BIODEGRADATION OF PESTICIDE-CONTAMINATED WASTEWATERS IN DENITRIFYING SEQUENCING BATCH REACTORS

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by

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Acknowledgements

Abstract

This research investigated the potential for industrial-strength 2-methyl-4-chlorophenoxyacetic acid (MCPA) degradation by activated sludge microorganisms in a sequencing batch reactor (SBR) under nitrate-reducing conditions.

The research was divided into four phases consisting of Phase I (a "proof-of-concept" phase); Phase II (an initial "tolerance" exploration phase); Phase III (an "effect of hydraulic retention time (HRT)" phase) and Phase IV (a "limits" phase). Prior to addition of the MCPA, base-line data was collected to ensure a stable operation of the SBR in terms of COD and nitrate removal. The SBR successfully and simultaneously removed the nitrates completely and around 98 % of the MCPA up to an initial concentration of 50 mg/L MCPA in the dimethylamine salt form (DMCPA) (Phases I, II and III); however, it took approximately 28 days to observe a steady, high-level removal of MCPA. When the concentration of DMCPA was increased to 75 mg/L (Phase IV) the MCPA removal efficiency dropped to 85 % but removal was observed only for a relatively short period of time, since the biomass appeared to eventually become saturated with the herbicide, stopping conversion of DMCPA to its acid form and halting biodegradation.

The bio-kinetic parameters for nitrate and acetate (COD) were quantified when the concentration of herbicide increased from 20 to 50 to 75 mg/L. The biodegradation kinetic model of COD changed from a first-order (baseline data) to a second-order kinetic model by the addition of increasing concentrations of the herbicide. The rate constant values (k_2) decreased from 1.51 ± 0.82 to 0.57 ± 0.14 to 0.25 ± 0.11 h^{-1} from 20 mg/L to 75 mg/L respectively. In regards to nitrate, the order of reaction remained the same as the baseline data (i.e. a first-order kinetic model) but the rate constant values (k_1) decreased from 2.58 ± 0.76 to

2.14 ± 0.40 to 1.24 ± 0.16 h^{-1} from 20 mg/L to 75 mg/L. Similarly, specific COD and nitrate uptake rates also decreased from 0.60 ± 0.12 to 0.39 ± 0.04 to 0.26 ± 0.07 mg/mg VSS d and 0.14 ± 0.01 to 0.12 ± 0.02 to 0.11± 0.01 mg/mg VSS d from 20 mg/L to 75 mg/L respectively.

Further to this, the bio-kinetic rate constants of DMCPA and MCPA were estimated by solving first-order modified differential equations (MDEs) using the function ode45 in MATLAB. This function implements a Runge-Kutta method with a variable time step for efficient computation after the initial conditions at time to, are specified. Thus, the "apparent" reaction rate constants for DMCPA and MCPA for 20 mg/L of herbicides were found to be k_D= 0.27 h^{-1} and k_M = 0.97 h^{-1} respectively; whereas, a three-fold decrease (k_D = 0.09 h^{-1}) in the apparent rate of DMCPA degradation and a two-fold decrease (k_M = 0.47 h^{-1}) in the rate of MCPA degradation was observed when the concentration increased from 20 to 50 mg/L.

The results of this study produced additional information on the biodegradability potential, limits and kinetics of MCPA under anoxic conditions; thereby providing supplementary information to an overall integrated pesticide-nitrate removal strategy.

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PART 1: BACKGROUND AND METHODOLOGY

Primary industries such as agriculture are the backbone of the New Zealand economy (Ballingall & Lattimore, 2004). This is because New Zealand's temperate climate and high soil fertility make it favorable for farming. For example, dairy products alone contribute 29% (NZ $ 15.5 billion) of total New Zealand exports (Statistics New Zealand, 2014) while horticulture accounts for 8% (NZ $ 3.9 billion) of New Zealand merchandise exports (Horticulture New Zealand, 2014). Recently, however, it has been acknowledged that the agriculture sector not only contributes to the economy of the country but also is a major source of environmental pressure caused by excessive use of agrochemicals and by agricultural run-off (Organization of Economic Co-operation and Development, 2007; Parris, 2011). This is because in-order to increase crop yield and to protect plants from deadly diseases, farmers often use fertilizers as well as pesticides. Through infiltration and percolation both nutrients and pesticides leach into the groundwater regime causing major pollution problems.

It has been estimated that about half of New Zealand's drinking water is pumped from groundwater reserves (Ministry of Health, 2013). One study on the pesticide contamination of groundwater has suggested that out of the 163 wells sampled as a part of national survey, 31 wells (i.e. 19%) tested positive for pesticides with 13 (8%) of the wells having two or more pesticides (Gaw et al., 2008). Of the 50 pesticides detected, 37 were herbicides. Another recent study conducted on 15 streams flowing through farmland in the South Island of New Zealand discovered that chlorinated herbicides were the most dominant form of pesticides used by farmers (Shahpoury et al., 2013). They reported that herbicides are transported either from agriculture soils to streams via runoff or through vapor drift where the pesticide has not been directly applied. Chlorinated pesticides are a global concern because of their long

persistence in the environment, their negative effect on non-targeted organisms and their bioaccumulation through different levels of the food chain (Li et al., 2013; Shahpoury et al., 2013). In particular, 2-methyl-4-chlorophenoxyacetic acid (MCPA) belongs to the chlorinated group of herbicides and is easily accessible in the New Zealand market. It is used to control broad-leafed weeds in lawns, sports grounds, golf courses and field crops (Massey University weed database, 2013).

Furthermore, fertilizer and pesticide manufacturing industries play a vital role in meeting the need for agrochemicals in New Zealand. For example, at present there are 75 and 18 officially registered fertilizer and pesticides manufacturing industries are in operation (Statistics New Zealand, 2015). As a result, generation of large amounts of wastewaters containing high nitrate and COD can be expected from such industries. Pre-treatment or on-site treatment of these micro-contaminants using denitrifying microorganisms in an SBR could be an effective and efficient bioremediation approach to manage the wastewaters from these sources.

Compounding the above pollution problem is the fact that the latest report issued by the Environment Canterbury Regional Council (2013) suggests that there is a high nitrate risk possibility in shallow groundwater areas of the Canterbury Plain (Figure 1). The report documents concerns where nitrate values can sometimes exceed the maximum acceptable value (MAV) of 11.3 mg/L nitrate-nitrogen (NO_3-N). A Ministry of Environment (2010) report also concluded that more than one-third (39%) of groundwater monitoring sites in New Zealand had levels of nitrate that are elevated above natural background levels, probably as the result of human activities such as the leaching of fertilizer and stock effluent. An OECD (2007) report also mentions that the New Zealand water quality in rivers and lakes has declined in regions dominated by pastoral farming, where high nutrient inputs and microbiological contamination destabilize natural ecosystems and pose risks to human health.

It seems axiomatic therefore that attempts must be made to protect the aquatic environment from over exposure to both these contaminants (i.e. nitrates and pesticides).
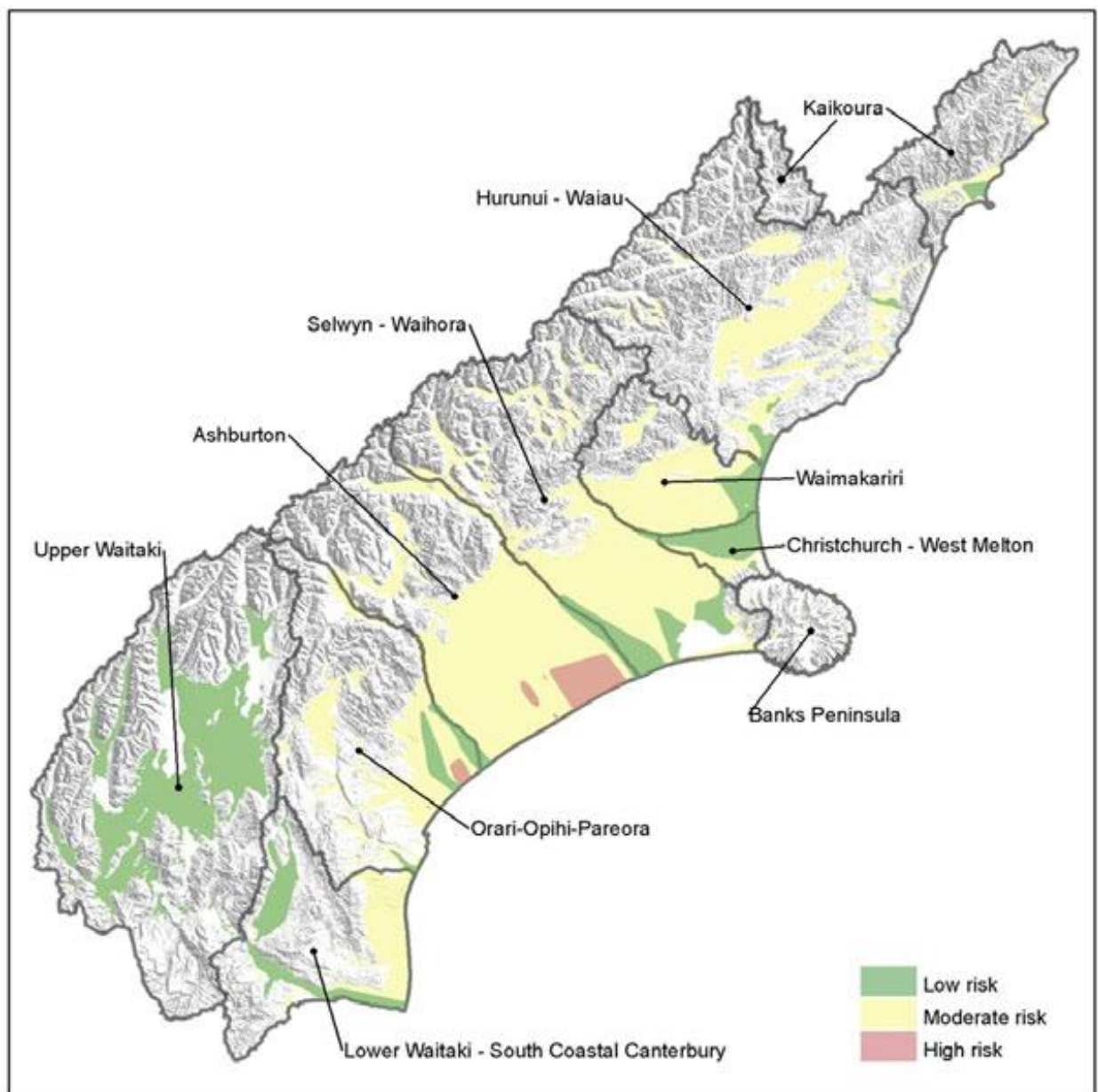
Figure 1.1. Map of the Canterbury region showing areas at low, medium and high-risk of nitrate concentrations exceeding the maximum acceptable value (MAV) in shallow groundwater (adapted from Environment Canterbury Regional Council, 2013).

This chapter consists of four sections. The first three sections describe in detail (i) the problem arising due to excessive use of pesticides (ii) the potential solution for simultaneous biodegradation of pesticides and nitrate and (iii) technologies used to deliver such solutions. The last section (iv) outlines the specific research objectives for this study.

2.1. Problems due to excessive use of pesticides

2.1.1. The increasing world-wide demand for pesticides

By 2050, the population of the world is projected to increase by 30% (from 7 billion to about 9.2 billion). Due to the increased population as well as a change in dietary patterns as developing countries pursue better quality food; the demand for food production is projected to increase by 70% (FAO, 2009). In order to meet these needs there is a challenge to grow more food on even less land while at the same time protecting crop yields from attack by pests, pathogens and weeds (Oerke & Dehne, 2004). In this scenario, pesticides make a significant contribution to maintaining and safeguarding the world's food production and supply. For example, in the US, corn was initially planted on hills, in "check" rows, which allowed farmers to cultivate the corn in two directions for weed control. With the advent of effective herbicides, farmers switched from hill planting to drilled, narrow-row planting and consequently the plant population increased from 10,000–12,000 plants per acre to 25,000–30,000 plants per acre (National Research Council, 2000). Furthermore, increased agricultural pesticide worldwide has nearly doubled the food crop harvests from a theoretical yield of 42% in 1965 to 70% by 1990 (Oerke, 2005).

At present, the worldwide consumption of pesticide is about 2 x 106 tonnes/year with the use of herbicides dominating other forms of pesticides (De et al., 2014). Figure 2.1 shows the

worldwide split in terms of pesticide use, i.e. 47.5% is the share of herbicides, 29.5% is the share of insecticides, 17.5% is that of fungicides and others, accounts for 5.5% only.
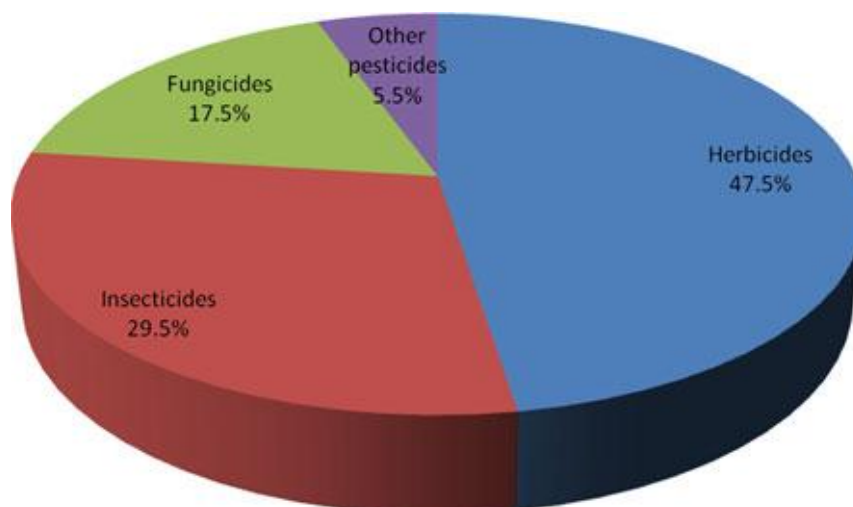
Figure 2.1. Worldwide distribution of pesticide groups by their consumption (De et al., 2014).

2.1.2. Pesticide poisoning to the environment: residue movement and accumulation

Even though the use of pesticides is vital in agriculture, most of their benefits are based on direct crop returns (Pimentel, 2005). Such benefits do not include the indirect environment and human health effects associated with pesticide use. As Pimentel et al. (1992) reported, human pesticide poisonings and illnesses are clearly the highest price people pay for pesticide use. Following release into the ecosystem, pesticide exposure to the public occurs primarily through eating foods and drinking water contaminated with pesticide residues. Toxicity to human beings by pesticides may be divided into three main groups, based on the pesticide exposure time and how fast the toxic symptoms develop (Calvert et al., 2008). For example, if a farmer is exposed to a single dose of a pesticide, the incident is referred to as acute and the effect is called acute toxicity. Acute toxicity is used to describe a toxic effect which typically appears immediately or within 24 h of exposure. Similarly, subchronic toxicity is the ability of a chemical compound to cause toxic health effects for over a year, but less than the lifetime of the exposed organism. Finally, chronic toxicity is the ability of a pesticide to cause adverse

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health effects over an extended time period, usually after repeated or continuous exposure, which may last for the entire life of the exposed organism. In any case, Calvert et al. (2008) reported that agricultural workers (mostly female) experience the most risk to acute pesticide poisoning in comparison to non-agricultural workers, particularly through drift, early reentry into the treated area and use in conflict with the pesticide's intended method of application and/or use. For example, Engel et al. (2004) examined breast cancer risk among farmer's wives who were exposed to different types of pesticides and found that there was strong evidence of an increased breast cancer risk with the use of 2,4,5-TP (2,4,5-trichlorophenoxyacetic acid; a chlorinated herbicide). In addition to cancer, long-term exposure to pesticides can cause other chronic problems; for example neurological effects, respiratory effects, reproductive effects, sensory disturbances as well as cognitive effects such as memory loss (Pimentel, 2002). Overall, a report by WHO and UNEP estimates that world-wide, roughly 200,000 people die while around three million are poisoned each year by pesticides (WHO, 1990).

It has been estimated that less than 0.1 % of the pesticide applied to crops actually reaches the target pest; the rest enters the environment, contaminating soil, water and air (Pimentel & Levitan, 1986). However, the negative effects to the environment depend on the toxicity of the pesticides, the measures taken during their application, the dosage applied and the weather conditions prevailing after application (Damlas & Eleftherohorinos, 2011). Some of these toxic compounds resist physical, biological, chemical and photochemical breakdown processes and thus persist in the environment for long periods of time (Mrema et al., 2013). For example, presence of organochlorine pesticides such as DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl ethane)) residues in the soil sample even though DDT has not been applied for over 20 years (Shivaramaiah et al., 2002). Residues of such persistent micro-pollutants are then transported by various means eventually entering the food chain, which ultimately

undergo "bioaccumulation" processes in the body tissues of organisms. Accumulation of methylmercury, a highly toxic organometallic compound in fish is a typical example of "bioaccumulation" or ''biomagnification" (Bloom, 1989).

The potential pathways for the transport of pesticide residues is through the "hydrologic cycle" (Figure 2.2) which supplies water for both humans and natural ecosystems (U.S. Geological Survey, 1995). In addition to this, transport of pesticides is also governed by various interdependent factors including land-use, soil/sediment biogeochemistry (such as pH, organic carbon content and microbial biomass and activity) and structure (Arias-Estévez et al., 2008). For example, Picton & Farenhorst (2004) reported that herbicide sorption generally increased with increasing soil organic content. Similarly, Nicholls (1988) mentions that the mobility of pesticides depend on soil pH. Permanent anions and weak acids can be very weakly adsorbed and hence increase the probability of leaching. Furthermore, Jahan et al. (2008) reported that some intermediate metabolite products of certain pesticide (e.g. nonylphenol, biodegradation product of nonylphenol ethoxylates) are more persistent and pose greater toxicity threats in the environment then its parental compound.

In any case, eventually some of the pesticide residues make their way to ground water bodies. There is increased concern about groundwater contamination by pesticides since one-half of the human population obtains its water from groundwater sources (Pimental, 2005). Once groundwater is contaminated, the pesticide residue remains for long periods of time.
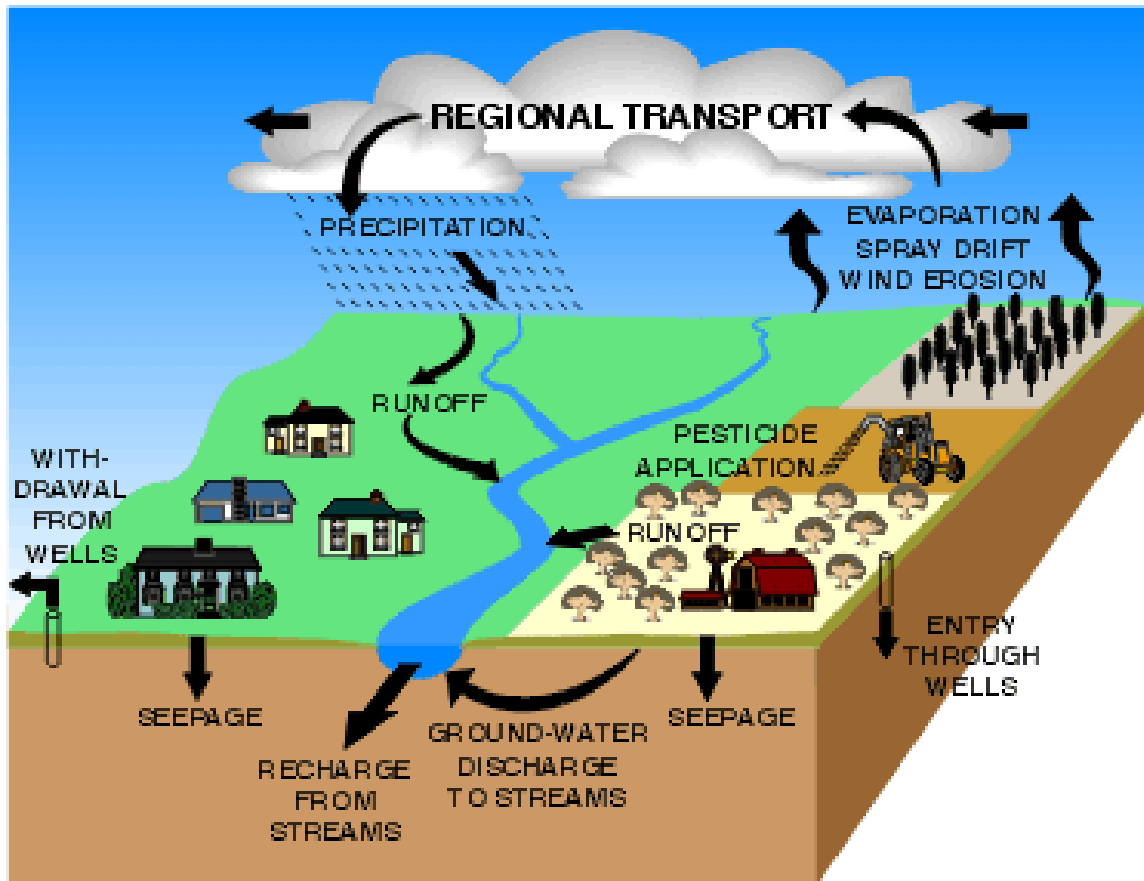
Figure 2.2. Pathways of pesticide movement in the hydrologic cycle (adapted from USGS Open-File Report 94-506).

2.2. The potential solution for simultaneous biodegradation of pesticides and nitrate

2.2.1. Biological wastewater treatment and the denitrification process

Biological wastewater treatment is aimed at the removal and stabilization of organic matter. It is accomplished by a variety of microorganisms and consists of two processes, synthesis and oxidation. That is, bacteria convert the organic matter to new bacterial cells (i.e. synthesis) while at the same time producing various gaseous end products via oxidation. The synthesis of new bacterial cells depends upon the source of carbon and energy; for example, heterotrophic bacteria reproduce well using dairy farming wastewater as a carbon source (Park & Yoo, 2009) because that type of wastewater contains a large amount of readily-degradable carbon. Degradation of the organic content in a wastewater diminishes most of the

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carbon but not the ammonia, the end product of nitrogen mineralization. When this ammonia enters water bodies, ubiquitous nitrifying bacteria converted it into nitrate (NO_3^-). Nitrate present in the aqueous environment is of great concern because of the following two reasons. First, the presence of nitrate in drinking water (even in minute amounts) may cause detrimental health effects; for example, methemoglobinemia in infants (the so-called ''blue-baby syndrome'') (Saeedi et al., 2012). Secondly, excess NO_3^- in stagnant water bodies such as lakes and estuaries causes eutrophication (deterioration of water quality and toxicity to aquatic life (Sun et al., 2010)).

The removal of nitrogen using different methodologies (e.g. ion exchange, adsorption, reverse osmosis, and electrodialysis) has been studied in the past (Yoon et al., 2001; Schoeman & Steyn, 2003; Annouar et al., 2004). Biological denitrification has been found to be the easiest and most economical way of removing nitrate after it has been produced by nitrification (De Lucas et al., 2005; Park & Yoo, 2009; Adav et al., 2010; Saeedi et al., 2012; Naik & Setty, 2012; Ye & Zhang, 2013).

The denitrification process (Figure 2.3) occurs in the environment by assimilatory and dissimilatory nitrate reduction. Assimilatory nitrate reduction involves the reduction of nitrate to ammonium for cellular synthesis and this process is normally found among higher green plants as well as microorganisms. Dissimilatory nitrate reduction however involves reduction of nitrate to nitrite which is mainly carried out by facultative anaerobes, as well as a few species of actinomycetes. The key difference between these two processes is the respiratory electron transport chain. In the assimilatory pathway, denitrification commonly occurs in the presence of oxygen and does not involve the respiratory electron transport chain. The dissimilatory pathway however is anoxic, occurring in the absence of oxygen. In this latter pathway, the reduction of nitrate ultimately to gaseous products of nitrogen is coupled with a

respiratory electron transport chain, thus oxidized inorganic compounds such as nitrate and nitrite can function as electron acceptors. The nitrate reduction reaction in biological denitrification involves the following pathway: bacteria such as Alcaligenes, Achromobacter, Micrococus, and Pseudomona, first reduce nitrate to nitrite and then produce nitric oxide, nitrous oxide and nitrogen gas (Naik & Setty, 2012).

$$NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$$
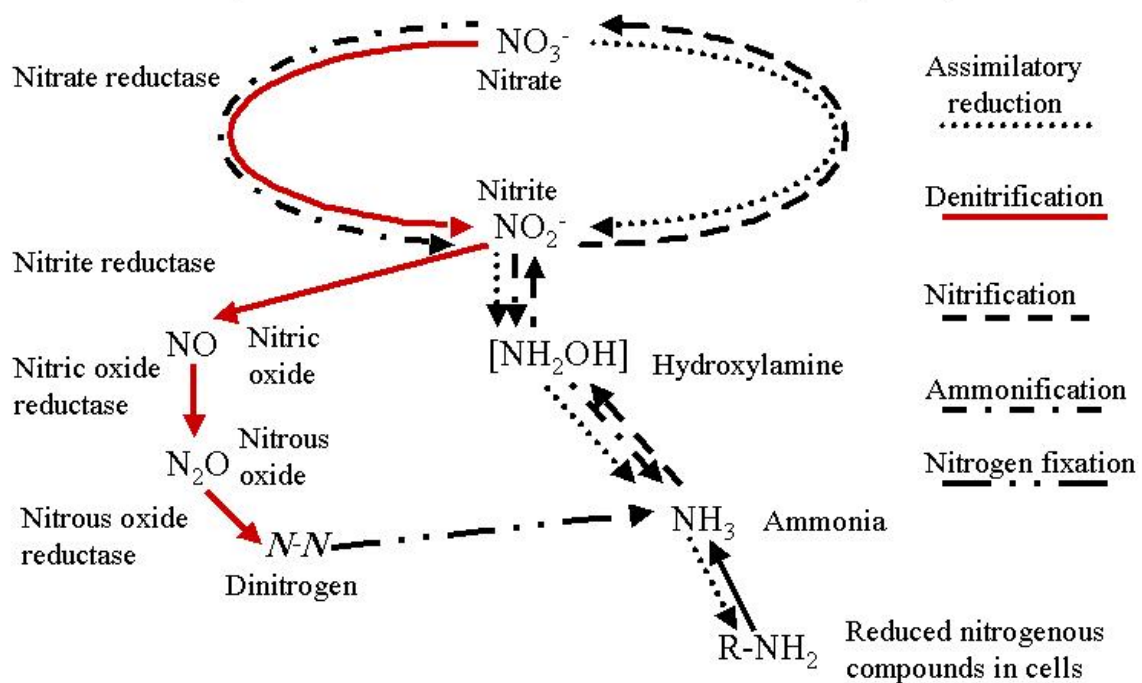
Figure 2.3. Transformation of nitrogen in the nitrogen cycle. Denitrification reactions are shown in red. The enzymes catalyzing each step in denitrification are listed next to the reaction they catalyze (adapted from Cornell University, 2014).

2.2.2. *Factors affecting the denitrification process*

The denitrification process is a heterotrophic process which is usually affected by various parameters including organic carbon source, the carbon to nitrogen ratio, temperature and pH (Li, 2001; Elefsiniotis & Li, 2006). In particular, it has been observed that complete

denitrification is hindered if there is insufficient organic carbon in the wastewater (Komorowska-kaufman et al., 2005; Adav et al., 2010). In such cases, an appropriate external carbon source must be used to maintain a balance between electron donor and acceptor (i.e. COD:NO_3-N ratio) (Randall & Barnard, 1998; Xie et al., 2012). The use of commercial organic carbon sources such as acetate, methanol, ethanol and glucose have all been found to be quite effective (Park & Yoo, 2009; Adav et al., 2010; Naik & Setty, 2012; De Filippis et al., 2013). For example, acetate is a readily biodegradable carbon source and is directly incorporated into the Krebs cycle to release energy for bacterial metabolism (Akunna et al., 1992; Khanitchaidecha et al., 2010). However, the cost associated with the use of exogenous carbon plays an important role in the ultimate selection of the carbon source. Consequently, inexpensive but effective external carbon sources for enhancing complete denitrification has been the interest of many researchers in recent years (Cherchi et al., 2009; Khanitchaidecha et al., 2010; De Filippis et al., 2013).

For acetic acid as a carbon source, the denitrification reaction without the cell synthesis reads as follows (Beun et al., 2000).

$$5CH_3COOH + 8NO_3^- \rightarrow 4N_2 + 10CO_2 + 6H_2O + 8OH^- \tag{2.1}$$
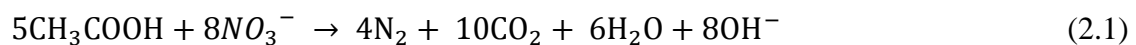
If no growths occurs this ratio would be 2.86 g substrate COD/ g N (eq. 2.1), which is theoretical minimum.

However, this ratio is often higher during the denitrification reaction and also depends on the types of carbon source used. For example, the chemical equilibrium equation including cell synthesis using acetic acid as a carbon source is suggested by Mateju et al. (1992) to be:

$$0.819\ CH_3COOH + NO_3^- \rightarrow 0.068\ C_5H_7NO_2 + HCO_3^- + 0.301\ CO_2 + 0.902\ H_2O +$$

$$0.466\ N_2 \hspace{6cm} (2.2)$$
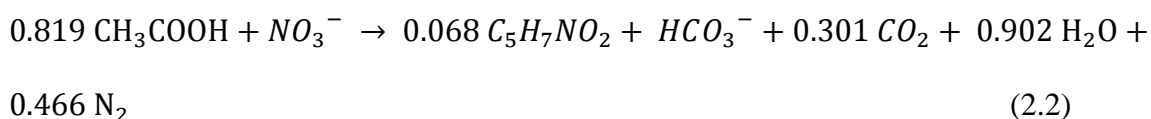
Based on equation (2.2), the reduction of 1 g NO_3-N theoretically consumes 3.51 g acetate to produce new cells (Mateju et al., 1992).

Environmental parameters such as pH and temperature also play a key role in influencing denitrifier growth, metabolism, denitrification gene expression and, subsequently, denitrification rates (Saleh-Lakha, 2009). Past studies have revealed that denitrification rates decrease with decreasing pH values (Šimek et al., 2009; Čuhel & Šimek, 2011). For example, Parkin et al. (1985) demonstrated a twofold decrease in the denitrification rate and a threefold decrease in the denitrification enzyme activity when the soil pH decreased from 6.02 to 4.08. In addition, Glass & Silverstein (1998, 1999) investigated the significance of pH on activated sludge denitrification systems using bench-scale SBRs and found that complete denitrification was achieved at higher pH values from 7.5 to 9.0. Similarly, there are several studies that have explored the effect of temperature on denitrification rates in soil and water. For example, Saleh-Lakha (2009) reported that low soil temperature negatively affects the denitrification activity while Elefsiniotis & Li (2006) reported a marked increase in the denitrification rates (approximately four to eight times) as the temperature increased from 10 to 20 °C. In the latter case, a further temperature change from 20 to 30 °C resulted in a less dramatic increase in denitrification rates. Furthermore, Carrera et al. (2003) studied the effect of temperature on denitrification rates at six different temperatures (6, 8, 10, 15, 20 and 25ºC) in batch mode and reported that the maximum denitrification rate was obtained at temperature of 25 ºC.

2.2.3. Ability of denitrifiers to degrade recalcitrant compounds

As mentioned, denitrifying bacteria have the ability to reduce nitrate and nitrite to nitrogen gas via biological denitrification in the presence of a carbon source. Past studies have shown that denitrifying bacteria can utilize a wide variety of recalcitrant organic substrates including herbicides (Katz et al., 2000; Aslan & Türkman, 2004, 2005; Chong & Chen, 2007; Elefsiniotis & Wareham, 2012; Cesar & Roš, 2013). In this case, both the herbicide is degraded and the nitrate is removed at the same time (Shawaqfeh, 2010). However, in order to do this effectively, it is generally acknowledged that the biomass requires an acclimatization phase which allows it to express degradative enzymes before it can begin to consume any so-called xenobiotic compounds (Chin et al., 2005; Chong & Chen, 2007). Katz et al. (2000) showed in batch tests that Pseudomonas species have the ability to degrade the herbicide atrazine in the presence of nitrate under both aerobic and anoxic conditions. Furthermore, Aslan & Türkman (2004) demonstrated high removal of several selected herbicides (trifluralin, fenitrothion and endosulfan (α+β)) via denitrification using wheat straw as a complementary electron donor. Similar results were shown by Aslan & Türkman (2005) who removed the above herbicides (up to 95%) via denitrification, even after changing the main electron donor from wheat straw to ethanol. Cesar & Roš (2013) discovered that biodenitrification can remove 99% of nitrate, 80% of deethylatrazine, 25% of atrazine, and 45% of metolachlor from polluted groundwater using sodium acetate as supplemented carbon source. In their study, they concluded however that there was no influence of increasing concentrations of nitrate on herbicide removal ability although very high concentrations of nitrate were used (up to 39 mg/L NO_3-N).

In summary, a review of the literature reveals sufficient evidence of the ability of denitrifers to degrade recalcitrant compounds and it provides impetus to conduct further research into integrated nitrate-herbicide removal strategies.

2.2.4. Potential biodegradation pathway of two commonly used chlorinated herbicides: MCPA and 2,4-D (2,4-Dichlorophenoxyacetic acid)

Aromatic compounds widely used to formulate pesticides are among the most prevalent and persistent organic pollutants (POPs) in the environment (Seo et al., 2009). In regards to the natural remediation of POPs, biodegradation processes play a pivotal role. By expressing various catabolic (biodegradative) pathways, microorganisms can use a wide range of aromatic compounds as carbon and energy sources (Harayama, 1992; Mangat & Elefsiniotis, 1999; Aslan &Türkman, 2004; Chong & Chen, 2007; Elefsiniotis & Wareham, 2013). Specifically, Fuchs et al. (2011) reported that the crucial step in degradation of aromatic compounds is overcoming the resonance energy that stabilizes the ring structure. Moreover, how microorganisms destabilize the aromatic ring depends on the presence or absence of oxygen; that is, on the redox condition since O_2 acts as a powerful means to oxidize and cleave the aromatic ring.

In anaerobic and anoxic conditions there is a lack of O_2 and the pathway is known to be reductive where inorganic electron acceptors such as NO_3^-, NO_2^- and SO_4^{-2} might serve as combined forms of oxygen sources (Evans & Fuchs, 1988). Nevertheless, for both aerobic and anaerobic processes, microorganisms use as many substrates as possible and transform them into a few key central intermediates that are then accessible to a reaction (such as central ring cleavage) which removes the aromatic character of the molecule. Fuchs et al. (2011), mentioned four specific microbial strategies, conditions and the key enzymes produced during the biodegradation of the aromatic compounds, these are presented in Table 2.1

Table 2.1 Different strategies for the degradation of aromatic compounds (adapted from Fuchs et al., 2011).

Strategy	Conditions	Key enzymes
O_2 - dependent ring cleavage of dihydroxylated aromatic compounds	Oxic	Ring-cleaving dioxygenases
O_2 - dependent ring epoxidation of CoA thioesters	Oxic but with fluctuating or low O2 concentrations	Ring epoxidases of the di-iron protein family
ATP-dependent ring reduction of CoA thioesters	Anoxic (during anaerobic respiration with a high ATP yield in facultative anaerobes, and during anoxygenic photosynthesis)	ATP-dependent benzoyl-CoA reductase
ATP-independent ring reduction of CoA thioesters	Anoxic (during anaerobic respiration with a low ATP yield in strict anaerobes, and during fermentation)	ATP-independent benzoyl-CoA reductase

Phenoxy herbicides, including MCPA and 2,4-D, are heavily distributed POPs in the environment because of their widespread use for weed control in agricultural crops (Sannino, 2015). Their structures is relatively similar and includes a phenyl (benzene) ring attached to an oxygen atom which is in turn attached to an acid and other substituents (e.g. chlorine, methyl group) (Anderson, 1996). Generally, the biodegradation of chlorinated aromatic compounds in the environment include two important steps i.e. the cleavage of the aromatic ring and the removal of the chlorine atom from the ring (Häggblom, 1990). In particular, Crespín et al. (2001) reported the biological breakdown of MCPA and 2,4-D herbicide by soil microorganisms (mostly under warm, moist conditions) can result in the formation of corresponding phenols (4-chloro-2-methylphenol and 2,4-dichlorophenol for MCPA and 2,4-D, respectively) (Figure 2.4a.). Furthermore and specific to 4-CP, the first step is the attack of phenol hydroxylase on 4-CP producing 4-chlorocatechol (Figure 2.4b.). Finally, the

conversion of 4-chlorocatechol via –meta cleavage pathway yields CHMS (5-chloro-2 hydroxymuconic semialdehyde), which is widely reported as a dead-end metabolite, at least when a pure culture is used in degradation (Buitrón et al., 2005).
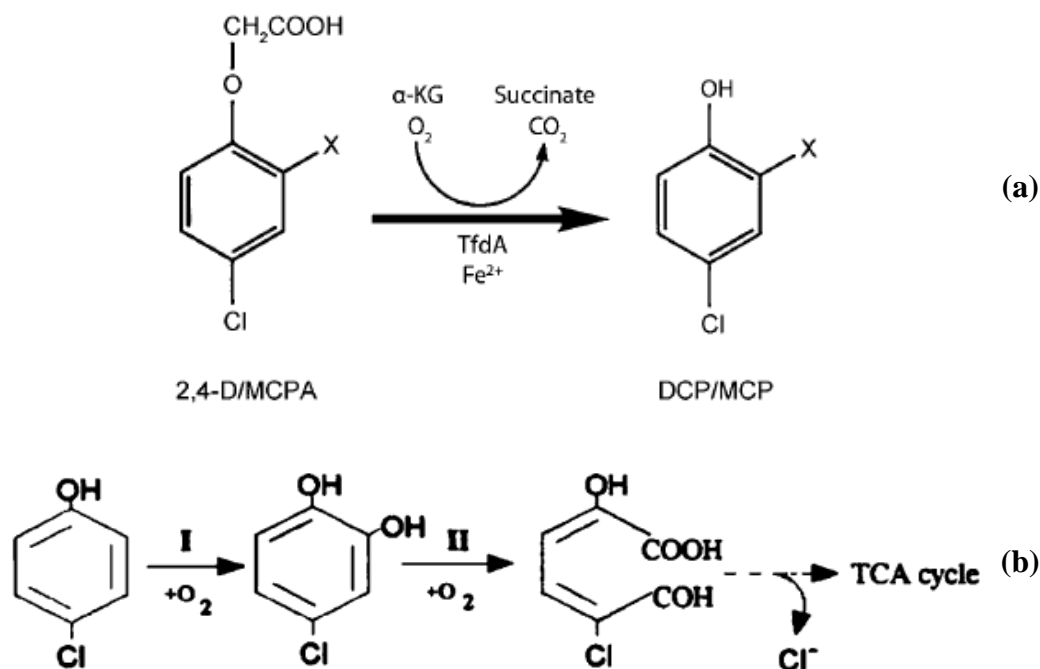
Figure 2.4. Proposed biodegradation pathway of (a) MCPA / 2,4-D and (b) 4-CP (adapted from Buitrón et al., 2005 and Baelum et al., 2008).

2.2.5. *Chlorinated herbicide MCPA in the environment*

Chlorophenoxy herbicides have been produced and used extensively in New Zealand since the 1950s. For example, New Zealand has been reported as the world's highest user of 2,4,5 trichlorophenoxyacetic acid (2,4,5-T) (Mannetje et al., 2005) although now it has been banned in New Zealand because of its extreme environmentally-hazardous nature. Nonetheless, other chlorophenoxy herbicides are still found in the market including MCPA. MCPA is a legally registered herbicide in New Zealand (Bourdôt & Saville, 1988) and commercially, it has been very successful because of its high ability to kill weeds and its relatively low price. MCPA is a selective, post-emergence agent frequently used to control unnecessary broad leaf weeds, primarily in cereal and grass seed crops (Costa et al., 2013). Foliar application is the general mode of use of this herbicide where it acts as a

phytohormone, taken up by broad leaf plants and resulting in nutrition deficiency and the subsequent death of plants (Schulz et al., 2012).
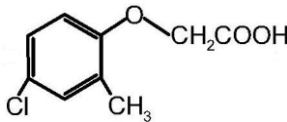
The structural and chemical properties of MCPA are found in Table 2.2; however, chemically, MCPA is a weak acid found in anionic form with a high mobility in the natural environment (soil and water). It therefore has potential for leaching into groundwater bodies (Hiller et al., 2006; Arias-Estévez et al., 2008) and, consequently, MCPA has been frequently detected at a concentration of 0.1-0.865 µg/L (ppb) (Donald et al., 2007; Gaw et al., 2008) in both surface and ground water bodies (WHO, 1996). There is also a growing concern about MCPA from a human health perspective since individuals occupationally and/or accidentally exposed to cholorophenoxy herbicides run the risk of increased cancers (Saracci et al., 1991; Mannetje et al., 2005). In addition, prolonged inhalation of MCPA residues can cause neurogenic affect, dizziness, burning in the chest and coughing (Crespín et al., 2001; Hiller et al., 2010).

The half-life of MCPA in soil is reported to be 4-16 days; (Crespín et al., 2001; Thorstensen et al., 2001; Hiller et al., 2006; Paszko, 2009) depending on the type and depth of soil, moisture content, pH and microbial population. Hiller et al. (2006) observed a high degradation of MCPA in soil that possessed a high organic content and microbial activity. Moreover, (Crespín et al. (2001) reported that microbiological degradation of MCPA is much more important than abiotic removal mechanisms, such as hydrolysis or photodecomposition. In biological degradation, microorganisms convert MCPA into 4-chloro-2-methylphenol and ultimately to harmless end-products such as CO_2 and H_2O.

Biological strategies to remove chlorophenoxy herbicides from wastewater have been investigated in the past. For example, Buisson et al. (1990) has suggested that MCPA can be degraded by microorganism in both aerobic and anaerobic environments. Similarly, Lechner

et al. (1995) demonstrated that gram-negative activated sludge microorganisms can degrade the 4-chloro-2-methylphenol, an intermediate product of MCPA, in batch culture studies. Furthermore, McAllister (1990) reported that an activated sludge system (consisting of Pseudomonas species) was capable of mineralizing landfill leachate containing high concentration of different chlorophenoxy herbicides (MCPA, 2,4-D and 2,4-T). Taking these studies into account, it is noted that activated sludge microorganisms have the ability to degrade the chlorophenoxy herbicide MCPA. However, to the best of the author's current knowledge, there does not seem to be any existing studies which focus on the removal of MCPA under denitrifying conditions.

Table 2.2 Physical and chemical properties of the pesticide MCPA.

Pesticide class	Phenoxy herbicide
Scientific name	2-methyl-4-chloro phenoxyacetic acid
Molecular formula	$C_9H_9ClO_3$
Structural formula	
Molecular weight (g/mole)	200.62
Appearance	White crystaline solid
Solubility in Water (mg/L)	273.9 (Hiller et al., 2010) at 25 °C
Dissociation constant (pKa)	3.07 (Vergili & Barlas, 2009)
Log Kow	2.73 (Environment Protection Agency, 2004;Vergili & Barlas, 2009)
Vapor Pressure (Pa)	2.3×10^{-5} Pa at 20°C (Roberts & Hutson,1999)
n-octanol-water partition coefficient	(WHO, 1996)

2.2.6. Abiotic loses

It should be noted than the removal of toxic compounds in the environment can also occur through abiotic means such as volatilization or bioadsorption.

a.   Volatilization: For volatilization, Henry's law is given by

$$m = HRT \tag{2.3}$$

Where:

H = Dimensionless Henry's law coefficient

m = Henry's law coefficient, atm-m^3/mol

R = Universal gas constant (8.2057 $\times$ 10^{-5} atm-m^3/mol-K)

T = Temperature, K

The value of the Henry's law coefficient is indicative of the tendency of a chemical to volatilize. A chemical can be considered to be volatile if m > 3$\times$ 10^{-7} atm-m^3/mol (H >1.2 $\times$ 10^{-5}), while a smaller value of m means that volatilization is usually an unimportant pathway of contaminant transport (Lyman et al., 1990). In the case of MCPA, Henry's law constant is < 10^{-7} atm-m^3/mol (Mabury & Crosby, 1996). Hence, the removal of MCPA occurs via volatilization would be expected to be very small.

b.   Bioadsorption

The n-octanol-water partition coefficient is an indicative of how likely a chemical compound dissolves in water or adsorbs onto organic particle (activated sludge in this case). The higher the n-octanol-water partition coefficient is, the higher would be the chance that a compound undergoes adsorption. For reference, the n-octanol-water partition coefficients of some organic compounds are presented in Table 2.3. From the table, it can be seen that n-hexane has the highest n-octanol-water partition coefficient which means it is very unlikely that n-hexane will dissolve in water if it was spiked in a jar containing both octanol (an organic compound) and water. The n-octanol-water partition coefficient of MCPA (26) is comparable to that of phenolic compound (28) indicating that there would be some chance of bioadsorption onto the cell wall of activated sludge microorganisms during a bioremediation process.

Table 2.3 n-Octanol-water partition coefficient of some organic compound.

Name of the compound	n-Octanol-water partition coefficient $\dfrac{(mole.L\ octanol^{-1})}{(mol.L\ water^{-1})}$	Affinity to absorption
n-Hexane	1300	Very high
Phenol	28	Slightly
MCPA	26	Slightly
Aniline	7.9	Low
Ethanol	0.48	Very low

Source: Collander, 1951; Schwarzenbach et al., 1993; WHO, 1996.

2.3. Technologies used to remove persistent micro-pollutants: Pesticides and nitrate removal in sequencing batch reactors

Sequencing Batch Reactors (SBRs) are a unit wastewater treatment operation consisting of 4 stages (Figure 2.5); filling, mixing plus reacting, settling and decanting (Tomei et al., 2010; Singh & Srivastava, 2011). Each SBR is a batch system (no flow-through during treatment) where specific operational parameters (such as sludge retention time (SRT) and hydraulic retention time (HRT)) and environmental parameters (e.g. temperature and pH) are normally set by engineers to optimize the process. Moreover, factors like organic loading rate, carbon to nitrogen (C:N) ratio, oxygen tension (aerobic, anaerobic or anoxic) strongly influence the treatment efficiency of an SBR. For example, Singh & Srivastava (2011) (citing Kuba et al., (1996)) mention that the anaerobic – anoxic sequencing batch reactor (A_2SBR) is an attractive process for domestic wastewater treatment since for complete removal of nutrients it produces less sludge and saves the aeration cost (as compared to an aerobic process). Because of their simple design and high reliability in respect to removing nutrients, SBRs have been widely accepted as an efficient method for treating wastewaters (Wilderer et al., 2001; Fernandes et al., 2013).
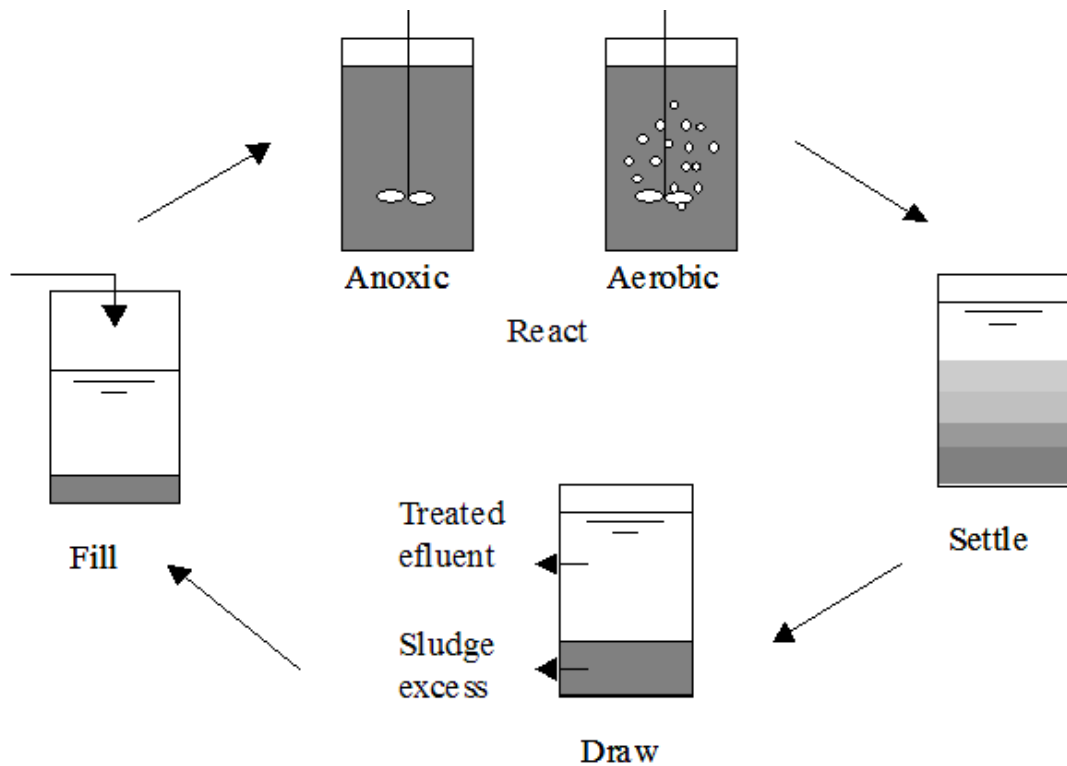
Figure 2.5. Operation of sequencing batch reactor (Source: www.inma.ucl.ac.be/EOLI/)

In the past decade, SBR technology has been extensively used for the treatment of xenobiotic compounds where it has proved to be a promising treatment strategy (Yoong et al., 2000; Chin et al., 2005; Celis et al., 2008, Elefsiniotis & Wareham, 2012). Generally, acclimatized biomass is produced in the SBR which is then used to degrade the compound (e.g. a pesticide) in the presence of an external carbon source. Mangat & Elefsiniotis (1999) however demonstrated that acclimatized biomass in an SBR could degrade a pesticide (2,4-D) both in the presence or absence of a readily biodegradable carbon source. Sanchis et al. (2013) demonstrated that SBR-acclimated biomass has the potential to degrade another chlorophenoxy herbicide MCPA. They reported that the biodegradability of this compound depends upon the biomass/substrate ratio as well as the testing time (i.e. a short biodegradability test using unacclimated activated sludge gave no biodegradation of the herbicide in 24 h whereas an acclimated biomass degraded the glucose (an external carbon

source) and herbicide simultaneously). Their results revealed the presence of a specialized bacterial population capable of using the herbicide as a carbon source.

There has also been some research carried out on the biodegradation of 4-chlorophenol (4-CP) in different SBR systems. For example, Buitrón et al. (2005) operated an SBR with high concentration peaks (shock loads) of 4-CP using two strategies; namely, variable-timing control (VTC) and observation-based, time-optimal control (OB-TOC). They concluded that the removal efficiency of 4-CP for both strategies was in excess of 98%, except during peak loads i.e. 1400 mg/L. In addition, shock loads caused complete shutdown of the VTC system whereas the OB-TOC strategy treated the 1400 mg/L concentration in less than 8 h without affecting the operation of the system. Moreover, Sahinkaya & Dilek (2007) showed that an SBR system can completely degraded 4-CP as well as 2,4-dichlorophenol (2,4-DCP) (chemical oxygen demand (COD) > 90%) suggesting that a long feeding time would be best for treating toxic wastewaters. Finally, Monsalvo et al. (2009) showed that 4-CP can be completely degraded in an SBR experiencing a wide range of influent concentrations (105-2100 mg/L) at a temperature between 25 and 350C.

In addition to pesticides, as mentioned nitrates are also a non-desirable constituent of municipal wastewaters. Removing both these constituents simultaneously is therefore a matter of great interest and several past studies have suggested that an SBR system can be designed to remove both of them in a simultaneous manner (He & Wareham, 2009; Lim et al., 2013; Kulkarni, 2013). In particular, Sarfaraz et al. (2004) demonstrated that granular denitrifying sludge produced in a sequencing batch reactor can remove 80% of both phenol and nitrate under anoxic conditions, up to an influent phenol concentration of 1050 mg/L. He & Wareham (2009) reported high removal of a pesticide (2,4-D) and nitrate in an SBR using naturally-generated volatile fatty acids as a supplemental carbon source. Another recent study

conducted by Lim et al. (2013) showed that acclimatized biomass produced in a moving bed sequencing batch reactor (MBSBR) can remove 4-chlorophenol and nitrate simultaneously, while Kulkarni (2013) reported that a simultaneous nitrification and denitrification (SND) SBR system can completely remove three different nitrophenols (2-nitrophenol; 2,4-dinitrophenol; and 2,4,6-trinitrophenol) (important building blocks and intermediates for large-scale production of pesticides) along with nitrogen under a regime in which concentrations of nitrophenols were gradually increased from 2.5 to 200 mg/L. To summarize, the literature suggests that an SBR is a simple, effective and cost efficient technology for biological removal of both nitrates and micro-pollutants (such as pesticides) from the environment.

2.4.   Research Objectives

Based on the above literature review, there seems to be a significant knowledge gap with respect to the biodegradation of the pesticide MCPA, particularly under nitrate-reducing conditions. In particular, the major aim of this research project is to investigate the potential for industrial-strength MCPA degradation, its limits and kinetics by activated sludge microorganisms in an anoxic sequencing batch reactor (SBR). To achieve this aim, the research project has been broken down into the following specific objectives. These specific objectives are correlated with four different experimental phases; namely, Phase I (a "proof-of-concept" or acclimatization phase); Phase II (an initial "tolerance" exploration phase); Phase III (an "effect of HRT" phase) and phase IV (a "limits" phase), as follows.

- **Phase I:** To investigate the simultaneous bio-degradation of industrial-strength concentration of MCPA and nitrate in the presence of a secondary carbon source (i.e. acetate).

- **Phases II:** To examine an initial tolerance capacity of anoxic microorganisms by increasing the concentration of herbicide.

- **Phase III:** To assess the effect of a change in hydraulic retention time (HRT) in the degradation of MCPA and other organic by-products.

- **Phase IV:** To explore the maximum limit of industrial-strength MCPA degradation by nitrate reducing microorganisms in an SBR.

Further to this, the denitrification ability of activated sludge microorganisms in a sequencing batch reactor was assessed in a baseline data collection phase. Moreover, the bio- kinetics parameters for MCPA, nitrate and acetate were quantified during each phase (I, II, III and IV).

This chapter describes details of the experimental setup, the sampling procedure and the methods of sample analyses that were followed during the collection of experimental data in the laboratory. Specific water and sludge quality tests were carried out according to Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Major experimental tests included total suspended solids (TSS), mixed liquor volatile suspended solids (MLVSS), chemical oxygen demand (COD), nitrate nitrogen (NO_3-N) and pesticide analysis via solid phase microextraction (SPME), gas chromatography (GC) and gas chromatography mass spectrophotometry (GC-MS). Occasionally, some other tests such as total organic carbon (TOC), nitrite-nitrogen (NO_2-N) and ammonia nitrogen (NH_4^+-N) were also carried out.

3.1.   Experimental set-up

3.1.1. Seed and feed.

The biomass (i.e. activated sludge microorganism seed) was sourced from the wastewater treatment plant (WWTP) located in the Bromley suburb of Christchurch, New Zealand. It was washed several times using tap water in order to remove unwanted grit before placing it into the reactors. To maintain the same COD throughout the research period (and for ease of operation), a synthetic wastewater was prepared using acetate (455 mg/L) as the carbon source with constituent elements (KH_2PO_4, 5.41 mg/L; $CaCl_2$, 0.26 mg/L; $NaHCO_3$, 0.59 mg/L; $MgSO_4 \cdot 7H_2O$, 0.13 mg/L; $FeSO_4.7H_2O$, 6.61 mg/L; $CuSO_4 \cdot 5H_2O$, 0.05 mg/L and $Al_2(SO_4)_3 \cdot 16H_2O$, 0.38 mg/L) mixed with tap water in order to make up a feed with approximately the same characteristics as municipal wastewater (i.e. 350 mg/L of COD) (Yoo et al., 1999; Aslan & Turkman, 2005 ; Chong & Chen, 2007 and Zela lab work, 2013).

3.1.2. SBR configuration.

Two cylindrical SBRs made of stainless steel were used, each with an internal diameter of 300 mm, total volume of 25 L and operating volume of 20 L. SBR-I (RC-I) was a control (i.e. no MCPA has been introduced) and SBR-II (RC-II) was a test reactor (i.e. incremental concentrations of the MCPA were injected). The SBRs were initially filled with washed biomass (10 L) and synthetic feed (10 L). Both reactors were operated in parallel, at room temperature (20-22º C) with a 40-60 day nominal sludge retention time (SRT) (Equation 3.1) and a 24 h hydraulic retention time (HRT) (Equation 3.2). A long SRT was used because the growth of biomass under anoxic conditions is presumed to be very slow from previous studies (Fongsatitkul et al., 2008) indicating that industrial strength wastewaters are readily treated in SBRs using long SRTs of up to 60 days.

Three inlet/outlet ports were installed in each SBR for feeding, decanting and sample collection/wasting. An external mixer was used for mixing to avoid air entrainment. Filling, mix plus react, settling and decanting functions (Table 3.1) were controlled via a programmable controller. In addition, oxidation reduction potential (ORP) probes were inserted into each reactor to monitor the denitrifying activity (Sabumon, 2008). One personal computer was attached to each SBR on which the "Lab View" software was run to monitor overall system parameters. At the end of each SBR cycle, biomass was allowed to settle and the supernatant was decanted. Effluent and mixed liquor samples were taken for analysis and the same amount of wastewater (synthetic feed) was fed to the reactor as fresh substrate while the source of electron acceptor (i.e. NO_3-N), in the form of sodium nitrate, was injected twice per cycle A photo of the experimental set-up is presented below (Figure 3.1) and a schematic of the experimental set-up is shown in Figure 3.2

Table 3.1 Timing of sequences over a 12-h cycle of the SBR.

Process	Fill	Mix 1	N(1)+MCPA pump	Mix 2	N-Pump 2	Mix 3	Settle	Decant
Time (min)	5	10	1	326	1	326	46	5

Equations for the design parameters SRT and HRT:

$$\text{SRT} = \frac{\text{Volume of tank (V)}}{\text{Sludge Wasteage Rate (Qw)}} = \frac{20\ \text{L}}{200\ \text{to}\ 500\text{ml/day}} = 40\ \text{to}\ 60\ \text{day} \qquad (3.1)$$

$$\text{HRT} = \frac{\text{Volume of tank (v)}}{\text{Effluent Decanted(Q)}} = \frac{20\ \text{L}}{20\ \text{L/day}} = \frac{20\text{L}}{\frac{20\text{L}}{\text{day}} * \frac{1\text{day}}{24\text{hr}}} = 24\ \text{hr} \qquad (3.2)$$
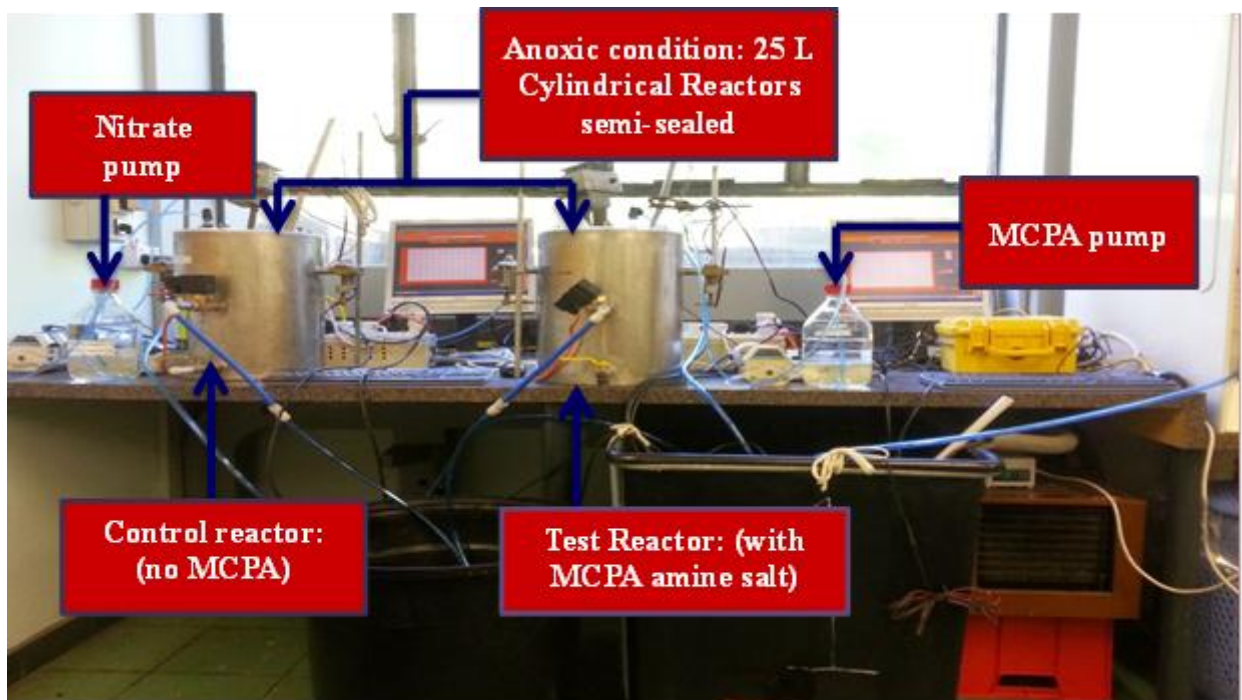
Figure 3.1. Experimental set-up.

Figure 3.2. Schematic representation of experimental setup of sequencing batch reactor.
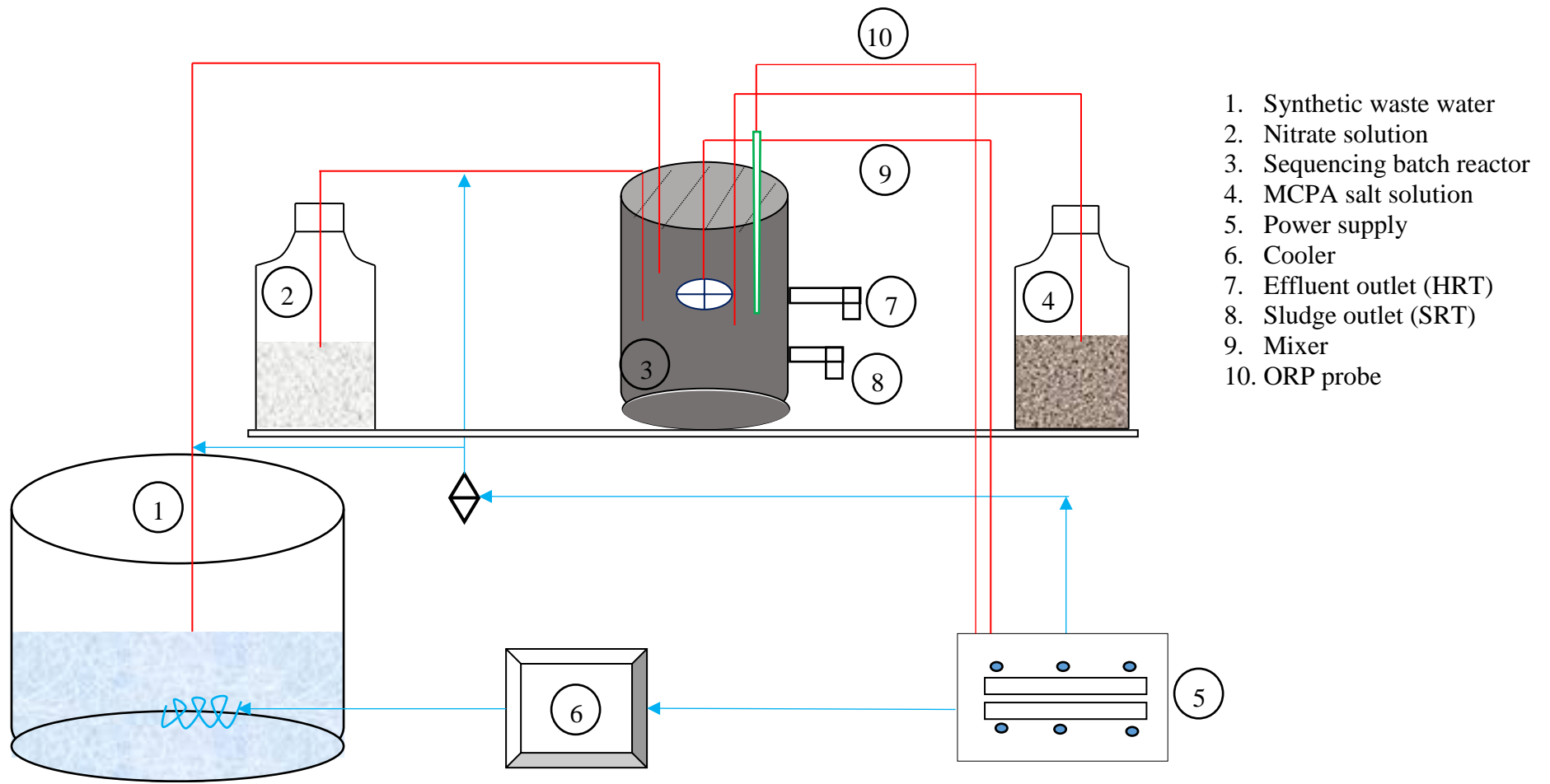
1. Synthetic waste water
2. Nitrate solution
3. Sequencing batch reactor
4. MCPA salt solution
5. Power supply
6. Cooler
7. Effluent outlet (HRT)
8. Sludge outlet (SRT)
9. Mixer
10. ORP probe

3.2. Feed supply system

In the initial stages of the study, 200 L of synthetic feed was prepared normally once every four days using the appropriate amounts of sodium acetate, potassium phosphate and other trace elements (mixed with tap water). However, track studies from 2 to 3 weeks of data regarding COD degradation inside the feed tank showed that the COD was dropping considerably (350 mg/L to 270 mg/L) by the third or fourth day. This was not desirable since when the COD varied in the feed tank, this directly influenced the COD inside the bioreactor which affected the COD: NO_3-N ratio making it difficult to stabilize the system. Thus, only 120 L of synthetic wastewater was prepared every two days and this was continuously cooled maintaining the desired COD of 350 mg/L. According to the timing of the SBR sequence (set by the operator), bacteria were fed 10 L of synthetic feed and 10 mL of trace elements via the feed pump during each feed. Similarly, the correct amount of nitrate (i.e. 100 mL of a 34 g/L $NaNO_3$ solution) was fed twice per cycle in order to maintain the reactors in anoxic conditions (i.e. ORP = -250 ± 25 mV).

3.3. Sampling procedure

The system was operated initially on a 24 h HRT basis; thus, every 24 h, two decant periods occurred. Decanted samples were collected in a clean vessel for analysis once or twice a week depending upon the requirements. Samples for soluble parameters (i.e. COD, NO_3-N, NO_2-N and NH_4-N etc.) were acidified with 1-2 drops of concentrated H_2SO_4 to arrest biological activity and filtered through a 0.45-µm membrane filter before analysis. For the purposes of COD and nitrate track studies, samples were taken at 0 min, 10 min, 20 min, 40 min, 1 h and every hour after that for five hours. Post-feed samples were quite murky since the reactors were in the mix/react stage when the samples were taken; thus, the samples were processed through multiple steps before analysis (i.e. centrifuged at 4400 rpm for 10 min, acidified to pH <2 and filtered using a 0.45-µm membrane filter).

3.4. Analytical methods

3.4.1. Total suspended solids (TSS) and mixed liquor volatile solids (MLVSS).

These were calculated daily for the first few months and 2-3 times per week after that, and always during a track study. Samples were taken when complete mixing occurred. A standard glass-fiber filter (Whatman glass fiber filter circles; grade GF/C; 90 mm ϕ) was used which had previously been oven dried at 103 to 105°C for at least 24 h prior to the test. The weight of the filter paper alone was first taken using an analytical balance (i.e capable of weighing to 0.1 mg). Then, the filter paper was wetted with a small volume of distilled water to seat it on the filtering apparatus. For a more rapid filtering process, a vacuum was applied. After that, a measured volume (normally 10 mL) of well-mixed sample was passed through the filter. The sample volume for TSS was chosen so that it normally yielded a residue of 10 to 200 mg. Then, the filter paper was put in the oven (103 to 105 °C) for at least one hour to evaporate the entire water content from the filter paper. Before reading the weight of the filter with the suspended solids, it was cooled down in a desiccator to achieve a constant mass. The difference in the mass of the filter before and after the filtration yielded the TSS. To obtain the MLVSS, the filter paper with the solid mass was ignited in a furnace at 550 °C for 1 h and cooled in a desiccator for 0.5 h before weighing. The weight lost per unit volume of sample on ignition was calculated as the MLVSS.

3.4.2. Chemical Oxygen Demand (COD).

COD is a measurement of the oxygen equivalent of the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant. The COD was measured by digesting the sample in a digester using potassium dichromate. A silver compound (Ag_2SO_4) was used as a catalyst to promote oxidation and a mercuric compound ($HgSO_4$) was used to reduce the interference of chloride ions on the oxidation by the dichromate. The oxidation

process was carried out by mixing 2 mL of a sample with 5 mL of high range (0 to 600 mg/L) digestion solution in glass tubes with a screw cap (10 x 100 mm). This was allowed to digest in a HACH Digital Reactor Block 200 (DRB200) at 150 °C for 2 h. After the oxidation step was completed, the amount of dichromate consumed was determined colorimetrically using a HACH digital DR 3900 spectrophotometer.

3.4.3. Nitrate (NO_3-N).

Nitrate was measured by the cadmium reduction method, which is a colorimetric method that involves contact of the NO_3^- in the sample with cadmium particles, which cause NO_3^- to be converted to NO_2^- (HACH, 2003; APHA, 2005). The NO_2^- produced is determined colorimetrically by diazotizing with sulphanilamide and coupling with N-(1-napthyl)–ethylenediamine dihydrochloride (NED dihydrochloride) to form a red color azo dye whose intensity is proportional to the original amount of NO_3^--N. The red color is measured by a spectrophotometer that measures the amount of light absorbed by the treated sample at a 500-nanometer wavelength. The absorbance value is then converted to the equivalent concentration of NO_3^--N by using a standard curve. The reagents used for this method were purchased from the HACH Company in packages. Among the available reagents in different ranges, almost all of the NO_3^--N values in this research were measured by using the high range (0 to 30 mg/L) reagent (NitraVer® 5, Nitrate reagent for 10 mL sample, Cat.21061-69). A spectrophotometer HACH DR 3900 was used to measure the absorbance and consequently the concentration of NO_3^--N. Testing was performed in accordance with the procedure described in HACH method 8039 (HACH, 2003).

3.4.4. Nitrite (NO_2-N).

Nitrite was measured using the ferrous sulfate method. In this method, the reagent ferrous sulfate is used in an acidic medium to reduce the NO_2^- to N_2O. Ferrous ions combine with the

N_2O to form a greenish–brown complex in direct proportion to the NO_2^- present. The procedure and reagent (NitriVer®2) were used in accordance with HACH method 8153 (HACH, 2003). The intensity of the color was measured by the HACH DR/3900 spectrophotometer.

3.4.5. *Ammonia (NH_4^+-N).*

Ammonia was measured using the salicylate colorimetric method. In this method, NH_4^+ compounds combine with salicylate to form monochloramine, which reacts with salicylate and forms 5-aminosalicylate. Then in the presence of sodium nitroprusside catalyst, the 5-aminosalicylate is oxidized and forms a blue colored compound. The blue color is masked by the yellow color from the excess reagent to give a final emerald-green complex (HACH, 2003). The emerald-green color developed in the reaction is proportional to the presence of NH_4^+ and measured at 655 nm. HACH Test N Tubes for high range (NH_4^+ to 50 mg/L NH_4^+-N) were used for all the tests during this research. A spectrophotometer HACH DR/3900 was used to measure the intensity of color and consequently the concentration of NH_4^+ present in the samples. Testing was performed by following the procedure steps described in HACH method 10031 (HACH, 2003). Several tests were done on different concentrations of the NH_4^+- N standard solution to check the sensitivity of the method before analyzing the sample.

3.4.6. *Oxidation Reduction Potential (ORP).*

The ORP probe used in this research was manufactured by the company YSI. The range of the ORP measurement was reported to be - 1999 mv to +1250 mv with an accuracy of $\pm$ 0.1 % $\pm$ 1 digit. The reference electrodes were Ag/AgCl and 3.5 M KCL gels, while the redox electrode was platinum. The probes were calibrated using quinhydrone buffer solutions (at pH = 4 and 7) according to the manufacturer guidelines before they were inserted into the

reactors. Two ORP probes were introduced into each reactor through a hole in the top of the reactor while a rubber stopper was used to prevent air from entering the reactor and to maintain anoxic conditions. Twice a month, the ORP probes were checked using the quinhydrone buffer solutions and cleaning of the platinum sensing surface was performed with deionized water and a soft cloth in order to prevent errors caused by sludge coating. The cleaning of the platinum sensing surface was conducted just after finishing the feed/waste cycle in order to minimize the effect on the ORP value from being cleaned by pure water (which has a high positive ORP compared to the negative ORP value experienced under anoxic conditions). The installation hole was temporarily blocked using another identical rubber stopper to avoid air entrainment during the cleaning periods. The ORP was continuously monitored by a Lab view program allowing real time data to be collected which was saved automatically via the process control system (to the computer hard drive). The reproducibility of the ORP profile and the sensitivity of the measured potential made ORP an ideal parameter for automatic monitoring.

3.4.7. Total Organic Carbon (TOC).

TOC was measured using a Shimadzu TOC-L CSH analyser with TOC-control L v1.01 software. Prior to the analysis of the sample, standards of Total Carbon (TC) and Inorganic Carbon (IC) were prepared for calibration, which are as follows:

TC 1000 ppm standard stock (KHP)

a.   Dry a few grams of reagent grade potassium hydrogen phthalate (KHP) for an hour at 110 ºC.

b.   After drying, place the KHP in a desiccator to cool (thermal currents from hot material may cause weighting inaccuracies).

c.   Accurately weight 2.125 g KHP and transfer quantitatively to a 1000 mL volumetric flask.

d.   Bring to volume with deionized (DI) water and stir. The carbon content is 1000 mg/L organic carbon.

IC 1000 ppm standard stock (sodium carbonate/bicarbonate)

a.   Dry several grams of reagent grade sodium hydrogen carbonate (sodium bicarbonate) over night in a silica gel desiccator. Dry several grams of reagent grade sodium carbonate for an hour at 250 ℃.

b.   Accurately weight 3.497 g sodium bicarbonate and 4.412 g sodium carbonate and transfer quantitatively to a 1000 mL volumetric flask.

c.   Bring to volume with DI water and stir. The carbon content is 1000 mg/L inorganic carbon.

d.   When tightly sealed and stored in a fridge, the stock solution may be retained for 2 months.

3.5.   Process control system

The SBRs were operated automatically using a microprocessor-based control system which ran on a 24 h basis. The process control system was composed of two key elements: a central control box and a personal computer. The central control box was microprocessor based and contained 16 analogue inputs, 16 digital inputs, and 16 digital outputs. This allowed complete and independent control of all the required SBR functions. In addition, the microprocessor was programmed to act as a stand-alone unit operating on a predetermined timer basis until being manually overridden by an operator. The personal computer ran a software program developed with the Lab View package from National Instruments. The software allowed the

computer to control and monitor the process using either an on board A/D card or via one of the computer communication ports. Furthermore, the ORP probes were joined to communication ports of the PC computer to indicate "biological activity". Figure 3.3 shows a schematic of the process control system.
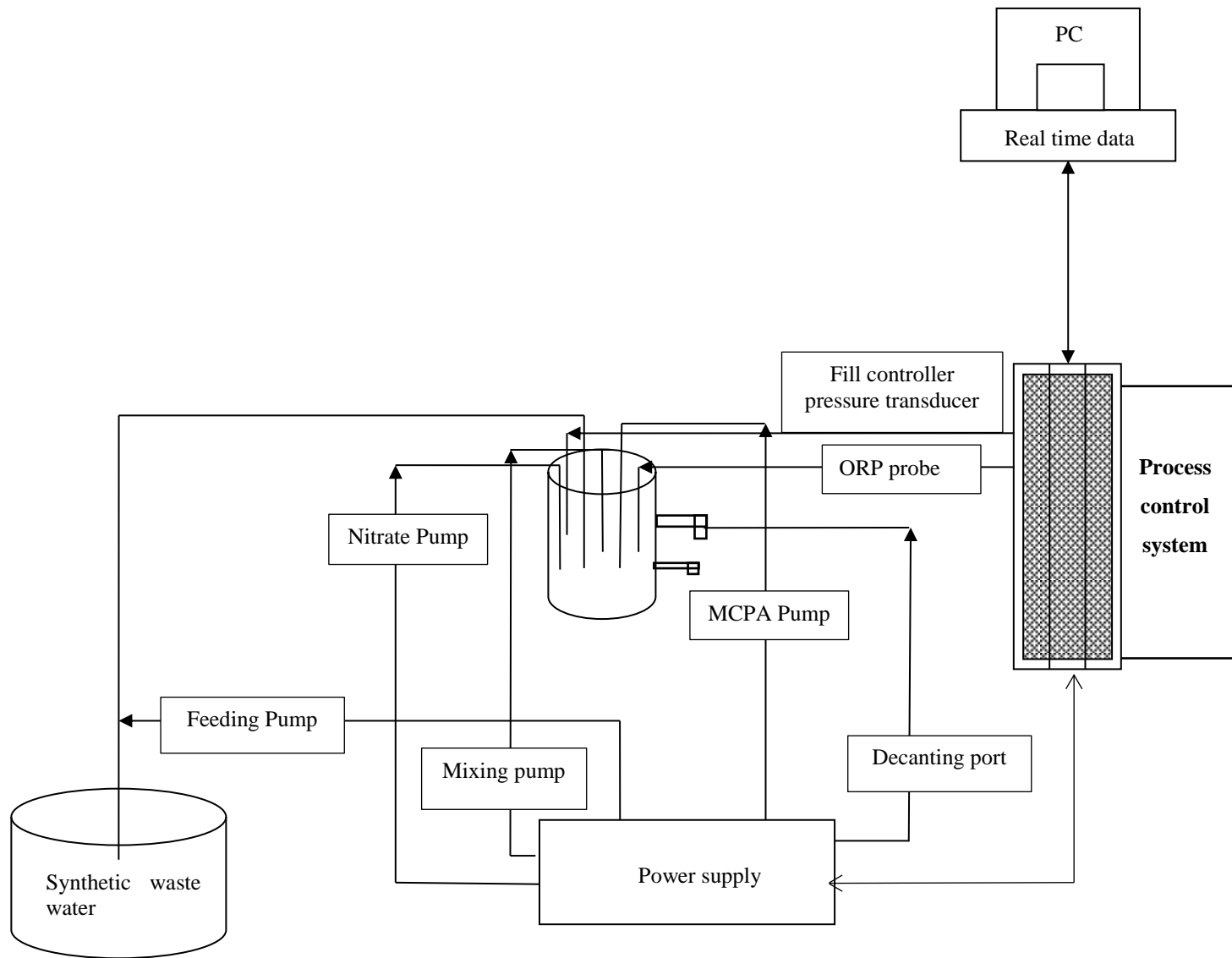
Figure 3.3. Schematic representation of the process control system.

3.6.   Pesticide injection strategy

During start-up, no pesticide was injected into the SBR to minimize toxic effects to the microorganisms; thus at this stage, acetate was the only organic carbon source. The start-up (preliminary) phase lasted for three to four months. After that, pesticide was added to the bioreactor since the biomass had exhibited stable behavior (i.e. steady denitrification activity with COD removal more than 95%; a stabilized anoxic range for the ORP value (-250 ± 25 mV) and, a consistent total (3600 ± 400 mg/L) and volatile (2800 ± 300 mg/L) suspended solids concentration).

MCPA acid of high purity is less soluble in water than the commercial product used by farmers (i.e. the dimethylamine salt of MCPA (DMCPA)) which is highly soluble (up to 750 g/L). When DMCPA is released into the environment, it is quickly converted into its acid form since MCPA salts are relatively unstable and acid is the herbicidal form (Thurston County Health Department, 2000). Therefore, this study was conducted by injecting DMCPA into the reactor which was then converted to MCPA acid in the reactor. The Dimethylamine salt of MCPA herbicide also known as Pasture Guard MCPA 750 (Ravensdown) is a commercial product used in New Zealand to control broad leaf weeds. The label states that the active ingredient contains 750 g/L MCPA as the dimethylamine salt in the form of a soluble concentration. However, besides the active ingredients there might be other additives used during the commercial formulation. As an example, Crosby and Bowers (1985) list a few of such constituents that are presented in Table 3.2.

Table 3.2 Typical analysis of MCPA amine formulation (adapted from Crosby and Bowers, 1985) 5

Constituent	Amount (%)	Method
4-chloro-2-methyl- Phenoxyacetate salts	>95	GLC, HPLC, GCMS
2-methyl- Phenoxyacetate salts	1	HPLC, GCMS
6-chloro-2-methyl- Phenoxyacetate salts	0.5	GCMS
Other dichloro-2-methyl phenols	trace	GCMS
4,6-dichloro-2-methyl-neutrals	trace	GCMS
(4-chloro-2-methylphenoxy)- N,N-dimethylacetamide	0.03	GLC

During the acclimatization period (i.e. the "proof-of-concept" phase) a relatively small but constant concentration (i.e. 20 mg/L) of DMCPA was fed to the biomass. Once MCPA degradation was observed in the SBR, the concentration of the herbicide was increased to 50 mg/L (Phase II and III) to test whether the system could tolerate that amount. Finally, in phase IV the concentration was increased to 75 mg/L to find the potential upper limit of MCPA degradation by anoxic microorganisms.

During each concentration, 3 to 5 track studies were carried out at 0, 0.5, 1, 2, 3, 4, 6, 9 and 12 hour intervals. For each track study, samples were first centrifuged (4400 rpm for 10 min) followed by filtering using a 0.45-µm membrane filter. The pesticide was extracted using the solid phase micro extraction (SPME) technique before being injected into the gas chromatograph. Some samples were preserved and stored (at 4º C) in the fridge following the EPA guidelines for pesticide analysis (EPA, 2007). Furthermore, data analysis of pesticide

degradation was done using Microsoft excel and modelling were carried out using Matlab version R2012b.

3.7. Pesticide extraction and analysis method

A solid phase micro extraction (SPME) technique (Figure 3.4) was used to extract the pesticide from the wastewater and this technique uses polydimethylsiloxane (PDMS) -coated SPME fibers (100 μm film thickness), a manual holder and 4-mL vials purchased from Supelco, New Zealand. The conditions for SPME extraction were similar to those used by Boussahel et al. (2002) and Krzyzanowski et al. (2008) for chlorinated pesticides/MCPA in water. New SPME fibers were first conditioned by heating at 250ºC for 0.5 h in the inlet port of the gas chromatograph. Two mL of the sample was then placed in a capped vial and the fiber was then exposed to the vial headspace while the sample was continuously stirred and heated at 50ºC with temperature control (Figure 3.4). When equilibrium was achieved, after 30 min, the fiber was taken out of the vial and introduced into the GC injector (Figure 3.5) where thermal desorption of the analyte was done for 3.5 min.
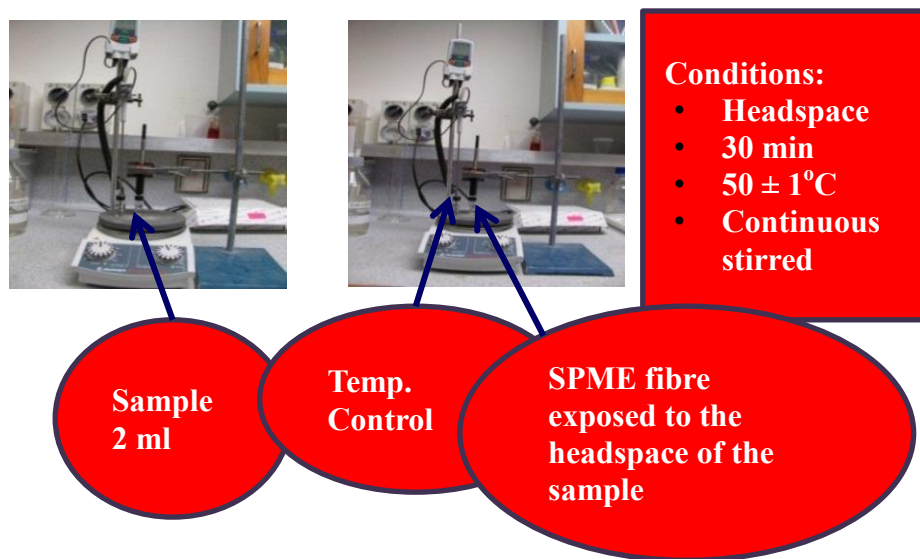
Figure 3.4. SPME technique.

Herbicide analysis was performed on a Hewlett-Packard gas chromatograph (HP 6890 series), equipped with a Ni-63 electron capture detector. A fused silica capillary column SPB-5TM (30 m x 0.25 mm x 0.25 μm film thickness) was used with a liner (Agilent 5181-3315;4mm ID dbl tap) appropriate for SMPE desorption. Helium of high purity (99.99%) was used as the carrier gas and nitrogen (99.99%) was used as the make-up gas. The column flow pressure was 54.60 psi and the flow rate was 2 mL/min. The injector and the detector temperatures were 200ºC and 280ºC, respectively. The initial oven temperature was set to 200ºC for 2 min; then it was programmed to increase up to 280ºC at a rate of 20ºC/min and held for 1.5 minute. The total run time of the analysis was 7.5 minutes. The peak area was used for the calculation of the herbicide concentration in mg/L.

Figure 3.5. Introducing SPME fiber into GC sample injection port.

The GC-MS analysis was performed on a Shimadzu QP2010 Plus instrument with manual injection. The GC was equipped with a SPB-5TM capillary column (30 m x 0.25 mm x 0.25 μm film thickness) with an appropriate glass liner (Shimadzu™ GC Models 17A (SPL-17 Injector)) for SMPE desorption in splitless mode. The oven temperature was programed with an initial temperature of 80 ℃ (for 2 min) followed by a ramp up from 80 ℃ to 280 ℃ (at 20 ℃/min). The final temperature (280º C) was held for 6 min. The column head pressure was 145.9 kPa and the injection temperature was 220℃. High purity grade (99.99 %) helium was used as the carrier gas at a flow rate of 2.8 mL/min. The GC was coupled to a mass spectrometer (GCMSQP2010 series) with capacity for scan data collection at up to 10,000 amu/sec and wide expanded mass range m/z 1.5 – 1090. Similarity search was done via retention Index (with the NIST library). MCPA detection was accomplished using the following fragment ions: m/z 141, 155, 214. Similarly, the major intermediate product of MCPA via microbial biodegradation (i.e. 4-chloro 2-miethyl phenol) was identified using the fragment ions: m/z 77,107,142.

3.7.1. MCPA peak identification and quantification

MCPA acid (purity > 99.9%) was purchased from Supelco, New Zealand and a calibration curve was prepared using the SPME-GC technique. Identification and quantification of MCPA in the effluent sample was done by comparison with the calibration curves. According to the physical structure, one mole of MCPA dimethylamine salt contains one mole of MCPA acid; therefore.

Molecular weight of dimethylamine salt = 245.7g/mole

Molecular weight of MCPA acid = 200.62g/mole

One gram of MCPA dimethylamine salt will produce $= \frac{200.627 g/mole}{245.77 g/mole} = 0.816$ gram of MCPA acid.

Thus, the theoretical yield of MCPA acid for various levels of MCPA salt fed to the bioreactor is shown in Table 3.2.

Table 3.3. Theoretical yield of MCPA.

Amount of DMCPA (mg/L)	Conversion factor	Theoretical yield of MCPA acid as Influent (mg/L)
20	0.816	20*0.816= 16.32
50	0.816	50*0.816= 40.8
75	0.816	75*0.816= 61.2

3.7.2. Method Verification

The reproducibility of this extraction technique for the studied chlorinated pesticide (i.e. MCPA) is illustrated in Table 3.3. The response factors obtained for a concentration of 30 mg/L is very satisfying, with a coefficient of variation 10.11%. Boussahel et al. (2002) reported coefficient of

variation (CV) of 8 to 18% for different chlorinated herbicide and stated that CV less than 20% for pesticides analysis indicates an acceptable precision.

Table 3.4. Coefficient of Variation.

Repetition (n=7)	Response (Hz)
1	8696.063
2	7584.25
3	6144.931
4	7522.756
5	8175.009
6	7122.128
7	8136.291
Average	7625.918
Std. Dev	771.5196
C.V (%)	10.11707

3.8. Batch tests

In order to understand the detailed kinetics of carbon consumption, nitrogen removal and MCPA formation and degradation, batch tests were carried out during each phase (Phases I, II, III and IV) with each set being replicated at least 3 times so as to produce reproducible results. Biomass from both SBRs (RC-I: control reactor and RC-II: MCPA test reactor) was taken separately and the experimental set up was constructed as shown in Figure 3.6.

3.8.1. Batch test procedure

a. Five hundred (500) mL of mixed sludge was taken at the end of the cycle from each SBR. This was then settled for 1 h and the supernatant was poured out. The "biomass" was washed two to three times (in order to bring the initial COD concentration to the same level for both reactor). After that, appropriate amounts of acetate, nitrate and herbicide solutions were injected as shown in the calculations below.

b. The headspace of a 1-L batch test bottle was then flushed with nitrogen gas to maintain anoxic conditions.

c. Thirty five (35) mL samples were taken at 0 min, 10 min, 20 min, 40 min, 1 h and every hour after that for five hours (sometimes until 12 h) and tests were run to calculate the COD, nitrate and herbicide uptake rate. The experiments were repeated for at least three times to acquire reproducible results.

d. Calculations and matrix

$$COD = 350 \text{ mg/L} * 0.494 \text{ L} = 172.9 \text{ mg}$$
$$NO_3\text{-}N = 14/85 * 34000 \text{ mg/L} * 0.005 \text{ L} = 28 \text{ mg}$$
$$COD: NO_3\text{-}N = 172.9 \text{ mg}/28 \text{ mg} = 6.17$$

The ratio of COD to NO$_3$-N is similar to the mother (SBR) reactor for the initial 5 hour period. Additionally, 0.05 mL of micro elements added to each batch reactor. The batch test matrix is presented in in Table 3.4.
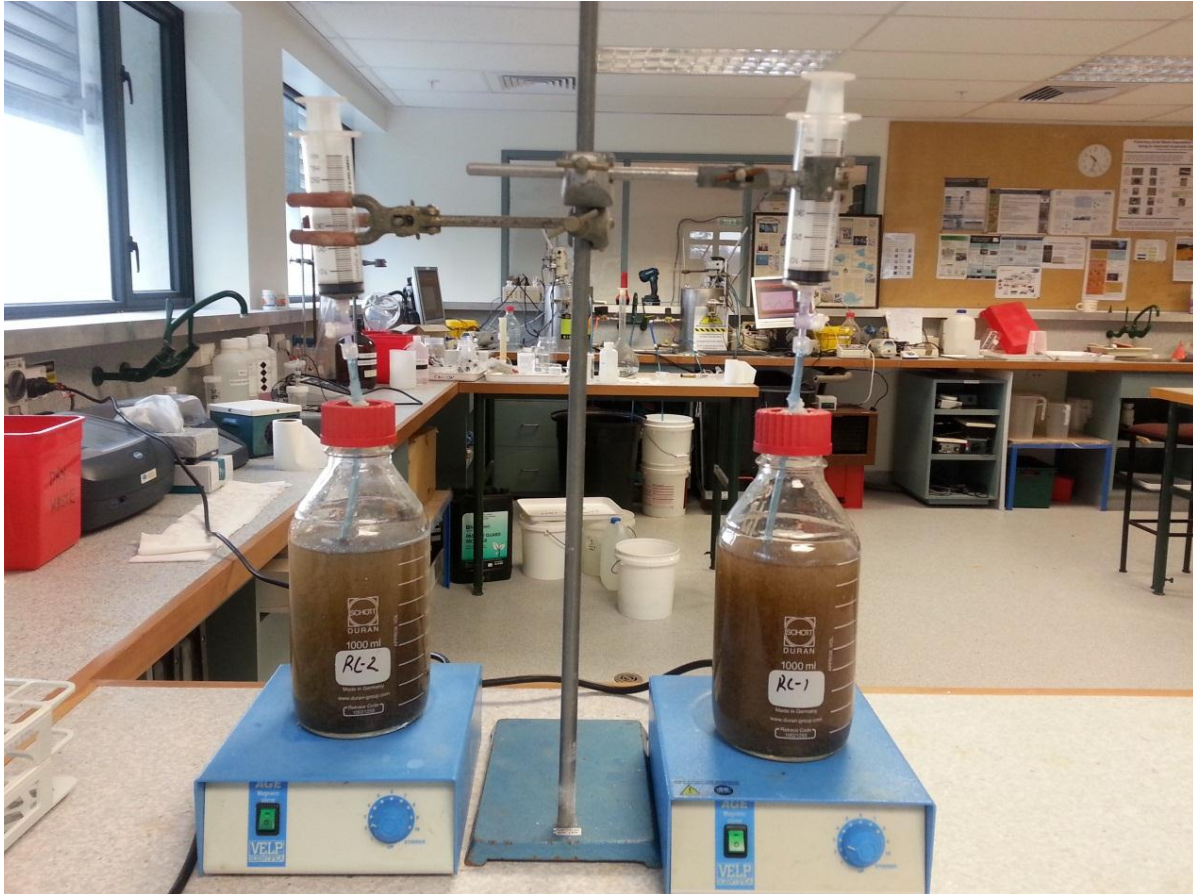
Figure 3.6. Denitrification batch test running in 1-L anoxic vessel at 24 h HRT.

Table 3.5. Batch test matrix.

	RC-1(Control)	RC-2 (MCPA feed)	COD: NO$_3$-N
Biomass (sludge)	500 mL	500 mL	
Acetate sol. (food)	494 mL	494 mL	
Nitrate sol.	5 mL	5 mL	
Total volume of single batch	1 L	1 L	6.17

3.9. Statistical methods

Statistical analyses such as average and standard deviations were calculated at each point to quantify the amount of variation or dispersion of a set of data values. Furthermore, single factor analysis of variance (ANOVA) and student t-tests were carried out to find out whether the effect of incremental concentrations of herbicides to anoxic microorganisms was statistically significant or not. In particular, single factor ANOVA investigated at least one inequality between the means of four bio-kinetic parameters (i.e. k_{COD}, K_N, r_{COD} and r_N) by the increasing concentration of herbicide whereas student t-tests examined the specific effect of herbicides at different phases (i.e. Phase I, II, III and IV).

3.10. Equipment and container cleaning procedure

Cross contamination of the lab equipment was potentially a major issue during the pesticide analysis and as such a specific cleaning procedure as outlined by Dec bureau of spill prevention and response (DECBSPR) was followed. This included:

a.   The equipment was washed thoroughly with laboratory detergent and hot water using a brush to remove any particulate matter or surface film;

b.   The equipment was then put in an acid bath for 12 h or overnight;

c.   The equipment was then rinsed thoroughly with hot tap water;

d.   The equipment was then thoroughly rinsed with deionized water;

e.   The equipment was then put inside the oven for 1-2 h to air dry; and,

f.   The equipment was stored in a plastic container to prevent airborne contamination.

PART 2: RESULTS AND DISCUSSION

This chapter summarizes the results obtained during the preliminary baseline data collection phase i.e. the period after the system started performing steadily and before injecting any pesticide into the bioreactor. The criteria for reaching pseudo-steady-state in the SBRS were (i) to reach a stable biomass concentration in terms of TSS and VSS (ii) to attain stable effluent characteristics (i.e. > 95 % COD and nitrate removal) and (iii) to obtain reasonably stable ORP data (ORP = -225 ± 25 mV) for anoxic conditions.

4.1.   TSS and VSS

The TSS and VSS values were large at the start of the experiment (5000 mg/L and 3000 mg/L) but dropped to 2000 mg/L and 1500 mg/L respectively as the reactor operation began (Figure 4.1). However, the TSS and VSS eventually stabilized at 4000 ± 400 mg/L and 2800 ± 300 mg/L as the system became established. The initial drop in both parameters is likely because the microorganisms were brought directly from the aeration tank of a full-scale biological wastewater treatment plant, where sufficient carbon and oxygen was always present. The bacteria were then exposed to an environment in which the COD concentration was different (as well as it being the only carbon source (i.e. acetate)). In addition, the biomass was undergoing electron acceptor acclimation in the sense of switching over to using NO_3-N as an electron acceptor instead of oxygen. As soon as the biomass adjusted to the new carbon source and a COD:NO_3-N ratio of 3.2 was obtained, then the SBR started performing consistently. The target COD:NO_3-N ratio of 3.2, was near that reported by Sarfaraz et al. (2004) for the treatment of phenolic wastewater under anoxic conditions.
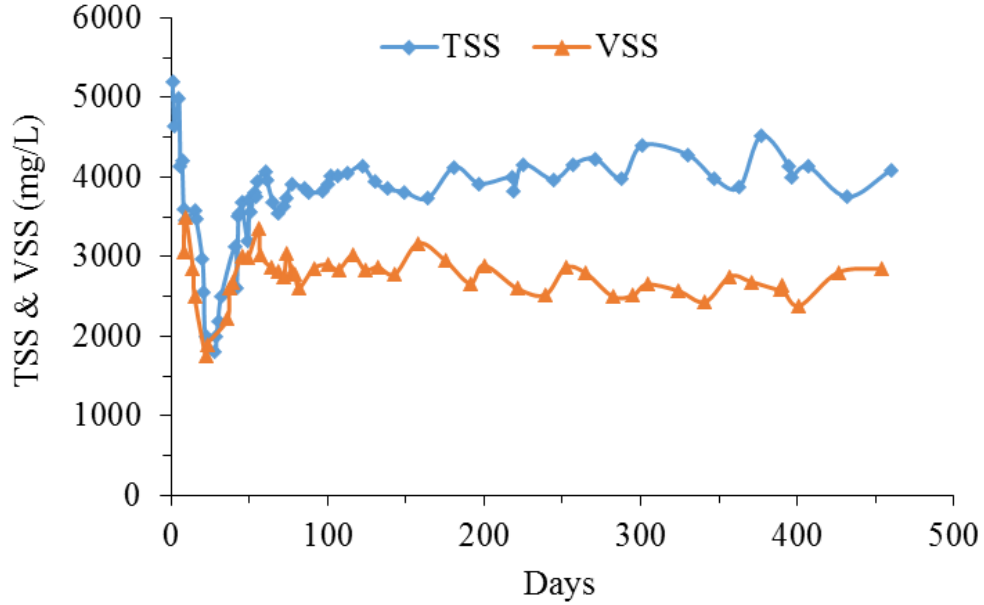
Figure 4.1. Stability of operation as shown by TSS and VSS.

4.2.   COD and Nitrate

Once stable solids concentrations were achieved, COD and nitrate track studies were carried out on five different dates. Average COD and nitrate values (normalized) were then plotted against time as shown in Figure 4.2. These results show that more than 95% of the COD and nitrate were effectively removed by denitrifying bacteria within the first five hours. Note that the system was designed to maintain the bioreactor in anoxic conditions (i.e. an ORP of -250 ± 25 mV), thus as mentioned, a second dose of nitrate was injected into the SBR after five hours of reaction time.
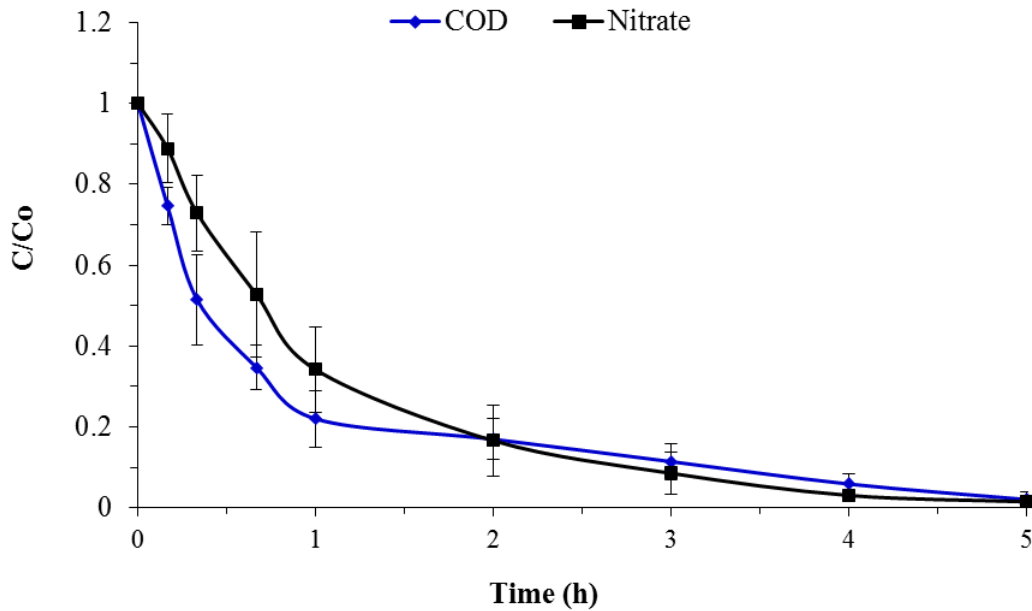
Figure 4.2. Stability of operation as shown by COD and nitrate track studies; average values

(n=5) and standard deviation (error bars) are shown.

Furthermore, a fresh sample of 1 L was taken from the reactor after feeding synthetic feed to the system for visual inspection (Figure 4.3). The figure indicates that small gas bubbles were formed in the system, pushing the biomass to the surface layer of the liquid in order to release the gas into the environment. Once, the gas was released, the biomass again moved to the bottom of the reactor and the cycle continued. Regular monitoring of the headspace of the bioreactor indicated that there was no methane present inside the reactor. Similarly, it was believed unlikely that significant amounts of CO_2 would be produced under effective denitrification activity (track study results above); thus it was concluded that the produced gas bubbles were most likely to be nitrogen gas.

Figure 4.3. Visual inspection of denitrification activity.

4.3. Typical ORP curve

As mentioned, ORP was monitored throughout the research period in order to maintain the bioreactor under anoxic conditions (ORP = -250 mV ± 25 mV). A typical plot of an ORP curve is presented in Figure 4.4. The figure suggests that as soon as the first dose of nitrate was injected into the bioreactor, the biomass started to reduce nitrate to nitrogen gas which can be seen by the descending portion of the ORP curve. The first denitrification process was completed in around five hours. At the end of nitrate reduction, the ORP curve quickly moved into an anaerobic zone (a theoretical sulfate reduction zone) and a knee type of structure was formed, known as the "nitrate knee" or "nitrate valley". Such a phenomena has been widely observed by various researchers in the past for denitrification processes operating in a sequencing batch reactor (Wareham et al., 1993; Tanwar et al., 2008). Finally, when the second dose of nitrate was introduced into the bioreactor, an increase in ORP values was observed and the reactor slowly moved back into the anoxic zone. The increase in ORP values can be attributed to the sensitivity

of the ORP probe to NO$_3$-N injection and in general, the ORP value remained in the anoxic zone

for most of remaining period.
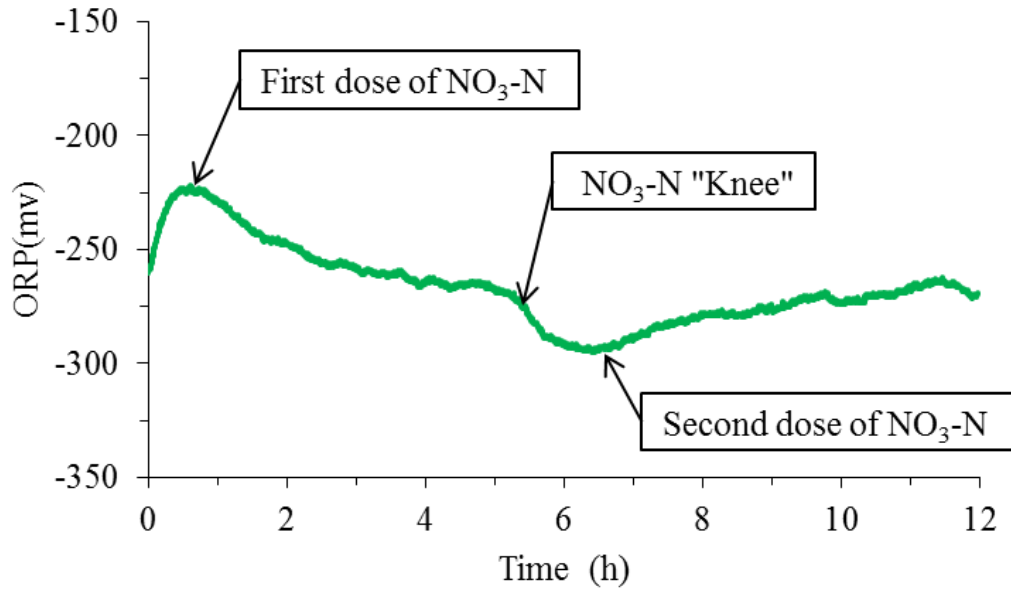
Figure 4.4. ORP data during one complete SBR cycle at 24 h HRT.

4.4. Cell yield

The net cell growth yield was estimated from the removal of nitrate as expressed in terms of

Nitrogen Oxygen Equivalents (NOE) according to Yang et al. (1995). NOE is defined as the

equivalent mass of oxygen involved in the same number of electron transfer when nitrate is

reduced to either nitrogen gas or nitrite, or both (Klapwijk et al., 1981). The coefficients of 2.86

and 1.71 are the equivalent mg O$_2$ per mg NO$_3$-N and per mg NO$_2$-N, respectively and can be

derived from the following reactions (Yang et al., 1995).

$$O_2 + 4H^+ + 4e^- = 2H_2O \qquad\qquad (4.1)$$

$$NO_3^- + 6H^+ + 5e^- = 0.5N_2 + 3H_2O \qquad\qquad (4.2.)$$

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$$NO_2{}^- + 4H^+ + 3e^- = 0.5N_2 + 2H_2O \hspace{3cm} (4.3)$$

Equations (4.1) and (4.2) yield: $(32/4)/(14/5) = 2.86$ mg O_2 per mg NO_3-N

Equations (4.1) and (4.3) yield: $(32/4)/(14/3) = 1.71$ mg O_2 per mg NO_2-N.

Contribution of NO_2-N to NOE is generally negligible in an efficient denitrifying reactor (Sarfaraz et al., 2004). Methane production under denitrifying conditions is unlikely while regular monitoring of the headspace of the reactor showed there was no methane present. The light brown color of granules and the absence of a sulfide odor indicated that sulfate reduction was at a minimum. Furthermore, ORP value in the bioreactor was maintain in between -225 ± 25 mV to obtain effective anoxic condition which support to the statement- sulfate reduction reaction was negligible (Zagury et al., 2006). Thus, it was concluded that NO_3-N was the only major electron acceptor.

As mention previously, the COD/ NO_3-N ratio maintained in this study was 3.2. Thus, the NOE can be calculated by dividing 2.86 by 3.2, which is 89 %. Similar results were given by Klapwijk et al. (1981) (78%); Yang et al. (1995) (83%); Sarafraz et al. (2004) (84%); and Bajaj et al. (2009) (82%). The remaining 11% of COD load was available for cell synthesis. Regular monitoring of solid loss during decantation process was about 3-5%; thus the remaining 6-8% of COD can assumed to be available for net sludge growth.

This chapter summarizes experimental results on the biodegradation of MCPA in the SBR under anoxic conditions. Specifically, the acclimatization period necessary for MCPA degradation is explained in Phase I (with 20 mg/L of DMCPA) which is also known as the "proof of degradation" concept phase. Once MCPA biodegradation behavior was observed, the capacity of the microorganisms to tolerate the herbicide is further reported in Phase II (with 50 mg/L of DMCPA) with the effect of HRT on the degradation of the herbicide described in Phase III (with 50 mg/L of DMCPA). Finally, the maximum limit of industrial strength concentration of MCPA degradation under anoxic condition was reached in Phase IV (with 75 mg/L of DMCPA). Along with this, the MCPA metabolites identified during the investigation and a likely MCPA degradation pathway is also reported.

5.1.   Phase I: MCPA acclimatization and degradation ("Proof of Concept")

After successful collection of baseline data, an industrial-strength concentration of 20 mg/L DMCPA was continuously fed to the SBR. In this experiment, it took around 28 to 30 days before the first sign of removal of MCPA in the SBR was observed. Similarly, González et al. (2006) reported that it took around 24 days for the primary degradation of MCPA spiked into a real wastewater treated in a fixed bed bioreactor (FBBR) under aerobic condition. The acclimatization period observed in this research is comparable with a related study on aerobic biodegradation of 2,4-D (having a similar structure as MCPA) by Celis et al. (2008) where they observed a 25- to 30-day acclimation period. Furthermore, it has been suggested that once the

necessary biochemical mechanisms for pesticide removal have been established, biodegradation of the recalcitrant compound then proceeds rapidly (Chin et al., 2005).

Once noticeable MCPA degradation appeared, three track studies (over a complete cycle each) were carried out on different dates in order to understand the MCPA degradation pattern. The average values of these three studies were plotted against time as shown in Figure 5.1. It is suggested that MCPA conversion (from salt to acid form) and degradation occurs simultaneously with the rate of formation of the acid larger than its removal rate in the first two hours, as seen by the rising peak (Figure 5.1). During the conversion of DMCPA to its corresponding acid both biological and abiotic process (such as hydrolysis) may play a role. In contrast, biodegradation of the acid was dominant after two hours, as revealed by the descending curve. Most importantly, the denitrifying microorganisms successfully degraded a significant part of the herbicide (around 80%) in the SBR within a few hours of reaction time and only a small part of the acid seemed to build-up. At the end of Phase I (after 90 days), more than 98 % removal of 20 mg/L of DMCPA was achieved, proving that degradation of MCPA could be realized under anoxic conditions. The probable denitrification reaction with MCPA as a carbon source can be written as

$$C_9H_9ClO_3 + \frac{20}{3}NO_3^- + H^+ \rightarrow \frac{10}{3}N_2 + 9CO_2 + Cl^- + 5H_2O \tag{5.1}$$
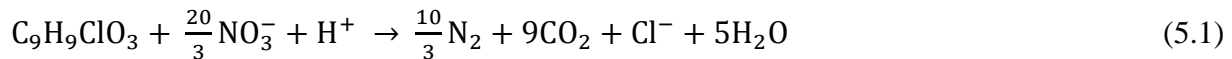
Three track studies for COD and nitrate consumption were also conducted to investigate if there was any hindrance to the biological activity caused by recalcitrant compound build-up. The data in Figure 5.2 indicates that there was no change in terms of nitrate removal; however, there seemed to be a slight effect on COD consumption compared to baseline conditions.

Figure 5.1. MCPA-formation and degradation pattern with 20 mg/L of DMCPA at 24h HRT; average values (n=3) and standard deviation (error bars) are shown.
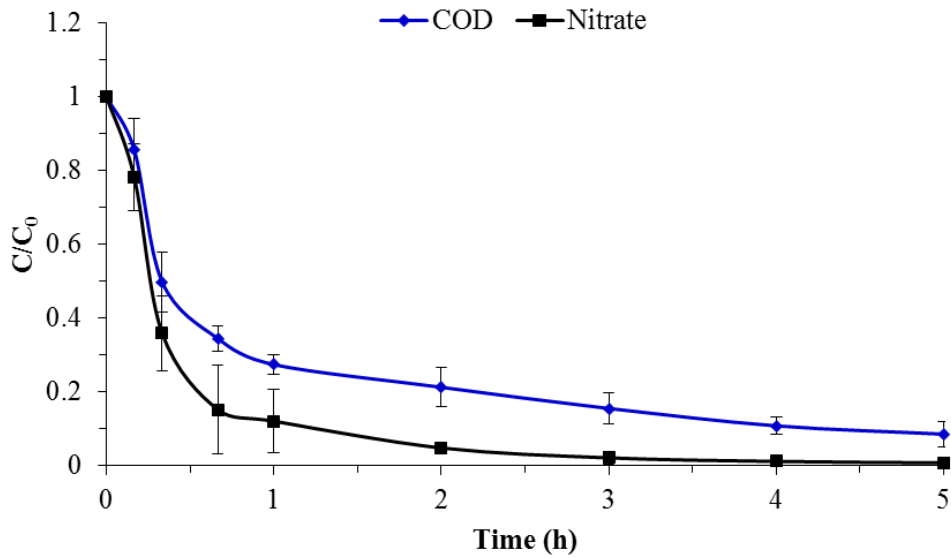
Figure 5.2. COD and nitrate track studies with 20 mg/L of DMCPA at 24h HRT; average values (n=3) and standard deviation (error bars) are shown.

Since acetate is a readily biodegradable substrate, as it was always completely removed within 5 h from the beginning (Figure 4.2), whereas the complete degradation of MCPA took 12 hour (Figure 5.1). This sequential utilization pattern of simple followed by complex organic carbon sources might have occurred. That is, diauxic growth is possible where enzymes that degrade a less rapidly metabolized energy source in the presence of a more rapidly metabolized one takes place (Saez & Rittmann, 1991; Mangat & Elefsiniotis, 1999; Elefsiniotis & Wareham, 2012).

5.2. Phase II: Effect of increased concentration of herbicide ("Tolerance phase")

In order to test the initial tolerance of the bacteria to increasing concentrations of MCPA, as mentioned, the amount of herbicide was increased from 20 mg/L to 50 mg/L. After two to three weeks, steady effluent COD concentrations were observed; thus four 12-h cycle track studies were carried out to observe the MCPA formation and degradation pattern in the new environment. The results (Figure 5.3) show that, compared to Phase I (2-3 h), the microorganisms took much longer (i.e. 8-9 h) to convert 50 mg/L of MCPA salt to acid. Eventually, incomplete biodegradation of the MCPA was observed in all three track studies. The increase in reaction time is likely due to the exposure of the bacteria to a higher MCPA concentration which inhibited microbial activity. Three track studies were also carried out to understand the COD consumption and nitrate removal patterns and, as depicted in Figure 5.4, the nitrate removal in the reactor was still > 95% within five hours, whereas the COD consumption level had dropped to 60%.
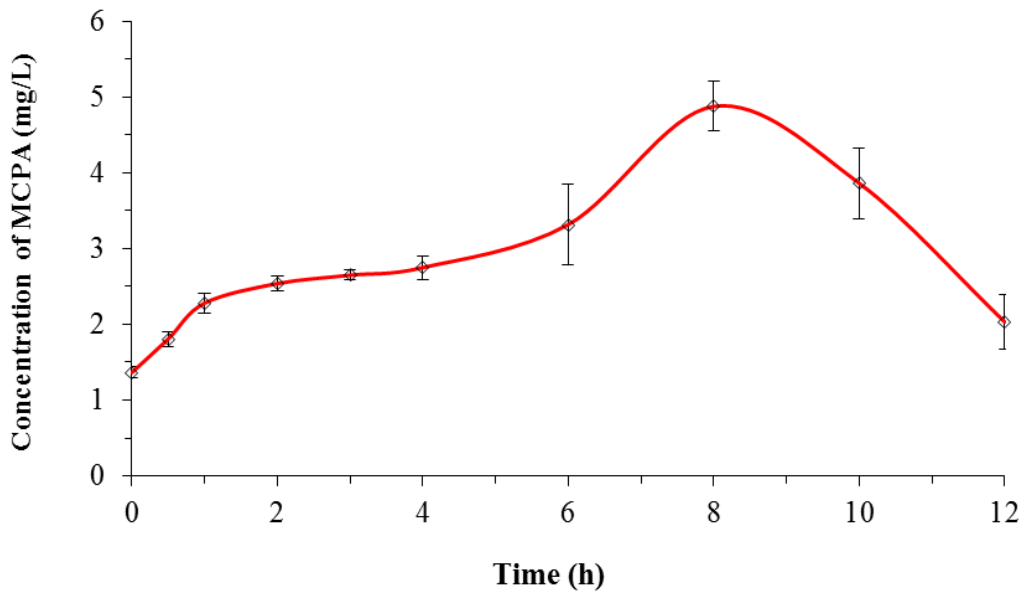
Figure 5.3. MCPA-formation and degradation pattern with 50 mg/L of DMCPA at 24h HRT; average values (n=4) and standard deviation (error bars) are shown.
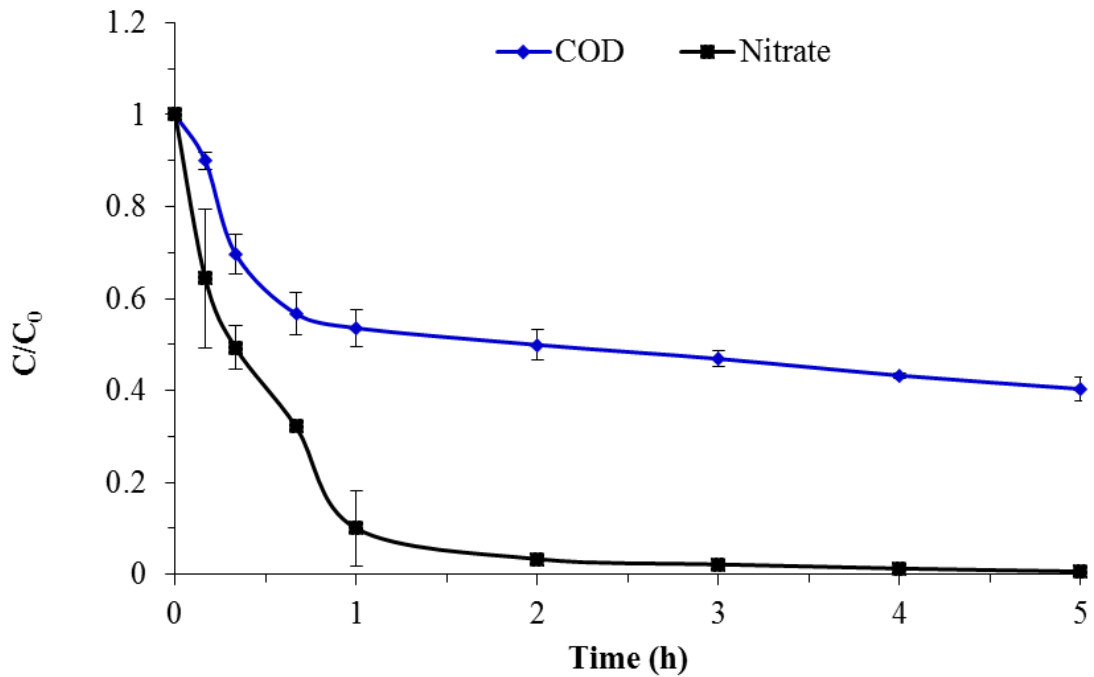
Figure 5.4. COD and nitrate track studies with 50 mg/L of DMCPA at 24h HRT; average values (n=3) and standard deviation (error bars) are shown.

The decrease in COD consumption and incomplete MCPA degradation pattern led to a check for the amount of nitrate available for denitrification in the bioreactor for the full SBR cycle. Consequently, full track studies for COD and nitrate were carried out again (Figure 5.5) with the results revealing that there was enough nitrate available for microorganisms in the second half of the cycle.

5.3.   Phase III: Effect of HRT on removal of herbicide

Following incomplete MCPA removal in a 12 h cycle (Figure 5.3), the biodegradation response was investigated at an HRT of 48 h (i.e. a 24 h cycle). Average values of the three repeated track studies are plotted against time in Figure 5.6. It is evident that a 48 h HRT was sufficient to degrade a concentration of 50 mg/L of DMCPA. More specifically, complete conversion of the MCPA salt to acid took a similar amount of time (8-10 h) as the 12 h cycle but obviously longer to degrade a significant portion of the MCPA acid. At the end of the cycle, the reactor achieved more than 98 % of MCPA acid (Table 5.1) removal by denitrifying microorganisms.

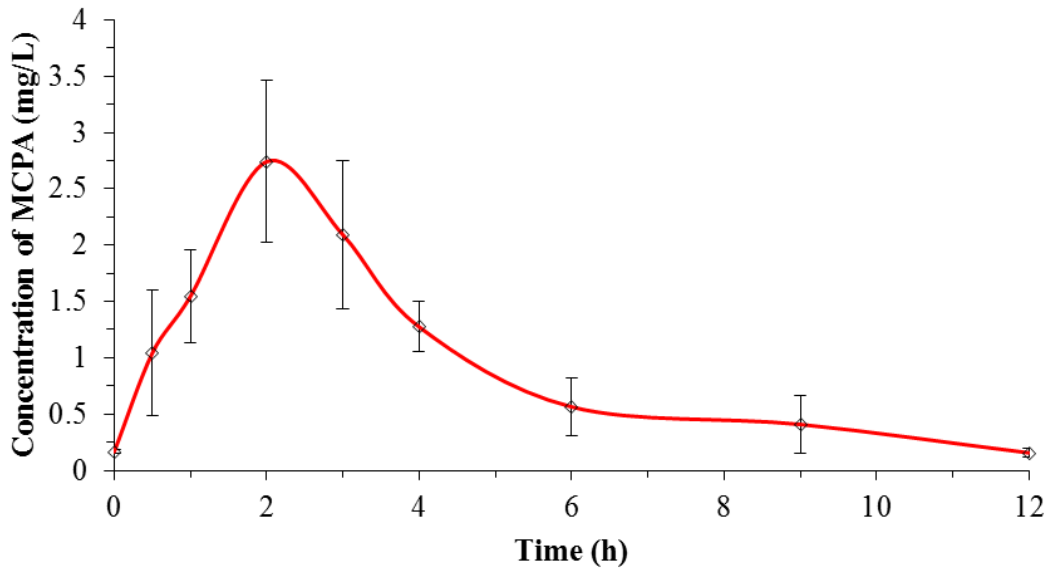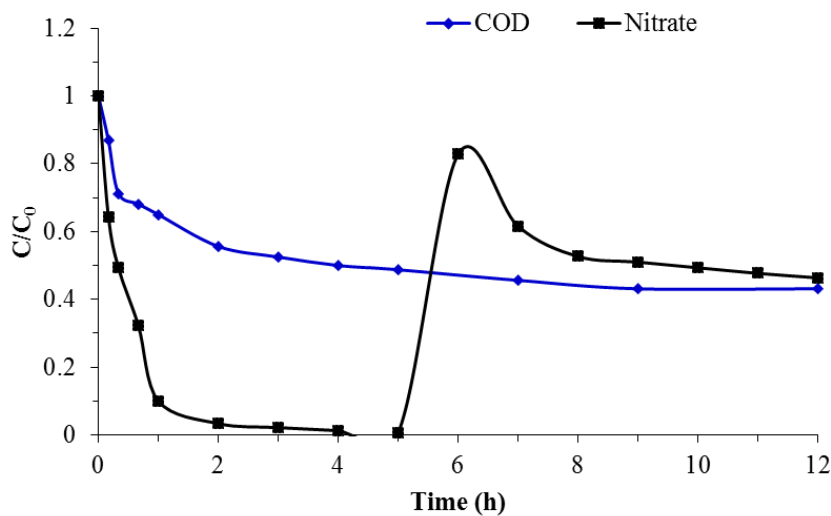Figure 5.5. COD and Nitrate track study for a full SBR cycle.
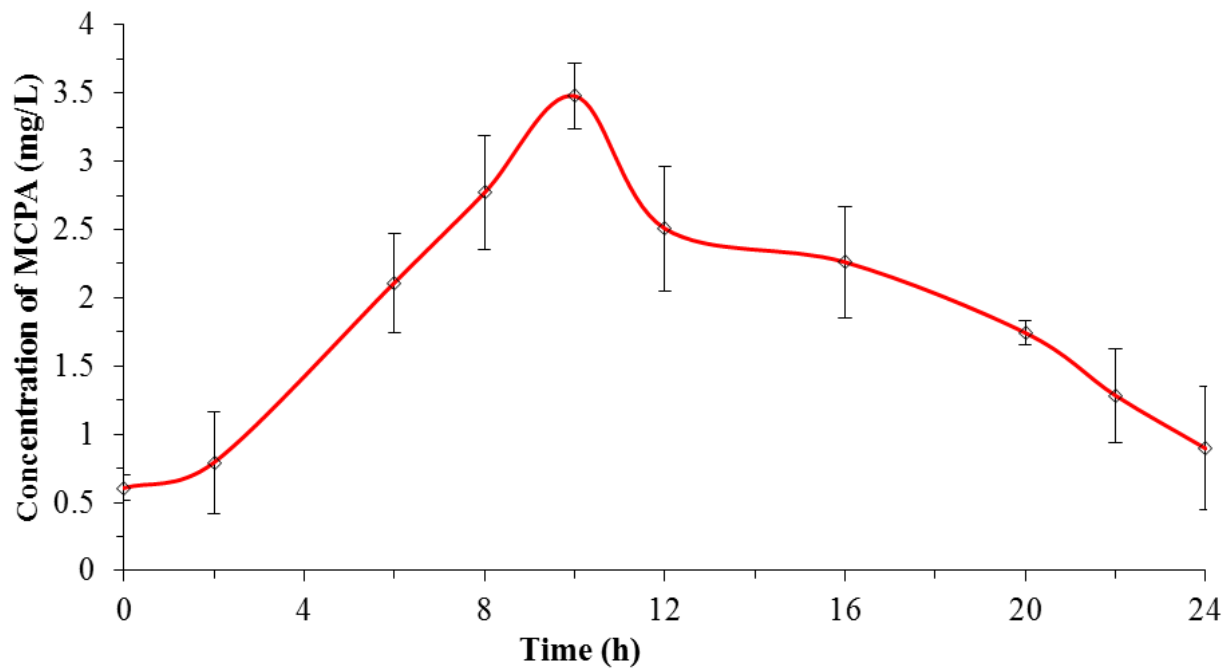
Figure 5.6. MCPA-formation and degradation pattern with 50 mg/L of DMCPA at 48 h HRT;

average values (n=3) and standard deviation (error bars) are shown.

Table 5.1 Percentage removal of MCPA.

Amount of MCPA acid as salt (mg/L)	Theoretical yield of MCPA acid; Influent (mg/L)	Actual MCPA acid; effluent (mg/L)	Removal (%)	HRT
20	16.32	0.25	$\frac{16.32-0.25}{16.32}= 98$	24 h
50	40.8	2	$\frac{40.8-2}{40.8}= 95$	24 h
		0.5	$\frac{40.8-0.5}{40.8}= 98$	48 h
75	61.2	6	$\frac{61.2-6}{61.2}= 90$	48 h

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However, even with a 48 h HRT, the COD removal achieved only 56% (Figure 5.7) with a constant effluent value of 69 mg/L for that last portion of the cycle. As such, an additional test (i.e. total organic carbon (TOC)) was carried out to investigate the proportion of organic carbon contributing to the COD in the effluent. The data suggests (not shown) that around 50-60% of the total organic carbon remained unused at the end of the cycle, most certainly contributing to the high effluent COD.

A possible origin for this organic carbon is the accumulation of intermediate degradation products of MCPA (e.g. 4-chloro-2-methylphenol (CMP) and 4-chloro-2-methyl-6-nitrophenol (CNMP)). Chiron et al. (2009) however suggested that CMP can disappear as quickly as MCPA, while CMNP seems to be only slightly more persistent than its parent compound. A GC-MS analysis for this research showed production of CMP which had not been degraded for some reason or another. Another explanation for the unused carbon is residual, formulation-related organic compounds (Crosby & Bowers 1985). That is, apart from MCPA acid, the commercial herbicide product contains other unknown organic compounds (such as long chain fatty acids) to make it soluble in water and to improve its adsorption to leaves when it is applied in the field. Thus, the commercial formulation of the DMCPA generates a considerable amount of residual, non-pesticide COD (i.e. 1 mg/L of commercial herbicide was found to give approximately 1.4 mg/L of COD). Crosby & Bowers (1985) reported several terminal residues during the formulation of DMCPA and these compounds could possible contribute to the residual COD in the bioreactor and may be difficult for denitrifying microorganisms to degrade.
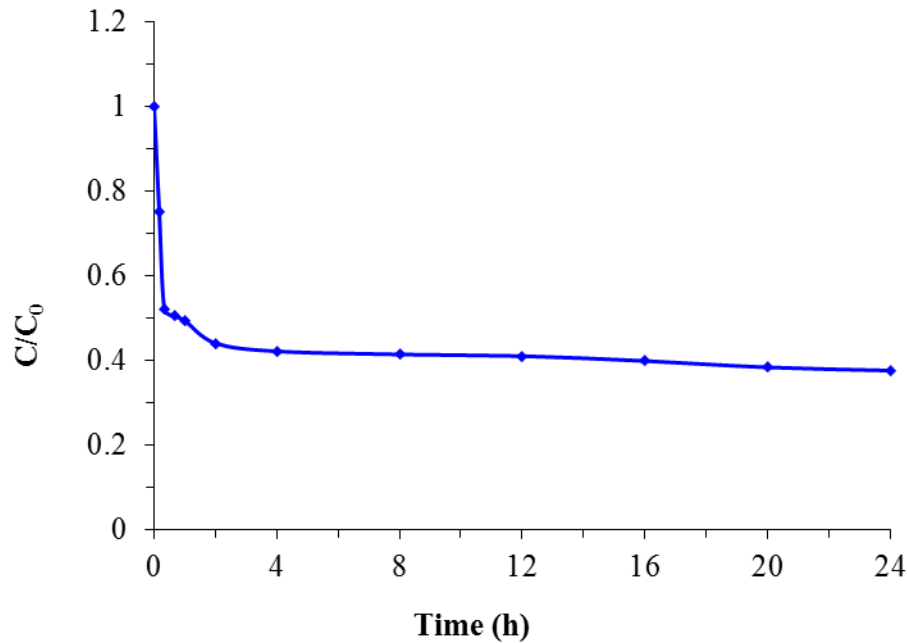
Figure 5.7. COD track studies with 50mg/L of DMCPA at 48 h HRT.

5.4.   Phase-IV: Limit Phase

To find the potential upper limit of biodegradation, the concentration of DMCPA was again increased, this time to 75 mg/L. The system was kept running at a 48 h HRT and 3 − 4 weeks were allowed to acclimate to the increased load before carrying out a detailed track study. Initial effluent data from samples collected every day as well as a preliminary track study conducted during the second week (Figure 5.8), indicated that the MCPA formation and degradation pattern was similar to the 20 mg/L and 50 mg/L pattern. Following this, detailed MCPA track studies were conducted 45 days after the initial injection of 75 mg/L of DMCPA into the bioreactor. However, surprisingly, this time all 4 track studies did not detect MCPA in any of the samples despite the promising results from the preliminary track study. After a rigorous check of all analytical protocols, it was concluded that the bacteria were no longer able to convert any further MCPA salt to acid and thus no MCPA formation/degradation pattern was being observed. A

further 3 weeks was allotted to the microorganisms to see if an extended period could assist with the increased concentration of DMCPA; however, again no MCPA was detected in the effluent samples. Two separate batch tests with an extended HRT (i.e. 72 h) were then conducted to confirm that HRT was not the problem, but again no MCPA was detected.

Interestingly, the denitrification activity was not affected even with the increased concentration (i.e. 75 mg/L) of the herbicide. Three track studies of nitrate (Figure 5.9a) indicated that nitrate was removed effectively within five hours, thus showing that the anoxic biomass was still active. Similarly, a study carried out by Schulz et al. (2012) reported that the majority of microorganisms were not affected by the addition of MCPA except for fungi which seemed more affected by the addition of MCPA than bacteria.
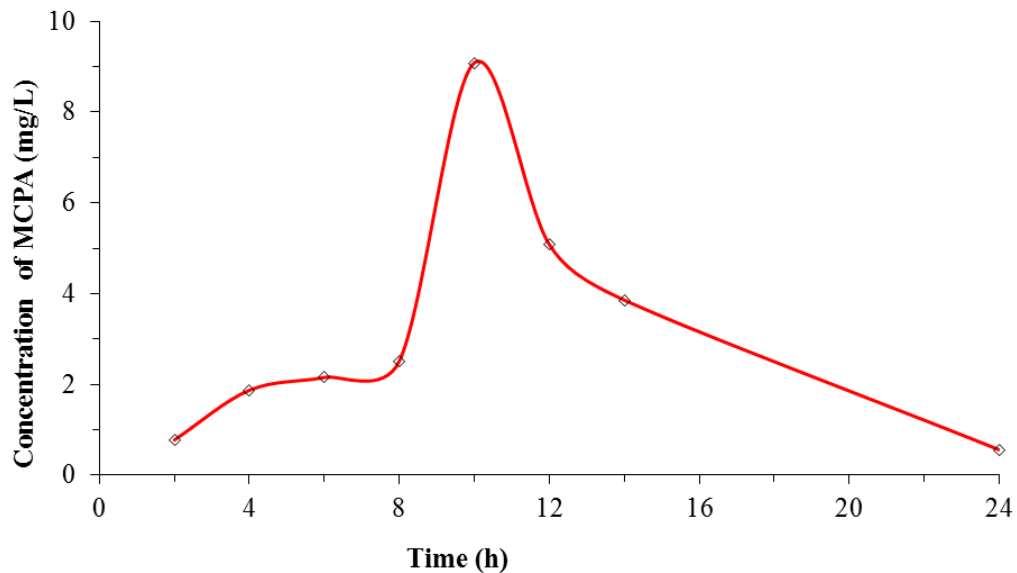
Figure 5.8. MCPA-formation and degradation pattern with 75 mg/L at 48h HRT (a preliminary track study conducted during the second week after the initial injection of 75 mg/L of DMCPA into the bioreactor).

One possibility for the cessation of MCPA conversion and degradation is that the biomass might have become affected in terms of their ability to degrade MCPA compounds, simply due to over-exposure to the herbicide. That is, the bacteria with the capability of degrading DMCPA had suddenly stopped producing the specific enzyme necessary for herbicide degradation. Another possibility is that the residual, non-degraded portion of the herbicide (as well as the complex organic compounds generated from the commercial formulation of the herbicide) might have accumulated in the reactor, reaching a saturation level and effectively hindering any further degradation of DMCPA.

To test the latter hypothesis, feeding of the DMCPA was halted for three HRT periods to flush any complex compounds out of the system. Results obtained during this period (Figure 5.9b) indicated the formation of MCPA acid up to a concentration of 4 mg/L followed by its removal down to 2.5 mg/L from day 1 to 3 respectively. This formation/degradation pattern had not been observed in the system while being fed with 75 mg/L DMCPA. This suggests that the bacteria still had some potential to convert small concentrations of MCPA salt to acid. Similarly, the effluent COD also dropped from 144 mg/L to 63 mg/L (Figure 5.9b) which was expected because during the flushing period, acetate was the only carbon source. DMCPA was then fed again to the system after the flushing period, but conversion/degradation of MCPA inside the bioreactor still did not take place. Although, it is difficult to predict the long-term behavior of the bioreactor, the observations associated with these conditions of the experiment suggest that the potential upper limit of MCPA degradation in anoxic conditions had been reached; namely that it lies somewhere between 50 and 75 mg/L. Furthermore, once that limit has been reached, at least

under the conditions of this experiment, the bacteria do not seem to easily recover their capability of degrading DMCPA/MCPA compounds.
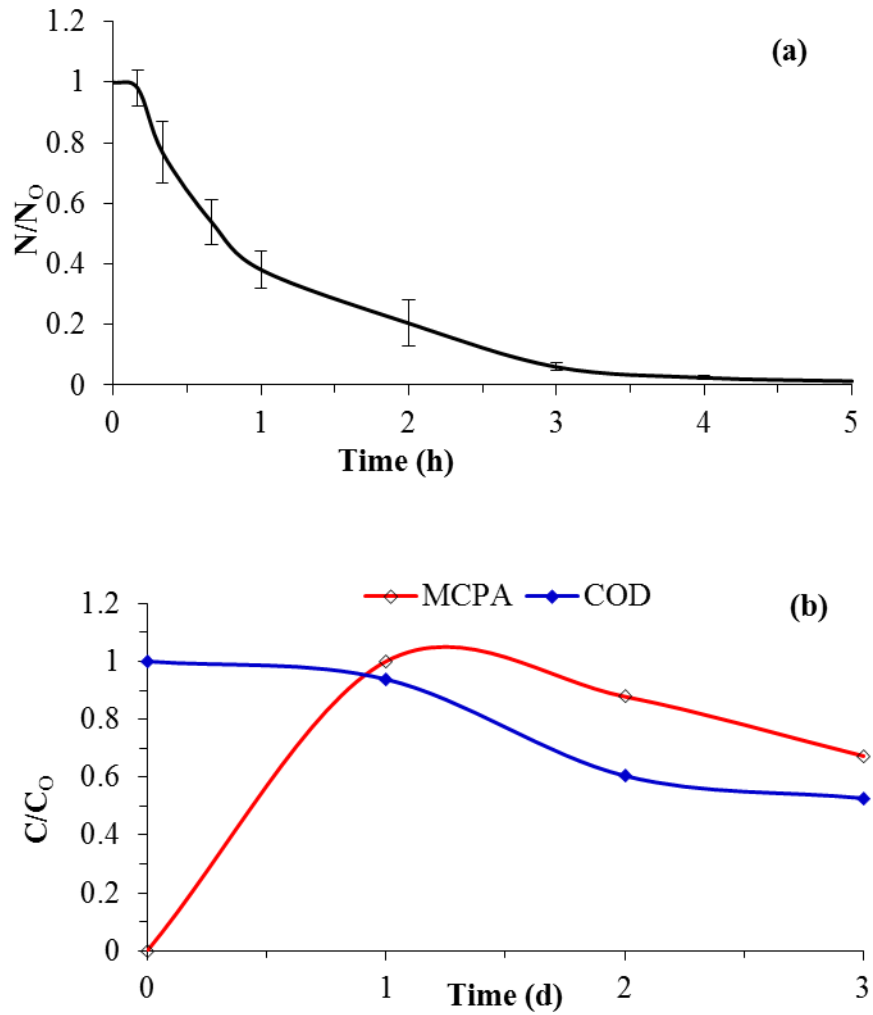
Figure 5.9. (a) Nitrate track study with 75 mg/L of DMCPA at 48 h HRT; average values (n=3) and standard deviation (error bars) are presented; and (b) MCPA and COD in the effluent during COD-flushing period.

5.5.   DMCPA/MCPA intermediate metabolites

Along with 4-chloro-2-methyl phenol (4CMP) several other DMCPA/MCPA metabolic intermediate products were detected during this investigation (Table 5.2). However, the detection of intermediate metabolites at 75 mg/L DMCPA represents a short period of time (i.e. before the preliminary track study) when the bacteria had not stopped producing specific enzymes that are capable of degrading DMCPA. Further, the presence of some phenolic compounds in the effluent may be an issue; however, Sarfaraz et al. (2004) reported that an SBR system under anoxic conditions could degrade phenolic wastewater at concentrations of up to 1050 mg/L. Furthermore, Buitrón et al. (2005) showed that 98 % of 4CP was removed with influent concentrations of up to 1000 mg/L in an SBR. Detection of the major metabolic intermediate product, 4-chloro-2-methyl phenol (4CMP), throughout this study suggests that MCPA biodegradation may occur via the Tfd pathway (Harker et al. 1989; Bælum et al. 2006). The degradation pathway of DMCPA/MCPA (Figure 5.10.) observed in this study is similar to the past literature (Buitrón et al., 2005; Baelum et al., 2008) as mention in Section 2.2.4.
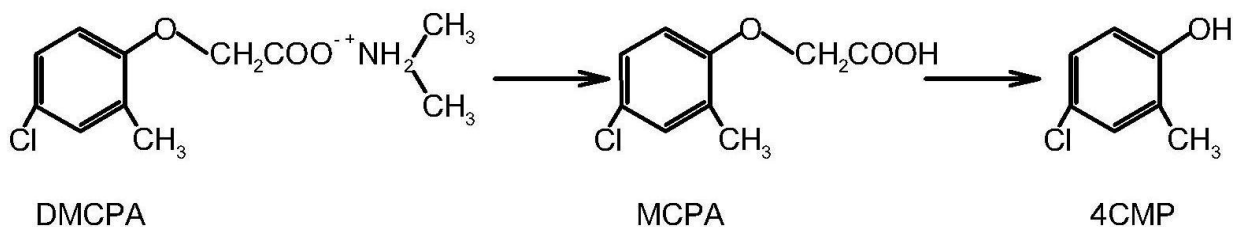
Figure 5.10. (a) Proposed degradation pathway of DMCPA/MCPA by microbial degradation.

Table 5.2 List of intermediate metabolites of MCPA at various times and concentrations.

Intermediate compound	Retention time (min)	Similarity percentage (%)	50 mg/L				75 mg/L	
			4h	8h	10h	24h	12h	14h
4-Chloromethyl phenol	5.2	83	√	√	√	√	√	√
Ethanol, 2-3,3-dimethylcyclohexylidene	6.5	78	×	×	√	×	×	×
Phenol, 2,4-bis(1,1-dimethylethyl)	6.94	85	√	√	√	√	√	√
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	7.45	94	√	√	√	√	√	√
Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	7.45	93	√	√	×	√	√	√
Cyclobutane, 1,2-diphenyl	8.45	91	√	√	√	×	√	√
Cyclic 3-(1,2-ethanediyl acetal	8.45	75	×	×	×	√	×	×
Cyclooctane, 1,5-dimethyl	9.36	79	×	√	×	×	×	√
(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans	12.18	76	√	√	√	×	√	√

5.6.   MCPA profile

Figure 5.11 shows the concentration of MCPA present in the SBR's effluent during the different phases. To summarize, after completion of the baseline data set, a concentration of 20 mg/L of DMCPA was injected into the SBR during the acclimatization period (Phase I). On the 28th day, the bioreactor exhibited the first sign of biodegradation, with an increasing trend until it removed around 98 % of the herbicide. When the DMCPA concentration was raised from 20 mg/L to 50 mg/L at the 90 day mark (Phase II), the concentration of MCPA in the effluent increased from 0.25 mg/L to 6 mg/L due to the increase in influent load. In other words, the MCPA degradation efficiency dropped from 98 % to 86 %; however, the biomass quickly adapted to the change in influent concentration with the removal efficiency gradually increasing, reaching approximately 95 % at day 110. During the HRT extension (Phase III) (changing the HRT from 24 h to 48 h), the SBR again exhibited around 98 % removal of herbicide. In this phase, a decrease in the concentration of MCPA in the effluent was directly attributed to an extension in the HRT. The final increment of MCPA from 50 mg/L to 75 mg/L (Phase IV) done on the 198th day led to sharp rise in effluent concentration and again decreased the MCPA removal efficiency to 85 %. On day 225, around 98 % removal of the MCPA was achieved. Although, the denitrification activity as well as the biomass concentration (as measured by TSS and MLVSS) was not affected after injecting 75 mg/L; the biomass appears to be become saturated with the herbicide, stopping conversion of DMCPA to its acid form and halting biodegradation.
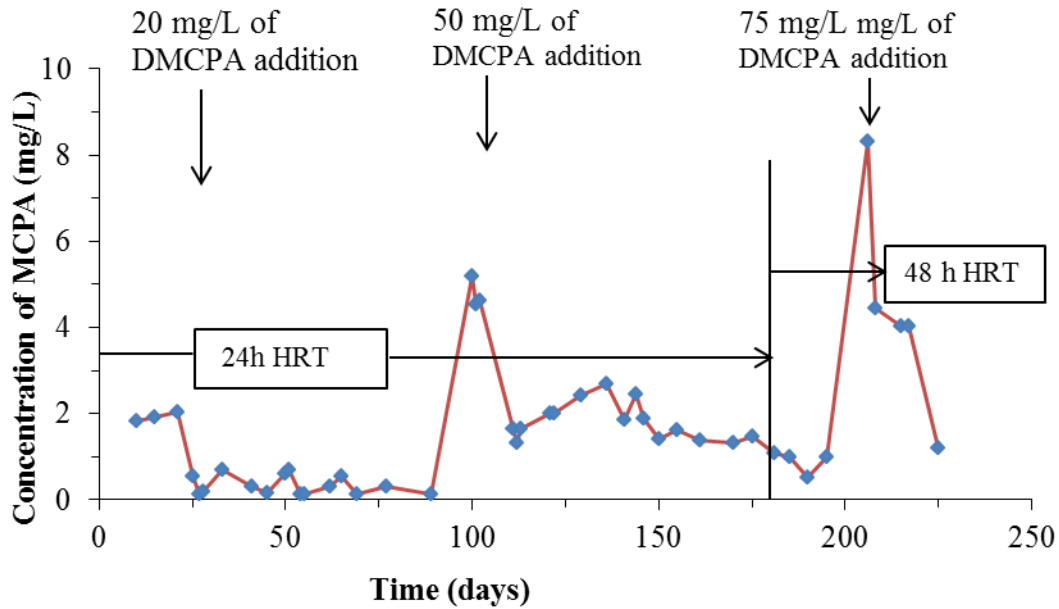
Figure 5.11. MCPA acid in the effluent after continuous addition of DMCPA at different concentration as noted above in the plot. During the entire period, the reactor was maintained in anoxic condition (ORP = -250 ± 25 mV) by injecting 56 mg/L of N- NO_3 twice per cycle.

5.7. COD profile

Influent and effluent soluble COD was measured throughout the research period and the result is presented in Figure 5.12. The figure suggest that the influent COD was around 110 mg/L which then dropped down to around 8 - 10 mg/L in the effluent, accounting for > 95% removal during the baseline data collection phase. When 20 mg/L of herbicide was injected to the system, in Phase I, the influent COD slightly increased from 117 to 120 mg/L, which then quickly went down to 20 - 25 mg/L in the effluent. It seems this initial concentration of herbicide did not affect the biomass much, for the COD removal efficiency was still between 85 - 90%. However, when the concentration of herbicide increased from 20 to 50 mg/L in Phase II, there was a sharp

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rise in the influent COD, from around 120 mg/L to 170 mg/L. The increase in COD in the influent can be attributed to the organic compounds that came with the commercial formulation of the MCPA salt. As mentioned earlier (in Section 5.3), 1 mg/L of DMCPA yielded around 1.4 mg/L of COD; thus 50 mg/L of DMCPA contributed around 70 mg/L of COD to the system. As a result, the SBR efficiency to remove the COD during Phase II dropped to around 60 - 65%. The decrease in COD removal capability of the microorganisms was also likely due to an increasing amount of MCPA and DMCPA intermediate metabolites/by-products in the system. Even during the HRT extension phase, (Phase III: from 24 h HRT to 48 h HRT) the effluent COD did not decrease. Furthermore, the increase in both influent and effluent COD continued during the "Limit Phase" with the final increment of DMCPA to 75 mg/L in Phase III yielding a COD removal percentage of only 40 - 45%.
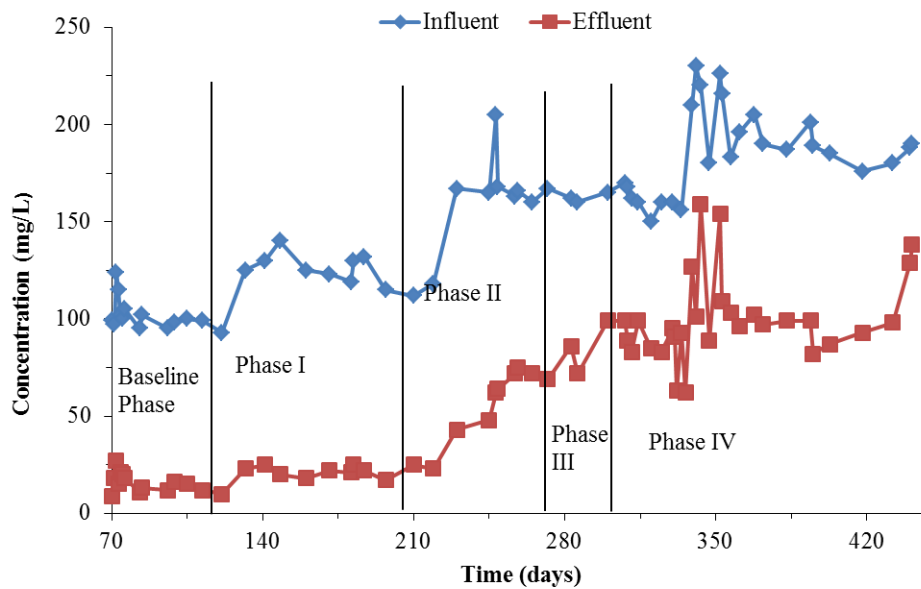
Figure 5.12. Influent and effluent CODs in the SBR during the entire research period (24 h HRT = Baseline Phase, Phase I and Phase II; 48 h HRT = Phase III and Phase IV).

5.8. Abiotic losses

5.8.1. *Volatilization*

Blank controls were carried out to quantify abiotic losses (such as volatilization) and these were estimated to be less than 2 %. Past literature (WHO, 1996) reported that MCPA did not volatilize from an aqueous solution (pH = 7) heated for 13 days at 34-35°C nor was it hydrolysed at neutral pH.

5.8.2. *Bioadsorption test*

Prior to the bioadsorption test, activated sludge microorganisms were stored in the dark and at low temperature (4°C) for three months to reduce biological activity to a minimum (Cooperative Chemical Analytical Laboratory (CCAL), 2006). On the actual day of analysis, the biomass pH was reduced to < 2 by adding a couple of drops of concentrated hydrochloric acid (HCl). This condition presumably stopped any possible biodegradation of MCPA in the batch reactor. Following that, three replicate batch runs were carried out by spiking a sample of MCPA to each batch reactor in order to estimate the removal of MCPA via bioadsorption. The normalized result is plotted in Figure 5.13 which indicates that, the general trend for MCPA removal via bioadsorption was consistent in all three replicates runs. It was estimated that around 13-15% MCPA removal occurred via bioadsorption meaning that the remaining 85% uptake of MCPA was likely due to biodegradation. As mentioned in Section 2.2.6, the theoretical n-octanol water partition coefficient of MCPA is 26, which means that there is some affinity for MCPA to get attached to the biomass cell wall. Moreover, previous research (He & Wareham, 2008) indicated that bioadsorption of 2,4-D (a sister herbicide to MCPA) in an SBR was around 10 % , which is near to the value obtained in this research.
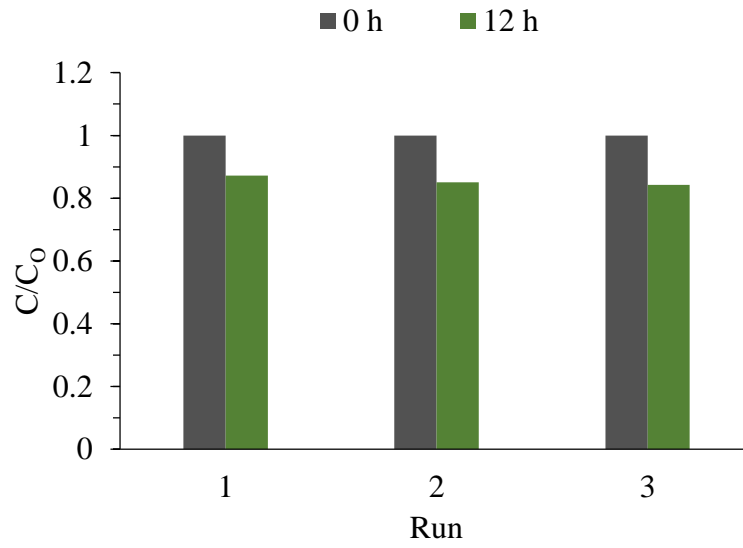
Figure 5.13 Removal of MCPA via bioadsorption process at 24 h HRT.

In this chapter, several bio-kinetic parameters (such as the kinetic rate constants and specific uptake rates for COD, nitrate and MCPA) are calculated and discussed. Appropriate models were fit to experimental data (track study data) obtained during the different Phases as well as from individual batch tests, which were then used to calculate the order/rate of reaction. Specific uptake rates were also calculated using the data from a targeted period of time when the biomass was highly active (i.e. when the substrate was not limiting). A specific uptake rate implies the highest possible "substrate consumption rate" by the biomass at that moment. Overall, Sections 6.1 and 6.2 provide an understanding of how these parameters have changed before and after the addition of the pesticide; whereas Section 6.3 focuses only on the rate of formation and degradation of the MCPA acid. As a reminder, acetate was the sole carbon source for the entire baseline test, whereas DMCPA was a secondary carbon source for Sections 6.2 and 6.3. Nitrate nitrogen was the sole electron acceptor in all cases.

6.1. Baseline data

 A typical plot of COD consumption and NO_3-N reduction is shown in Figure 6.1 which highlights the experimental vs theoretical fit. It appears from the figure that the biodegradation of acetate and nitrate before injection of any pesticide into the SBR system follows very closely a first-order kinetic model ($C = C_O * e^{-kt}$).
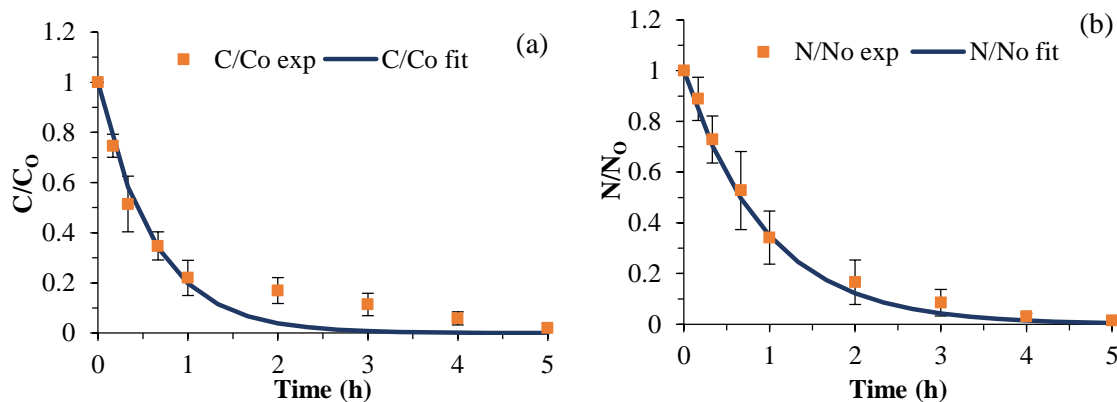
Figure 6.1. Curve fitting with first-order reaction: (a) COD and (b) Nitrate.

The kinetic rate constants and specific COD and nitrate uptake rates associated with the baseline track study data (average of five repetitions) are summarized in Table 6.1. For both COD and NO_3-N, the R^2 value was found to be more than 0.95 when fitted with a first-order reaction.

The specific denitrification rate (0.12 ± 0.02 mg/mg VSS d) obtained in this study is at the lower end of the range reported by other researchers (Table 6.2). Variation in the denitrification rate might be due to differences in the COD: NO_3-N ratio, differences in the primary carbon source used for denitrification and the reactor configuration (e.g. CFSTR versus batch). For example, Ge et al. (2012) (Table 6.2) demonstrated that denitrification rates vary with the biodegradability characteristics of the electron donor used and the final COD/ NO_3-N ratio in the bioreactor. They observed a change in denitrification rate when they altered the organic carbon source (acetate, methanol and glucose) and COD/NO_3-N ratio. They noted a three-fold increase in the denitrification rate when they increased the COD/ NO_3-N ratio from 6 to 25, keeping the same organic carbon source (acetate and methanol) in an SBR. As such, the low denitrification rate obtained in the present study can likely be attributed to the low COD: NO_3-N ratio used (3.2) in

comparison to the Table 6.2 values. Further to this, He & Wareham (2008) noted that denitrification rates are generally higher in continuous, flow-through systems, as compared to batch systems. This is primarily attributed to acclimation of bacteria to carbon sources at steady state conditions.

Table 6.1 Kinetic rate constants and specific COD and Nitrate uptake rate without pesticide injection (average of five repetitions with standard deviation).

Run	First-order kinetic constant, k_1 (h^{-1})		Specific uptake rate (mg/mg VSS d)	
	COD	NO_3-N	COD	NO_3-N
Baseline data	1.62 ± 0.41	1.02 ± 0.28	0.36 ± 0.02	0.12 ± 0.02

Table 6.2 Summary of denitrification rates achieved with various organic carbon sources.

Organic carbon source	COD/NO_3-N	Type of reactor	Denitrification rates	Literatures
Methanol	4	SBR	0.54 mg NO_3-N/mg VSS d	Fernández-Nava et al., (2008)
Acetate	6	SBR	0.28 g NO_3-N/g VSS d	Ge et al., (2012)
Methanol	6	SBR	0.13 g NO_3-N/g VSS d	Ge et al., (2012)
Glucose	6	SBR	0.24 g NO_3-N/g VSS d	Ge et al., (2012)
Acetate	25	SBR	0.60 g NO_3-N/g VSS d	Ge et al., (2012)
Methanol	25	SBR	0.40 g NO_3-N/g VSS d	Ge et al., (2012)
Glucose	25	SBR	0.02 g NO_3-N/g VSS d	Ge et al., (2012)
Acetate	C:N = 2.05	CMR	0.60 mg NO_3-N /mg VSS d	Xu et al., (1996)
Mixed VFA	C:N = 2.37	CMR	0.75 mg NO_3-N /mg VSS d	Xu et al., (1996)
Natural VFA	N/A	SBR	0.02 g NO_3-N/g VSS d	He & Wareham, (2008)
Acetate	3.2	SBR	0.12 ± 0.02 mg NO_3-N/mg VSS d	This research

6.2. Kinetic constants and specific uptake rates of COD and nitrate in the presence of pesticide (Phase I, II, III & IV)

After reaching "steady state" operation in the SBR, the biodegradation kinetics of acetate (COD) and nitrate were calculated during the incremental changes in the concentration of the herbicide.

6.2.1. Phase I : 20 mg/L of pesticide

Figure 6.2 shows that the biodegradation kinetics of acetate has slightly changed due to the initial injection of pesticide into the SBR and; instead, it more closely followed a second order kinetic model ($C = C_o/1 + k_2*C_o*t$). Figure 6.2a,b shows the difference between first and second order kinetics model fitted against the experimental data. The corresponding R^2 values were found to be 0.94 and 0.98 respectively.

The kinetic constants and the specific COD and nitrate consumption rates (an average of three repetitions) were again calculated and are presented in Table 6.3. It is evident from the data (a comparison between baseline data (Table 6.1) and Phase I (Table 6.3)) that the overall COD kinetic rate constant remained practically unchanged (a relatively similar value given the large standard deviations around the mean) by the addition of the initial concentration of pesticide. However, the specific COD uptake rate increased by 64%. This is because a small concentration of herbicide did not interfere (i.e. does not inhibit COD consumption) with the activity of a large group of denitrifying microorganisms, but instead simply functions as a secondary carbon source which ended up increasing the specific COD uptake rate of the biomass.

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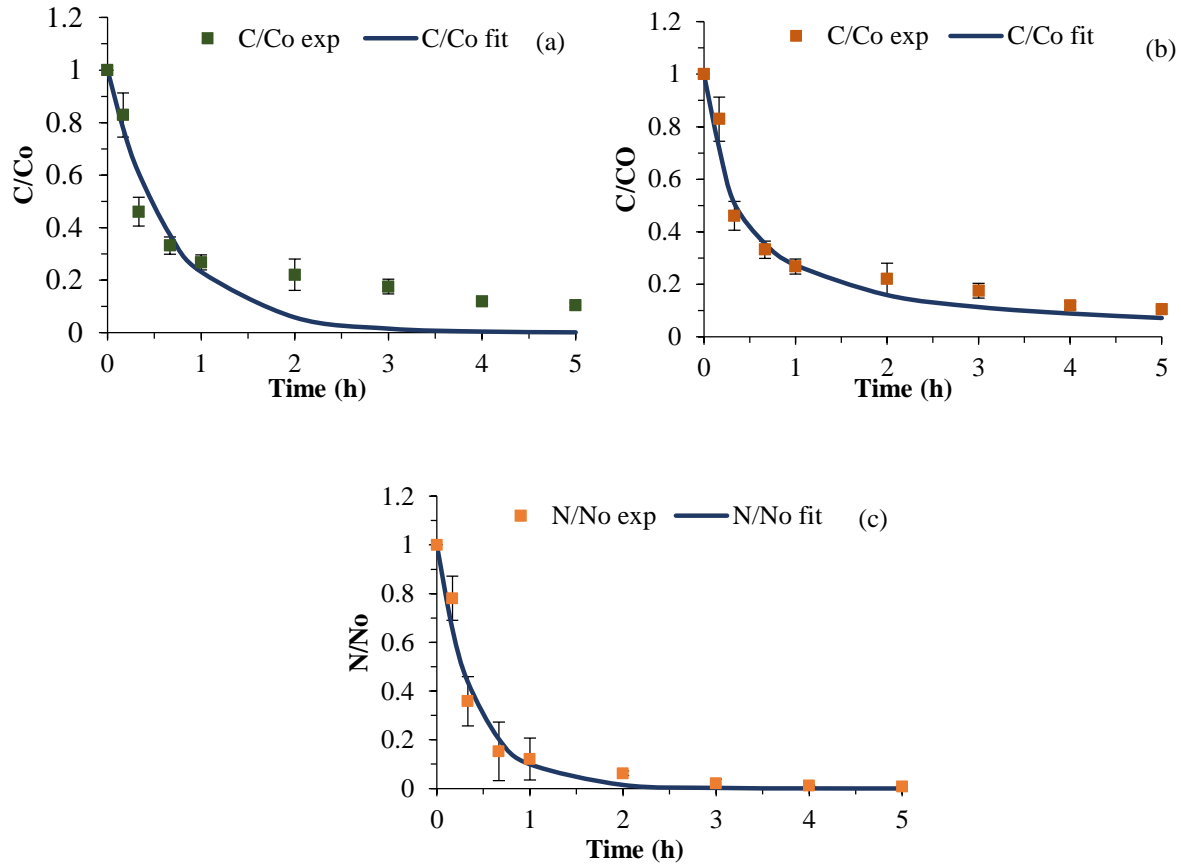
Figure 6.2. Curve fitting: (a) COD with first-order reaction, (b) COD with second-order reaction and (c) Nitrate with first-order reaction.

In regards to nitrate, the order of reaction remained the same as the baseline data (i.e. a first-order kinetic model) (Figure 6.2c). The figure in fact indicates that the denitrification process occurred rapidly after the injection of herbicides, removing NO_3-N completely within 2 h. As such, the kinetic constants and specific nitrate uptake rate increased sharply from 1.02 ± 0.28 to 2.58 ± 0.76 h^{-1} and 0.11 ± 0.02 to 0.14 ± 0.01 mg/mg VSS d (Table 6.3) respectively during this period. The data suggests that, similar to the COD consumption profile, the denitrification kinetics was not inhibited by the initial concentration of pesticide. Rather, it increased

significantly (around 60%). It was due to the fact that as the herbicide provided extra carbon source to the microorganisms; and as such, corresponds to an increase in denitrifying activity.

Table 6.3 Kinetic rate constants and specific COD and Nitrate uptake rates after pesticide injection (average of three repetitions with standard deviation).

Run	Kinetic constants, k_1 and k_2 (h^{-1})		Specific uptake rate (mg/mg VSS d)	
	COD: k_2	NO_3-N: k_1	COD	NO_3-N
Phase I	1.51 ± 0.82	2.58 ± 0.76	0.60 ± 0.12	0.14 ± 0.01
Phase II & III	0.57 ± 0.14	2.14 ± 0.40	0.39 ± 0.04	0.12 ± 0.02
Phase IV	0.25 ± 0.11	1.24 ± 0.16	0.26 ± 0.07	0.11 ± 0.01

6.2.2. Phase II & III : 50 mg/L of pesticide

Keeping the acetate concentration constant (at 350 mg/L), an increase in DMCPA concentration from 20 mg/L to 50 mg/L (Phase II) resulted in a substantial decrease in both the COD kinetic constant and the specific COD consumption rate by 62% and 35% respectively (Table 6.3). This denotes an adverse effect on the acetate utilization pattern. The observation of "inhibition in consumption" of a simpler organic source (acetate) by the presence of complex (relatively toxic) carbon source (DMCPA) in this study is not surprising as Chin et al. (2005); Celis et al. (2008) and Elefsiniotis & Wareham (2012) all observed a similar influence on glucose utilization by activated sludge microorganisms when they injected increasing concentrations of the herbicide 2,4-D. Furthermore, both chlorinated herbicides (2,4,D and MCPA) come from the chlorophenoxy group and have relatively similar physical structures; thus, it was anticipated that they might exhibit similar characteristics when increasing concentrations were injected into the biomass.

Overall, as can be seen from Figure 6.3, the acetate biodegradation kinetics followed the same trend as the corresponding second-order kinetics model.

The rate of nitrate consumption continued to follow first-order kinetics even after the concentration of herbicide increased to 50 mg/L (Figure 6.3). However, the kinetic rate constant slightly decreased by around 17 % compared to the Phase I (Table 6.3). When the concentration of the herbicide gradually increased, the rate of reaction of the bacteria slowed down which can be seen by the decreasing trend in the denitrification kinetics (Table 6.3). It is possible that the accumulation of non-degraded toxic organic compounds suppressed the biomass activity which eventually slowed down the microbial metabolism.

6.2.3. *Phase IV: 75 mg/L of pesticide*

A representative plot of COD and nitrate uptake rate is presented in Figure 6.4. It is apparent from both Figure 6.4 and Table 6.3 that the inhibition trend for COD consumption was more pronounced when the DMCPA concentration increased to 75 mg/L. It is clear that both kinetic constants (modelled second-order kinetics) and specific COD uptake rates further decreased by 56% and 32% respectively compared to Phase II. It is possible that the accumulation of undegraded toxic organic compounds suppressed the biomass activity which eventually slowed down the microbial metabolism.

In a similar fashion, both the nitrate kinetic constant and specific uptake rate decreased from 2.14 ± 0.40 to 1.24 ± 0.16 h-1and 0.124 ± 0.02 to 0.11 ± 0.01 mg/mg VSS d (Phase II & III to IV) respectively. This indicates slower denitrifying activity in this period despite complete consumption of NO_3-N. The change in denitrification rate can be attributed to either the

denitrifying microorganisms reaching a saturation point (in terms of denitrification (i.e. they had already achieved their maximum denitrification rate)) or the increased recalcitrant concentration of the pesticide inhibited their biological performance (i.e. a toxicity effect). Further in-depth microbiological studies would be needed to explain this behavior.
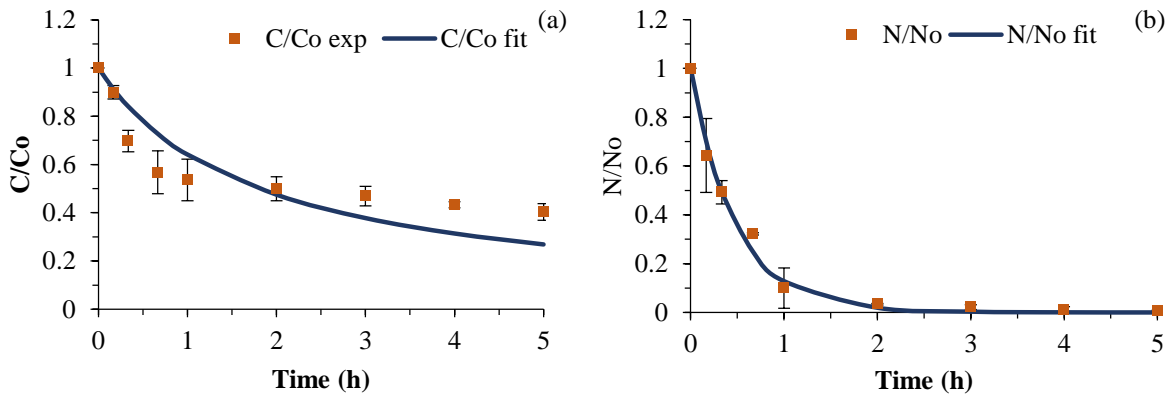
Figure 6.3. Curve fitting: (a) COD with second-order reaction and (b) Nitrate with first-order reaction.
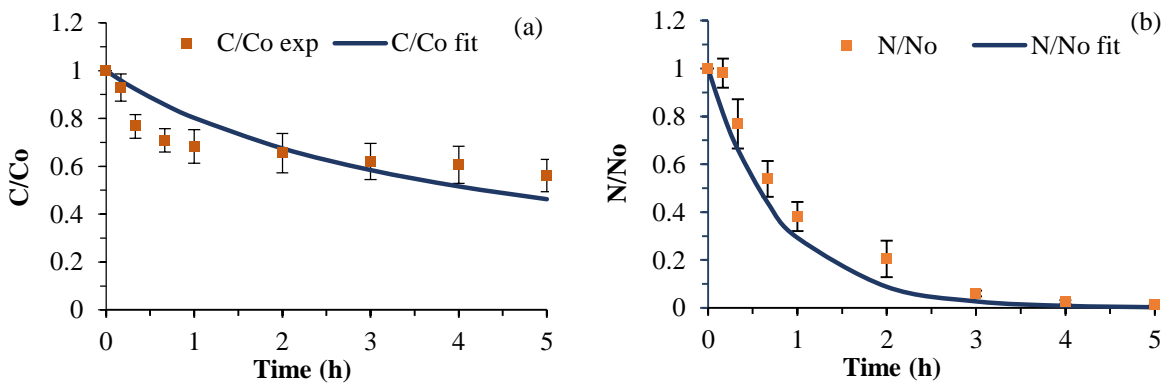
Figure 6.4. Curve fitting: (a) COD with second-order reaction and (b) Nitrate with first-order reaction.

6.2.4. *Statistical analysis*

Outcomes of the single factor ANOVA suggest that there was a significant difference in the means of different bio-kinetic parameters (except specific nitrate consumption rate, r_N) as a result

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of increasing concentration of herbicide. However, this difference in the means of COD and nitrate uptake rates are likely due to the cumulative effect of various concentrations of herbicide. Furthermore, student t-tests results are is presented in Table 6.4. The result suggests that there was no effect on the COD and nitrate uptake rates by the addition of 20 mg/L herbicide. The effect was apparent in terms of k_{COD} and r_{COD} when the concentration of herbicide further increased to 50 and 75 mg/L (it supports the assumption of the effect of increasing concentration of DMCPA in COD uptake). Apart from a few cases (baseline vs 50 mg/L and 50 vs 75 mg/L), there was no effect on the nitrate uptake rate by the increasing concentration of herbicide. Obviously, due to the limited number of data it is very hard to develop a visible pattern of the effect of herbicide on the microorganisms.

Table 6.4 Student t-tests outcomes.

Concn (mg/L)	Baseline				20 mg/L				50 mg/L			
	k_{COD}	k_N	r_{COD}	r_N	k_{COD}	k_N	r_{COD}	r_N	k_{COD}	k_N	r_{COD}	r_N
20	0.87	0.1	0.06	0.22								
50	0.01*	0.03*	0.49	0.77	0.18	0.52	0.04*	0.48				
75	0*	0.28	0.19	0.94	0.16	0.13	0.01*	0.23	0.07	0.04*	0.1	0.79

Note: * H_1 accepted (values differ significantly) when Pcal < 0.05.

6.3. MCPA formation and degradation kinetics

The experimental data indicates that when the dimethylamine salt of MCPA (i.e. DMCPA) was fed to the bioreactor, the biomass quickly converted it to MCPA acid that was then steadily degraded. The conversion reaction occurring inside the bioreactor can be written as follows:

DMCPA (D) ⟶ MCPA (M) ⟶ different intermediate and final products *(e.g chloromethyl phenol)*
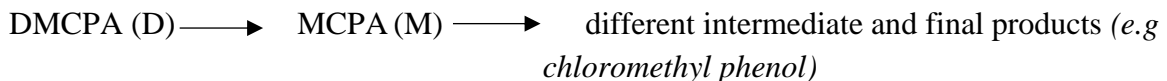
Assuming a first-order reaction-rate for each transformation, a materials balance for each of the primary compounds leads to the following set of ordinary differentials equations (ODEs):

$$\frac{dD}{dt} = -k_1 D \tag{6.1}$$

$$\frac{dM}{dt} = k_1 D - k_2 M \tag{6.2}$$

This research was limited to the potential biodegradation kinetics of MCPA acid; thus, the conversion of MCPA to other intermediate products and their associated equations were not considered. Equations 6.1 and 6.2 are valid only if one mole of DMCPA yields one mole of MCPA acid. In addition, using units of mg/L in Equations 6.1 and 6.2 and including the molecular weight of each individual compound, yields the following set of modified differential equations (MDEs):

$$\frac{d[D] * \frac{1}{Mw.D} * \frac{1}{1000}}{dt} = -k_1 * [D] * \frac{1}{Mw.D} * \frac{1}{1000}$$

$$\frac{d[D]}{dt} = -k_1 * [D] \tag{6.3}$$

Similarly,

$$\frac{d[M] * \frac{1}{Mw.M} * \frac{1}{1000}}{dt} = \{k_1 * [D] * \frac{1}{Mw.D} * \frac{1}{1000}\} - \{k_2 * [M] * \frac{1}{Mw.M} * \frac{1}{1000}\}$$

$$\frac{d[M]}{dt} = k_1 * [D] * \frac{Mw.M}{Mw.D} - k_2 * [M] \tag{6.4}$$

Where:

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[D] = Concentration of the dimethylamine salt of MCPA, mg/L

[M] = Concentration of MCPA acid, mg/L

Mw. D = Molecular weight of DMCPA = 245.7 g/mole;   Mw. M = Molecular weight of MCPA = 200. 62 g/mole; k_1 = rate constant for DMCPA and k_2 = rate constant for MCPA.

Equations 6.3 and 6.4 were solved simultaneously using the function ode45 in MATLAB for each concentration of herbicide. This function implements a Runge-Kutta method with a variable time step for efficient computation after the initial conditions at time t_o, are specified.

6.3.1. For 20 mg/L of DMCPA

Using initial concentration of both DMCPA and MCPA, equations 6.3 and 6.4 were solved simultaneously and the result is plotted against experimental data in Figure 6.5. Figure 6.5 shows that the degradation of DMCPA and MCPA seems to follow a first order kinetic model. Hence, the MCPA acid modelled curve gave a good fit with the experimental data. However, it was not feasible to directly quantify the DMCPA in the lab (as explained earlier in Section 3.6 and 3.7), thus the model obtained by the best fit of experimental data of MCPA acid demonstrates the possible degradation pattern of DMCPA at that specific concentration in the SBR.
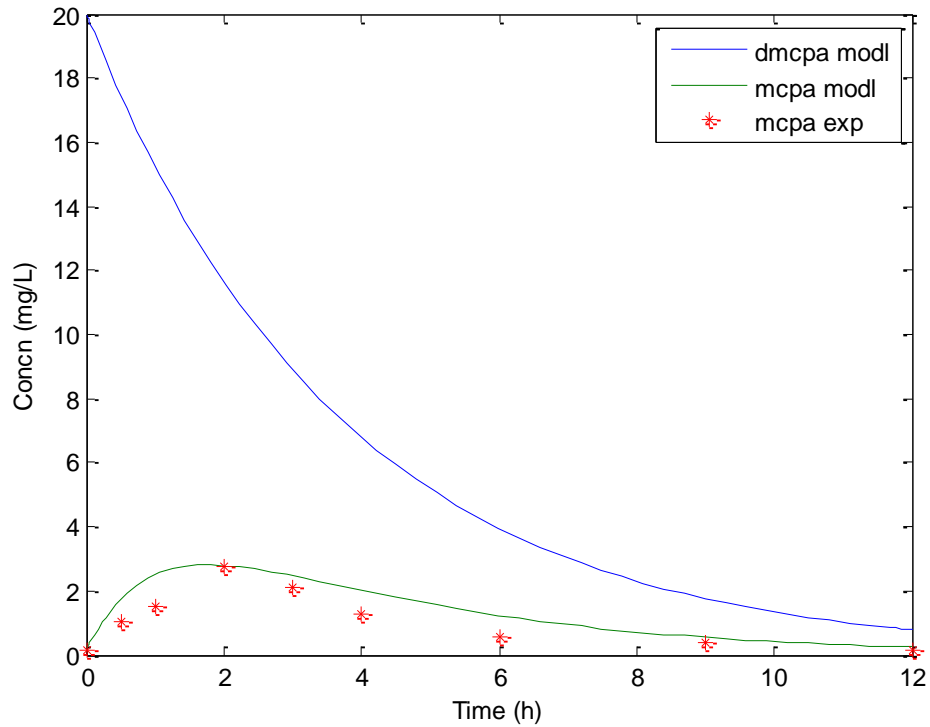
Figure 6.5. DMCPA and MCPA degradation kinetics: model vs experimental data at 24 h HRT.

As anticipated, the conversion of DMCPA to MCPA acid and the degradation of MCPA acid took place simultaneously inside the SBR. Initially, there were relatively large concentrations of DMCPA available in the bioreactor; thus, the formation rate of MCPA acid was faster than its degradation. As a result, accumulation of MCPA acid took place which can be seen by the increasing trend in the concentration of MCPA acid. However, after two to three hours; most of the DMCPA had been converted into acid (and other intermediate products). These continued to degrade as reflected in the descending portion of the curves. Furthermore, the model suggests that for 20 mg/L of herbicide, ≥ 95% degradation of both DMCPA and MCPA had taken place in a 12 hour time period.

The "apparent" reaction rate constants (obtained by fitting with the developed model) were found to be $k_1 = 0.27$ h^{-1} and $k_2 = 0.97$ h^{-1} respectively for DMCPA and MCPA. The "apparent" rate of reaction gives the cumulative (or lump-sum) degradation rate of DMCPA and MCPA inside the bioreactor.

6.3.2. For 50 mg/L of DMCPA

The biodegradation kinetics of DMCPA and MCPA were further investigated with 50 mg/L of herbicide at 48 h HRT (24 h cycles). The same first-order kinetic model (as for 20 mg/L) was applied for 50 mg/L. The model curve against experimental data is plotted in Figure 6.6.
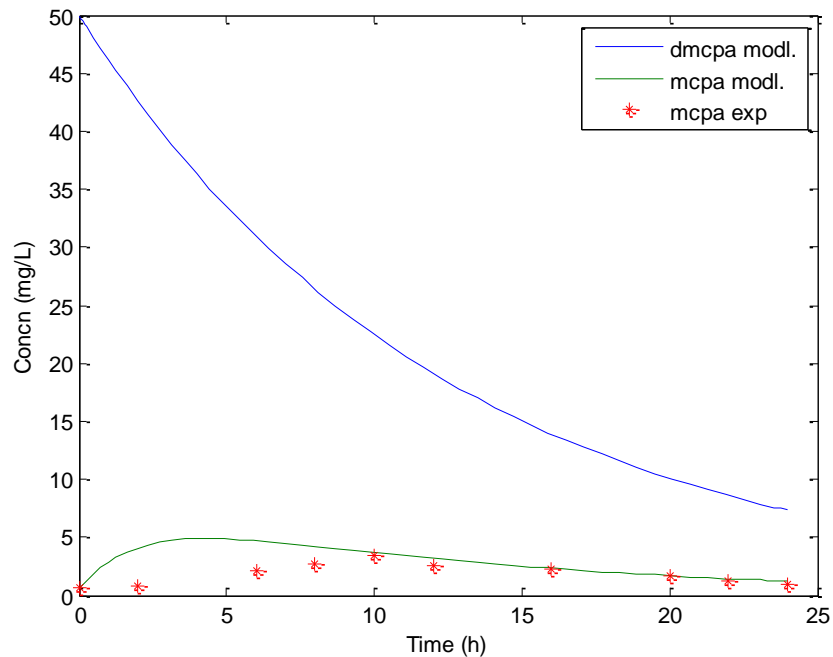
Figure 6.6. DMCPA and MCPA degradation kinetics: model vs experimental data at 48 h HRT.

Figure 6.6 shows that the developed model curve does not accurately fit first-order kinetics i.e. it is not as good fit as 20 mg/L (Figure 6.5). Further, the R^2 value was found to be 0.87 and 0.30 for 20 and 50 mg/L respectively when fitted with first-order kinetic model. A trial model with

second order kinetics (for 50 mg/L) was also tested but that did not fit well either. It is likely therefore that the DMCPA and MCPA degradation rates were affected by the increased concentration of herbicide (50 mg/L). That is, as the concentration of the herbicide increased, the rate of reaction of at least one compound slowed down (either DMCPA or MCPA) leading to the MCPA concentration reaching a peak after 10 h instead of 2 h as occurred when DMCPA in the influent was 20 mg/L (Figure 6.5). It is also possible that two separate groups of microorganism were involved in the conversion of DMCPA to MCPA acid and the degradation of MCPA acid to its end products. As such, one of two potential hypotheses might be occurring. The first hypothesis is that, the rate of conversion of DMCPA to MCPA slowed down at the beginning (because of the high concentration of herbicide and/or the microorganisms might be saturated with DMCPA) whereas the MCPA degradation rate remained unchanged. As a result, the rate of formation of MCPA acid was less than its degradation rate; thus, it took some time to develop the MCPA peak. In contrast, the second possibility is that the rate of conversion of DMCPA to MCPA remained unchanged, whereas the rate of degradation of MCPA acid was faster than its formation (because bacteria were acclimated to the herbicide). As a consequence, the MCPA peak was delayed. In both cases, as the reaction proceeded, the MCPA degradation rate slowed down due to either microorganism saturated with the herbicide or the accumulation of intermediate compounds inhibiting the degradation process (i.e. an inhibition effect).

Further to this, the first hypothesis is the more likely explanation in the bioreactor because when the concentration of the herbicide was increased to 75 mg/L, the biomass converted very little DMCPA to MCPA acid (as described in Section 5.4). Moreover, after 3 weeks of the initial

injection of 75 mg/L, detailed lab observations indicated that the conversion of DMCPA to MCPA acid had completely shut down.

In any case, a first-order kinetic model is unable to predict the inhibition or biomass saturation behavior in the bioreactor, thus, a more complex model (such as Michaelis Menten (MM) or Haldane equation) is required. Even then, modification of these models are often needed (e.g. Marques et al., 2014) in order to adequately describe the inhibitory effect associated with the biodegradation of xenobiotic compounds.

In this research, the complexity of the investigated pesticide (i.e. commercially-available DMCPA) meant that it was very hard to quantify the biodegradable and non-biodegradable portions of the compound (i.e. other residual products used during the commercial formulation of the herbicide). As such, it was not possible to precisely determine whether the inhibition that occurred was either due to the herbicide itself or other additives used during the formulation of the herbicide. As such, it is not feasible to incorporate inhibition factors in equations 6.3 and 6.4. Nevertheless, even with a first-order kinetic model, the removal of both DMCPA and MCPA was more than 80 % at the end of 24 hour period in the anoxic SBR.

The "apparent" reaction rate constants for 50 mg/L of DMCPA were found to be $k_1 = 0.09$ h^{-1} and $k_2 = 0.47$ h^{-1} respectively for DMCPA and MCPA. This suggests a threefold decrease in the apparent rate of DMCPA degradation (this is substantial and tends to imply the first hypothesis is true) and a two- fold decreases in the rate of MCPA degradation as the concentration of the herbicide increased from 20 to 50 mg/L.

Furthermore, most previous research describing the biodegradation kinetics of MCPA have been done on soil samples and/or on contaminated land sites (Crespin et al., 2001; Mortensen & Jacobsen, 2004; Jensen et al., 2004; Hiller et al., 2009; Paszko, 2009). For example, Hiller et al. (2009) carried out a comparative study of MCPA biodegradation in two different soil types (Chernitsa - sandy loam and Regosol – sandy soil). They reported that the degradation of MCPA in the soil was affected by many factors such as, the organic carbon content of the soil, the source of nutrients available and the activity and size of the microbial population. By fitting their observed data with a first-order rate equation ($C=C_o e^{-kt}$) they obtained MCPA degradation rates of $k_1 = 0.31$ d^{-1} (Chernitsa) and $k_2 = 0.06$ d^{-1} (Regosol). The rates reported by Hiller et al. (2009) are quite low; however, possible reasons for the higher reaction rates obtained ($k = 0.97$ h^{-1} for 20 mg/L and $k = 0.47$ h^{-1} for 50 mg/L) in the present research are (i) a large microbial community population which has been acclimatized to the recalcitrant compound; (ii) a relatively longer period of time in the SBR, and (iii) plenty of easily degradable organics (acetate) available in the system (which was continuously supplied with an excess of needed micro-nutrients).

Crespin et al. (2001) and Paszko (2009) also concluded that MCPA degradation kinetics in the soil surface layer followed a first-order kinetic model. In particular, Crespin et al. (2001) studied the degradation of two commonly used herbicides 2,4-D and MCPA at different depths in an agriculture soil and the degradation rates were found to be 0.142 and 0.135 day^{-1} respectively. They reported that there was a direct correlation between the degradation parameters and the microbial biomass content (the rate of degradation of herbicide decreased with increasing soil

depth (i.e. the decrease in biodegradation resulted from a decrease in the number of microbial degraders)).

The overall result suggests that, similar to the biodegradation of MCPA in soil, biodegradation kinetics of MCPA by activated sludge microorganisms also follows a first-order kinetic model up to a concentration of 20 mg/L. As the concentration of the herbicides increases, there is high probability that microorganisms become saturated with potentially toxic material meaning that the reaction rates of both parent compounds and the resulting metabolite decreases. Thus, it may mean that a more complex model is required to adequately describe the biomass saturation and inhibitory effect during the degradation of industrial-strength concentrations of pesticides.

As mentioned in Chapter 3 (Section 3.1.2), two sequencing batch reactors were designed and operated simultaneously for 19 months; the control reactor (RC-I) and the MCPA test reactor (RC-II). The purpose of the control reactor was to ensure all other parameters were running smoothly, since the lab conditions were identical for both reactors except the herbicide feeding strategy in RC-II. This chapter compares changes occurring during the experimental period in RC-I and RC-II along with the results from the 3 batch tests.

7.1.   TSS and VSS

TSS and VSS in both reactors were monitored for the entire period of the study. The result presented in Figure 7.1 indicates that both reactors exhibited similar characteristics when the sludge switched from being in a large full-scale aerated wastewater treatment plant to 20 L anoxic SBRs. That is, the TSS of both reactors dropped from approximately 5000 mg/L to 2000 mg/L. However, once the $COD:NO_3$-N was held at 3.2, both reactors started performing steadily. As such, stable TSS and VSS were attained and around 4000 ± 400 and 2800 ± 300 mg/L respectively. Regular observation of TSS and VSS data shows that there does not seem to be any change encountered between RC-I and RC-II even after injection of the pesticide in RC-II. This indicates that the biomass was not affected very much by the presence of the pesticide, at least in terms of the amount of solids. One possible reason might be the concentration of herbicide injected (i.e. 20, 50 and 75 mg/L) was not enough to significantly interrupt the metabolic behavior of the anoxic microorganisms.
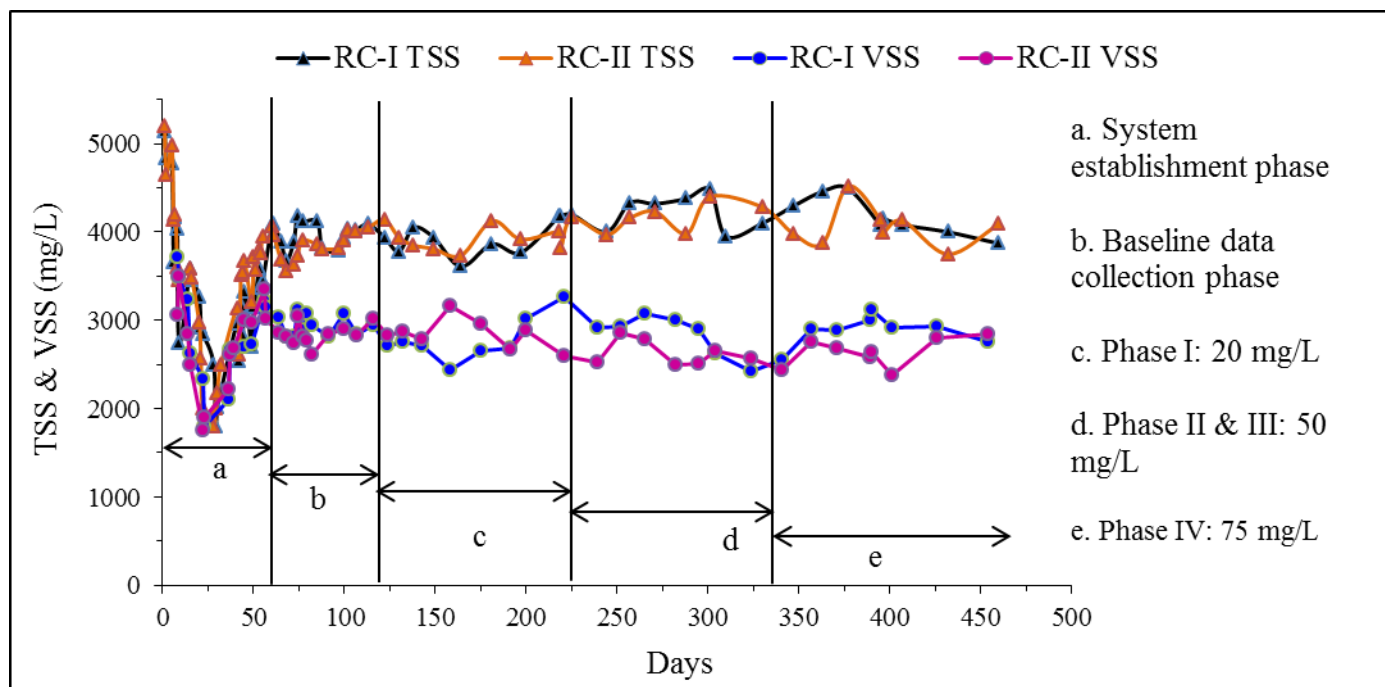
Figure 7.1. TSS and VSS in the control (RC I) and test (RC II) reactors.

7.2. COD and nitrate consumption before and after pesticide injection

Comparative track study results of COD and nitrate consumption in both reactors before and after the herbicide injection (into RC-II) are plotted in Figure 7.2. This shows that the COD and nitrate were consumed completely within five hours' time in both reactors during the baseline period (Figure 7.2a and b). However, the substrate utilization pattern (especially COD consumption) changed immensely with more than 50 % of the COD remaining unused in RC-II compared to RC-I, after a series of herbicide injections into the RC-II (Figure 7.2c). This inhibition is likely due to the accumulation of MCPA intermediate metabolites and additives or formulation agents used for the commercial formulation of DMCPA. In the case of nitrate reduction, Figure 7.2d indicates the nitrate utilization pattern was slightly delayed in RC-II after

herbicide injection; however, at the end of five hours' time, complete reduction of nitrate took place in both reactors.
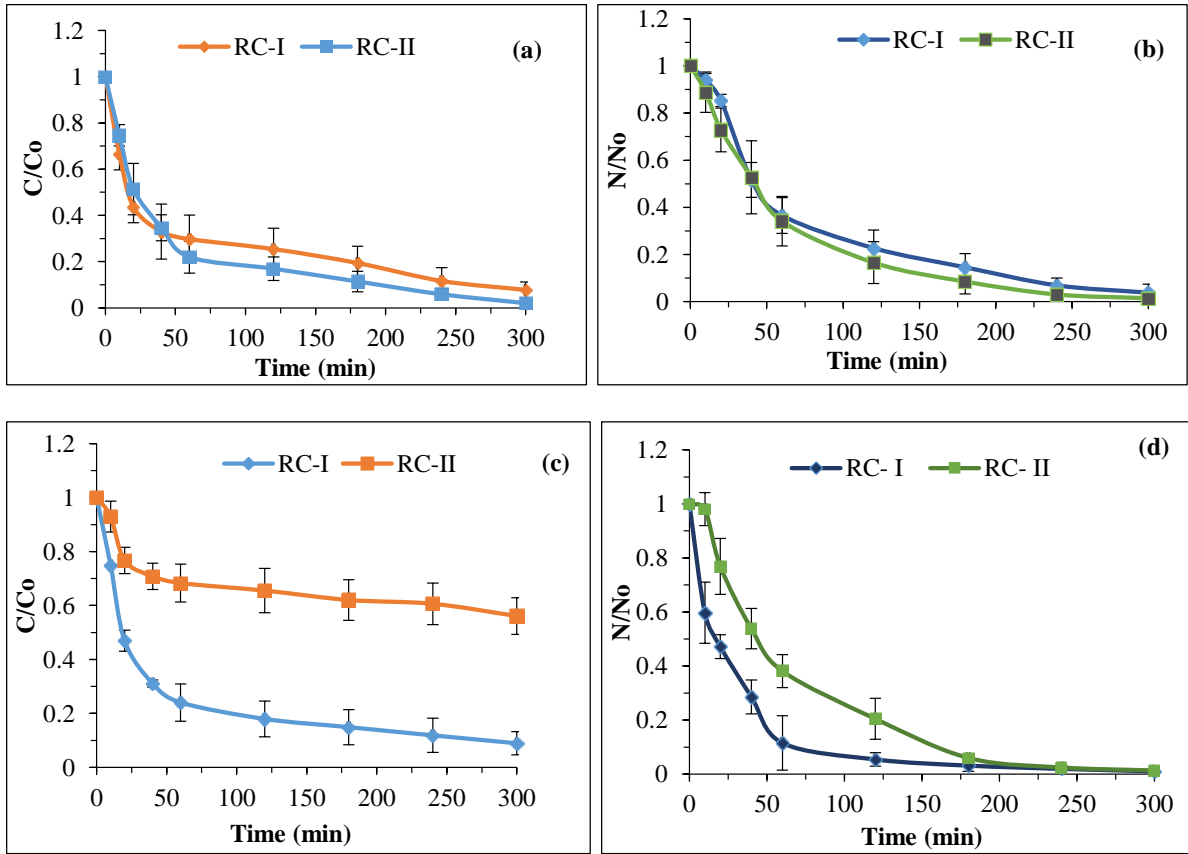
Figure 7.2. (a) COD and (b) Nitrate consumption pattern before pesticide injection and (c) COD and (d) Nitrate consumption pattern after pesticide injection.

7.3. TOC

Total Organic Carbon (TOC) present in the effluents of RC-I and RC-II were examined and the results are plotted in Figure 7.3. The results demonstrates that more than 98 % of the TOC had been used by the microorganisms in RC-I, however, there was a substantial amount (i.e. 55 to 60

%) of non-degradable organic carbon present in RC-II after a series of herbicide injections into the reactor. The presence of large amount of unused organic carbon in the effluent of RC-II was further evidence of the presence of MCPA intermediate metabolites and/or additives or formulation agents of DMCPA inside the bioreactor.
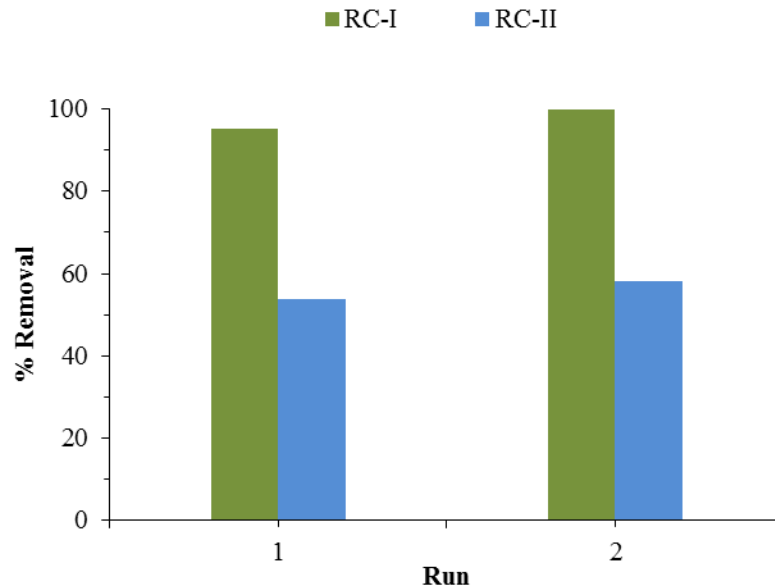
Figure 7.3. Percentage removal of Total Organic Carbon (TOC) in the effluent of the control (RC-I) and test (RC-II) reactors at 48 h HRT.

7.4. ORP and pH

On-line monitoring parameters such as ORP and pH accurately detect the state of various biological processes (Tanwar et. al., 2008) and can be used as relative operational parameter for better control of reactor systems. As such, in this research, ORP was manipulated by injecting nitrate solution (twice each cycle) into the bioreactor. The pH however was continuously monitored (not controlled) throughout the study period. The ORP for the first five hours of the cycle for both reactors I and II are plotted versus the nitrate reduction in Figure 7.4. The figure

shows there is a direct correlation between the ORP and nitrate curve as shown by R^2 values ($\geq$ .90) for both reactors.
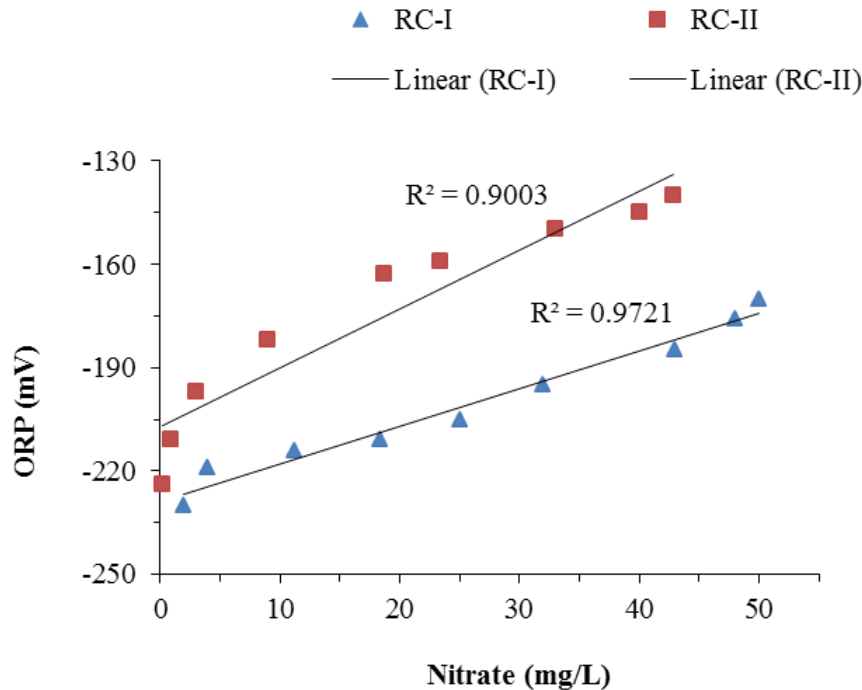
Figure 7.4. ORP vs Nitrate in RC-I and II.

Similarly, the pH results from the entire period are plotted in Figure 8.5. The observed result indicates that the pH of the SBR system stabilized around a relatively basic pH of 9.0 (despite some irregular points). A study carried out by Glass & Silverstein (1998, 1999) suggest that a pH around 8.5 to 9 can often be favorable for anoxic microorganisms. Their investigation on the effect of pH during denitrification revealed that at a pH value $\leq$ 7.0, denitrification of high initial nitrate concentration (i.e. 1350 mg/L) was completely inhibited. As the pH increased from 7.5 to 9.0, the accumulation of nitrite increased significantly; however the overall time required to completely reduce both the nitrate and nitrite was approximately constant. Regular checks of

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both bioreactors associated with Figure 7.5 indicated that there were occasionally negligible amounts of ammonium (NH_4 -N$\leq$ 0.5 mg/L) and nitrite ($\leq$ 5 mg/L).
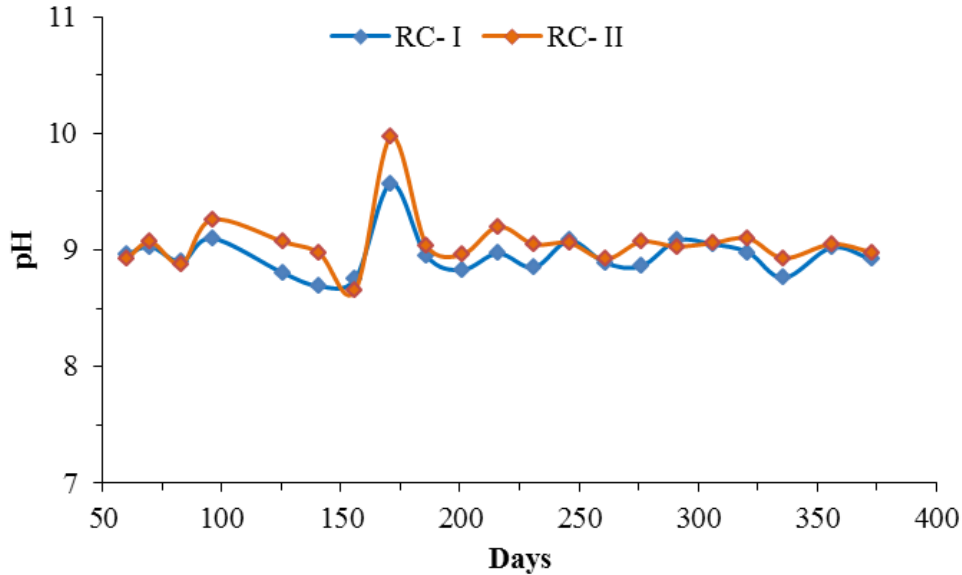
Figure 7.5. pH value in RC-I and II.

7.5.   Biomass characteristics

Microscopic analysis of the biomass flocs after gram staining indicated the presence of gram positive rods with an average size of the rod about 1 μm. Further to this, scanning electron micrograph (SEM) images were taken and these are presented in Figure 7.6 (RC-I) and Figure 7.7 (RC-II). The objective of the SEM images was to see if there was any visible difference in the shape and size of anoxic microorganisms before and after pesticide additions. The figure is inconclusive however perhaps DNA extraction would be able to see any differences. Such techniques were however beyond the scope of this work.
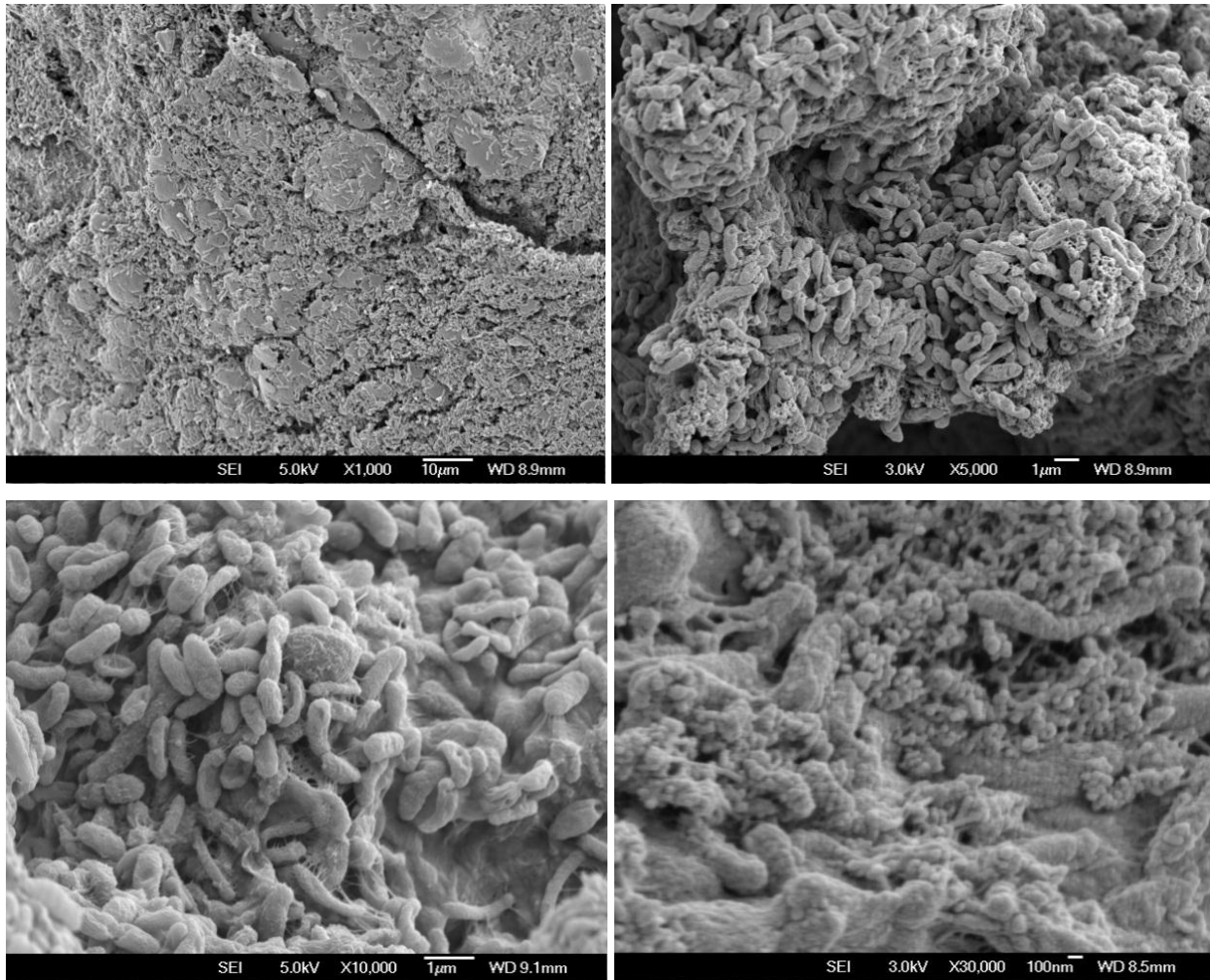
Figure 7.6. SEM images RC-I.
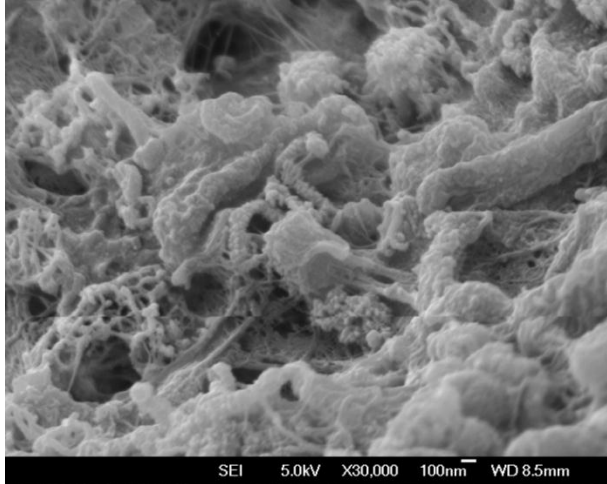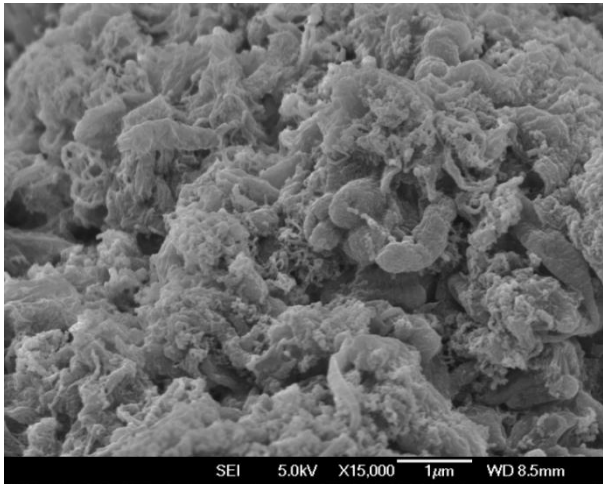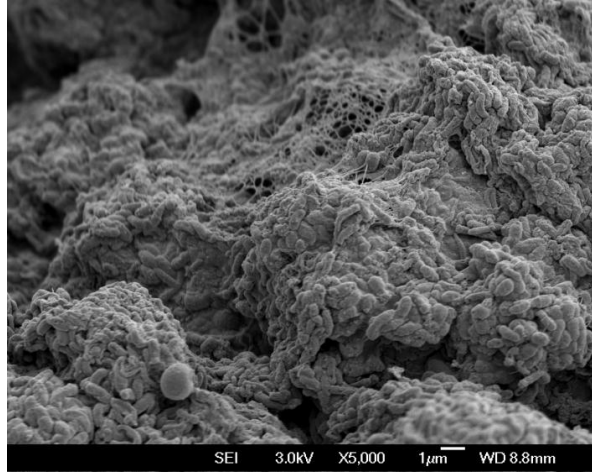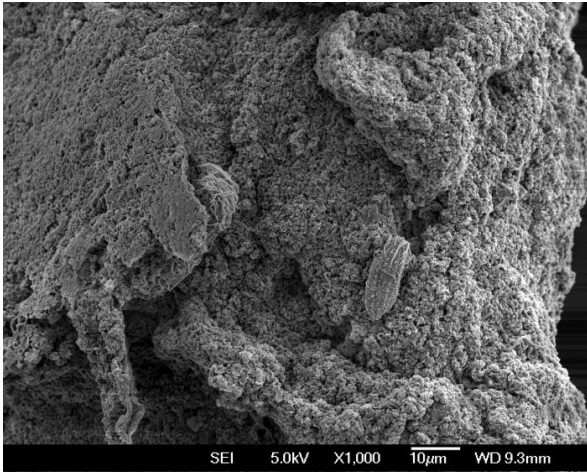
Figure 7.7. SEM images RC-II.

7.6.   Spiking of MCPA to non-acclimatized biomass

In order to better understand the microorganism's response to a sudden exposure of the herbicide, three further sets of batch tests were carried out. As such, 20 mg/L of DMCPA was spiked into non-acclimatized biomass (i.e. biomass from RC-I) which yielded around 16.25 mg/L of MCPA acid (detailed calculations are contained Table 3.2). Figure 7.8 show that the anoxic microorganisms completely (with some standard error represented by error bars) converted 20 mg/L of DMCPA to MCPA acid within 2 hours' time. Unlike the MCPA acclimatized biomass (Section 5.1), the non-acclimatize biomass could not degrade MCPA immediately (i.e. during the initial two-hour period) since the conversion of DMCPA to MCPA acid had not taken place at that time. As soon as the DMCPA was converted to MCPA acid, degradation occurred quite rapidly removing more than 70 % of the MCPA acid within 4 hours. As the reaction proceeded, track studies revealed that the biomass was unable to degrade the final part of the MCPA acid even in 24 hours' time (i.e. a 48 h HRT) (Figure 8.8).
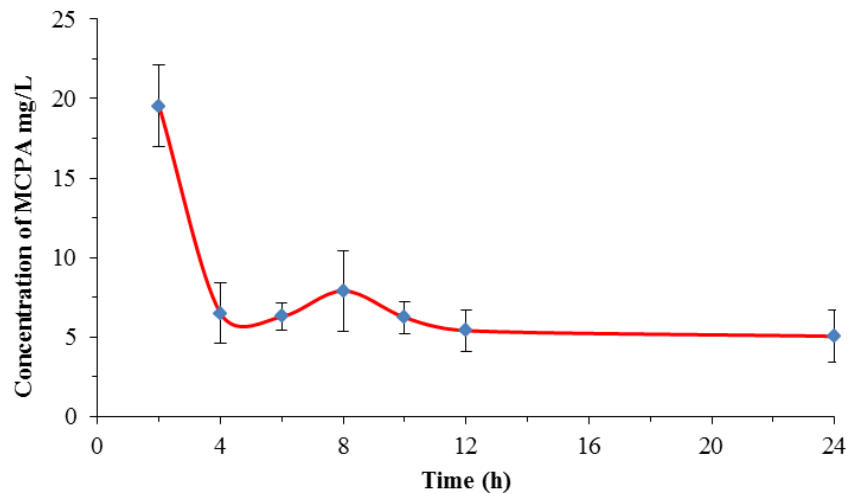
Figure 7.8. DMCPA/MCPA degradation by non-acclimatize biomass at 48h HRT. Errors bars representing (n=3) average values with standard deviation.

PART 3: CONCLUSION AND RECOMMENDATIONS

Conclusions

A number of conclusions can be drawn from this research and these are summarized below:

1. During the baseline data collection phase, the anoxic SBR system performed successfully and obtained pseudo-steady-state conditions at a COD:NO_3-N ratio of 3.2 as reflected by i) stable biomass concentrations in terms of TSS and MLVSS with 4000 ± 400 mg/L and 2800 ± 300 mg/L respectively (ii) stable effluent characteristics (i.e. > 95 % COD and nitrate removal) and (iii) stable ORP data (ORP = -225 ± 25 mV).

2. During the MCPA acclimatization period (Phase I), an industrial-strength concentration of 20 mg/L DMCPA was continuously fed to the system. In this experiment, it took around 25 to 30 days before the first sign of removal of MCPA in the SBR was observed. Detailed MCPA track studies revealed that the conversion (from salt to acid form) and subsequent degradation occurred simultaneously in the SBR, with the rate of formation of the acid larger than its removal rate in the first half of the cycle and biodegradation of the acid dominant in the second half of the cycle. At the end of Phase I (after 90 days), more than 98 % removal of 20 mg/L of DMCPA was achieved, indicating that degradation of MCPA could be realized under anoxic conditions. Three track studies for COD and nitrate consumption indicated that there was no change in terms of the COD and nitrate removal pattern during this period.

3. The amount of DMCPA was increased from 20 mg/L to 50 mg/L in Phase II and; compared to Phase I (2-3 h), the microorganisms took much longer (i.e. 8-9 h) to convert 50 mg/L of MCPA salt to acid. Eventually, incomplete biodegradation of the MCPA was observed. The COD consumption and nitrate removal patterns during Phase II revealed that the nitrate removal in the reactor was still > 95% within five hours, whereas the COD consumption level had dropped to 60%.

4. Phase III investigated an HRT of 48 h (a 24 h cycle) and showed that it was sufficient to degrade a concentration of 50 mg/L of DMCPA. More specifically, complete conversion of the MCPA salt to acid took a similar amount of time (8-10 h) as the 12 h cycle but obviously longer to degrade a significant portion of the MCPA acid. At the end of the cycle, the reactor achieved more than 98 % of MCPA acid removal by denitrifying microorganisms. However, even with a 48 h HRT, the COD removal achieved only 56%.

5. The concentration of DMCPA was increased to 75 mg/L during Phase IV; however detailed track studies did not detect MCPA in any of the samples. After a rigorous check of all analytical protocols, it was concluded that the bacteria were no longer able to convert any further MCPA salt to acid; thus, no MCPA formation/degradation pattern was likely to be observed. Interestingly, the denitrification activity was not affected even with the increased concentration (i.e. 75 mg/L) of the herbicide.

6. The observations associated with these experimental conditions indicate that the potential upper limit of MCPA degradation in anoxic conditions lies somewhere between 50 and 75 mg/L. Furthermore, once that limit has been reached, at least under the conditions of

this experiment, the bacteria do not seem to recover their capability of degrading DMCPA/MCPA compounds.

7. Along with 4-chloro-2-methyl phenol (4CMP) several other DMCPA/MCPA metabolic intermediate products were detected during this investigation.

8. Blank controls were carried out to quantify volatilization and these were estimated to be less than 2 %. Furthermore, it was found that around 13-15% MCPA removal occurred via bioadsorption meaning that the remaining 85% uptake of MCPA was likely due to biodegradation.

9. The kinetic rate constants of COD and nitrate associated with the baseline track study data follow a first-order kinetic model and the rate constants values are 1.62 ± 0.41 and $1.02 \pm 0.28 h^{-1}$ respectively. Similarly, specific COD and nitrate uptake rates are found to be 0.369 ± 0.02 and 0.118 ± 0.02 mg/mg VSS d correspondingly.

10. The biodegradation kinetic model of COD changed from a first order (baseline data) to a second order kinetic model by the addition of increasing concentrations of herbicide. The rate constant values (k_2) decreased from 1.51 ± 0.82 to 0.57 ± 0.14 to 0.25 ± 0.11 h^{-1} when the herbicide concentration increased from 20 to 50 to 75 mg/L respectively. In regards to nitrate, the order of reaction remained the same as the baseline data (i.e. a first-order kinetic model) but the rate constant values (k_1) decreased from 2.58 ± 0.76 to 2.14 ± 0.40 to 1.24 ± 0.16 h^{-1} from 20 to 50 to 75 mg/L. Similarly, specific COD and nitrate uptake rates decreased from 0.60 ± 0.12 to 0.39 ± 0.04 to 0.26 ± 0.07 mg/mg VSS d and 0.14 ± 0.01 to 0.12 ± 0.02 to 0.11 ± 0.01 mg/mg VSS d during 20 to 50 to 75 mg/L.

11. The "apparent" reaction rate constants for DMCPA and MCPA for 20 mg/L of herbicides were found to be k_D= 0.27 h^{-1} and k_M = 0.97 h^{-1} respectively. As the concentration of DMCPA increased from 20 mg/L to 50 mg/L, a three-fold decrease (k_D = 0.09 h^{-1}) in the apparent rate of DMCPA degradation and a two-fold decrease (k_M = 0.47 h^{-1}) in the rate of MCPA degradation was observed.

12. This study concluded that the biomass was not substantially affected by the presence of the MCPA in the SBR, at least in terms of the amount of solids. However, the substrate utilization pattern (especially COD consumption) changed immensely with more than 50 % of the COD remaining unused in the MCPA fed reactor, as compared to the control reactor.

13. Unlike the MCPA-acclimatized biomass, the non-acclimatized biomass could not degrade MCPA immediately. The anoxic microorganisms took around 2 hours of time to completely convert 20 mg/L of DMCPA to MCPA acid. However, as soon as the DMCPA was converted to MCPA acid, degradation occurred quite rapidly removing more than 70 % of the MCPA acid within 4 hours. As the reaction proceeded, track studies revealed that the biomass was unable to degrade the final part of the MCPA acid even in 24 hours of time.

Recommendations

After successful completion of this research, the following recommendations are made:

1. In order to further investigate MCPA, some pre-treatment methods (such as acid or alkali treatment) are recommended, as these potentially increase MCPA solubility, as well as helping with the extraction method. The higher the solubility of the compound, the higher would be the chance of particles to dissolve in the feed. This would correspondingly increase the probability of microorganisms being able to easily degrade the compound (providing there is sufficient electron acceptor available).

2. The SBR was found to be an effective technology in regards to the concurrent biodegradation of pesticide and nitrates. However, further research could be carried out to increase its efficiency by optimizing some operational and environmental parameters such as HRT, SRT, pH, and temperature etc.

3. Research could be carried out in an SBR with respect to the biodegradation efficiency of 4CMP (a major metabolite of MCPA) in both aerobic and anaerobic conditions. As a result, a complete mineralisation scheme with all biochemical pathways of MCPA as a treatment operation could be understood.

4. A comprehensive molecular analysis such as polymerase chain reaction (PCR) assay could be carried out independently to investigate the possible MCPA and 4CMP

degrading microbial consortia. The PCR technique involves the use of a single primer set (which targets a specific gene) to detect an organism. The primer set can be designed for specific species and can detect the target organism in the presence of others.

5. One could develop a theoretical mathematical model on simultaneous pesticide and nitrate degradation strategy in SBRs. Depending upon the complexity of the recalcitrant compound, different mathematical models (such as Michaelis Menten (MM) or Haldane equation) could be tried. The inhibitory effect associated with the biodegradation of xenobiotic compound could also be explored by adjustments of these models.

6. One could investigate the biodegradation capability of some other structurally similar chlorinated herbicides such as Mecoprop [2-(4-chloro-2-methylphenoxy)propanoic acid] or Dicamba (3, 6-dichloro-2-methoxybenzoic acid) in an SBR.

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Appendix-I: Raw data

a. COD and Nitrate track study data (baseline)

Time (min)	COD					Average	COD/COD$_0$	Standard Deviation
	Day-1	Day-2	Day-3	Day-4	Day-5			
0	81	94	95	97	97	92.8	1	6.0133186
10	47	67	74	57	64	61.8	0.665948	9.1956511
20	27	53	39	44	40	40.6	0.4375	8.4047606
40	11	51	33	30	28	30.6	0.329741	12.753039
60	11	45	33	24	25	27.6	0.297414	11.2
120	9	39	26	20	24	23.6	0.25431	9.6871048
180	6	30	20	19	15	18	0.193966	7.7717437
240	3	21	13	10	7	10.8	0.116379	6.0794737
300	1	12	8	9	6	7.2	0.077586	3.6551334

Reactor-I (table above)

Time (min)	COD					Average	COD/COD$_0$	Standard deviation
	Day-1	Day-2	Day-3	Day-4	Day-5			
0	97	106	90	99	102	98.8	1	5.344155686
10	80	77	70	72	69	73.6	0.74494	4.223742416
20	42	72	43	35	64	51.2	0.51822	14.21829807
40	26	40	32	29	45	34.4	0.34818	7.059745038
60	13	32	25	12	27	21.8	0.22065	7.93473377
120	10	22	18	10	24	16.8	0.17004	5.878775383
180	6	16	17	7	10	11.2	0.11336	4.53431362
240	1	9	8	6	5	5.8	0.0587	2.785677655
300	0	1	2	6	1	2	0.02024	2.097617696

Reactor-II (table above)

Time (min)	Reactor-I					Average	$NO_3\text{-}N/NO_3\text{-}N_0$	Standard deviation
	$NO_3\text{-}N$							
	Day-1	Day-2	Day-3	Day-4	Day-5			
0	65	40	66	65	50	57.2	1	10.45753317
10	60	39.4	60	60	48	53.48	0.934965035	8.435733519
20	58	33.4	57	53	43	48.88	0.854545455	9.383901108
40	32.4	16.6	35.4	32	32	29.68	0.518881119	6.662852242
60	25.4	12.8	18.5	22	25	20.74	0.362587413	4.68
120	16.2	6.8	10	13	18.3	12.86	0.224825175	4.139371933
180	13.3	2.9	8	7	11.2	8.48	0.148251748	3.58295967
240	8	1.5	3	4	4	4.1	0.071678322	2.154065923
300	7	0.9	0.7	0.8	1.9	2.26	0.03951049	2.408817137

Time (min)	Reactor-II					Average	$NO_3\text{-}N/NO3\text{-}N_0$	Standard deviation
	$NO_3\text{-}N$							
	Day-1	Day-2	Day-3	Day-4	Day-5			
0	36.2	42.8	26	37	25	33.4	1	6.84807
10	35.6	40	18.5	33	23	30.02	0.898802395	8.02007
20	31.4	33	14.4	27	18	24.76	0.741317365	7.3489
40	29.6	14.4	12.7	22	10	17.74	0.531137725	7.14524
60	20	8.7	8	12	8	11.34	0.339520958	4.57629
120	11.9	5	0.9	7	4	5.76	0.17245509	3.64779
180	6.6	1.6	0.6	4	1.9	2.94	0.088023952	2.13878
240	1.9	0.9	0.4	0.9	1	1.02	0.030538922	0.48744
300	0.4	0.2	0.4	0.7	0.6	0.46	0.013772455	0.17436

b. MCPA track study data:

With 20 mg/L of DMCPA at 24h HRT

Time (h)	Area (Hz)				
	Day-1	Day-2	Day - 3	Average	Std
0	68	72	67	69	2.16025
0.5	276	123	134	177.6667	69.677
1	308	231	182	240.3333	51.8609
2	330	322	515	389	89.1553
3	247	254	424	308.3333	81.8386
4	183	191	246	206.6667	28.004
6	97	94	164	118.3333	32.3144
9	68	87	142	99	31.3794
12	64	75	64	67.66667	5.18545

With 50 mg/L of DMCPA at 24 h HRT

Time (h)	Area (Hz)				Average	Std
	Day-1	Day-2	Day-3	Day-4		
0	223	219	201	229	218	10.440307
0.5	250	282	286	274	273	13.96424
1	318	361	335	313	331.75	18.753333
2	350	373	352	380	363.75	13.00721
3	366	377	375	392	377.5	9.3407708
4	417	357	392	394	390	21.435951
6	454	393	412	583	460.5	74.089473
8	669	612	613	723	654.25	45.909558
10	548	451	490	624	528.25	65.17045
12	352	252	250	350	301	50.009999

With 50 mg/L of DMCPA at 48 h HRT

Time (h)	Area (Hz)			Average	Std
	Day-1	Day-2	Day-3		
0	112	120	139	123.667	11.3235
2	107	122	212	147	46.3681
6	296	264	372	310.667	45.2941
8	453	327	398	392.667	51.5773
10	515	484	443	480.667	29.4882
12	439	307	334	360	56.9386
16	399	279	310	329.333	50.8615
20	262	253	280	265	11.225
22	149	225.667	249	207.889	42.7164
24	135	107	237	159.667	55.8649

Appendix- II: Calibrations curves

a.   Calibration curves for MCPA using SPME-GC.

$y = 124.31x + 48.234$

$R^2 = 0.999$
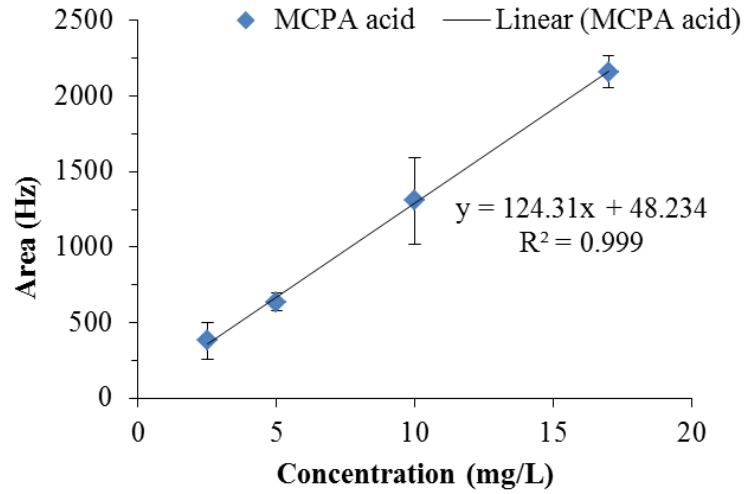
Figure AII.1. Pure MCPA acid calibration curve; average values (n=3) and standard deviation (error bars) are shown

b.   Calibration curves for Total and Inorganic Carbon using a Shimadzu TOC-L CSH analyzer:

$y = 3.0665x - 51.5$

$R^2 = 0.9997$
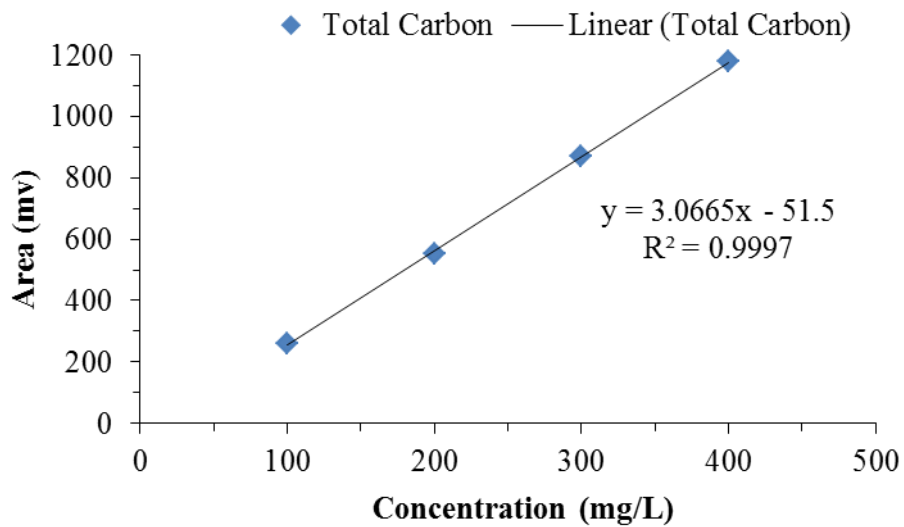
Figure AII.2.   Calibration curves for Total Carbon using a Shimadzu TOC-L CSH analyzer
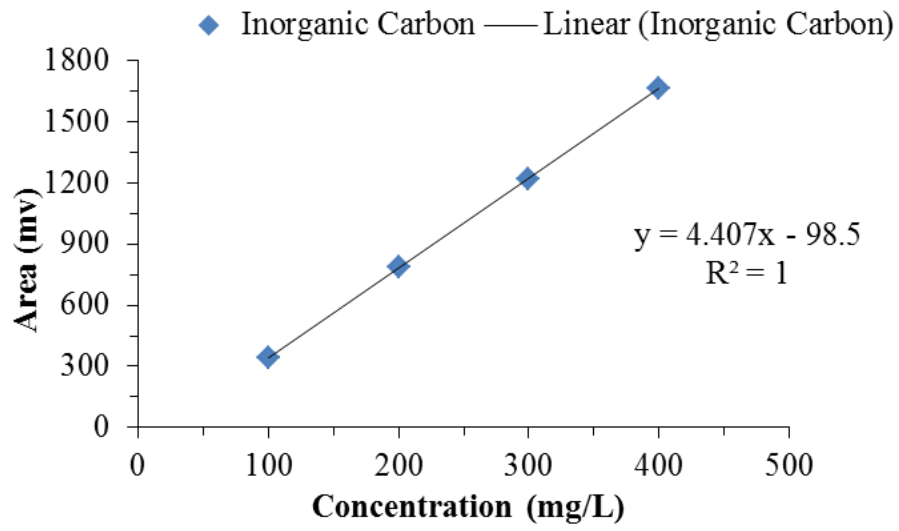
Figure AII.3.   Calibration curves for Inorganic Carbon using a Shimadzu TOC-L CSH analyzer

Appendix- III: Important Chromatographs
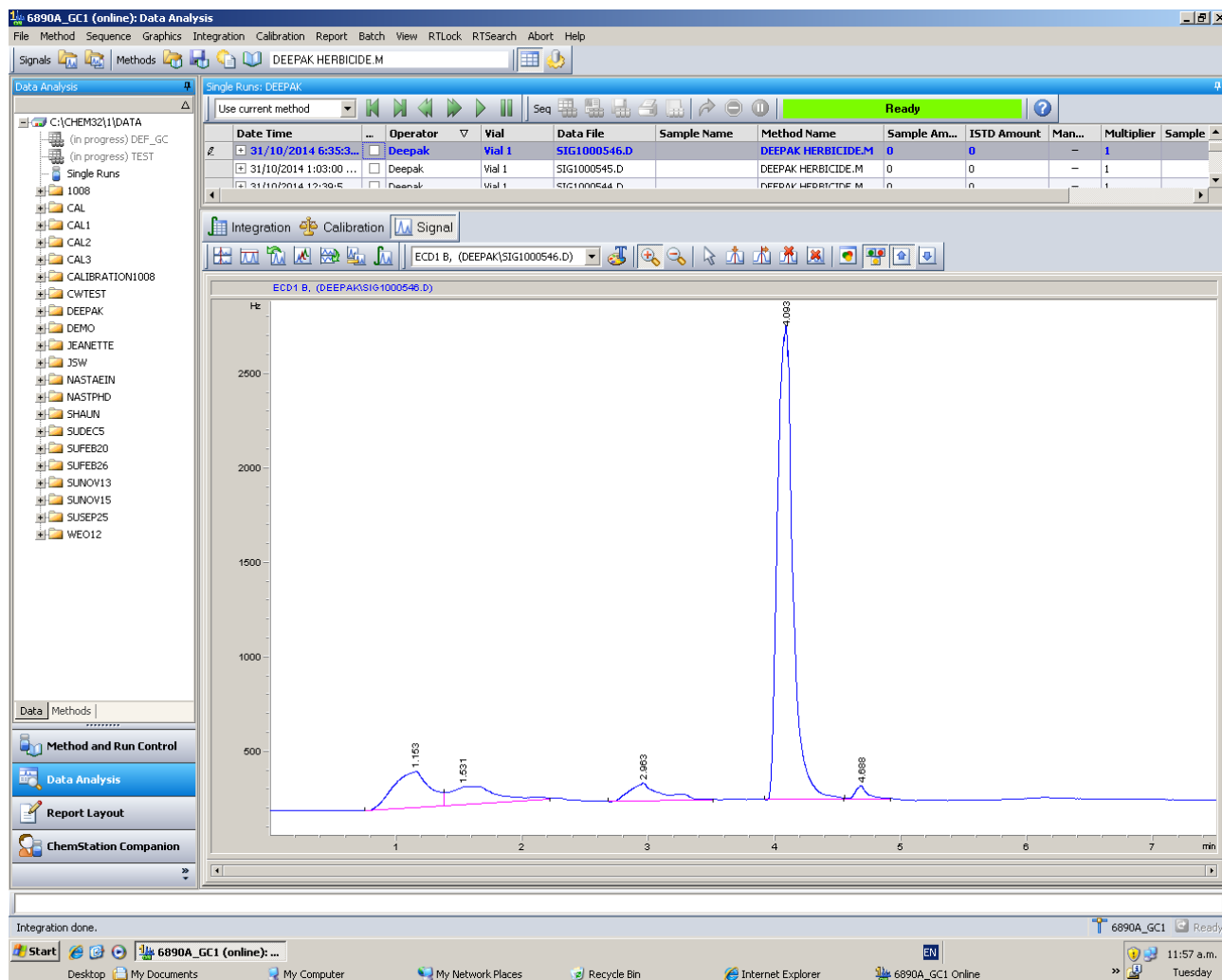
Figure AIII. 1. Pure MCPA acid
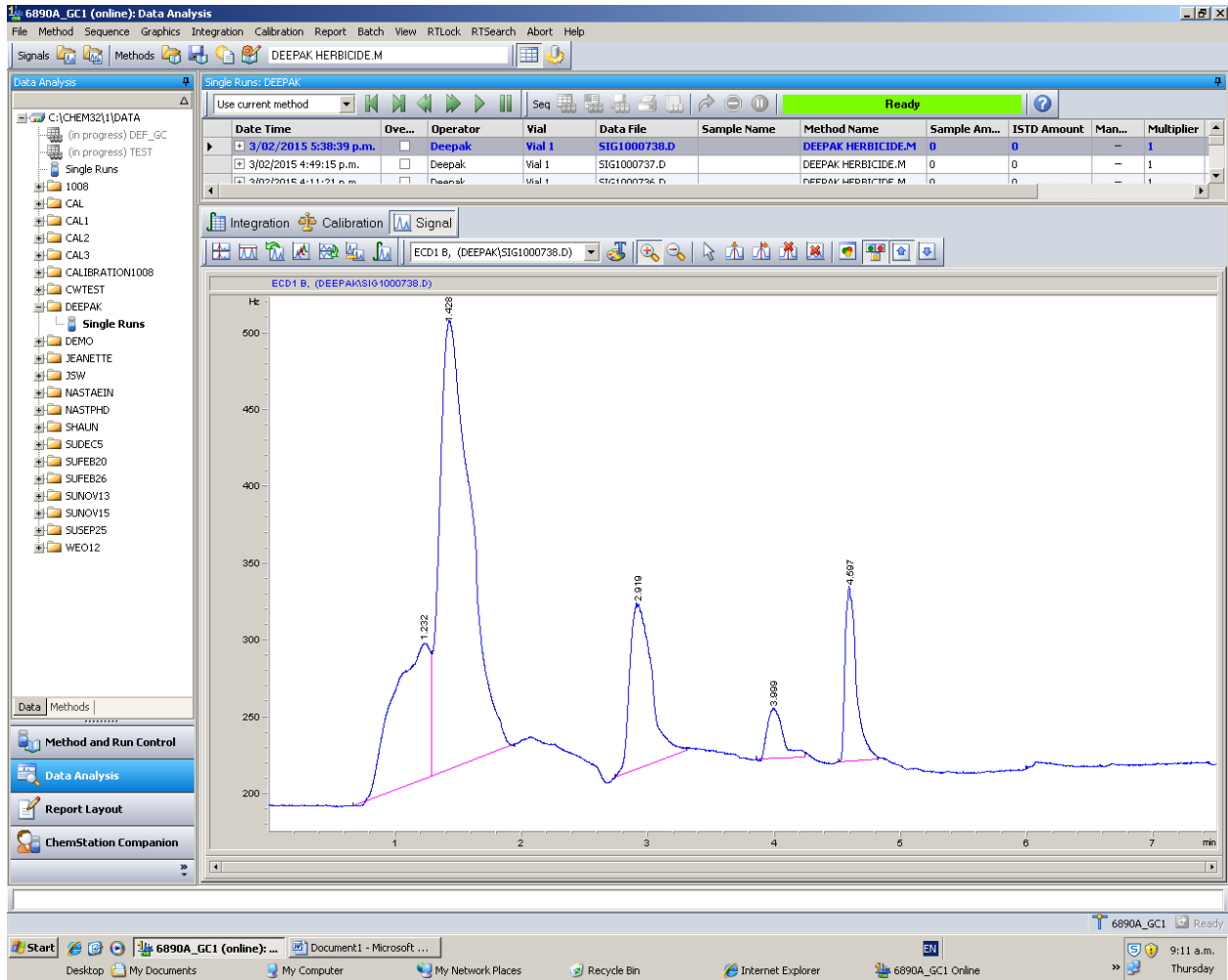
Figure AIII. 2. MCPA in the influent with 50 mg/L of DMCPA at 24h HRT
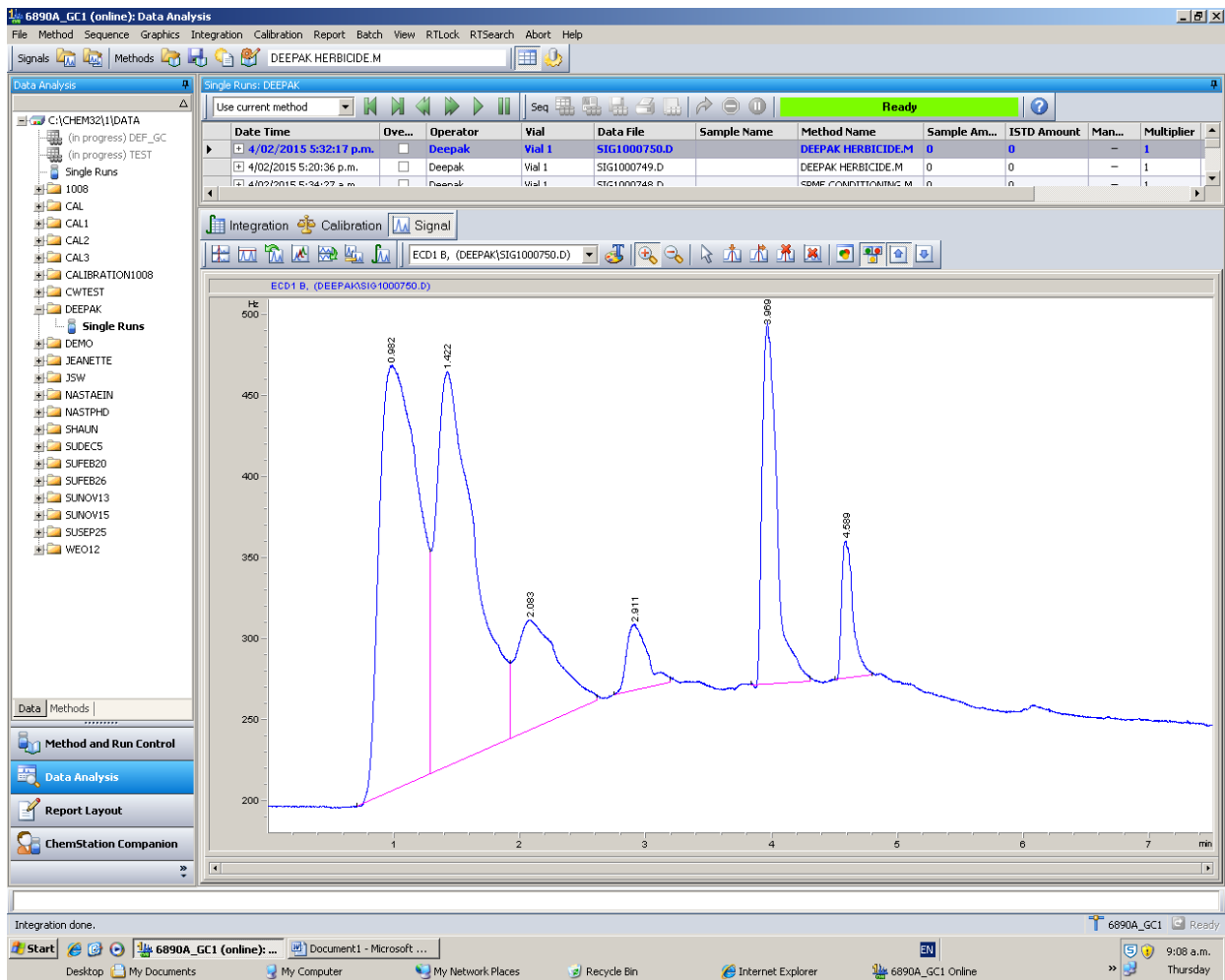
Figure AIII. 3. MCPA during the peak (maximum formation) with 50 mg/L of DMCPA at 24h HRT
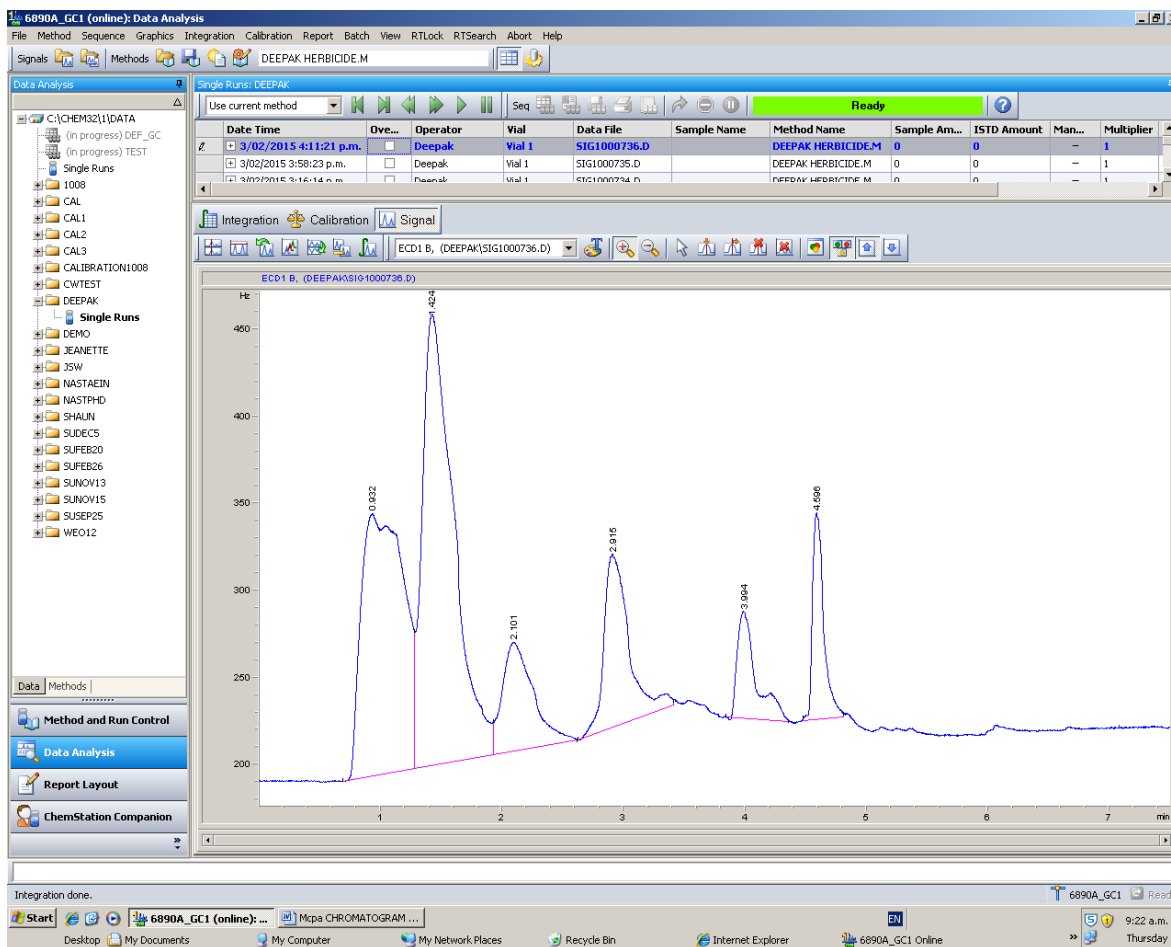
Figure AIII. 4. MCPA in the effluent with 50 mg/L of DMCPA at 24h HRT

Appendix- IV: Chemical structure and fragmentation pattern of the Intermediate products

4-Chloro 2-methyl Phenol

Line#:1 R.Time:5.255(Scan#:952)
MassPeaks:425
RawMode:Averaged 5.235-5.295(948-960) BasePeak:107.05(27479)
BG Mode:None Group 1 - Event 1
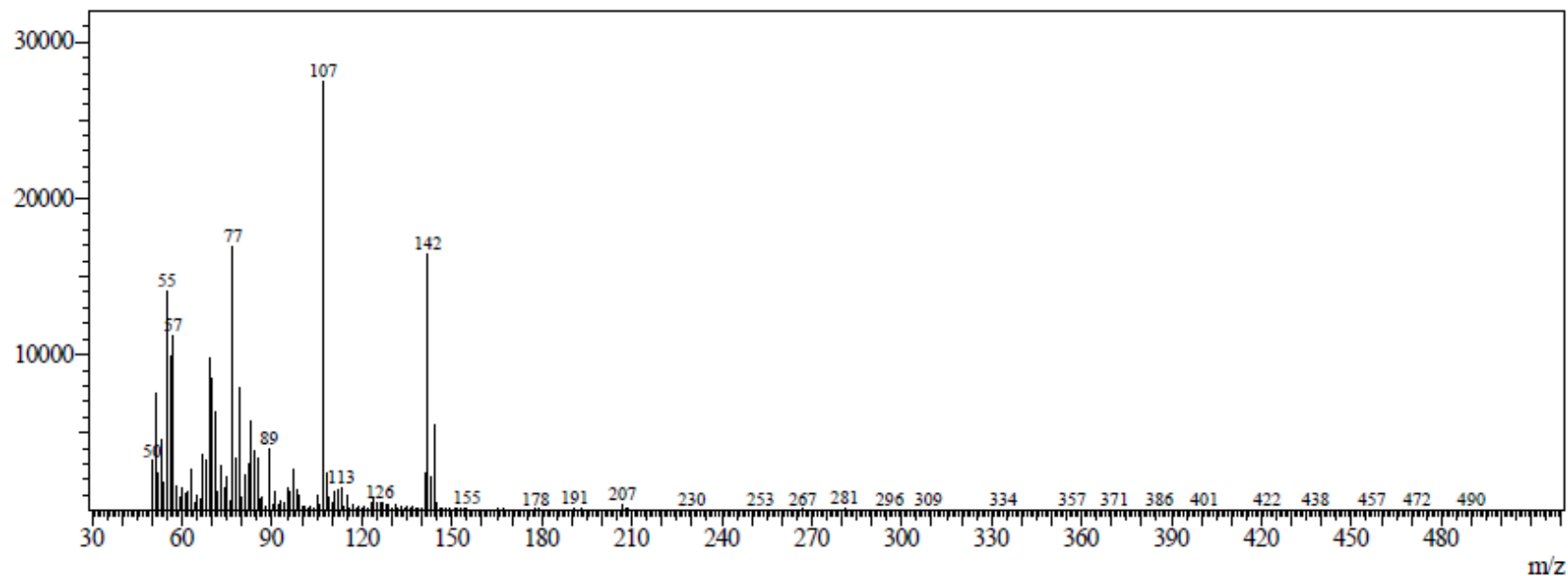
Phenol, 2,4-bis(1,1-dimethylethyl
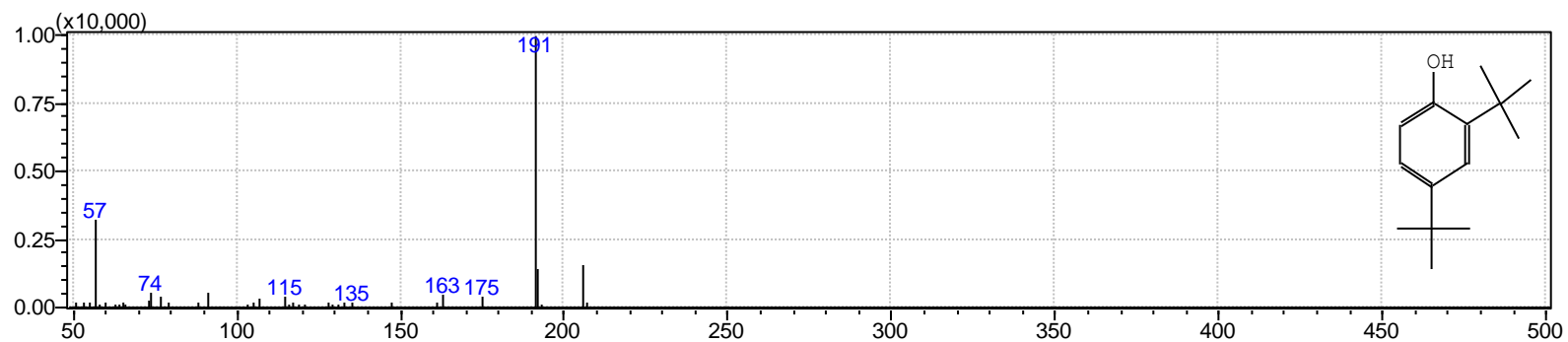
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester
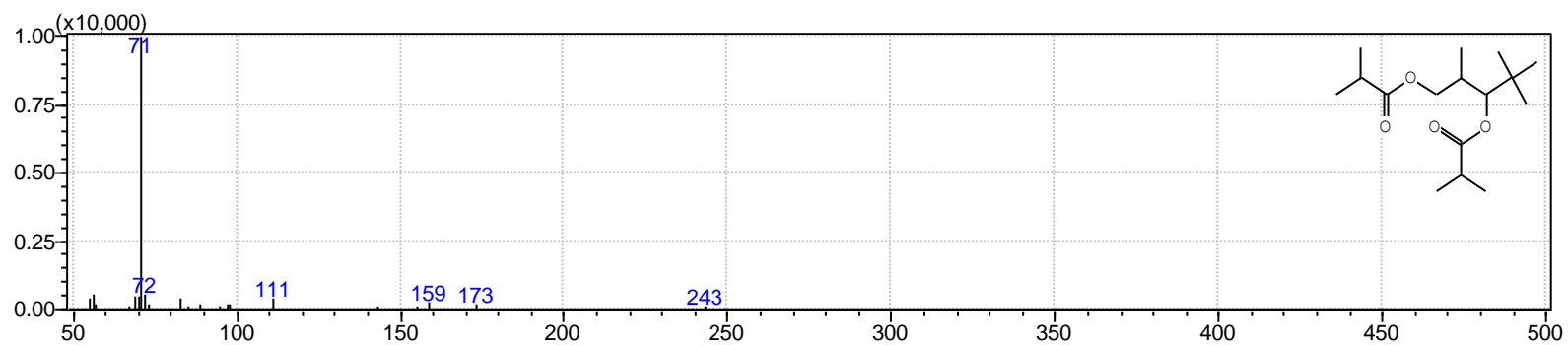
Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester
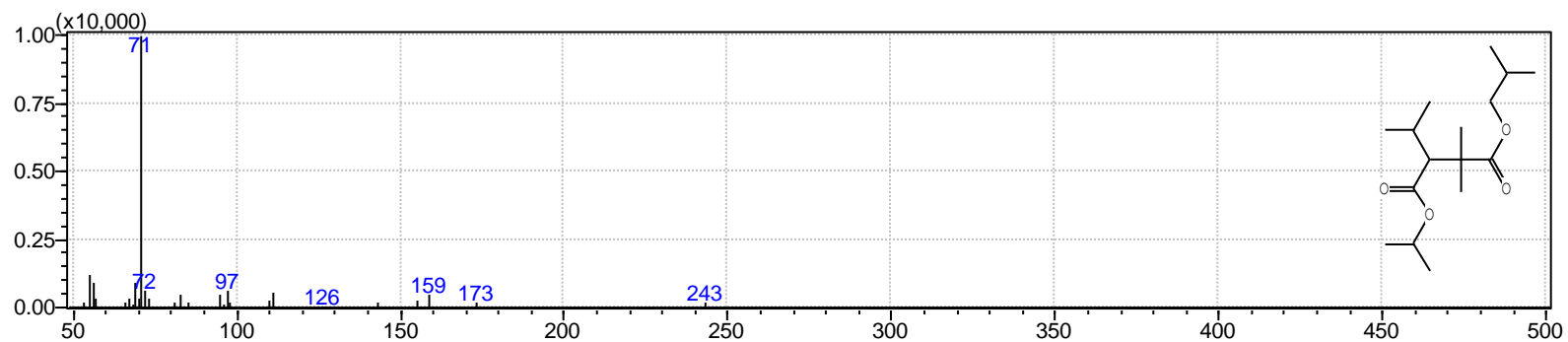
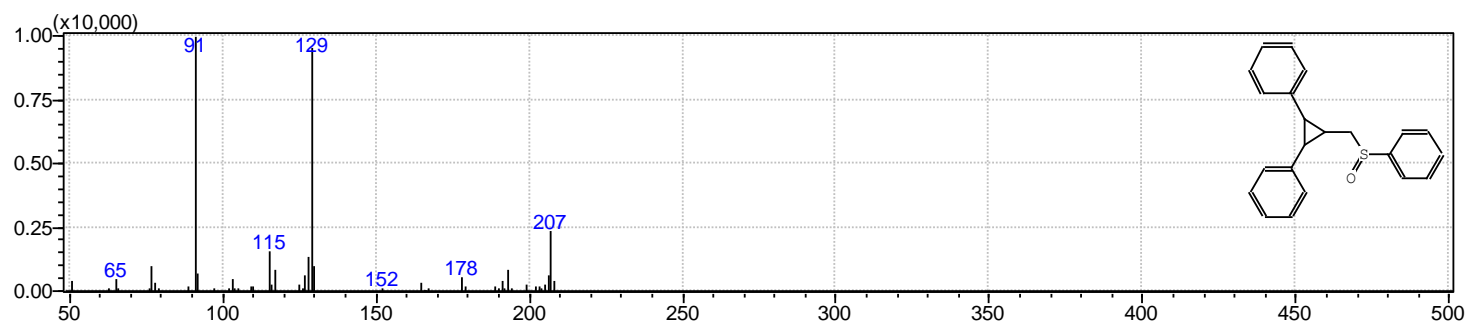Cyclobutane, 1,2-diphenyl- $$ (2-Phenylcyclobutyl)benzene

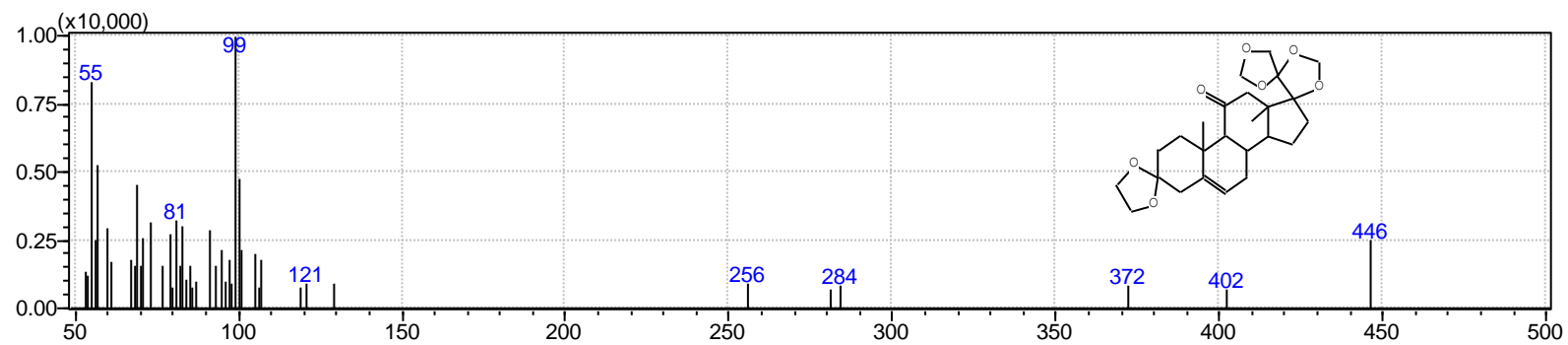Cyclooctane, 1,5-dimethyl- $$ 1,5-Dimethylcyclooctane
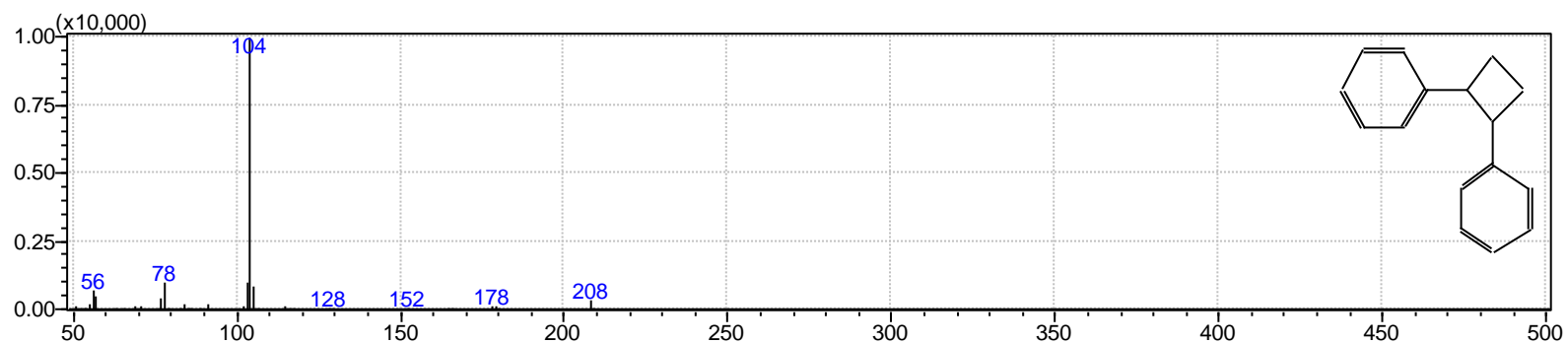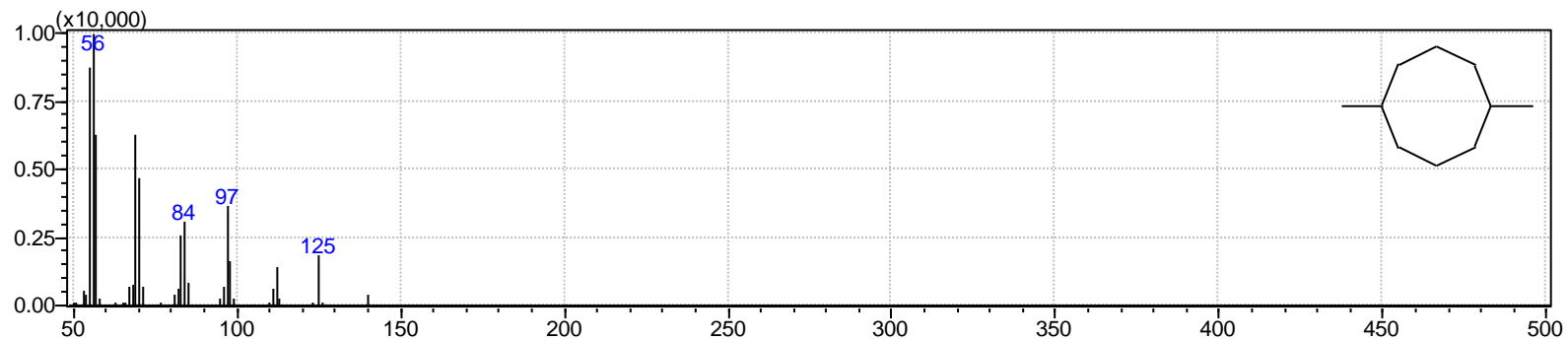
Ethanol, 2-3,3-dimethylcyclohexylidene
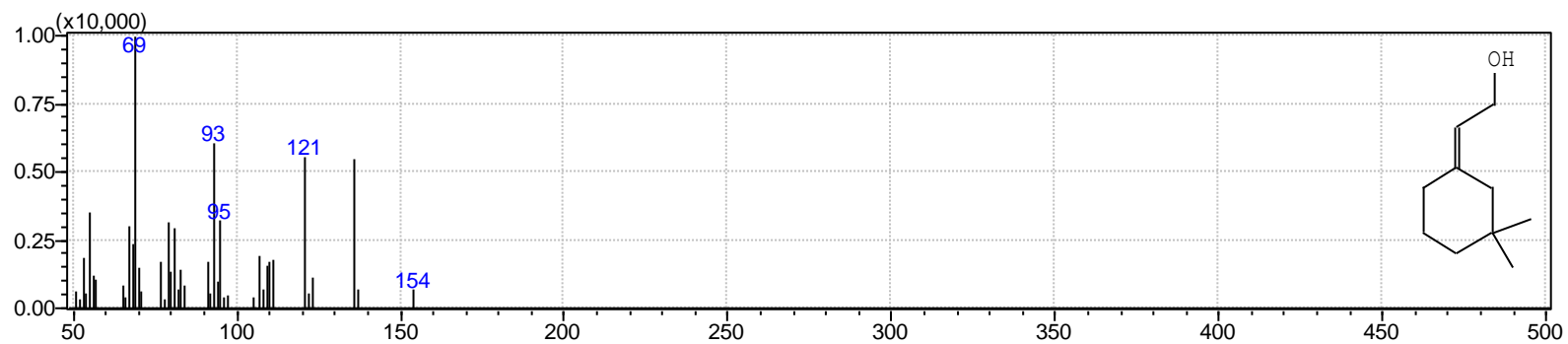
(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans

Pregn-5-ene-3,11-dione, 17,20:20,21-bis[methylenebis(oxy)]-, cyclic 3-(1,2-ethanediyl acetal)

Appendix-V: Lab Photos

Figure AV.1. Experimental lab (left) and Peter and Dave attaching GC instruments (right)
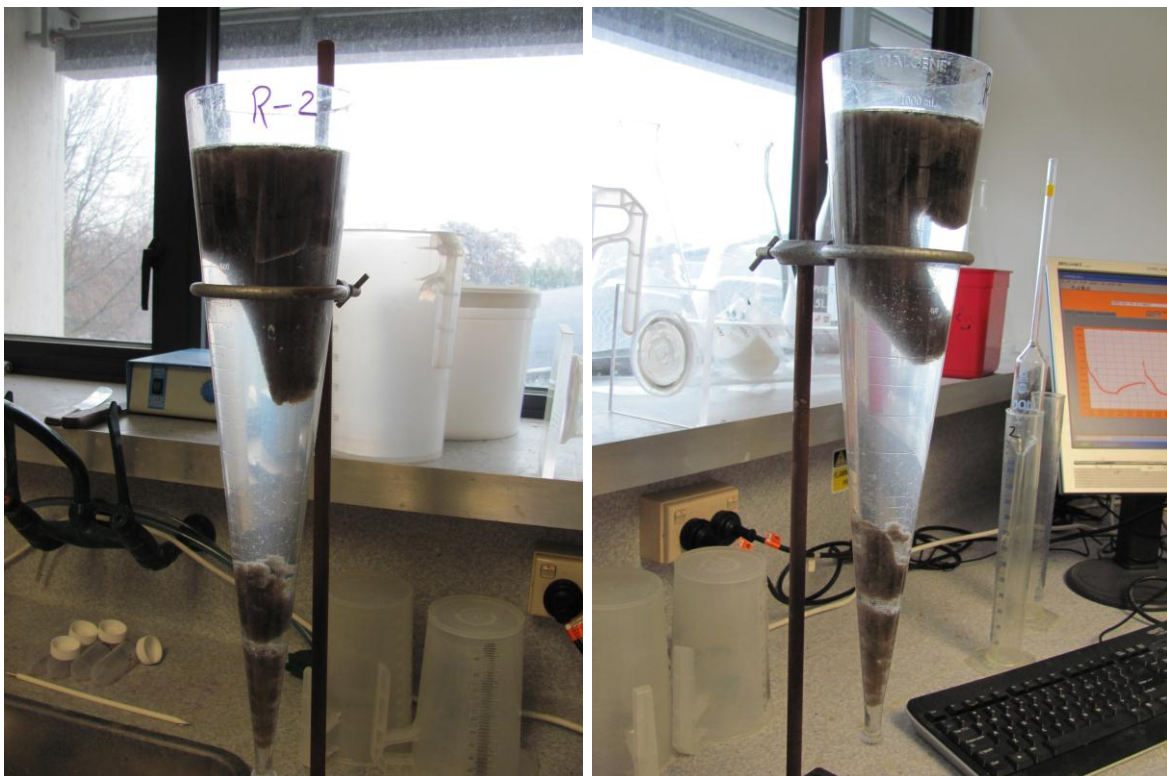

Figure AV.2. Visual inspection of denitrification process in imhoff cone

Figure AV.3. SPME set-up

Figure AV.4. Deepak injecting sample in GC injecting port