above the hot surface of GC. This kept the gas sample at an average of 45 ^{o}C as it passed through the CO_2 sensor.

A.5 Experimental flow diagram

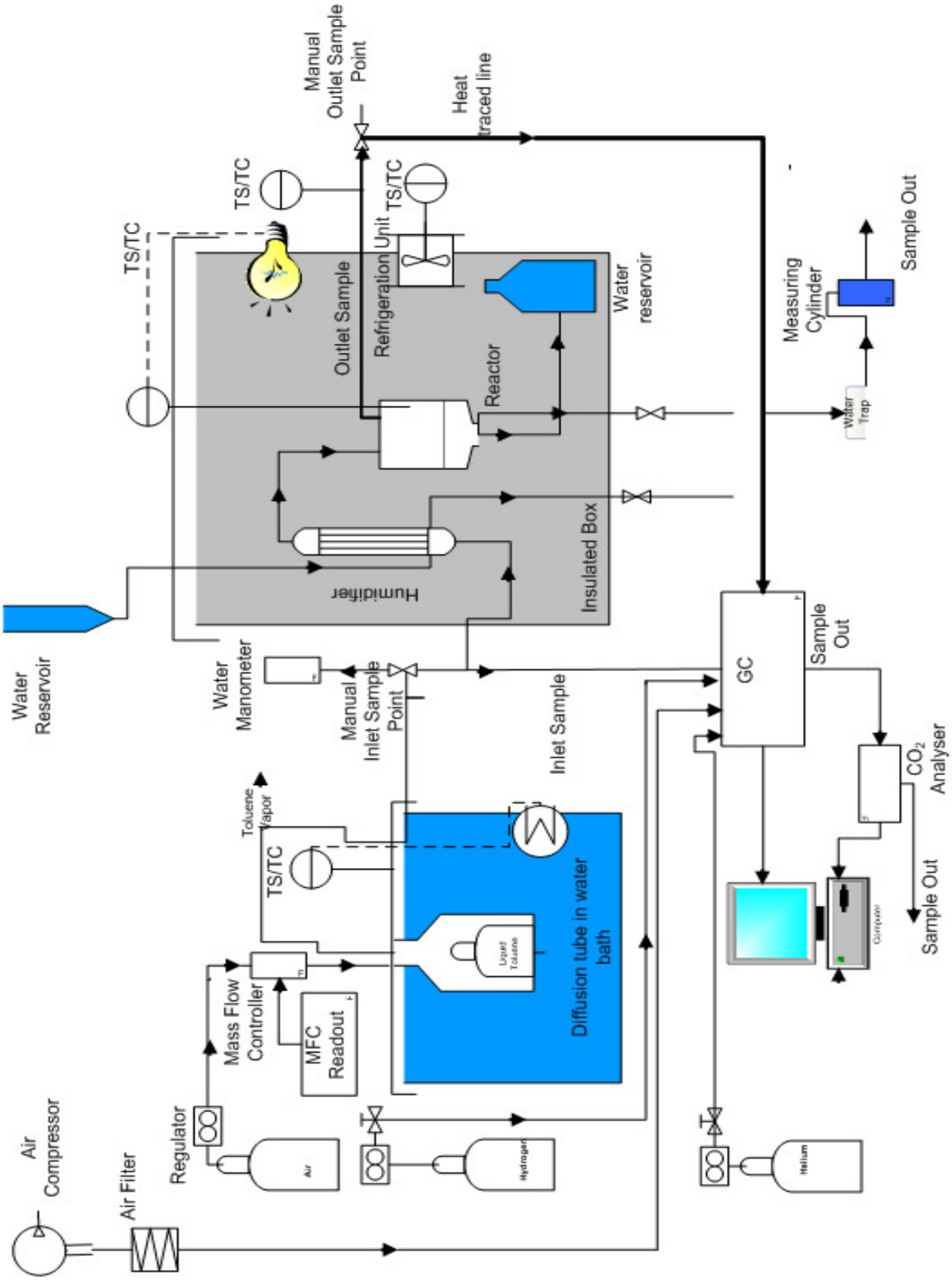


Figure A.2: Experimental set-up

A.6 Nomenclature

c	concentration of water in gas phase	g. m^{-3}
e_S	saturation vapour pressure	mbar
F_g	gas flow rate	$m^3.h^{-1}$
M_W	molecular weight of water	$g.mol^{-1}$
n	moles of water	-
R	gas constant	$J.K^{-1}.mol^{-1}$
T	temperature	K
t	time	h
V	volume of gas	m^3

Appendix B

B.1 Growth medium

For the initial isolation of toluene degraders from soil, minimal salt media (MS medium) with 1.5% agar was used (Tab. B.1). The composition of the medium was based on the hydrocarbon degradation minimal salt medium (Shen et al., 1998). A standard serial dilution procedure (MacLowry et al., 1970) was followed and a 1 ml sample from 10^{-5} dilution was used to inoculate the agar plates using the spread plate method (Buck and Cleverdon, 1960). Approximately 430 ppm of toluene in the head space of the dessicator was maintained, where the agar plates were incubated.

Table B.1: Composition of MS medium adapted from Shen et al. (1998)

Chemical	**Concentration (g. L^{-1})**
$NaNO_3$	4
NaH_2PO_4	2.6
K_2HPO_4	1.2
$FeSO_4.7H_2O$	0.0035
$MgSO_4.7H_2O$	0.4
$CaCl_2.2H_2O$	0.02
Agar	15

(pH adjusted to 7.0 before adding the agar)

Luria Bertani (LB) agar medium (Clermont et al., 2000) (Tab. B.2) was used to subculture the toluene degraders grown in the MS agar plates. LB broth was used to grow the *S. maltophilia* and *P. putida* for preparing using in the biofilm reactor. However, later MS broth was also used in growing these two toluene degraders in order to compare the acclimation time of toluene degraders in the biofilm reactor. PBS composition used in the experiment is shown in Tab. B.3.

Table B.2: Composition of LB medium adapted from Clermont et al. (2000)

Chemical	Concentration (g. L^{-1})
Tryptone	10
Yeast Extract	5
Sodium Chloride	10

(pH adjusted to 7.0 before adding the agar)

Table B.3: Composition of 1X PBS adapted from Clermont et al. (2000)

Chemical	Concentration (g. L^{-1})
Sodium chloride	8
Potassium chloride	0.2
Disodium hydrogen phosphate	1.44
Potassium dihydrogen phosphate	0.24

B.2 Serum bottle studies

MS medium along with appropriate concentration of metabolic uncoupler was used in the serum bottle batch screening experiments. Approximately 0.5 µL of HPLC (High Performance Liquid Chromatography) grade liquid toluene was injected in to the serum bottle to generate approximately 700 ppm of toluene vapour in the head space sealed with a bung and a cap.

B.3 Toluene degrader identification

Following the primary identification, the five isolates were subjected to taxonomical identification through amplification and sequencing of the 18s rDNA for the fungus and 16s rDNA for the four bacterial isolates. The sequences were compared with the database of known 18s rDNA and 16s rDNA sequences through blast search for identification. The gene sequences of all the five identified toluene degraders are shown below.

18s rDNA sequence of *Aspergillus versicolor*

TGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTACTGAGTGCGGGCTGCCTCCGGGCGCCCAACCTCCCACCCGTGAATACCTAAC
ACTGTTGCTTCGGCGGGGAACCCCCTCGGGGGCGAGCCGCCGGGGACTACTGAA
CTTCATGCCTGAGAGTGATGCAGTCTGAGTCTGAATATAAAATCAGTCAAAACTT
TCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAACTGCGAT
AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGC
GCCCCCTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAG
CCCGGCTTGTGTGTTGGGTCGTCGTCCCCCCCCGGGGGACGGGCCCGAAAGGCAG
CGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGACTA
GGGCCGGCCGGGCGCCAGCCGACGTCTCCAACCATTTTTCTTCAGGTTGACCTCG
GATCAGGTAGGGATACCCGCTGAACTTAAGCATATCA

16s rDNA sequence of *Stenotrophomonas maltophilia*

TAACACATGCAAGTCGAACGGCAGCACAGAGGAGCTTGCTCCTTGGGTGGCGAG
TGGCGGACGGGTGAGGAATACATCGGAATCTACTCTGTCGTGGGGGATAACGTA
GGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGATCTTC
GGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAA
GGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTG
GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGAC
AATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGT
TGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCTAATACCCGGTTGGGATG
ACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA
CGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGT
CGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACT
GGGCGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATG
CGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTG
ACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGA
AGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGC

AACGCGAAGAACCTTACCTGGCCTTGACATGTCGAGAACTTTCCAGAGATGGAT
TGGTGCCTTCGGGAACTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAG
CACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGT
GGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTACTAC
AATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATCCCAGAAAC
CCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCT
AGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACAC
CGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTTAACCTTCG
GGAGGGCGCTTGCCACGGTGTGGCCGATGACTGGGGTGAAGTCGTA

16s rDNA sequence of *Pseudomonas citronellolis*

TAACACATGCAAGTCGAGCGGATGAAGGGAGCTTGCTYCCKGATTCAGCGGCGG
ACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTCCGAAA
GGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCT
CACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTAGGTGGGGTAATGGCTCA
CCTAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG
CGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAA
GCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTA
CCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG
GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTA
AGATGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCATAACTGCCTG
ACTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAG
ATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACT
GAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAAC
GCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCTGGCCTTGACATGTCCGGAATCCTGCAGAGATGCGGGAGTGCC
TTCGGGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTA
TGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG
ACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTC
GGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCAGAAAACCGATCGT
AGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATC
GTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGCAAGGGGGACG
GTTACCACGGAGTGATTCATGACTGGGGTGAAGTCGTA

16s rDNA sequence of *Ochrobactrum tritici*

TAACACATGCAAGTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGGTGAGTAAC
GCGTGGGAACGTACCTTTTGCTACGGAATAACTCAGGGAAACTTGTGCTAATACC
GTATGTGCCCGAAAGGGGAAAGATTTATCGGCAAAGGATCGGCCCGCGTTGGAT
TAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCCATAGCTGGTCTGA
GAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTG
AGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGG
TAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAA
GGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGGCGGACTTTT
AAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACTGGAA
GTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTA
GATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGACGC
TGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAATGTTAGCCGTTGGGGAGTTTACTCTTCGGTGGCGCAGCTAAC
GCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGC
AGAACCTTACCAGCCCTTGACATACCGGTCGCGGACACAGAGATGTGTCTTTCA
GTTTGGCTGGACCGGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTT
AGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGAT
GACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGT
GGTGACAGTGGGCAGCGAGCACGCGAGTGTGAGCTAATCTCCAAAAGCCATCTC
AGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCG
CGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCATGGGAGTTGGTTTTACCCGAAGGCGCTGTGCTAACCGCAAGGAGGCAG
GCGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACA

16s rDNA sequence of *Pseudomonas putida*

TAACACATGCAAGTCGAGCGGATGAAGTAAGCTTGCTTAYGGATTCAGCGGCGG
ACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTTCGAAA
GGAGCGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCT
TGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCAC
CAAGGCTACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGC
GAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
CACTTTAAGTTGGGAGGAAGGGCAGTAAGCTAATATCTTGCTGTTTTGACGTTAC
CGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGG
TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAA
GTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAG

CTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGA
TATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTG
AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAAC
GCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCC
TTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTA
TGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG
ACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCG
GTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCACAAAACCGATCGTA
GTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCG
CGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACG
GTTACCACGGTGTGATTCATGACTGGGGTGAAGTCGTA

B.4 References

Buck, J.D., Cleverdon, R.C., 1960, The spread plate as a method for the enumeration of marine bacteria. Limnology and Oceanography, 78-80.

Clermont, O., Bonacorsi, S., Bingen, E., 2000, Rapid and Simple Determination of the *Escherichia coli* Phylogenetic Group. Applied and Environmental Microbiology 66, 4555-4558.

MacLowry, J.D., Jaqua, M.J., Selepak, S.T., 1970, Detailed methodology and implementation of a semiautomated serial dilution microtechnique for antimicrobial susceptibility testing. Applied Microbiology 20, 46-53.

Shen, Y., Stehmeier, L.G., Voordouw, G., 1998, Identification of hydrocarbon-degrading bacteria in soil by reverse sample genome probing. Applied and Environmental microbiology 64, 637-645.

Appendix C

C.1 Pentacholorophenol (PCP) & 2, 4, 6 trichlorophenol (TCP) analysis

Following soil studies, the tested liquid samples (PCP and TCP) were sent for analysis at Hill Laboratories, New Zealand. The results showed that only 18.4% of PCP did not end up in the removed liquid whereas 68.4% of the TCP was missing from the liquid. Similar analysis was performed only for PCP following the biofilm studies (*S. maltophilia*). The results showed that only 7% PCP was missing from the liquid sample. Table C.1 shows the results of these analyses in detail.

Table C.1: Results of PCP & TCP analysis in the liquid sample (Source: The Hill Laboratories, NZ)

Metabolic Uncoupler	**Biofilter bed**	**Quantity tested in differential biofiltration reactor (g)**	**Quantity reported from the liquid sample by "The Hill Laboratories, NZ" (g)**	**% change**
PCP	Soil	0.038	0.031	18.4%
TCP	Soil	0.79	0.25	68.4%
PCP	Biofilm (*S. maltophilia*)	0.038	0.0353	7.1%

Appendix D

D.1 Soil characteristics

Soil Analysis		Value
Total Organic Carbon	g/100g dry wt	8.43 ± 1.01*
Total Inorganic Carbon	g/100g dry wt	1.52(one measurement)
Organic matter	%	18.1
Total Nitrogen	%	0.91
C/N Ratio		11.5
Moisture content (dry basis)	%	30
Ammonium –N	mg/kg	6
Nitrate –N	mg/kg	416
Mineral N (sum)	mg/kg	421

Appendix E

E.1 SEM protocol

The samples were immersed in liquid nitrogen and freeze dried. The freeze dried samples were then mounted on a stub with double sticky tape, sputter coated with carbon paint in a sputter coater (SCD 050, Balzers, Liechtenstein) and examined under a scanning electron microscope (Wetzlar, Germany).

E.2 Confocal protocol

The samples were stained with acridine orange (0.01% in 0.1 M phosphate buffer, pH 7). Stained sample was placed on a curved glass slide and examined with a Leica model TCS SP5 confocal microscope (Wetzlar, Germany) equipped with an Ar/Kr laser. Samples were exited by an Argon laser beam at 488 nm and emitted light was selected by filters detecting cell wall at 530-565 nm. Digital images were processed and analyzed with Leica application suite.