BIOLOGICAL TREATMENT OF INDUSTRIAL STRENGTH CLOPYRALID IN WASTEWATERS: BIODEGRADATION & TOXICITY

A thesis submitted in partial fulfilment of the requirements for the
Degree of Doctor of Philosophy
in Civil and Natural Resources Engineering
at the University of Canterbury

by
Amarpreet Kaur Hura

Department of Civil and Natural Resources Engineering
University of Canterbury, New Zealand
July, 2019

ACKNOWLEDGEMENTS

It is a matter of immense pleasure to acknowledge my debt to my respected supervisors Dr. Ricardo Bello Mendoza and Dr. David Wareham Associate Professor, Department of Civil and Natural Resources Engineering (CNRE), University of Canterbury, New Zealand. It is because of your priceless intellectual guidance and innovative and constructive ideas in my experimental and write-up work that I found my PhD tenure fantastic. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. Your advice on both research as well as on my career path have been invaluable. It is indeed my privilege to work under both of you.

Similar, profound gratitude goes to Dr. Hossein Alizadeh, Research Officer at Lincoln University, New Zealand who has been a truly dedicated mentor outside my University. I am particularly indebted to Hoss for his constant faith in my work and for his support by so generously hosting me in the bio-protection lab of Lincoln University. I have very fond memories of my time there.

Special appreciation and thanks to my technical advisors in the Department of CNRE, Mr. Peter McGuigan, Mr. Manjula Premaratne and Mr. Dave MacPherson for their extraordinary support in setting-up my experimental reactors and being always able to solve any problems that might occur during the lab work.

I also hugely appreciate Dr. Marie Squire, Department of Chemistry and Dr. David Leung, Department of Biology, University of Canterbury, New Zealand, for sharing their expertise so willingly i.e. chemistry and microbiology, respectively and for their guidance towards my research work.

I am grateful to the College of Engineering and the department of CNRE (especially to Elizabeth Ackermann) for providing financial and administrative support.

I take this opportunity to thank all my UC friends for their help and moral support including Deepak Chouhan, Fabio Silveira, Ethar Al-Essa, Lei Zhang, Dana Abudayyeh, Ana Sarkis Fernandez, Salina Dhakal, Vineet Shah, Tushar Chowdhary, Helmy Hermawan, Forrest Bilek,

Mayank Srivastava, Mayank Tripathi, Abhirup Basu Roy Chowdhary, Farideh Jamalibehnamand, Vishvendra Bhanu and Ashu Kedia. All the precious moments that I spent with you all will stay in my heart forever.

Deep heartedly, I thank my parents, my husband and my other family members for their encouragement, blessings and motivation at each and every step. They are the most important people in my world and I dedicate this thesis to them.

Last, but not least, I thank God who has guided me as a pole star and gave me strength to overcome this challenge on my way.

Thank you for making this a reality.

Amarpreet Kaur

ABSTRACT

Pesticides are used worldwide to increase crop production and to remove unwanted weeds. In New Zealand, clopyralid (3,6 dichloro-2-pyridinecarboxylic acid) is one of the most widely used pesticides to control broadleaf weeds. There is very little research however reporting the removal of clopyralid in wastewaters by biological means; thus, the purpose of this research was to examine the potential biological degradation of industrial strength clopyralid wastewaters under aerobic conditions.

The study was divided into six phases, namely: Phase I (a Preliminary Phase), Phase II (an Acclimatization Phase), Phase III (a Batch Phase), Phase IV (a Kinetic Modelling Phase), Phase V (an Intermediate Identification Phase) and Phase VI (a Microbial Inhibition Phase). In the first phase, prior to the addition of clopyralid, stability parameters were assessed, these included suspended solids, COD removal, ORP and DO in a sequencing batch reactor operated under aerobic conditions. Once stability in the SBR was achieved, a commercial formulation of clopyralid at 50 mg/L of concentration was introduced. The first sign of degradation in the SBR was identified in the acclimatization phase and the results showed that 98 % of the clopyralid had been removed effectively during 5 days of acclimatization. After that, COD removal of 50 mg/L of clopyralid was measured and it was found that 70 % of the COD was removed. However, when the concentration of clopyralid was increased to 100 mg/L then the removal of clopyralid was 98 % but COD removal dropped to approximately 60 %. Due to this, batch reactors were used in phase III with a pure form of clopyralid since it was suspected that the large COD in the SBR effluent was due to additives in the commercial formulation that are not disclosed by the manufacturer due to proprietary interests.

Batch reactors were run for 24 h with initial clopyralid concentrations from 50 to 300 mg/L. It was observed that the biomass successfully treated the clopyralid up to the 250 mg/L concentration mark with 98 % removal at 100 mg/L. At the highest initial concentration of 300 mg/L, no further degradation was observed. In parallel with clopyralid degradation, COD removal was also observed; however results indicated that increasing concentrations of clopyralid created a potentially toxic effect on the biomass, with bacterial lysis most probably contributing to soluble microbial products which affected the effluent residual COD. It was also predicted that clopyralid

degradation may form different transformation products during the degradation which increased the effluent COD. Consequently, the COD removal efficiency decreased with increasing clopyralid concentration.

Phase IV checked the toxic effect of clopyralid on the biomass. It was identified that clopyralid did not have any inhibitory effect between 50 to 225 mg/L of clopyralid concentration; however, at 250 mg/L the clopyralid utilization rate dropped reaching zero at 300 mg/L, which reflects the fact that clopyralid at higher concentrations is toxic to biomass. For this experimental phase, a mathematical model was also investigated and the Luong Model (1985) proved to be the best fit.

In phase V, inorganic compounds (such as nitrate, nitrite and ammonia) and organic compounds (such as TOC), transformation products and SMPs were evaluated to test their possibility of contributing to the residual COD. It was determined that out of all these compounds, only SMPs were likely contributing to residual COD.

Finally, microbial growth inhibition was studied in the presence of clopyralid by two ways i.e. by measuring the Oxygen Uptake rate (OUR) for all species present in the sludge and microbiologically for the species cultured on media in laboratory environment. The results showed that some of the species were inhibited immediately upon contact with clopyralid as the OUR dropped suddenly to approximately half when clopyralid was introduced. However, some of the species were resistant at higher concentrations of clopyralid according to *in vitro* assays.

Overall, this study concludes that in batch tests (run for 24 hrs) with clopyralid injected having initial concentrations between 50 mg/L and 300 mg/L, the biomass successfully treated the clopyralid (98 % removal at 100 mg/L) up to the 250 mg/L concentration mark. At the highest initial concentration of 300 mg/L, no further degradation was observed. In parallel with clopyralid degradation, COD removal was also observed; however results indicated that increasing concentration of clopyralid created a potential toxic effect on the biomass, with bacterial lysis forming soluble microbial products that contribute to the effluent residual COD. Consequently, the removal efficiency of the COD decreased with increasing clopyralid concentrations.

Table of Contents

List of Figures

List of Tables

CHAPTER-1 – INTRODUCTION

New Zealand's climate conditions and high soil fertility make it favorable for farming with agriculture being the largest sector of New Zealand's tradable economy, contributing to about two-thirds of exported goods (Brazil, 2008). For instance, dairy products contribute around $15.5 billion (Statistics New Zealand, 2014) while horticulture contributes $3.9 billion of New Zealand's total exports (Horticulture New Zealand, 2014). Although it has been reported that New Zealand generates much of its wealth from agricultural activities; unfortunately this comes at a cost to the environment (Hutching, 2006), mainly because pollution arises by the use of excessive agrochemicals and agricultural run-off (Sagasta, et al., 2017). Agrochemicals such as pesticides and fertilizers are used to increase crop productivity and protect plants from deadly diseases. It is understood that through infiltration and percolation, these compounds leach into the groundwater and create water pollution (Agrawal et al., 2010). It should also be noted that groundwater is an important source of drinking water in New Zealand (Ministry of Health, 2017); particularly because approximately half of New Zealand's drinking water supplies originate from below ground (The New Zealand Drinking-Water Process, 2018). National surveys have detected pesticides in drinking water sources; and in the year 2014, pesticides were detected in 28 out of 165 groundwater wells. Among these, 10 wells contained more than two types of pesticides (Close & Humphries, 2016). In total 80 types of pesticides were detected with 75 of them classified as herbicides. Research has also been conducted in regard to the varieties of pesticides used in New Zealand and it was found that among all pesticides (herbicides, fungicides, and insecticides) the most dominant class are herbicides (Manktelow et al., 2005). Furthermore, it turns out that most of the herbicides used in New Zealand are in the form of chlorinated organics (Shahpoury et al., 2013) which are transported to streams either from agriculture soils via runoff or through vapor drift (where the pesticides have not been applied directly).

Chlorinated herbicides are a global concern since they remain for long periods in the environment, are stable within living organisms and are difficult to treat (Mrema et al., 2013). The most common chlorinated herbicide widely used in New Zealand, Australia (Rolando et al., 2015) and the USA is clopyralid (Pohanish, 2014), since it is effective and easily accessible in the market. Clopyralid (3,6-dichloro-2-pyridinecarboxylic acid) is a selective herbicide used to control broadleaf weeds

such as thistles and clovers in lawns and in a variety of crops such as spinach, corn, wheat, barley and oats (Haskell, 2003).

Clopyralid is part of the picolinic acid family of herbicides. It is slightly toxic to humans and causes severe eye and skin irritation (U.S. EPA, 1990a). Some reproductive and developmental effects have also been noted in laboratory animals, as indicated by a change in liver and kidney weights (WSDOT, 2006). Moreover, it is hazardous for some plant species such as peas and beans (Berberidou et al., 2016).

Clopyralid is highly soluble in water with a maximum solubility concentration of around 1000 mg/L (Tu et al., 2001). Because of this, clopyralid is readily transported by storm water and irrigation runoff; thereby, increasing the potential to contaminate water bodies. Cox et al., (1998) advised that 30 days from initial application, clopyralid had been found in 6 ft (2 m) deep soil water samples, thereby confirming the high potential of clopyralid to contaminate ground water (Tu et al., 2001).

Moreover, from an industrial perspective, the main source for pesticide-contaminated water is the various points in the manufacturing process, with some points in the process producing large quantities of wastewater. More specifically, the main sources of wastewater are the process residues, spillages and wash waters from the manufacture/formulation process; as well as unused spray solutions and equipment washings from pesticide applications (Mahmood et al., 2016). The wastewater generated from the pesticide manufacturing industry contains large amounts of pesticide both in the dissolved and/or in the suspended form (Sahu & Chaudhari, 2013) and these concentrated effluents therefore need to be treated by a specific treatment approach.

It would be advantageous to treat clopyralid contaminated wastewater by biological means, since the biological processes are generally less expensive that their chemical counterparts. However, biological treatment of clopyralid can be challenging, since clopyralid is classified as a non-readily biodegradable substance (European Food Safety Report, 2006). It is also stable under hydrolysis and photolysis (Berberidou et al., 2017). Little if any research appears to have been done on the potential for biological degradation of clopyralid in water; thus, this research will seek to add to that body of knowledge by considering a specific treatment option for this important pesticide.

CHAPTER-2 – LITERATURE REVIEW

2.1 Use of Pesticides and Associated Problems

In order to meet the ever increasing needs for food, farmers endeavor to improve their agriculture production. In this situation, pesticides play an important role, since by the use of pesticides, plants can be maintained and protected and the loss in crop yield will be smaller with food production meeting the population demand (Oerke & Dehne, 2004). It has been reported that since 1990 the use of pesticides around the world, has nearly doubled the food crop yield from 42% to 70% (Jozsef & Krisztina, 2011).

It is also noted that world-wide production and consumption of pesticides has increased massively since the 1990s (Rajendran, 2003). Recent research suggested that the average pesticide application rate is around 11 kg/ha around the world (Roser & Ritchie, 2017). It is also reported that among all categories of pesticides, herbicides are the most commonly used (Manktelow et al., 2005). Pretty & Bharucha (2015) suggested that the total use of pesticides can be accounted for by herbicides 42%, insecticides 27%, fungicides 22% and other agrochemicals 9%.

The use of pesticides significantly improves agriculture production; however, their use also pollutes environmental components such as soil, water and air. It has been reported that different characteristics of organochlorine pesticides, such as high lipophilicity, bioaccumulation, long half-life and potential for long-range transport, increase the chances of contaminating environmental media, even after many years of application (Jayaraj et al., 2016). Furthermore, Pimentel (1995) found that only a small percentage (i.e. 0.3%) of applied pesticide actually reached the targeted pest, while the other 99.7% was transferred to the environment.

Non-target plants are affected by pesticides since nitrogen fixation which is required for the growth of plants is hindered. Pesticides also kill bees that pollinate plants. Many kinds of animals are harmed by pesticides and it is expected that all animals may be poisoned somewhat by pesticide residues that remain on food, for example when wild animals enter sprayed fields or nearby areas shortly after spraying (Palmer et al., 2007). For example, every year 67 million birds are killed by the use of pesticides in the U.S.A. (Manea et al., 2017). Pesticide run-off into rivers and streams

is very harmful for aquatic biota. That is, pesticides enter into water bodies and kill fish and plants and when dead matter decay, bacteria consume the water's oxygen (Toughill, 1999).

Pesticides can also cause acute and chronic health effects to humans which vary from simple skin and eye irritation to severe effects such as cancer, reproductive problems and damage to the nervous system (Nicolopoulou-Stamati et al., 2016).

The use of all kinds of pesticides is problematic for non-targeted species, however organochlorine pesticides are the worst (Jayaraj et al., 2016). One example of organochlorine pesticides is clopyralid (Pubchem, 2018). Clopyralid is persistent in soil, water and vegetation and when applied to soil, can rapidly dissociate, becoming very soluble in water and thereby sparingly binding to soil particles. Low soil adsorption means that clopyralid can contaminate surface and ground water via leaching and sub-surface water flows (Cox et al., 1998).

Moreover, clopyralid is also known for its ability to persist in dead plants and compost (Moody & Oliver, 2013). Due to this reason, the use of clopyralid is banned in domestic lawns in the U.S.A. (Haskell, 2003) and in New Zealand (New Zealand Environment Risk Management, 2007).

2.2 Pesticides Dissipation and Degradation Pathway

Pesticides can either be degraded or dispersed in the environment through various different pathways as shown in Figure 2.1. Pesticide occurrence, fate and degradation depend upon the pesticide's properties as well as the environment in which it is applied. Important properties of pesticides are their adsorption capacity, volatility, leachability, abiotic resistance and biotic resistance; while the environment conditions that affect pesticide fate and degradation constitute the moisture content, temperature of soil and presence of sunlight (Buyuksonmez et al., 2000).

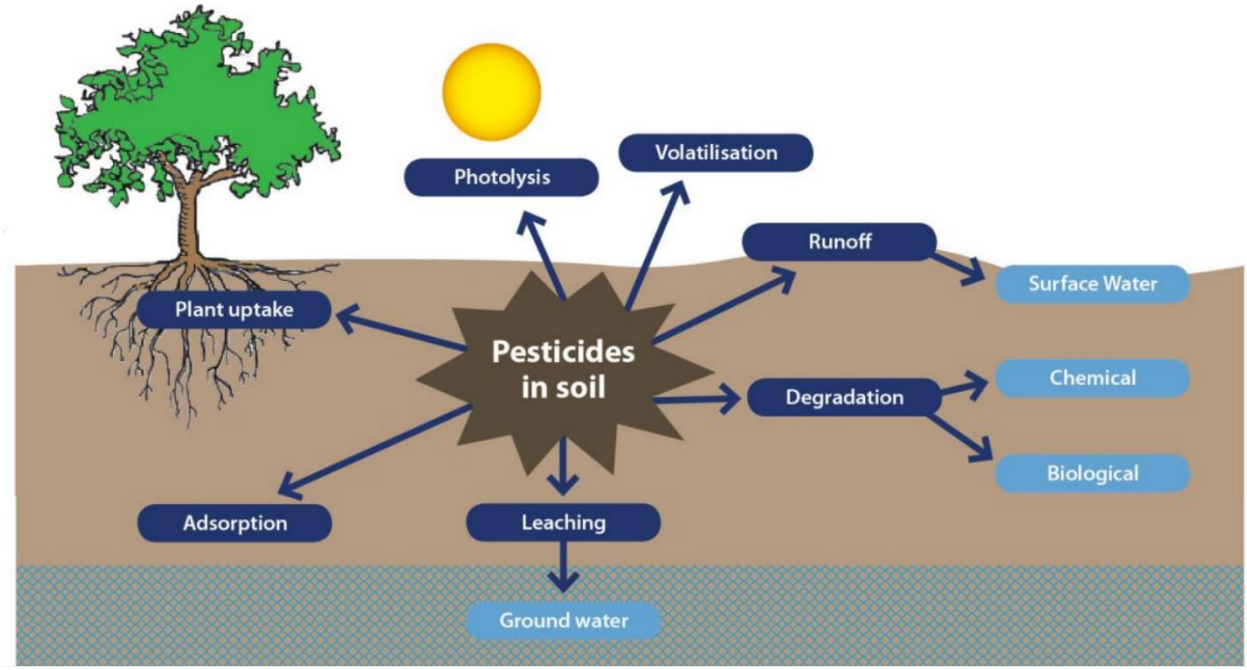


Figure 2. 1. Pesticides fate and transport when applied to soil (derived from the Recycled Organics Unit, 2007b)

It has been observed that clopyralid can degrade faster when soil moisture and temperature are high and application rates are low (Büyüksönmez et al., 2000). It has also been reported that clopyralid in water is resistant to sunlight photolysis ($t_{1/2}$ = 261 days) as well as hydrolysis (stable to direct hydrolysis and classed as very persistent) (Berberidou et al., 2016). Finally, insignificant degradation of clopyralid has been reported in an aquatic environment under aerobic and anaerobic conditions (Dow AgroSciences, 1998).

2.3 Properties of Clopyralid

2.3.1 Physico-chemical properties

Clopyralid is a pyridine carboxylic acid compound with a molecular mass of 192 g/mol. Its empirical formula is $C_6H_3Cl_2NO_2$ and its chemical name is 3,6-dichloro-2-pyridinecarboxylic acid. Clopyralid is available in the market in the form of salts. Figure 2.2 shows the clopyralid structural formula in different salt forms.

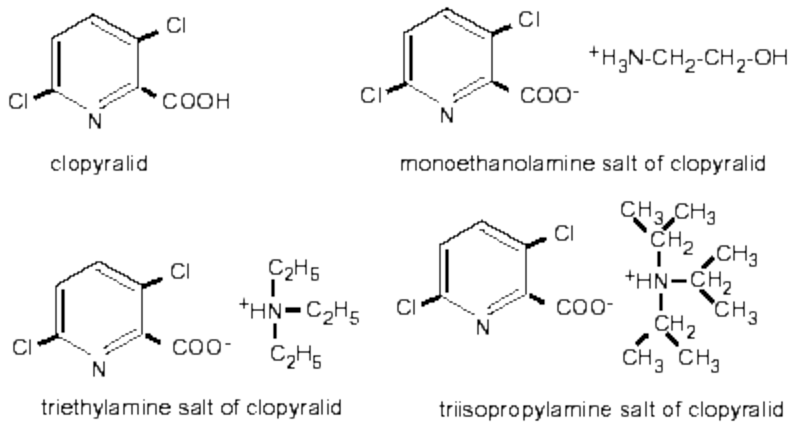


Figure 2. 2. Clopyralid structural formula in salt forms (Dow AgroSciences, 1998).

Clopyralid does not volatilize readily in the field but its volatility increases with temperature and soil moisture and decreases with organic matter content (Helling et al., 1971). It is highly soluble in water with a water solubility of 1000 ppm, and does not bind strongly to soil particles (Cox et al., 1996). The K_{oc} of clopyralid; that is, its organic carbon sorption constant, is estimated as 5 mL/g (i.e. very mobile) and this value decreases with increasing pH (Pik et al., 1977). Due to this, clopyralid has high leachability (Groundwater Ubiquity Score = 5.06) (DowElanco, 1997). Its adsorption potential is also very low but increases with time, which can limit long term leaching.

2.3.2 Toxicity

Clopyralid toxicity has been evaluated by the U.S. EPA (Environmental Protection Agency) and has been categorized as having acute toxicity, sub-chronic toxicity, chronic toxicity and effects on reproduction. It was reported that acute exposure of rats to clopyralid produced severe eye irritation (U.S. EPA, 1990a) and in some cases, permanent impairment of vision and blindness (E.I. du Pont de Numerous and Co., 1998). In addition, clopyralid causes skin irritation and its repeated exposure caused skin allergies in some individual rats (Dow Agrosciences, 1998).

Medium term exposure of mice to clopyralid for three months at high doses resulted in an increase in the size of the animals liver cells as well as increased liver weights (U.S. EPA, 1997a). A similar experiment was conducted on dogs for six months and liver weights were observed to increase in the case of females, while urinary tract problems were detected in males (U.S. EPA, 1997b).

Chronic studies (i.e. long term exposure) on laboratory animals have identified effects on stomach, blood, liver and body weight at low doses (U.S. EPA, 1997b). A two year study on rats found

hyperplasia (increase in the amount of organic tissue that results from cell proliferation) in the stomach lining, while a one year study on dogs found a decrease in the number of red blood corpuscles and an increase in liver weights (U.S. EPA, 1997c). Furthermore, a two year study on mice reported body weight reduction in males (U.S. EPA, 1997a).

Clopyralid has not been classified as a cancer-causing pesticide but exposure of clopyralid can cause substantial reproductive problems (U.S. EPA, 1998). For instance, a laboratory test on rabbits showed a decrease in the weight of fetus at low and high doses and an increase in skeletal abnormalities in fetuses (U.S. EPA. Office of Pesticides and Toxic substances, 1991).

2.3.3 Mode of action

Clopyralid is a synthetic plant growth hormone with physical and chemical properties similar to those of auxin, a natural plant growth hormone. This type of pesticide kills the target plant by binding to the receptors sites (i.e. indole acetic acid) for the plant growth hormone auxin. It is suggested that clopyralid enters into the plant through roots and leaves and is translocated through the plants over the xylem and phloem tissues, effectually impacting all parts of the plant (Dow Agrosciences, 1998). At higher concentrations, clopyralid inhibits cell division and growth of certain plants, which ultimately cause the death of the plant (Tu et al., 2001). An example of clopyralid-induced damage to a tomato plant is shown in Figure 2.3.

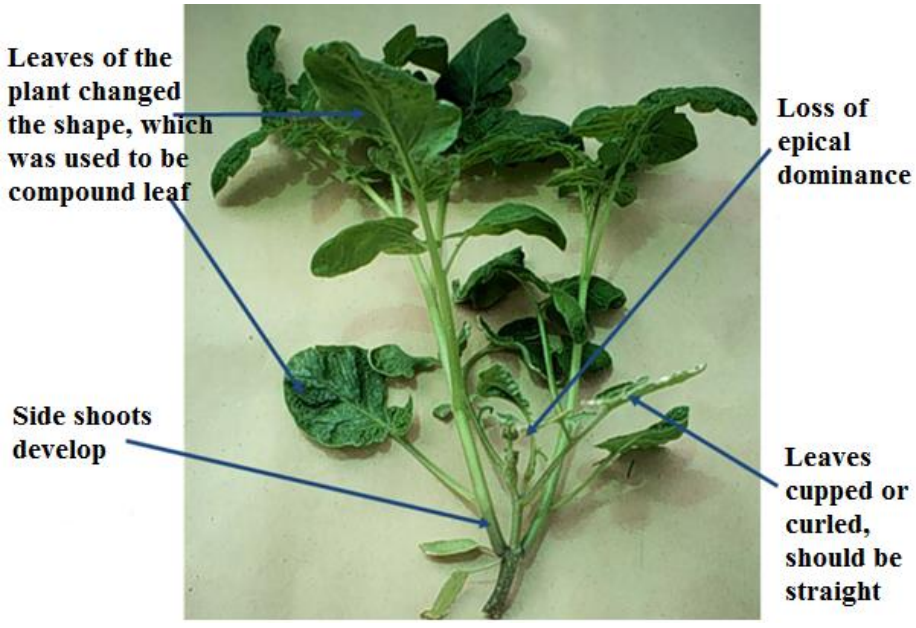


Figure 2. 3. Clopyralid-induced damage to a tomato plant (Fauci et al., 2002)

2.4 Biological Treatment of Pesticides in Wastewater

2.4.1 Biological wastewater treatment

In general, water quality is usually affected by chemicals such as heavy metals, solvents, dyes and pesticides, which enter into the aquatic medium in several different ways, either dumped directly by industries or from wastewater treatment plants (WWTP). Chemicals may also enter into the water indirectly through the use of plant health products, such as biocides and fertilizers in agriculture (Oller et al., 2011). The two main routes for eliminating chemical compounds in waters are chemical and biological oxidation. In chemical oxidation processes, reaction mechanisms change both the structure and the chemical properties of the organic substances. For example, organic compounds are oxidized by ozone or hydroxyl radicals (•OH) which break the molecules into smaller fragments in the form of oxygen and carbon dioxide (Akmehmet Balcıoğlu & Ötker, 2003). However, chemical oxidation for complete mineralization is generally expensive because the oxidation intermediates formed during treatment tend to be resistant to complete chemical degradation. Furthermore, all methods consume energy (radiation, electricity, etc.) as well as chemical reagents (catalysts and oxidizers) which increase (in terms of use) with treatment time (Muñoz & Guieysse, 2006).

Biological oxidation, on the other hand is cost effective, simple to operate and also eco-friendly (Valdez & Maradona, 2013). In general, biological oxidation refers to the elimination of the pollutant by the metabolic activity of living organisms such as microorganisms and in particular autotrophic and heterotrophic bacteria, protozoa and fungi (Madoni at al., 2000), that live in natural water and soil environments (Cox et al., 2013). When the wastewater comes in contact with these microbes, they utilize the organic waste present in the water for their catabolic activity in order to grow, while the organic waste is converted into simple molecules, such as carbon dioxide and methane. The main purpose of biological treatment is the reduction of organic matter via different oxygen tensions. Accordingly, biological treatment is further divided into three processes i.e. anaerobic, anoxic, and aerobic wastewater treatment.

Anaerobic treatment processes are characterized by the absence of free oxygen and are typically used for the treatment of waste that have high concentrations of biodegradable organic material. Such processes convert the organic material by anaerobic microorganisms to gas, containing

methane and carbon dioxide, known as "biogas". In the case of anaerobic treatment, both the electron acceptor and electron donor are the organic matter itself (Lettinga, 1995).

In contrast, anoxic wastewater treatment uses nitrate as an electron acceptor while the electron donor is still the organic matter. An anoxic process is therefore typically used for the removal of nitrogen from wastewater, through a process known as denitrification. Denitrification requires that organic nitrogen be first converted to nitrate, which typically occurs in an aerobic treatment process. The nitrified water is then exposed to an environment without free oxygen and in the end, nitrogen is released in the form of nitrogen gas or nitrogen oxides (Sarfaraz et al., 2004).

Finally, aerobic wastewater treatment is a biological process that takes place in the presence of oxygen and therefore encourages the growth of naturally-occurring aerobic microorganisms, which will assist in the restoration of wastewater. This in turn creates more bacteria for the biodegradation of organic material in wastewater. In the case of aerobic treatment, bacteria consume oxygen as an electron acceptor in order to convert organic compounds into carbon dioxide (Marsolek et al., 2007).

2.4.2 Microbial inhibition in wastewater treatment systems

It has been noted that the structure of the microbial community plays an important role in the degradation pattern of recalcitrant compounds including pesticides (Shchegolkova et al., 2016). Although some microbes die immediately when coming into contact with particular compounds; others have their growth inhibited. Therefore, it is important to identify both the taxonomy (as well as the diversity of that taxonomy) within the microbial community in addition to observing any inhibitory effects from the presence of pesticide.

Shah (2016) has studied the microbial structure of activated sludge in anaerobic-anoxic-aerobic reactors using denaturing gradient gel electrophoresis (DGGE). The communities were evaluated by using two different systems with the polymerase chain reaction followed by denaturing gradient gel electrophoresis using amplified gene fragments, 16S rRNA of bacteria. This technique is capable of directly characterizing the bacterial population in many kinds of wastewater samples, mostly for cultured bacteria. The results under DGGE sequence demonstrate the diversity of microorganisms in activated sludge. For example; 17 bands for Proteobacteria, 6 bands for

Bacteroidetes, 2 bands for Actinobacteria, 1 band for phylum TM7 and 1 band for Acidobacteria have been found.

These microbes are inhibited in the presence of toxic or recalcitrant compounds and inhibitory effects can be usually identified by two simple techniques such as by measuring the oxygen uptake rates as well by measuring the degradation capability of activated sludge (Tobajas et al., 2016). Rott et al., (2017) investigated the bacterial inhibition in activated sludge by a wastewater containing pesticide collected from pesticide processing plant. One of these technique used was the respiration test (Rott et al., 2017) and they observed that even a small amount of pesticide wastewater cause an inhibitory effect (especially on nitrifying bacteria) eventually reducing the purification capacity of the wastewater treatment plant. It was also noticed that in comparison to autotrophic nitrificants, heterotrophic bacteria were more resistant to the pesticide wastewater.

Microbial inhibition was also studied for different kinds of pesticides including 2, 4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chloro-phenoxyacetic acid (MCPA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Allister et al., 1991). Microbial inhibition was observed for the pesticides 2,4,5,-T, MCPA and 2,4-D, at 2 g/L, 930 mg/L and 500 mg/L, respectively as the overall degradation capability of activated sludge was reduced.

2.4.3 Formation of soluble microbial products

It is very common in the biodegradation of some recalcitrant compounds that transformation or intermediate products are formed (Grady et al., 2011). Substrate metabolism and biomass decay in biological wastewater treatment plants also release various colloidal and soluble organic compounds in the effluent. These compounds are categorized as soluble microbial products (SMPs). SMPs are the main fragment of contaminants in the effluent (Kornboonraks and Lee, 2009) and it is very important to identify these compounds, if you wish to remove them from the effluent. Previous studies indicate that among all post-treatment processes of water, activated granular carbon is the most useful technique to treat SMPs; however, research to better understand the complexity of SMP products is still in progress (Azami et al., 2012).

SMPs are usually composed by carbohydrates, amino acids, antibiotics, humic acid, proteins, polyethylene glycol, polysaccharides and extracellular enzymes etc. depending on the complexity of the effluent composition (Azami et al., 2012). Consequently, it is very difficult to identify all

the SMPs in the effluent by one analytical method (Le-Clech et al., 2006). However, UV-Vis spectrophotometry was used by Kunacheva and Stuckey in 2014 to identify several SMPs, such as total protein at 595 nm, total carbohydrates at 490 nm and polyethylene glycol at 280 nm.

The presence of SMPs can be detected by calculating the COD in the effluent (Kunacheva and Stuckey, 2014). That is, higher effluent COD values in comparison to the influent COD indicates the occurrence of SMP products. In addition, a physical difference in settling of influent and effluent can also reflect the presence of SMPs; that is, settling properties of the biomass have been found to deteriorate with SMP formation (Azami et al., 2011).

2.4.4 Kinetics modelling associated with biodegradation

Kinetic studies of the biodegradation of toxic or recalcitrant compounds in wastewater treatment systems are considered to be an important factor in an engineering system. As such, different mathematical models tend to be tested to see the pattern of biological degradation. In general, kinetics can be described by a cell growth or substrate (toxic or recalcitrant compounds) utilization model.

Various types of mathematical models have been used to identify the inhibitory effect on cell growth in the presence of recalcitrant compounds as well as on the substrate in their own transformations. Most of the model equations have been extracted from models of substrate inhibition of enzymatic reactions and involve a common substrate inhibition term (K_i) (Tziotzios et al., 2008).

Tazdait et al. (2013) studied different kinetic models for pesticide inhibition i.e. malathion in an aerobic batch reactor and compared these models in a curve fitting exercise. The model equations used were the Andrews's equation (1968), the Webb equation (1963), the Teisser equation (Edwards, 1970), the Yano and Koga equation (1969), the Aiba-Edwards equation (1968) and the Loung equation (1987); these are defined as follows:

The Andrews equation (Edwards, 1970) is the simple and most commonly used equation modelling the growth inhibition kinetics of microorganisms it is described as follows:

$$\mu = \mu_{max} \frac{S}{(K_s + S + (\frac{S^2}{K_i})} \qquad (2.1)$$

Where,

μ= specific growth rate (day^{-1}),

μ_{max} = maximum specific growth rate (day^{-1}),

S = substrate concentration (mg/L),

K_s = half saturation constant (mg/L), and

K_i = inhibition constant (mg/L)

The Andrews (1968) equation describes substrate utilization as follows:

$$r_s = r_{s\,max} \frac{S}{(K_s + S + (\frac{S^2}{K_i})} \qquad (2.2)$$

Where,

r_s = specific substrate consumption rate (day^{-1})

$r_{s\,max}$ = maximum specific substrate consumption rate (day^{-1})

The Webb equation described in equation (2.3) is the modified version of the Andrews equation and is derived from enzyme kinetics integrating an allosteric effect with β being dimensionless. The equation is applicable for allosteric substrate inhibition.

$$r_s = r_{s\,max}\, S \frac{1+(\beta + \frac{S}{K_i})}{(K_s + S + (\frac{S^2}{K_i})} \qquad (2.3)$$

Teissier (1970) suggested an equation to describe the stimulation of bacterial growth by substrate concentration and use to predict substrate inhibition at higher substrate concentration.

$$r_s = r_{s\,max}\, (\exp(\frac{-S}{K_i}) - \exp(\frac{-S}{K_s})) \qquad (2.4)$$

While, Yano and Koga (1969) proposed a model on the basis of growth inhibition at higher concentrations of rate-limiting substrate described as:

$$r_s = r_{s\,max} \frac{S}{(K_s + S + \left(\frac{S^2}{K_i}\right) + \left(\frac{S^3}{K_i\, x\, K}\right))} \qquad (2.5)$$

Where,

K = positive constant (mg/L)

Aiba-Edwards (1968) described an empirical correlation for substrate inhibition s shown in equation (2.6). Nevertheless, simulated data with substrate inhibition settles well with experimental data.

$$r_s = r_{s\,max}\, S \frac{\exp(\frac{-S}{K_i})}{(K_s + S)} \qquad (2.6)$$

While Luong (1987) described substrate inhibition to microorganism growth as follows:

$$r_s = r_{s\,max} \frac{S}{S+K_s} \left[1 - \left(\frac{S}{S_m}\right)\right]^m \qquad (2.7)$$

In addition, in 1985 Luong proposed another equation on substrate inhibition, when the substrate consumption rate is stimulated at low concentrations and inhibited at high concentrations. This is mentioned in Mulchandani et al. (1989) research and described in equation (2.8)

$$r_s = r_{s\,max} \frac{S}{S+K_s} \left[1 - \left(\frac{S}{S_m}\right)^n\right] \qquad (2.8)$$

Where,

S_m= substrate concentration above which net growth ceases (mg/L)

n = dimensionless fitting parameter with no physical significance

It is considered that in all these models substrates act as inhibitors at higher concentrations and activators at lower concentrations. However, the best way to compare the models is the coefficient of determination (R^2), as if the R^2 for a model score near to 1 implies that the particular kinetic model being the best fit (Tazdait et al., 2013).

2.4.5 Clopyralid in the environment and its biodegradation

It is reported that clopyralid is moderately persistent in soils and can be degraded completely by soil microbes at certain soil conditions (a function of soil temperature and moisture) (DowElanco, 1997). For example, clopyralid dissipates in comparatively short time periods in warm and moist soil while it can persist for several years in cold, dry and waterlogged soils (Pik et al., 1977). Due to this, the half-life of clopyralid in soil can vary anywhere between one week to one year, depending upon the soil conditions (Bovey & Richardson, 1991).

Research has been done on clopyralid dissipation in allophanic soil, which has both high moisture and temperature (Ahmad et al., 2003). Clopyralid was applied to sterilized and non-sterilized soil under laboratory and field conditions. The herbicide in the soil was maintained under laboratory conditions at 60% soil moisture content and incubated at 10^{o}C, 20^{o}C and 30^{o}C. Although, clopyralid was sprayed directly on to the field soil, it was assessed under different soil management regimes such as shaded pasture, pasture open to direct sunlight, shaded bare ground and bare ground open to direct sunlight. Results indicated that herbicide degradation was high for the sterilized soil (in the laboratory at 30^{o}C) and also high in the field for unshaded bare ground, as well as unshaded pasture. In contrast, at 20 ^{o}C laboratory experiments showed that the dissipation rate of clopyralid was faster with non-sterilized soil (a half-time of 7.3 days) than in sterilized soil (half-time of 57.8 days) (Ahmad et al., 2003). The study suggested that the rate of dissipation was directly proportional to the temperature and moisture content of the soil while inversely proportional to the organic matter concentration in the soil (Tomco Patrick L. et al., 2016).

Smith and Aubin (1989) also investigated the degradation of clopyralid in three soils i.e. clay, clay loam and sandy loam at different temperatures. Results showed that degradation increased with increasing temperature from 10^{o}C to 30^{o}C and the half-life can vary anywhere between 10 to 47 days. The half-life was the same for clay and sandy loam but higher for clay loam because of the high moisture content (as compared to the sandy loam and clay).

It has also been documented that clopyralid is very persistent in compost and manures (Solid waste operations, 2003). Clopyralid levels in compost as low as 10 ppb is harmful to plants such as beans, peas, potatoes, tomatoes, sunflowers, clovers, lettuce, peppers and lentils (Michel & Doohan, 2003). Degradation of clopyralid in compost has been studied and it is suggested that clopyralid dissipation in compost is highly variable (Lubytė et al., 2007). Firstly, the degradation is biphasic i.e. in two separate phases (one phase includes the adsorption of clopyralid by the organic matter present in the compost and the second phase includes the microbial degradation of adsorbed material as organic matter decomposes) (Vandervoort et al., 1997). Secondly, the rate of degradation is faster at high temperatures and lower with high initial concentrations (Brinton & Blewett, 2004).

Studies suggested that clopyralid's half-life in compost is in the range of 10 and 30 days for different composting feedstocks. For example; 30 days for leaf-garden waste (Brinton and Blewett,

2004) and 10 days for grass for clopyralid concentrations 0.01 mg/kg and 0.9 mg/kg (Miltner et al., 2003), respectively.

Finally, in water, clopyralid is highly soluble and does not bind with suspended particles in the water column. It is estimated that around 0.01% of clopyralid applied on agriculture land was leached into a stream in the period of rainfall which appeared after three days of application (Leitch & Fagg, 1985). In water, clopyralid can be degraded by microbial activities of aquatic sediments; however, as clopyralid does not bind with sediments readily, it tends to persist in aquatic medium and its half-life in water can vary in the range of 8 to 40 days (DowElanco, 1997).

2.4.6 Abiotic losses of clopyralid

The abiotic loss is the loss of contaminants or compounds from the environment without any involvement of biological activities. It should be noted that toxic compounds such as clopyralid can be removed from the environment through other natural processes such as volatilization or bio-adsorption. Volatilization plays an important role in the loss of contaminants through soil and water into the air. The physical process of volatilization can be described mathematically by Henry's law of volatilization i.e.

$$H = \frac{V_t}{S_t} \qquad (2.9)$$

Where:

H is Henry's law constant (P-m^3/mol),

V is vapour pressure of chemical at temperature t (Pascal), and

S is solubility of the chemical in water at temperature t (mol/m^3)

The value of Henry's law constant defines the tendency of a chemical to volatilize and if the value of Henry's law constant is > 10.14 P-m^3/mol, then the compound is highly volatile (United States Environmental Protection Agency, 1996). The reported value of Henry's law constant for clopyralid is 1.8 x 10^{-11} P-m^3/mol (Pesticide Properties Database-clopyralid, 2018), which makes clopyralid a non-volatile compound. Hence, any removal of clopyralid occurring through volatilization would be expected to be negligible.

2.4.7 Transformation products of clopyralid

Research on clopyralid degradation by advanced oxidation processes (AOPs) has suggested the formation of various transformation products. For example; photocatalytic degradation of clopyralid in wastewater formed various intermediates such as ammonium, nitrate and different carboxylic acids (3-chloro-6-hydroxypicolinic acid, 6-chloro-3-hydroxypicolinic acid, short-chain carboxylic acids and 3,6-tetrachloro-2,4-bipyridine-2-carboxylic acid). These intermediate compounds were determined using liquid chromatography mass spectrometry (LC-MS) (Berberidou et al., 2016).

Furthermore, the same intermediate compounds were detected using LC-MS during clopyralid degradation by solar photocatalytic as well as electron-Fenton processes (Berberidou et al., 2016). It is also reported that these intermediates degrade to simple molecules and mineralization can be described according to the reaction:

$$C_6H_3O_2NCl_2 + 13/2\ O_2 \rightarrow 6CO_2 + HNO_3 + 2HCl \qquad (2.10)$$

However, it has been suggested that these transformation products degraded faster when these AOPs are combined with constructed wetlands (Berberidou et al., 2017). The reduction in concentration of these intermediates is due to the presence of plants in wetlands; that is, the micro-environment around the root area of these plants supports the growth of specific microorganisms which decompose xenobiotic organic matter and consumes carbon and nitrogen as the source for their growth (Lv et al., 2016; Runes et al., 2003).

2.4.8 Toxic effect of clopyralid on microorganisms

The acute toxicity of untreated clopyralid solutions, as well as transformation products, was evaluated by Berberidou et al. (2016) by using a bioluminescence eco-toxicity test. The test organism was the marine bacteria *Vibrio fischeri*. Samples were collected and luminescence was recorded after incubation at different time intervals (i.e. 5, 15 and 30 minutes). The inhibition percentage of bioluminescence was calculated using a microtox calculation software. In the case of clopyralid, it was found that 40 mg/L was highly toxic to *V. fischeri* marine bacteria. The research reported that after 15 minutes of incubation, clopyralid induced 83 % inhibition; however,

multiple transformation products formed during clopyralid degradation by AOPs, created 100 % inhibition, when they attained almost their maximum concentrations.

2.5 Use of SBR Techniques to Treat Chlorinated Pesticides

2.5.1 Sequencing batch reactor

The sequencing batch reactor (SBR) is a type of biological process used to treat wastewater that is characterized by operating in batches of fill, react and decant. The most important difference between SBRs and continuous activated sludge processes is that in the former reacting and settling take place in the same reactor while a separate clarifier is required in the case of the conventional activated sludge process.

The Sequencing Batch Reactor has five phases carried out in the following series:

1. Fill: Filling of influent in the reactor.
2. React/Mix: The reaction in the tank while mixing i.e. biomass in the reactor consumes the substrate.
3. Settle: When mixing is stopped, settling of the biomass occurs at the bottom of the reactor resulting in clarified supernatant.
4. Decant: Removal of clarified supernatant from the tank to separate the sediment.
5. Idle: This is the time between decant and fill. It is basically used to adjust the time between multi-tank systems and this is not a necessary phase for all SBRs. Also, in this phase, sludge comes out from the bottom of the reactor along with water, a process called wasting.

The main advantage of using SBRs is their operational flexibility; that is, they can be operated under aerobic, anaerobic as well as anoxic conditions as shown in Figure 2.4. Moreover, there is potential capital cost savings, since SBRs do not use clarifiers (Poltak, 2005).

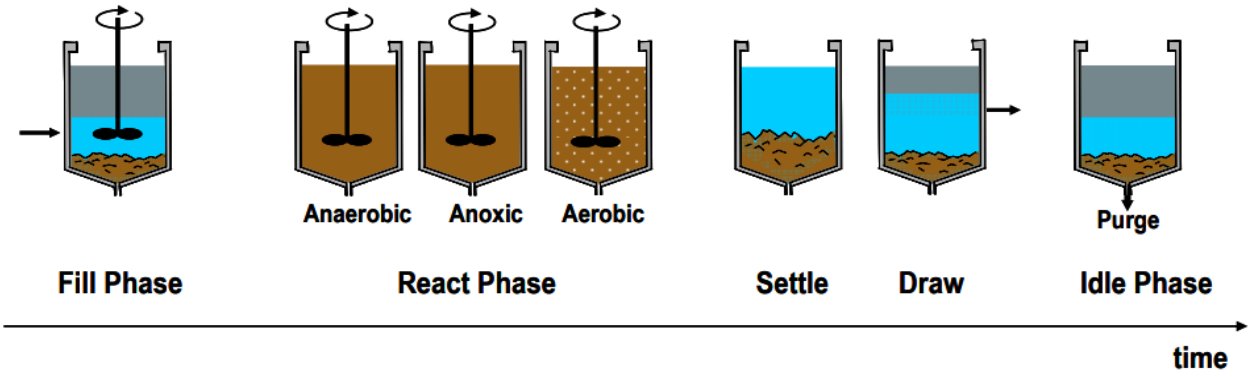


Figure 2. 4. Typical sequence phase of SBRs

2.5.2 On-line monitoring during the SBR process

Physical and chemical changes occur during the biological removal of organic matter and nutrients in SBRs and these can be followed on by online monitoring of dissolved oxygen (DO) and oxidation reduction potential (ORP). Variations in the values of ORP and DO can provide the necessary information for process state evaluation and control (Spagni et al., 2007).

Oxidation-reduction potential monitors the capacity of chemical species to release or accept electrons during the microbial reaction in the reactor (Yu & Bishop, 2001). In SBR, ORP values vary in the range between – 250 to + 250 millivolts (mV) (Gao et al., 2003). A solution with higher value (i.e. positive value) of ORP reflects a potential to oxidize organic matter and vice versa.

Oxidation takes place when electrons release and a free radical accepts an electron from a cell or chemical species i.e. the cell or chemical species is oxidized and a free radical is reduced. Reduction takes place when acceptance of electrons occur as shown in Figure 2.5 (Ebbing et al., 2009).

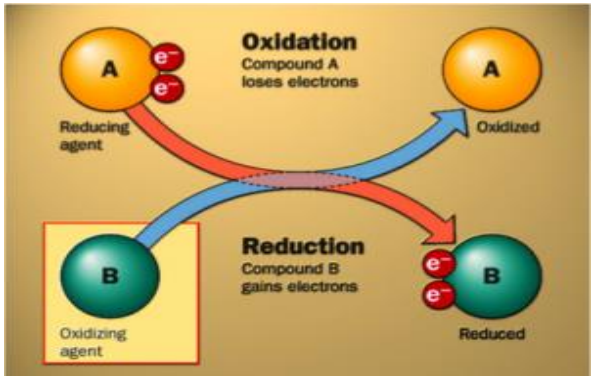


Figure 2. 5. Mechanism of Oxidation-Reduction Reaction (Ebbing et al., 2009)).

Dissolved oxygen reflects the gaseous oxygen dissolved in water. Oxygen enters into the water directly by the absorption from atmosphere while water temperature can affect the DO level (high temperature and low DO level). It is reported that good COD and nitrogen removal in aerobic processes happen only when DO range varies between 1.5 mg/L to 5.5 mg/L in SBRs (Cao et al., 2017). Hence, tracking the value of DO during the aerobic SBR process is very important in the monitoring of the reactor's performance.

2.5.3 Use of biological treatment and batch reactors for the treatment of chlorinated herbicides

All types of biological processes have been used to treat chlorinated pesticides and particularly for the degradation of pesticides having similar physicochemical properties to clopyralid, such as 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA) and picloram (Shawaqfeh et al., 2010; Tu et al., 2001). For example, the biodegradation of 2,4-D along with dichlorodiphenyltrichloroethane (DDT) has been studied in packed glass columns (Santacruz et al., 2005). The biofilter was packed with *tezontle* (a low cost basaltic scoria) and inoculated with a 24 hours culture of *Pseudomonas fluorescens* in YPG (yeast, peptone and glucose) medium. Removal of 2,4–D was achieved to a level of 99% at an initial concentration of between 100 and 500 mg/L, while the removal of DDT was as high as 55 - 99 % at an initial concentration of up to 150 mg/L (Santacruz et al., 2005).

The degradation of 2,4-D and picloram (commonly called tordon) has also been studied in a packed bed column, which was operated continuously (Ordaz-Guillén et al., 2014). The column was packed with alternative layers of soil, fragments of tezontle and filled with MS-T medium (i.e. tordon and mineral salt medium). The reactor was initially incubated for 7 days in batch mode and then the same medium was supplied at a constant flow rate. Changes in picloram and 2,4–D were evaluated spectrophotometrically and the highest average removal efficiency obtained was 94.58 ± 2.62 % and 99.56 ± 0.44%, respectively corresponding to hydraulic retention times between 0.236 days and 1.087 day (Ordaz-Guillén et al., 2014).

Some research treating chlorinated pesticides that have similar physicochemical properties to clopyralid has already been done in SBRs. For example, a study on the biodegradation of the herbicide 2,4-D investigated the effect of supplemental substrates (presence or absence of phenol and dextrose) in an aerobic SBR (Mangat & Elefsiniotis, 1999). In these experiments, three

reactors were run simultaneously, first with 2,4-D and phenol, second with 2,4-D and dextrose and third with 2,4-D as a sole carbon source. The results showed that in the presence of supplemental substrates, the removal rates of 2,4-D were affected by the type of additional substrate, being significantly lower in the case of dextrose. The final results found that the removal rate was fastest in the presence of phenol. However, the removal efficiency in all three reactors was approximately the same (i.e. > 99%) under appropriate HRT and biomass concentrations.

Another study investigating the biodegradation of 2,4-D in SBRs under both aerobic and anaerobic conditions, used glucose as a supplemental carbon source. The reactors were run under different temperatures, 30 ± 2^{o}C for the anaerobic reactor and 22 ± 2^{o}C for the aerobic reactor. Complete removal of 2,4-D (concentrations up to 500 mg/L) was observed in aerobic reactors but after an acclimation period of 30 days; however, anaerobic reactor was able to degrade 120 mg/L of 2,4-D in acclimation period of 70 days (Celis et al., 2008).

Furthermore, biodegradation of MCPA was observed in an anoxic SBR maintained under nitrate reducing conditions. It was determined that with an initial MCPA concentration of 50 mg/L, 98% removal was achieved and with increasing concentration to 75 mg/L, 85% removal was achieved. After that, no further removal occurred. However, nitrate was found to be completely removed from the effluent (Chouhan et al., 2017).

One SBR study investigated the salt form of clopyralid (monoethanolamine) and involved the removal of monoethanolamine (MEA) in an aerobic SBR. The results indicated that up to 9000 mg/L of MEA were removed with the efficiency of 92% but with a relatively long hydraulic retention time of 10.5 days. The COD was also reduced in the same HRT; however, the COD effluent values were higher than the MEA values (Kim et al., 2010).

Biological degradation was also studied in the small batch test of 2 L capacity. The results indicated that pesticides such as 2,4-D and TCP were degraded significantly after the adaption period of 1-2 months. However, no threshold concentrations from adaptation was observed and the degradation pattern followed the first-order kinetics (Nyholm et al., 1992). Further, a combined process was studied for the degradation of 2,4-D, where electrochemical cells were used as pre-treatment and small batch tests (250 mL of flasks) holding activated sludge were used as post-treatment method. The results revealed that the coupled treatment shortened the length of total 2,4-

D degradation to two days; however no mineralization was observed before 7 days, when only biological treatment was used (Fontmorin et al., 2013).

Although apparently no evidence has been found for the treatment of clopyralid in wastewater by biological means, the above studies illustrate that SBRs are able to treat a variety of recalcitrant pesticides; mainly because of their flexibility in providing a variety of oxygen tensions. As such, the SBR is a good candidate for investigation into the treatment of a clopyralid-contaminated wastewater.

2.6 Research Objectives

The evidence above suggests that clopyralid can be biologically degraded since there is some evidence that clopyralid has been degraded aerobically in compost (Gilbert et al., 2010) and in soil (Ahmad et al., 2003). However, to date, little research on the treatment of clopyralid in wastewaters has been done; thus of particular interest for this research is whether clopyralid can be degraded in biological wastewater treatment systems and especially whether clopyralid can be removed at industrial strength concentrations that occur through accidental spills, effluents from manufacturing plants and leaking storage sites. The major aims of this research therefore were to investigate:

1. The potential for aerobic degradation of clopyralid in wastewaters in the presence of a secondary carbon source (i.e. acetate).
2. The maximum industrial strength concentration of clopyralid that can be degraded under aerobic conditions.
3. The potential for aerobic degradation of clopyralid in wastewaters as a sole carbon source.
4. The kinetic modelling associated with aerobic degradation of clopyralid.
5. The identification of intermediate compounds formed during the degradation of clopyralid.
6. The identification of culturable microbes in activated sludge and their inhibition at different clopyralid concentrations.

CHAPTER-3 – MATERIALS AND METHODS

This chapter describes the experimental set-up and design, including the synthetic feed preparation and sampling procedures. It also describes the analysis of various parameters such as DO, ORP, TSS, MLVSS, total organic carbon (TOC), SMPs, microbial isolation as well inhibition and clopyralid extraction by LPME-SPME and gas chromatography electron capture detector (GC-ECD) technique.

3.1 Seed and Feed

For this research, activated sludge was collected from the wastewater treatment plant (WWTP) located in the Bromley suburb of Christchurch, New Zealand. In order to remove unwanted grit, the biomass (i.e. sludge) was washed several times with tap water (chlorine free). As per its origin, the biomass had a wide variety of heterotrophic bacteria with the ability to degrade wastewater. This was a necessary condition to increase the possibility of establishing bacteria capable of degrading clopyralid, once they were exposed to the pesticide for a sufficient period of time.

To maintain a similar COD throughout the research period (for ease of operation), a synthetic wastewater was prepared in the lab. The compounds in Table 3.1 were mixed with tap water in order to make up a feed sewage which had approximately the same characteristics as municipal wastewater (e.g. 300 - 350 mg COD/L). However, it was also observed that with time COD was degraded in the feed tank (350 mg/L to 270 mg/L by third day). To prevent this degradation as well as to maintain a COD: NO_3-N ratio of 10:1 only 120 L of synthetic feed was prepared at a time with the synthetic feed being kept in a refrigerated tank where the average temperature was maintained at 10^0C.

Table 3. 1. Major and micro nutrients in the synthetic wastewater

Chemical	**Molecular formula**	**Concentration (mg/L)**	**Source of**
Sodium acetate	CH_3COONa	455	Carbon
Monopotassium phosphate	KH_2PO_4	5.41	Phosphorous and potassium
Trace elements			
Calcium chloride	$CaCl_2$	0.26	Calcium and Chloride
Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	0.13	Magnesium and Sulphur
Ferrous sulphate	$FeSO_4.7\ H_2O$	6.61	Iron
Copper sulfate	$CuSO_4 \cdot 5H_2O$	0.05	Copper
Aluminium sulfate	$Al_2(SO_4)_3 \cdot 16H_2O$	0.38	Aluminium

[Sources: (Yoo et al., 1999; Aslan, 2005; Chong & Chen, 2007)]

3.2 SBR Experimental Set-up

A schematic representation of the experimental set-up is shown in Figure 3.1. Two 25-L stainless steel cylindrical reactors with an internal diameter of 300 mm were used in parallel. Both of them were maintained under aerobic conditions, whereby one reactor acted as a test reactor (i.e. clopyralid was injected) and the other as a control (i.e. no clopyralid was injected). For purpose of clarity, only one schematic diagram is shown.

The reactors had a working volume of 20 L and were operated in sequencing batch mode, at room temperature (20-22^{0}C) with a 40-60 day SRT (Equation 3.1) and 24 h HRT (Equation 3.2). A long SRT was used in consideration of previous research in which industrial wastewater was more readily treated in SBRs using long SRT of up to 60 days (Fongsatitkul et al., 2008). The SBRs were initially filled with 10 L of seed biomass and 10 L of synthetic feed (Section 3.1) whereas, 10 L of synthetic feed was fed daily twice according to the HRT. Three inlet/outlet ports were installed in each SBR for feeding, biomass wasting and decanting/sample collection. Moreover, a diffused aeration device was introduced from the top of the reactor along with an impeller since it was aerated with the top being left open. Filling, mixing plus reacting, settling and decanting functions were controlled via a programmable controller (Lab View version, National Instruments,

Austin, TX), which ran throughout the experiments on a personal computer for each reactor. In addition, ORP and DO probes were placed in the reactor to monitor the microbial activity (Sabumon, 2008) and their readings were also visible in Lab View. At the end of the reacting period, the biomass was allowed to settle, before decanting the supernatant.

The decanted or treated samples were taken for analysis and the same amount of wastewater (10 L) was fed to the reactor as fresh substrate.

Equations for the design parameters HRT and SRT are as follows:

$$\text{SRT} = \frac{Volume\ of\ tank\ (V)}{Sludge\ Wasteage\ Rate\ (Qw)} = \frac{20\ L}{200\ to\ 500\ ml/day} = 40 - 100 \text{ days} \qquad (3.1)$$

$$\text{HRT} = \frac{Volume\ of\ tank\ (V)}{Effluent\ Decanted(Q)} = \frac{20\ L}{20\ L/day} = \frac{20L}{\frac{20L}{day} * \frac{1day}{24hr}} = 24 \text{ hr} \qquad (3.2)$$

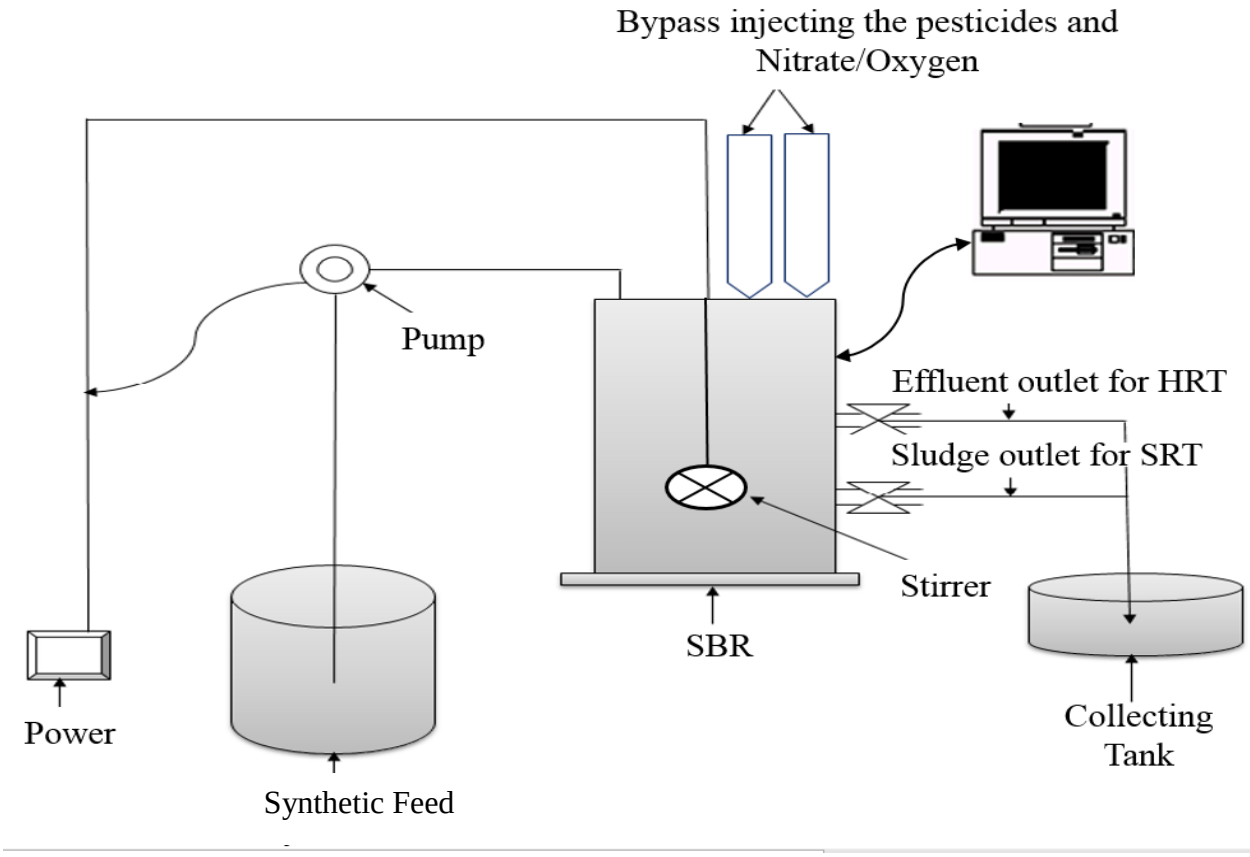


Figure 3. 1. Schematic representation of Sequencing Batch Reactor

3.3 Process Control System

The SBRs were connected to a microprocessor-based control system for automatic operation on a 24 hours basis. This system included two key elements, namely a central control box and a personal computer with the Lab View software from National Instruments. The central control box consisted of 16 analogue inputs, 16 digital inputs and 16 digital outputs that allowed the control of the SBR functions independently. Additionally, the microprocessor was also programmed to perform as a single unit on a predetermined time basis unless manually altered by an operator. Besides controlling the SBRs, the Lab View software was also used to monitor the SBR process by using an on board A/D card on the computer communication port. Moreover, the ORP and DO probes were also attached to the communication port of the computer to monitor the microbial activity in the SBRs.

3.4 Sampling Procedures

Samples were taken at different times for the analysis of various parameters. For example, for the analysis of total suspended solids (TSS) and mixed liquor volatile suspended solids (MLVSS), samples were collected during the reactor's mixing period three times per week. Samples for the analysis of chemical oxygen demand (COD) and total organic carbon (TOC) were taken at fixed interval of 0, 15, 30, 60, 90, 120, 250 and 590 min in one cycle to follow-up these parameters during track studies. Samples for the analysis of suspended solids were measured as collected whereas samples for the analysis of COD and TOC were processed through various steps to stop the biological activity. These included centrifugation at 4400 rpm for 10 min then acidification with H_2SO_4 to pH < 2 and finally filtration through 0.45µm membrane filter (APHA et al., 2005).

3.5 Pesticides Injection Strategy

In the first or preliminary phase of the research no pesticide was introduced in the reactor in order to minimize any toxic effects on microorganisms while their population stabilized. As such, the microorganisms only had acetate as carbon source. Once stable conditions in the reactor were achieved (that is after three to four months) the injection of the pesticide started. The reactors were regarded to be in stable condition when key parameters such as suspended solids (4000 ± 500 mg/L), COD removal (> 95%), ORP (50 to 250 mV) and DO (maximum concentration 4.5 mg/L for aerobic conditions) were above or within acceptable values.

As noted above, the injection of clopyralid to the SBRs started after stable conditions were achieved. To this end, a clopyralid solution of 50 mg/L was prepared from a commercial formulation of the pesticide (Transline, Dow Agrosciences, HQ Australia) that contained 300 g/L of clopyralid in amine salt form according to the manufacturer. It has been reported that the commercial formulations of clopyralid amine solution contain other additives along with the active compound (El dorado National Forest, 2010); for example, monoethanolamine, isopropyl alcohol and polyglycol. Both monoethanolamine and isopropyl alcohol are food additives and there is little evidence that these compound can affect the risks associated with using clopyralid (Villalvazo, 2010).

A spike of clopyralid solution was injected from the top of the reactor via a peristaltic pump (in one minute) after the fill stage and at the beginning of the mix stage. The day after the injection of clopyralid, a sample of the reactor's mixed liquor was collected (at the end of mix stage) and analyzed to assess any degradation of clopyralid. This was done on a daily basis. Once pesticide degradation was observed and stabilized, the analysis of other parameters such as COD, DO and ORP, began. After that, the concentration of clopyralid was increased to 100, 150, 200 mg/L and so on until the threshold level of degradation was achieved. However, although clopyralid was still degraded at higher initial concentrations, the presence of other additives in the formulation led to increased COD values in the effluent (Section 4.2.1). Therefore, it was decided to use pure clopyralid instead of the commercial formulation and due to the financial limitation as pure clopyralid was found to be very expensive, smaller batch tests were used instead a full-scale SBRs having a capacity of 20 L.

3.6 Batch Tests

Batch tests were used to evaluate the rate of clopyralid degradation and each batch test consisted of a 500 mL reactor filled with 250 mL of biomass and 250 mL of synthetic feed containing clopyralid. To this end, 500 mL of sludge was collected from one of the SBRs and allowed to settle for 1 hour. Then, the supernatant was discarded and the settled 250 mL volume of biomass was used in the batch test. A working solution of clopyralid standard (99.99% purity) was prepared by dissolving 25 mg of powdered clopyralid in 250 mL of synthetic wastewater for one hour and then this solution was mixed with the settled 250 mL biomass, therefore reaching an initial clopyralid concentration of 50 mg/L.

After that, once degradation of initial clopyralid concentration and COD was achieved, the initial clopyralid concentration was increased from 50 to 100 mg/L and so on till the maximum degradation concentration limit was achieved. As a result, seven individual concentrations of batch tests were tried i.e. 50, 100, 150, 200, 225, 250 and 300 mg/L for reaching the threshold degradation concentration.

The reactor had an inlet to introduce air for aerobic batch tests, an outlet to collect the sample from, and the solution in the bottle was mixed continuously by means of a magnetic stirrer at a constant speed of 500 rpm.

The batch tests were done with three replicates for each concentration of clopyralid as well as one control reactor containing no clopyralid. The batch reaction time was 24 h with the sample collected at intervals of 0, 15, 30, 60, 90, 120, 400, 590 and 1200 minutes. The track studies in the case of batch tests were also useful in generating the kinetics of herbicides.

3.7 General Analytical Techniques

3.7.1 Oxidation reduction potential and dissolved oxygen

ORP and DO were monitored continuously using probes and their signal was recorded by the Lab View software on a personal computer thus allowing the real-time collection of data which was automatically saved on a daily basis. The ORP probe was made of platinum and the reference electrode was Ag/AgCl and 3.5 M KCl. The DO probe used polytetrafluoroethylene membrane (YSI, Yellow Springs, OH).

The ORP probe was calibrated as per the manufacturer guidelines (before putting it into the reactor) by using quinhydrone buffer solution at pH 4 and 7. The DO probe was calibrated using tap water saturated with oxygen. Both the probes were hung from the top of the reactor and fixed to the top surface with a plastic wire. For better results, both probes were washed once per month with deionized water to prevent sludge coating.

3.7.2 Total suspended solids (TSS) and mixed liquor volatile solids (MLVSS)

Suspended solids were measured 3 times per week. A 5-mL sample was collected during the mixing stage and a glass-fiber filter circle (grade GF/C 90 mm ϕ, Whattman) was used to filter the sample. The filter circle was dried at 103-105°C for at least 24 h before its use. The initial weight

of the filter circle was measured using an analytical balance (which was capable of weighing to 0.1 mg) and then the filter was assembled in the filtration apparatus where a vacuum pump was used to increase the rate of filtration. The sample was diluted with deionized water to a final volume of 50 mL which was then passed through the filter circle. A vacuum was applied for two minutes for faster filtration and then the filter circle was taken out and put in an oven for drying at 103-105°C for 1 hour. After drying and cooling down, the filter circle was weighed again. The difference in the mass of the filter circle before and after the filtration process reflected the TSS content of the 5-mL sample. To determine the MLVSS, the same filter circle was placed in a furnace at 550°C for 1 hour and the weight lost per unit volume of sample was calculated from the weights before and after ignition.

3.7.3 Chemical oxygen demand (COD)

For COD analysis, the sample was first digested in a closed reflux digester unit (Digital Reactor Block 200, HACH). HACH pre-prepared high-range reagent was used which contains potassium dichromate for the oxidation purpose, silver compound (Ag_2SO_4) as a catalyst to speed-up the oxidation, and mercuric compound ($HgSO_4$) to reduce the interference of chloride ions. The sample of 2 mL was mixed with 5 mL of high-range reagent in the COD vials (glass tube with a screw cap; 10 x 100 mm). After that, the COD vials were placed into the digestion unit at 150°C for 2 hrs. In the digestion unit, dichromate oxidized the sample completely and the value of COD was measured by dichromate consumption. The amount of dichromate consumed was equivalent to the COD in mg/L and was measured by a change in absorbance with a HACH Digital DR 3900 spectrophotometer. It is noted that H_2SO_4 was added to the samples collected at different time intervals to drop the pH and to stop the reaction (APHA, 2005).

3.7.4 Total organic carbon (TOC)

TOC was measured with a Shimadzu TOC-L CSH/CSN analyzer fitted with a PC, the TOC-L control software version 1.04 and an automatic liquid sampler. Samples for TOC analysis were collected from the reactors at different time intervals. Ten mL of each water sample was placed into a TOC bottle and diluted with 30 mL of deionized water before analysis.

3.7.5 Oxygen uptake rates (OUR)

To measure the OUR, a DO probe was used and calibrated according to the manufacturer guidelines. After that, mixed liquor suspended solids (MLSS) sample was collected in 500 mL of a bottle from sequencing batch reactors. The sample was allowed to settle for 45 minutes and the supernatant was discarded. Then new synthetic feed (250 mL) was added into the bottle. Later, the sample was aerated for 15 minutes while the bottle left uncovered. After 15 minutes of aeration, the bottle was tightly closed with a lid and placed on the magnetic stirrer (at a speed of 250 rpm) while DO probe left inside. DO was recorded for another 15 minutes in 30 seconds time intervals.

Once all the DO was recorded, the graph was plotted DO v/s time and a possible straight line was constructed through the points. The slope was calculated and multiply by 60 min/hr, which was equal to OUR and expressed in mg O_2/L/hr.

3.7.5 Soluble microbial products (SMPs)

To detect SMPs, two samples were collected from batch reactors i.e. at 15 min and 24 h for the 3 conditions of acetate only, acetate plus clopyralid and clopyralid only. Each sample was centrifuged at 13000 rpm for 10 min and then filtered by using a 0.20 µm micro filter (Kunacheva and Stuckey, 2014). After that, the samples were placed in a UV-Vis spectrophotometer measuring at five different wavelengths i.e. 230, 254, 280, 490 and 595 nm micro filter (Kunacheva and Stuckey, 2014) corresponding to polyethlene glycol (PEG), amino acids (254 and 280 nm), total carbohydrates and proteins, respectively.

3.7.6. Transformation products

The samples were collected at different time intervals (0, 15, 30, 60, 90, 120, 590 and 1200 min), centrifuged and then filtered through 0.2 µm membrane filters. After that, one drop of HCl was added to the filtered solution to drop the pH (Berberidou et al., 2017). The next step was to place the sample in a small 2-mL vial covered with foil to prevent exposure to light and preserve it in a fridge at -4ºC. After that, the samples were sent to the Department of Chemistry of the University of Canterbury for the assessment of transformation products. LC-MS was used in an effort to identify these products from the biological degradation of clopyralid.

High resolution accurate mass samples were analysed on a maXis 3G UHR-Qq-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) coupled to a Dionex Ultimate 3000 LC system (ThermoFisher). Furthermore, a 5-µL sample was injected into a flow of 50:50 water (0.5% formic acid): acetonitrile at 0.2mL/min. 5µL ESI-L Low Concentration Tuning Mix (Agilent Technologies) was injected after each sample as a calibrant. Data was processed using Compass software (Bruker Daltonik GmbH, Bremen, Germany)

3.7.7 Clopyralid extraction

A liquid phase micro-extraction followed by a solid phase microextraction technique (LPME-SPME) was used to extract clopyralid from water. The conditions for extracting the clopyralid from the aqueous samples were the same as the ones used by Ciucanu et al. (2014). This method involves the use of a chemically bonded polydimethylsiloxane (PDMS) coated SPME fiber of 7-µm thickness, a manual holder, and 4-mL vials, all purchased from Supelco, New Zealand.

To extract clopyralid from water by SPME, it is important to condition the fiber first, which was done by heating at 330^{o}C for 0.5 hour at the inlet port of the gas chromatograph. In the meantime, the sample solution of clopyralid was acidified to pH 1 by the addition of HCl. A 4.5-mL vial was filled with 4 mL of acidified sample solution and 85 µL of dichloromethane. Then the fiber was introduced into the sample which was agitated at 500 rpm at room temperature. The fiber was left immersed in the sample for 10 minutes and then introduced into the inlet of the GC, where thermal desorption of the analyte took place for 10 minutes.

3.7.8 Clopyralid identification and its accuracy

Clopyralid 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 (30m x 0.25 mm x 0.25µm film thickness) was used with a liner (Agilent 5181-3315; 4mm ID dbl tap) appropriate for SPME 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 40.98 psi and the initial flow was 2.1 mL/min. The injector was in split mode at 220^{o}C with split ratio 30:1. The GC oven temperature started at 80^{o}C for 1 minute and then increased to a final temperature of 170^{o}C

first at a rate of 40°C per minute, from 80°C to 150°C, and then at a rate of 3°C per minute, from 150°C to 170°C. The total run time of the analysis was 10.42 minutes.

Standard clopyralid acid (purity ≥ 99.99%) was purchased from Supelco, New Zealand and a calibration curve was prepared using the LPME-SPME-GC-ECD technique described above. Standard clopyralid comes in the form of powder that was dissolved in deionized water at concentrations of 5, 10, 30, 40, and 50 mg/L to produce the calibration curve. The peak area appearing in the chromatogram was used for the calculation of the herbicide concentration in mg/L. Identification and quantification of clopyralid in the effluent sample was done by comparison of the retention time of the peaks in the chromatograms and by the calibration curves.

Table 3.2 shows the coefficient of variation (CV) of the GC-ECD method to a clopyralid solution of 50 mg/L. Boussahel et al. (2002) reported CV values of 8 to 18% for a different chlorinated herbicide and stated that a CV of less than 20% for pesticides analysis indicates an acceptable precision.

Table 3. 2. Repeated value of response factors in Hz for concentration 50 mg/L of clopyralid to calculate the coefficient of variation

Repetition (n=7)	Response (Hz)
1	2699
2	3583
3	3895
4	4295
5	4432
6	3031
7	3649
Average	3650
Standard Deviation	578
Coefficient of Variation (%)	16

To find out the efficiency of the method, two theoretical concentrations 2 mg/L and 25 mg/L were measured in the GC-ECD. The area appeared in the form of peak was then placed (in red) on the

calibration curve (Figure 3.2). It was further noted that the calculated calibration curve was acceptable and that the limit of detection for the GC-ECD method was 2 mg/L.

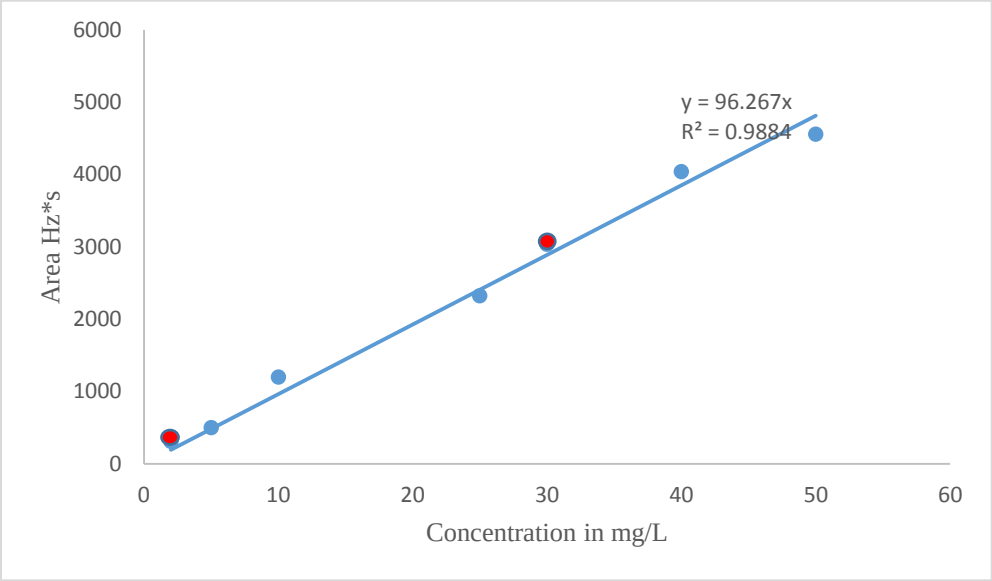


Figure 3. 2. Calibration curve efficiency

3.8 Microbial Analytical Techniques

3.8.1. Media preparation and microbial isolation

Duplicate grab samples of wastewater (20 mL) were collected in test-tubes from the aerobic SBR during the mix stage. These test tubes were covered by aluminum foil to avoid light transmission into the wastewaters and quickly transferred to a microbiology lab where the samples were stored in a refrigerator before use for experiments.

Preparation of media

Two media were prepared i.e. Nutrient Agar (NA) to culture bacteria and Potato Dextrose Agar (PDA) for fungi. Both media (NA and PDA) were purchased from Oxoid, UK and prepared as per the manufacturer instructions. The media were autoclaved for 30 minutes at 121ºC and 15 *psi*. Once the media were autoclaved, they was stored in a water bath at 55ºC to be maintained in liquid state. To isolate bacteria and fungi resistant to clopyralid, different amounts (such as 20, 30, 40 and 50 µL) of the herbicide were added to both media under a laminar flow cabinet to make a

serial concentration of the herbicide. All media both with or without clopyralid were dispensed in Petri plates (8 cm) under a laminar flow cabinet and left in ambient temperature to be solidified.

Isolation of microbes from wastewater

To make a serial dilution, one mL of sample collected from the test tube was diluted with 9 mL of sterile water in a universal container and six subsequent dilutions were prepared. Then 100 µL from each container was spread over the NA and PDA medium to isolate culturable bacteria and fungi, respectively. The Petri plates were incubated at 24°C and individual colonies of both were isolated and purified as per Alizadeh et al., (2017).

3.8.2. Identification of microbes

Total genomic DNA extraction

Single colonies growing on PDA or NA media were used for DNA extraction using Chelex® and the purity of the extracts containing DNA was assessed using a Nanodrop spectrophotometer (Alizadeh et al., 2017).

PCR (polymerase chain reaction) amplification:

The bacterial 16S rRNA genes and fungal internal transcribed spacer (ITS) region of ribosomal operon were amplified using primers f8–27 & r1510 and ITS1F & ITS4R, respectively, as described by Alizadeh et al. (2017) before. The amplification products were visualized by electrophoresis before they were sequenced at Lincoln University sequencing facility. Sequence files generated from both 16s rRNA or ITS sequencing were edited and assembled using the ChromasPro software and compared to the nucleotide database at EzTaxon-e identification service for bacteria (https://www.ezbiocloud.net/) or the US National Centre for Biotechnology Information (NCBI) to find the nearest relatives (Alizadeh et al., 2017).

3.8.3 Microbial inhibition assays

To identify whether inhibition had occurred from a microbial perspective in the presence of clopyralid at different concentrations, two methods were used: paper disk and incorporation of the herbicide into broth medium methods.

In the paper disk method, Petri plates containing 10 mL of either PDA or NA were used. Inoculum consisting of a 5 mm diameter PDA plug excised from the margin of a three-day old fungal colony was placed at the center of a PDA plate and incubated at 25°C for three days. Four paper disks (GE Healthcare, Life Sciences, UK) were placed equidistantly in each plate (Ye and Ng 2002) and three disks were impregnated with the clopyralid formulation at three different concentration i.e. 10, 20 and 50 µL (containing 300 gm/L of clopyralid) while the fourth disk was the control. For bacteria, NA plates were inoculated with a lawn of a bacterium and four paper disks were placed on four different corners of the media and were impregnated with the clopyralid formulations. The plates were incubated for one day further and the zones of inhibition were measured and compared with the control disks.

In the incorporation of the herbicide into nutrient broth method, nutrient broth medium (purchased from APHA DIFCO) was prepared by dissolving 1.6 gm of nutrient broth in 200 mL of deionized water. Then, 5 mL of medium was placed in each universal tube of 5 tubes. After that, the universal tubes were autoclaved for 20 minutes at 121ºC and 15 *psi*. Similar steps were repeated for potato dextrose broth method (purchased from Scharlau) and the media was prepared by dissolving 4.8 gm of potato dextrose broth in 200 mL of deionized water. The bacteria as well as fungi were inoculated in nutrient and potato dextrose broth universals, respectively with different clopyralid formulation concentrations i.e. 20, 30, 40 and 50 µL and zero as control. The inoculated tubes were then placed on a shaker at 120 rpm for 24 h. The solutions were then transferred to centrifuge tubes and centrifuged at 10,000 rpm for 10 min before the supernatant was discarded. Each tube was filled with 3 mL of deionized water and vortexed, before the absorbance was measured at 600 nm in a Genesys 10 UV scanning spectrophotometer (Thermo Scientific, Helios gamma)**.**

Assay of the Bactericidal/Fungicidal and Bacteriostatic/Fungistatic effect of Clopyralid:

The clopyralid was assayed for bactericidal/fungicidal and bacteriostatic/fungistatic effect as described by Theis et al. (2005) with some modifications:

Bacteria

Bacteria were collected from a 2-day old isolated culture grown on NA by adding 5 mL of sterile water in a laminar flow cabinet and the suspension was collected and transferred to a tube before adjusting the CFU (colony forming unit) to 10^6 per ml. Strile YPG medium [0.3% yeast extract, 1 % peptone and 2% glucose] containing different concentrations of clopyralid (up to 200 µL) was

prepared and 4.9 mL were dispensed in each sterile centrifuge tube before inoculating with 100 µL of the bacterial suspensions. The tubes were incubated at 24ºC on a shaker (120 rpm) for 24 h and were then centrifuged at 10,000 rpm for 10 min. The supernatant was removed under aseptic conditions in a laminar flow cabinet. The settled bacteria were washed three times with sterile YPG and then end 5 mL of sterile YPG were added to the bacterial pellet and incubated again at 26 ºC on a shaker for three days. For a control, the media without clopyralid were used. Any delayed growth of bacteria was considered as a bacteriostatic effect and no growth after removal of the clopyralid was considered as a bactericidal effect.

Fungi

For fungi the sampling was the same as bacteria but instead a conidial suspension of fungi was collected from a PDA culture.

3.9 Abiotic Processes

Abiotic processes may contribute to the loss or removal of clopyralid from water, the extent of which has been studied in two different ways:

1. Volatilization for 24 hours
2. Bio-adsorption of clopyralid on dead biomass for 24 hours

To assess the extent of the volatilization of clopyralid, two batch tests (one with aeration and other without aeration) were run together in 500 mL bottles kept open for the entrainment of air and to collect samples. Samples were prepared from the pure form of clopyralid at 50 mg/L of concentration, dissolved in deionized water with all batch reactors run for 24 hours. Samples were collected 4 times i.e. 0, 120, 600 and 1200 minutes.

Bio-adsorption of clopyralid has been studied in two different ways; firstly, two concentrations i.e. 50 and 100 mg/L of pure form of clopyralid were dissolved in 500 mL batch reactors in synthetic feed and samples were collected three times i.e. 0, 120 and 1200 minutes. After collecting the samples, the cell walls of the microorganisms were ruptured through ultra-sonication, so that adsorbed clopyralid on biomass was released into aqueous phase. After that the sample was centrifuged at 4400 rpm for 15 minutes and the supernatant was filtered through a 0.45 µ filter (clopyralid was measured using the GC-ECD).

In addition, after inactivating the biomass with sodium azide, the pure form of clopyralid was dissolved in synthetic feed at two different concentrations 50 and 100 mg/L, these concentrations were introduced in 500 mL batch reactors and the reactors were monitored for 24 hours. Samples were collected at different time intervals (0, 30, 60, 90, 120, 240, 590 and 1200 mins) to see whether clopyralid was removed through bio-adsorption to the sludge.

3.10 Experimental Design and Phases

This research is primarily designed in six different phases i.e. Phase I – Preliminary Phase , Phase II – Acclimatization Phase , Phase III – Batch Phase, Phase IV – Kinetic Modelling, Phase V – Intermediate Study Phase and Phase VI – Microbial Inhibition Phase as shown in Table 3.3.

The preliminary phase (Phase I) assessed the stability of the SBRs, where all the parameters such as TSS, MLVSS, ORP, DO and COD removal, achieved stable values as mentioned previously in Section 3.5. The stability of the SBRs was measured in the absence of clopyralid, with only the presence of acetate as a sole carbon source. Once stability of all the required parameters were achieved, the formulated clopyralid at 50 mg/L was injected in the test reactor as part of the acclimatization phase (Phase II). In this phase, other parameters at that concentration of clopyralid (i.e. 50 mg/L) were also examined to assess stability (such as good COD removal). Once stability in COD and clopyralid removal were attained, track studies were performed for both parameters. Later, the clopyralid concentration in the reactor was increased to 100 mg/L and the same parameters of interest were monitored.

In Phase III (the batch phase), instead of formulated clopyralid, a pure form of clopyralid was used in small reactors of 500 mL volume. In the batch phase, clopyralid was introduced in different batch bottles at various concentrations i.e. 50, 100, 150, 200, 225, 250 and 300 mg/L to observe the threshold level of degradation, in the presence or absence of acetate. Track studies were done to measure clopyralid as well COD removal. Once the threshold level of concentration in clopyralid degradation was determined, kinetic modelling (Phase IV) was done. Kinetic modelling was a curve fitting exercise, to understand the pattern of clopyralid degradation as well as to compare the experimental data against a particular mathematical model.

Afterwards, residual compounds were identified under phase V that includes all the intermediate compounds such as organics (TOC), inorganics (TKN), transformation products (TPs) and SMPs.

These compounds are helpful to explain the COD taxonomy in the effluent. To measure these compounds, samples were collected at different time intervals for different compounds; for example, for TOC and transformation products, samples were collected at time intervals i.e. 0, 15, 30, 60, 90, 120, 590 and 1200 min, while for inorganic compounds, samples were collected two times i.e. at 0 min and 590 min. At the end of phase V, samples were collected at 15 min and 24 h for measuring the SMPs.

Microbial activities and communities were identified in the microbial inhibition phase (Phase VI). In this phase, microbial activities were identified by measuring degradation capability and OUR. Degradation capability can be determined by observing clopyralid as well COD removal and if the removal is large (near to 80%) that implies good degradation capability, which means healthy biomass in activated sludge. On the other hand, OUR measured the respiration rates of biomass. Culturable microbes were isolated and then identified using Polymerase Chain Reaction (PCR)-based molecular method and effect of Clopyralid on their growth was assessed using the Paper Disk/Incorporation of Clopyralid into Nutrient Broth Methods.

Table 3. 3. Experimental design for aerobic reactor

Phase Number	**Description**	**Measure Parameters**	**Objectives achieved**
Phase I (Preliminary Phase	Stable conditions were achieved in the SBRs to test the stability of the operations.	Suspended solids, COD removal, ORP and DO.	
Phase II (Acclimatization Phase)	Commercial clopyralid solution along with acetate at two different concentrations (50 and 100 mg/L) was introduced into the SBR to assess the degradability of the compound.	Clopyralid removal along with COD removal	1
Phase III (Batch Phase)	Standard form of clopyralid (in the presence or absence of secondary carbon source acetate) was introduced into the batch reactor at various concentrations from 50 to 300 mg/L to determine the threshold level of degradation.	Clopyralid removal along with COD removal.	2 and 3
Phase IV (Kinetic Modelling)	Track studies generated kinetic modelling data for different concentrations and to observe the pattern of clopyralid degradation.	Track removal of clopyralid.	4
Phase V (Intermediate Study Phase)	Samples were collected at different time intervals and then the inorganic as well as organic compounds were identified.	TOC, TKN, TPs and SMPs.	5
Phase VI (Microbial Inhibition Phase)	Microbes present in the activated sludge with the ability to be cultured were identified, alsomicrobial inhibition assays were performed at different clopyralid concentrations.	OUR, COD, Bacteria and fungi	6

CHAPTER-4 – BASELINE STUDIES IN THE REACTOR AND CLOPYRALID DEGRADATION

4.1 Baseline Conditions in the Aerobic Sequencing Batch Reactor (Phase I)

This section summarizes the preliminary baseline data collection to assess the performance and stability of the sequencing batch reactors before injecting the herbicide. Performance and stability of SBRs refer to four key parameters: (i) stable biomass in terms of total suspended solid concentration values i.e. 3500 ± 500 mg/L, (ii) consistent effluent COD removal (≥ 95%), (iii) stable ORP range between 50 mV and 250 mV, and (iv) acceptable DO range between 1.5 and 4.5 mg/L.

During the reactors start up, it was observed that the TSS dropped in both reactors from an initial value of approximately 4700 mg/L to an average value of 3500 ± 500 mg/L as the system became established (Figures 4.1 and 4.2). It is presumed that the initial TSS drop was due to the change in the biomass environment from full scale biological wastewater treatment plant conditions to lab reactor conditions in respect to oxygen tension and substrate composition as well as concentration. In particular, in the SBRs the biomass was exposed to a single carbon source (i.e. acetate) contrary to what happens in a full scale WWTP treating real municipal wastewater. Moreover, initially the reactor was not in equilibrium conditions as the rate of loss of biomass was higher than the rate of biomass growth, due to the new environment. This was evident from visual inspection i.e. biomass washout observed during the decant process. As soon as the biomass adapted to the new environment and attained equilibrium, the suspended solids concentrations stabilized at 3500 ± 500 mg/L which represents a 15 % variation. A variation of 10 to 20 % in the concentration of suspended solids is typical in aerobic SBRs and defines the expected level of stability in these systems (Mangat & Elefsiniotis, 1999). Hence, it can be observed from Figures 4.1 and 4.2 that stable reactor performance with respect to suspended solids was achieved approximately a month after the reactors were first started. Nonetheless, the reactors were kept running without clopyralid until the stability of other parameters, such as COD removal, ORP and DO were also assessed.

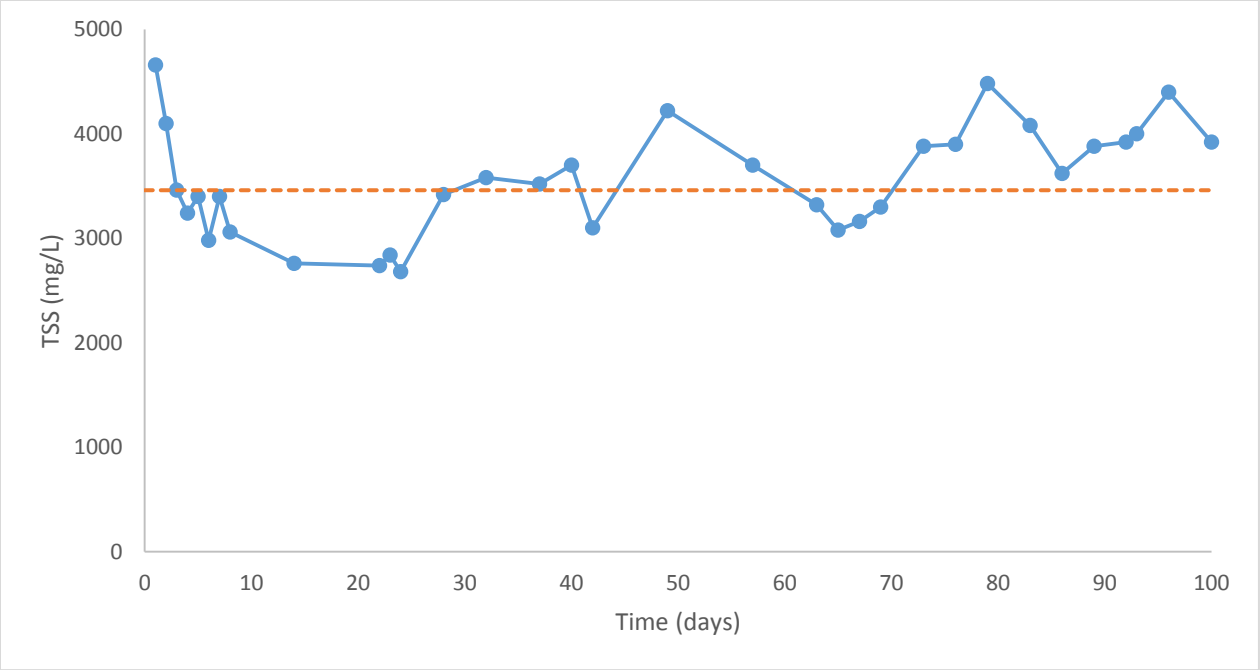


Figure 4. 1. Change in total suspended solids (TSS) concentration over time for reactor 1

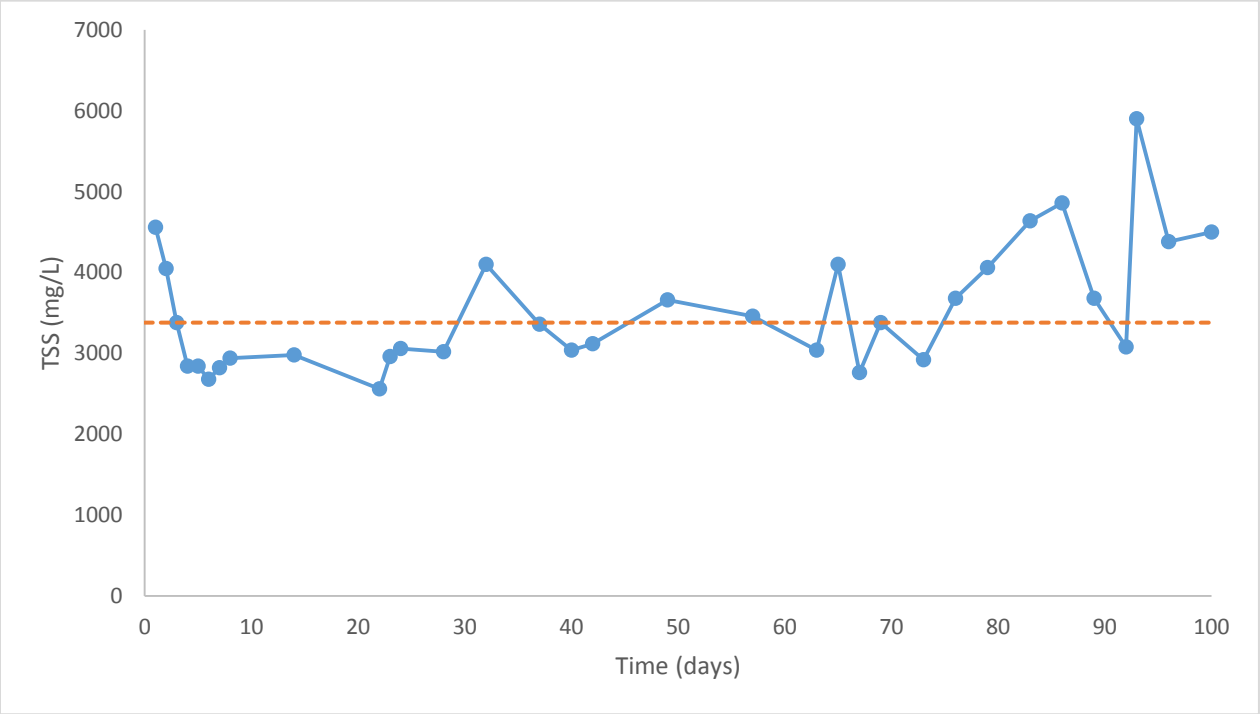


Figure 4. 2. Change in total suspended solids (TSS) concentration over time for reactor 2

Once the reactors were deemed to be stable in terms of TSS concentrations, COD track studies were carried out on three different days. Average normalized COD values were then plotted against time as shown in Figures 4.3 and 4.4. The results show more than 80 % reduction of COD (from approximately 300 to 50 mg/L), within the first two hours of mixing in both SBRs. After that, a further 15 % of COD reduction was observed with the lowest level reached after the settling of the biomass for two hours as biomass activity continued (although at a much lower pace).

It can be observed from Figures 4.3 and 4.4 that COD degradation occurred in two distinct stages reflecting Monod kinetics in which microbial growth rate depends on substrate concentration in the aqueous environment. The mathematical expression of Monod kinetics used to describe the specific microbial growth rate is shown by equation 4.1:

$$\mu = \mu_{max} \frac{S}{K_s + S} \qquad (4.1)$$

Where, μ is specific growth rate of microorganisms in mg/L/day, μ_{max} is the maximum specific growth rate in mg/L/day, K_s is the saturation constant in mg/L and S is the substrate concentration in mg/L.

The specific substrate utilization rate (r_s) in mg/L/day is related to the specific growth rate of microorganisms (μ) by a yield coefficient (Y) according to Equation 4.2:

$$Y = \frac{dX}{dS} \qquad (4.2)$$

$$\mu = \frac{Y}{X} . \frac{dS}{dt} \qquad (4.3)$$

$$\mu = Y . r_s \qquad (4.4)$$

Also,

$$\mu_{max} = Y . r_{s\,max} \qquad (4.5)$$

$$r_s = \mu_{max} \frac{S}{K_s + S} . \frac{1}{Y} \qquad (4.6)$$

Where, X is the biomass concentration in mg/L and $r_{s\,max}$ is the maximum specific substrate utilization rate.

During the first stage (i.e. first 120 minutes), S is much higher than a typical K_s values as fresh substrate is introduced into the reactor at a relatively high concentration i.e. approximately 300 mg/L. This means the term [$S/(K_s+S)$] approaches a value of one and $r_s \sim (\mu_{max}/Y) \sim r_{s\,max}$ in Equation 4.6. In other words, the observed specific rate of substrate uptake (or COD removal) (r_s) approaches the maximum specific substrate uptake rate ($r_{s\,max}$) and is independent of substrate concentration. This results in a high COD removal over a short period of time.

During the second stage (after 120 minutes), the S value drops and approach K_s and the observed substrate degradation rate is only a fraction [$S/(S+K_s)$] of the maximum specific substrate uptake rate. During this stage, COD removal is limited by the substrate concentration itself and it takes substantial time to observe a slight decrease in COD concentrations.

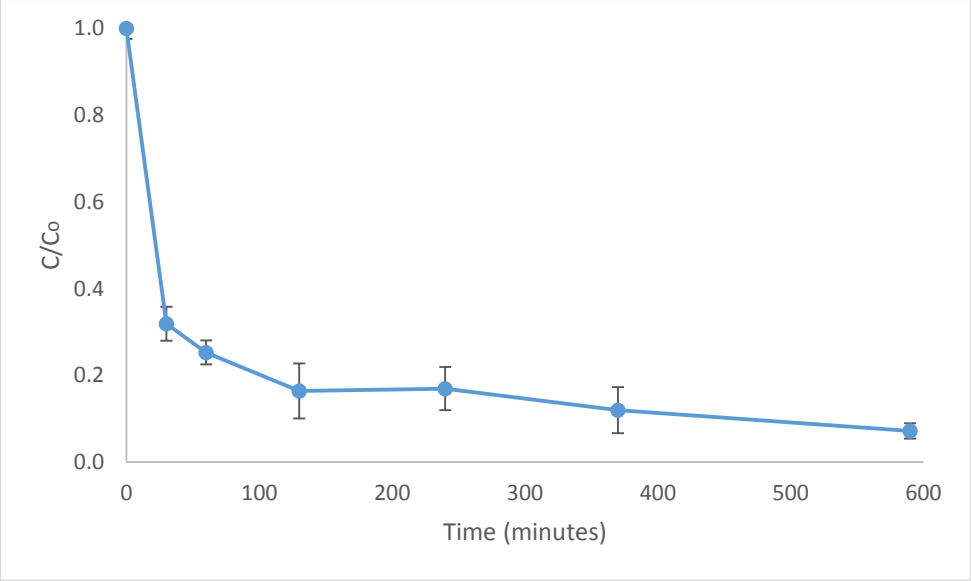


Figure 4. 3. Normalized COD concentration from mixing/reaction to decanting stage of aerobic SBR cycle for reactor 1. Average values (n = 3) and standard deviation (error bars) are shown.

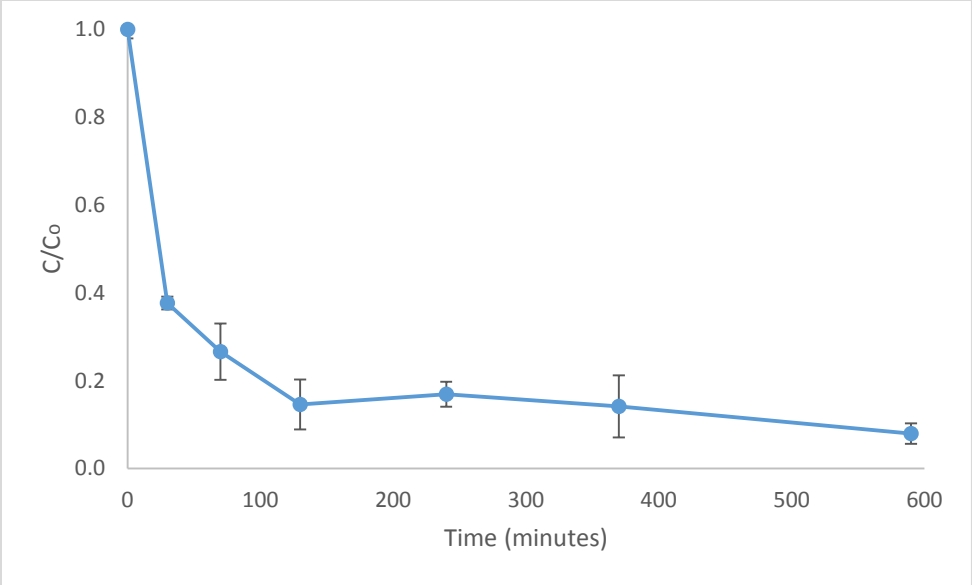


Figure 4. 4. Normalized COD concentration from mixing/reaction to decanting stage of aerobic SBR cycle for reactor 2. Average values (n = 3) and standard deviation (error bars) are shown.

As mentioned, ORP and DO were also monitored while the system was running to ensure aerobic conditions of the reactors. Typical ORP & DO curves for one complete cycle of a 24-hour HRT are shown in Figures 4.5 and 4.6, respectively. The graphs show ORP and DO values increasing from approximately 50 mV to 250 mV and from 0 to 4.5 mg/L respectively, during the mix/react stage of the SBR cycle. Once ORP and DO reached their maximum level, they remained steady until dropping during the biomass settling phase, when aeration was adjourned.

Under aerobic conditions, the DO is affected by the oxygen supply as well as microbial activities for utilizing oxygen as an electron acceptor, in the reactor. Hence, during the filling phase, it can be observed from the graphs that DO was at its lowest level as organic matter required a large amount of oxygen for respiration at this stage. During the mix/react stage, when air was supplied to the reactor, the DO rose slowly as organic matter was receiving continuous oxygen for respiration and then reached their highest possible level. At the uppermost value of DO, oxygen supply and consumption both became equal and achieving a stable value of DO. However, related to the DO profile the ORP follows the same trend in the graph with the initial difference between ORP and DO curve due to the variation in response of ORP and DO probes. Also, in the beginning DO values are low as it was consumed by the microbes; however, ORP values were significantly more in comparison to DO as the reduced compound released due to the oxidation reaction such

as ammonia and acetate. DO and ORP decreased during settling as mixing and aeration were stopped.

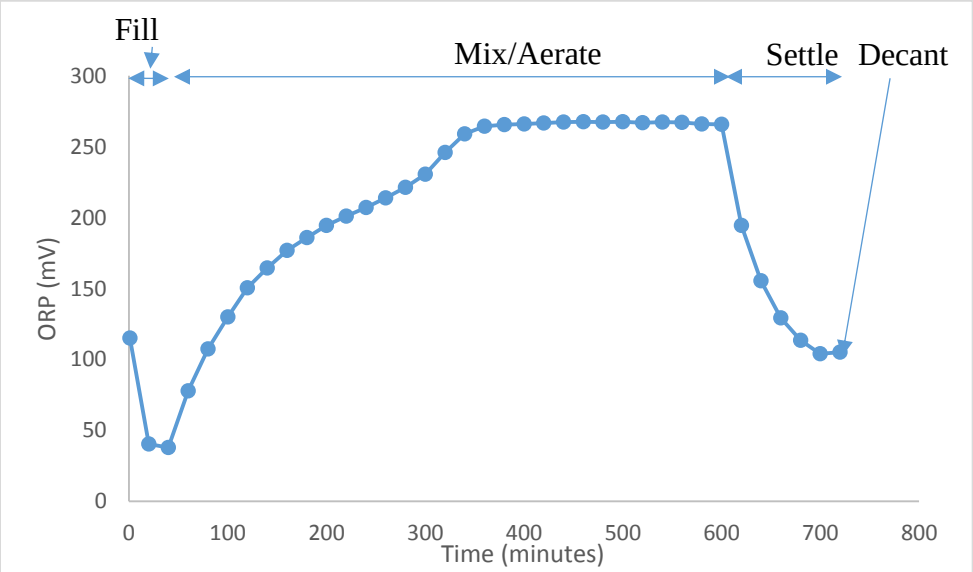


Figure 4. 5. Typical ORP values for one complete 24-h cycle in an aerobic SBR reactor.

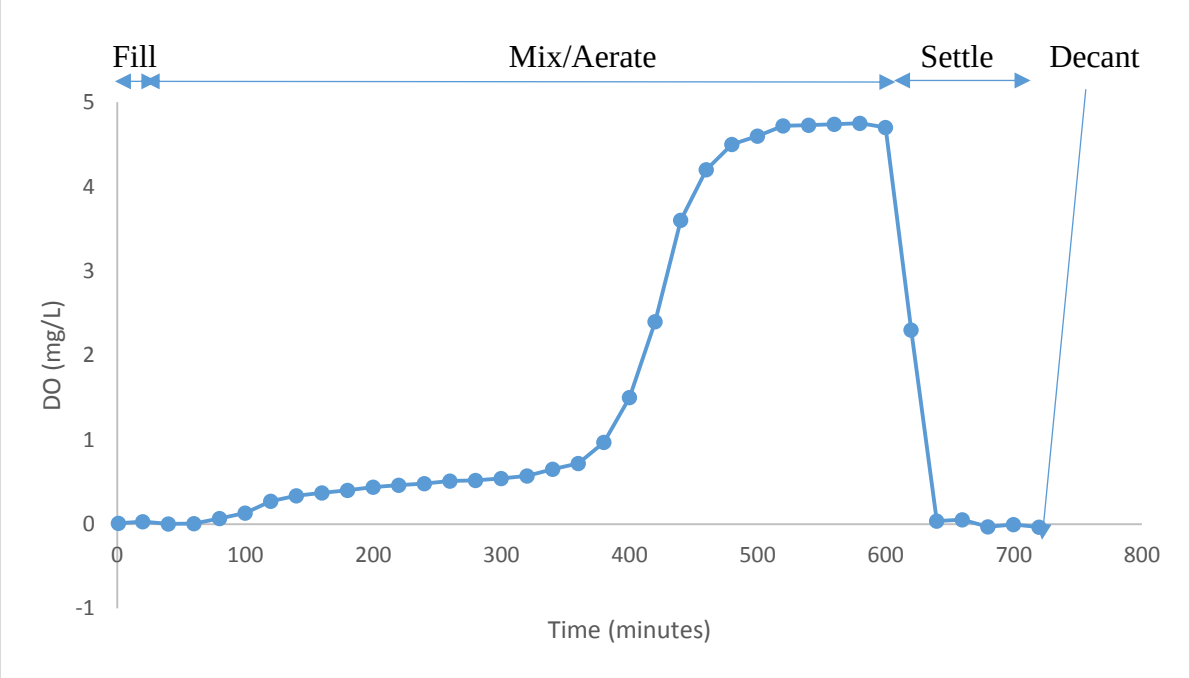


Figure 4. 6. Typical DO curve for one complete 24-h cycle in an aerobic SBR reactor

Therefore, it can be determined from all of the above results that the required parameters for aerobic sequencing batch reactor such as suspended solids, COD removal, ORP and DO attained stable conditions as mentioned in the experimental design (Table 3.2).

4.2 Evaluate the Degradability of the Compound (Phase II and III)

4.2.1 Degradation of commercial clopyralid in the SBR (Phase II)

After collecting the baseline data, commercial clopyralid was introduced into the SBR at an initial concentration of 50 mg/L. The first sign of degradation appeared in the reactor after one day and further to this, every day thereafter the amount of clopyralid was measured in the influent as well in the effluent as shown in Figure 4.7. It was observed from Figure 4.7 that after 5 days 98% of the clopyralid had been degraded by a very low effluent concentration i.e. 2 mg/L and the removal of clopyralid reflects the consistency in their degradation pattern after 5 days. This is in contrast with the commonly reported observation that biomass exposed to xenobiotics requires a certain time period (typically 20 to 50 days for aerobic systems) to induce the necessary enzymes for xenobiotic biodegradation (Celis et al, 2008). However, few research suggested that the acclimation of microorganisms on a xenobiotic compounds depends upon the microbial mechanisms of induction of enzymes (Ascon-Cabrera & Lebeault, 1993; Linkfield, 1989; Wiggins et al., 1987). Hence, it can be predicted that possibly activated sludge from Bromley contain those enzyme which have capability to degrade the clopyralid on first day of introduction.

Afterwards, track studies of clopyralid were performed and the results are shown in Figure 4.8. Results of track studies for the initial concentration of 50 mg/L indicated that 90% of the clopyralid was removed in the first two hours and the pesticide was essentially eliminated by the end of the cycle. A similar pattern was observed when a higher concentration of clopyralid (100 mg/L) was introduced to the reactor (Figure 4.8), thus the first objective was considered to be achieved (namely that clopyralid could be degraded aerobically in a wastewater). This pattern is also similar to that shown by the COD removal as described in Section 4.1.

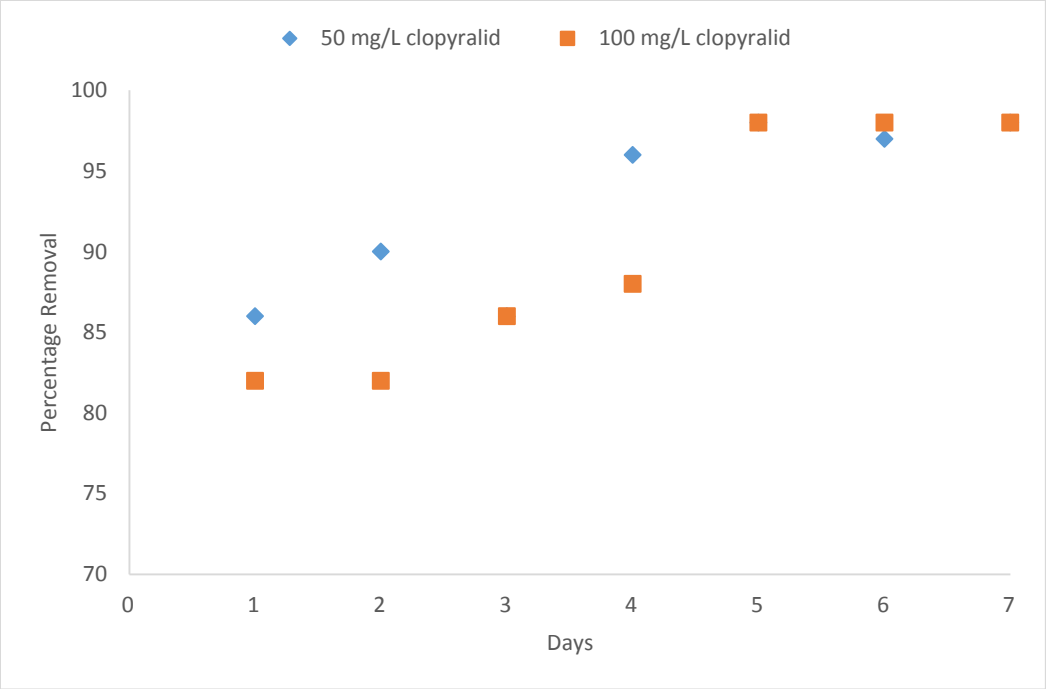


Figure 4. 7. Clopyralid degradation pattern in the SBR for first 7 days

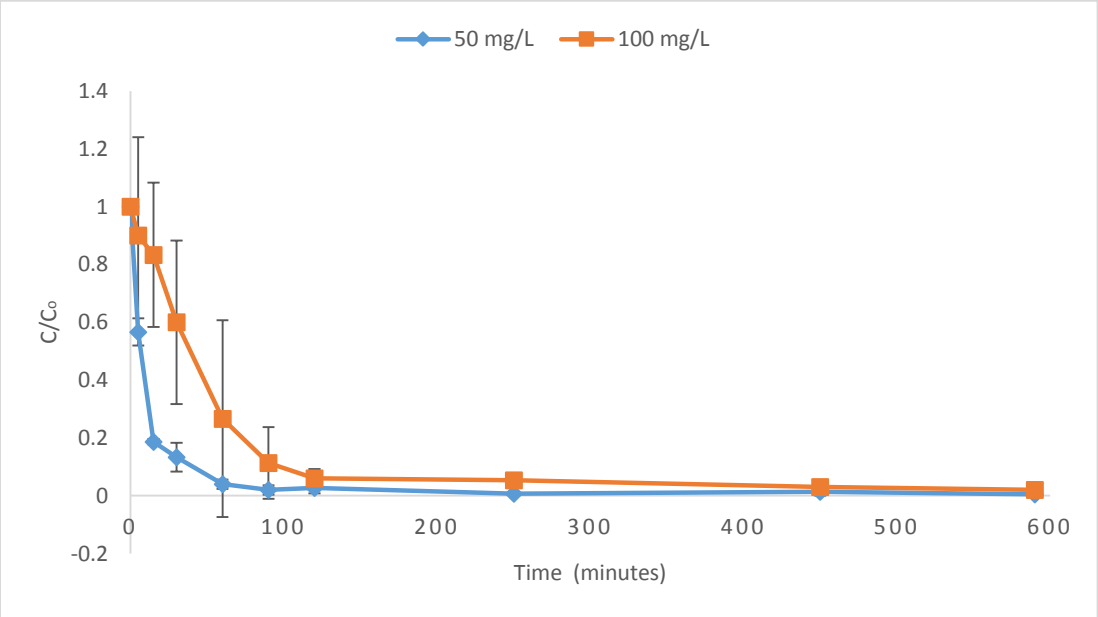


Figure 4. 8. Normalized curve for commercial clopyralid degradation in the SBR

Despite the excellent degradation of clopyralid, track studies of COD (at the initial concentration of 50 mg/L of clopyralid) revealed that the final COD concentration was still very large (i.e. 150 mg/L). Since the total initial COD concentration in the reactor was 490 mg/L, out of which the COD contributed by 50 mg/L of clopyralid was only about 60 mg/L (as measured in the lab); it

was thought that the large residual COD was due to the presence of other compounds in the commercial formulation of clopyralid such as monoethanolamine salt, isopropyl alcohol and polyglycol, which are reported as major additives in the formulation of commercial clopyralid (Villavazo, 2010). Moreover, research on the aerobic biodegradation of MEA by Kim et al. (2010), also reported that during degradation of MEA, the COD values in the effluent were high. Independent measurements of the 50 mg/L clopyralid formulation showed an approximate COD value of 900 mg/L, meaning the contribution to the COD by these additives was substantial.

It can be further observed from Figure 4.9 that the introduction of 100 mg/L of commercial clopyralid to the SBR resulted in an influent COD concentration of 680 mg/L and an even higher residual COD (250 mg/L). Furthermore, for both concentrations of clopyralid (i.e. 50 mg/L and 100 mg/L) the COD degradation efficiency was ≥ 65% but not 95% as compared to the reactor without clopyralid.

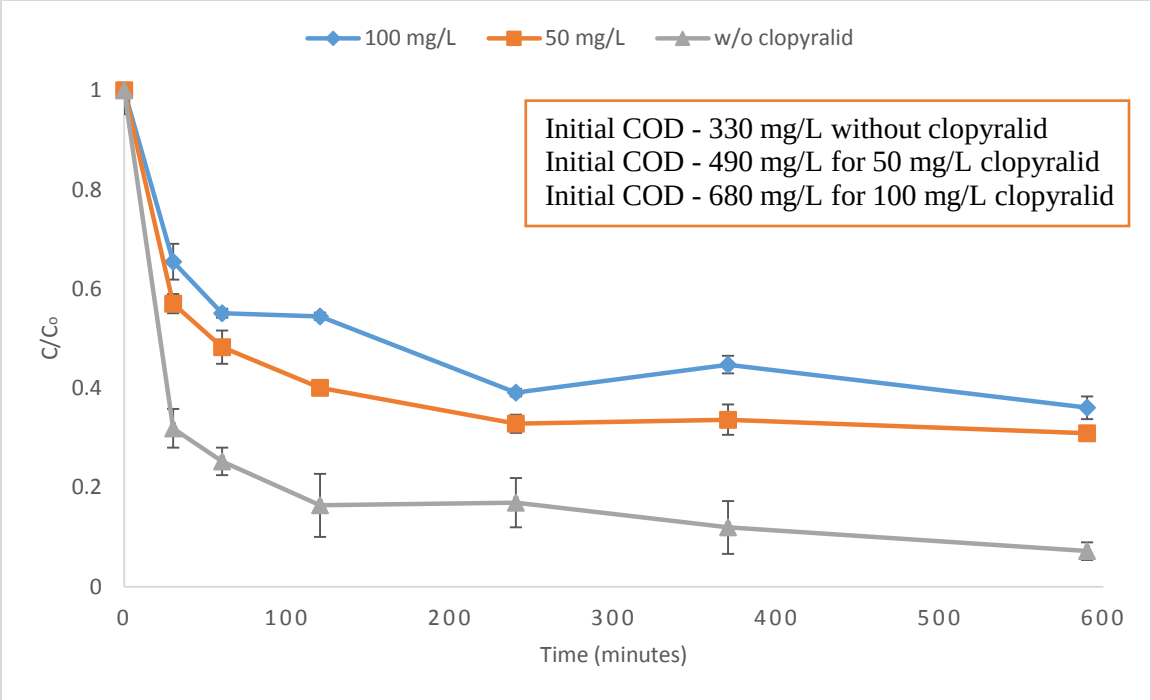


Figure 4. 9. Normalized curve for COD degradation in the presence or absence of commercial clopyralid solution in the SBR

The theoretical COD of all the significant compounds in the clopyralid formulation, i.e. monoethanolamine, polyglycol and isopropyl alcohol along with clopyralid, were calculated. The

results suggest that 50 mg/L of each of these compounds would contribute 115, 80, 120 and 60 mg/L of COD, respectively. However, the initial measured COD in the laboratory formulation at 50 mg/L of active clopyralid compound was around 900 mg/L (out of which only 60 mg/L of COD was contributed by clopyralid). Hence, it can be assumed that there must also be further formulation compounds in addition to monoethanolamine, polyglycol and isopropyl alcohol.

To decrease the residual COD (associated with the formulation compounds), the HRT was increased from 24 to 34 h, but the COD was then measured as 400 mg/L for the 100 mg/L of clopyralid concentration, up from 250 mg/L at an HRT of 24 h. A possible reason for increasing residual COD with HRT was biomass lysis. Further increase in the HRT to remove the residual COD was deemed to be impractical; thus from there on, experiments concentrated on investigating whether effluent COD could be reduced, if pure clopyralid was used.

That is, it was decided to use a pure form of clopyralid for further experiments (in batch reactors) to eliminate the interference of additives in subsequent experiments, since it was difficult to identify the COD content in the commercial solution because of proprietary interests. As such, the degradation of the analytical standard of clopyralid was examined in smaller (500 mL) batch reactors due to the financial limitation of using pure clopyralid.

4.2.2 Removal of clopyralid under abiotic conditions

Studies suggested that bio-adsorption is basically dependent on these n-octanol-water partition coefficient or logKow value (ChemsafetyPro, 2018). LogKow is unitless and defined as the ratio of substance concentration in octanol and in water. In other words, logKow is an indication of how easily a chemical compound dissolves in water or adsorbs onto organic particles (activated sludge in this case). Previous research indicates the logKow value is an important parameter to predict the distribution of organic substances in water, soil, air and biota (ChemsafetyPro, 2018). Substances with >4.5 logKow value tend to adsorb more readily on organic matter (such as biomass) and have low affinity for water. The reported logKow value of clopyralid is 1.06 (Pubchem, 2018) which is very low in comparison to 4.5 and indicates a low chance of adsorption on biomass. Bio-adsorption of clopyralid was studied practically by two different ways as follows:

1. Volatilization of clopyralid in abiotic conditions: To evaluate the volatility of clopyralid, two different experiments were performed (one with aeration and the other without aeration) as

mentioned in Section 3.9. The results of the experiments reveals that there was no removal of clopyralid at 0, 120 and 590 mins of volatilization, under both the conditions. However, it was detected that over the 24 hours, 3-5% of clopyralid was removed. Therefore, it can be deduced that there was insubstantial loss of clopyralid through volatility and the most likely mechanism for removal was through active biomass degradation.

2. Bio-adsorption of clopyralid on biomass: This experiment was evaluated under two different conditions. In these first the biomass cell wall was ruptured after the sample collection, using ultra-sonication while in the other, the metabolic activity of biomass was inactivated by adding sodium azide at 0.2 gSAZ/gTSS (Bardot et al., 2010).

The results are shown in Table 4.1 and 4.2. When the cell wall of microbes were ruptured, they release all the adsorbed clopyralid into aqueous phase. Due to this Table 4.1 shows the clopyralid concentrations at zero minute has no drop in the placed concentrations. This is in contrast to what happened in the SBR system where there was an immediate drop in the initial clopyralid concentrations (i.e. it dropped from 100 to 87 and 50 to 41 mg/L). Consequently, it can be deduced that approximately 15 % of the clopyralid was immediately adsorbed on to the biomass. At 120 mins and at 24 h (1200 min), clopyralid was removed to 90 % and 99 %, respectively according to Figure 4.8. However, when clopyralid was measured after for the bio-adsorption test at 120 mins and at 24 h, the percentage of removal changed to 68% and 90 % for 50 mg/L of clopyralid concentrations while for 100 mg/L of clopyralid degradation changed to 78 % and 83 %, respectively. Therefore, it was determined that clopyralid was bio-adsorbed by approximately 22 % (90 minus 68) and 12 % (99 minus 90) for 50 and 100 mg/L of clopyralid concentrations in the first two hours, respectively. On the other hand, the bio-adsorption was very little in 24 hours, approximately varying from 10 – 15 % for 50 to 100 mg/L, respectively.

Table 4. 1 Removal of clopyralid for 24 hours when bio-adsorption was assessed after the cell wall of microbes was ruptured

Time	**Clopyralid - 50mg/L**	**Clopyralid - 100 mg/L**
0	50	100
120	16	22
1200	5	17

Under the second condition (when biomass activity was stopped by adding sodium azide) the clopyralid removal fluctuated as shown in Figure 4.11. The significant point however was that the removal ended up being 50 %. Initially, it was thought that all the removal would be attributed to adsorption, but this is much greater than the 15 % observed in sonication experiments. However, previous research suggests that sodium azide is only able to inactivate the metabolic activity of gram-negative bacteria, while gram-positive bacteria are mostly resistant to any bacteriostatic effects of sodium azide (Delorit, 2012). Therefore, it can be anticipated that not all of the 50 % of clopyralid was adsorbed by the bacteria present in the activated sludge, but only some of the clopyralid was degraded by gram-positive bacteria.

Table 4. 2 Removal of clopyralid for 24 hours when bio-adsorption was assessed by putting sodium azide in the batch reactor and deactivating the microbial activity

Time	Clopyralid - 50mg/L	Clopyralid - 100 mg/L
0	50	100
30	42	81
60	35	78
90	38	55
120	41	66
600	25	55
1200	22	53

In summary, it is estimated that active biomass is able to degrade most of the clopyralid present in the system; however, 10 – 15 % of the clopyralid may have accumulated on the biomass without being degraded completely.

4.3 Aerobic Batch Tests (Phase III)

4.3.1 Degradation of the pure form of clopyralid in batch reactors

To evaluate the aerobic degradation of clopyralid in a batch reactor, as mentioned in section 3.10 500 mL of biomass was collected from the 20-L SBR and settled for 45 minutes. Following that settling period, 250 mL of supernatant was discarded. Batch tests were run together in triplicates by using an initial concentration at 50 mg/L of the pure form of clopyralid mixed with 250 mL of synthetic feed and 250 mL of biomass. Afterward, the initial concentration of clopyralid was increased from 50 to 300 mg/L with various concentrations in between, such as 100, 150, 200, 225

and 250 mg/L. Each concentration was run as a separate series of batch tests. The results obtained are shown in Figure 4.12.

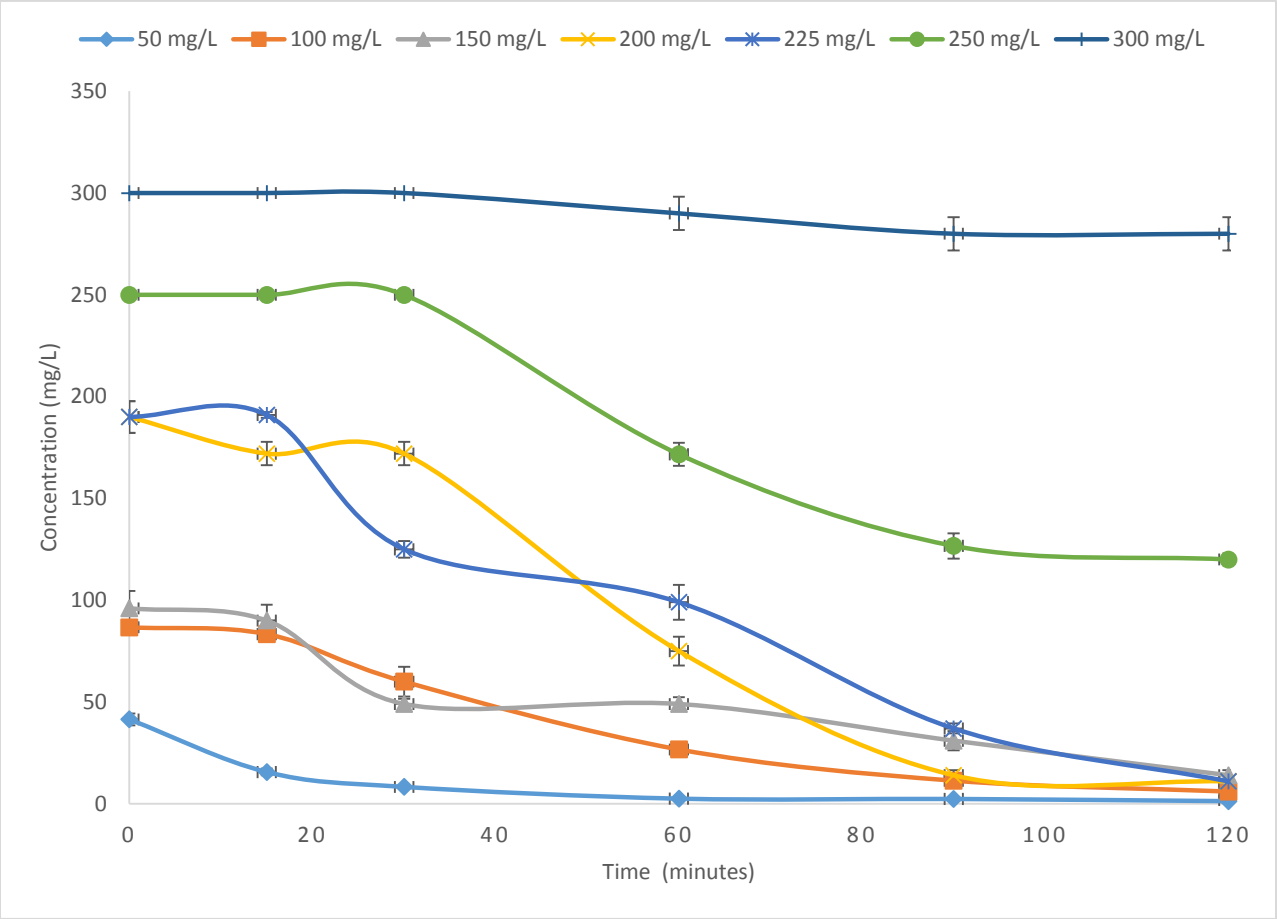


Figure 4. 10. Clopyralid degradation in first 120 minutes

The results show that under aerobic conditions, the majority of pure clopyralid for all initial concentrations except (250 and 300 mg/L) was removed in the first 2 h. As can be expected after settling for 45 minutes, the bacteria were very likely carbon deficient and started utilizing influent feed/substrate rapidly to provide energy for subsequent catabolic reactions. During the remaining 22 h of the batch tests (beyond the figure time scale), less than 10% degradation occurred for the 50, 100, 150, 200 and 225 mg/L concentrations. Figure 4.12 shows that the removal efficiency was affected and reduced from 99% to 40% with an increasing clopyralid concentration from 50 mg/L to 250 mg/L. Around 25 % and 5 % clopyralid degradation occurred for the 250 mg/L and 300 mg/L concentrations, respectively. Therefore, it seems for the conditions of this research, the

second objective was achieved; namely, that the maximum threshold level for complete degradation of pure clopyralid was approximately 250 mg/L (in a 120-minute timeframe). However, the degradation efficiency for 2,4-D having similar physical properties as clopyralid is double (i.e. threshold level of degradation is 500 mg/L) under aerobic reactors (Celis et al., 2008), which implies that clopyralid is a difficult compound to treat.

4.3.2 Removal of COD in batch reactors

In parallel with the clopyralid degradation, the removal of COD was also studied for the aerobic batch reactors and the results are shown in Figure 4.13. In the reactor, the COD value was mainly contributed by two sources i.e. clopyralid (60 mg/L) and synthetic feed containing acetate (i.e. 320-350 mg/L). However, the COD values due to the synthetic feed varied and was sometimes as low as 250-270 mg/L even one day after its preparation (as mentioned in Section 3.1). As such, when the batch reactors were not run on the same day when the feed was prepared, then the COD associated with the synthetic feed was reduced. The loss in COD associated with synthetic feed was calculated before mixing the feed with the pure form of clopyralid. The calculated COD values for synthetic feed were 240, 248, 256, 283, 278, 291 and 276 for the different batch tests which later contained clopyralid concentrations of 50, 100, 150, 200, 225, 250 and 300 mg/L, respectively. Moreover, when this feed containing clopyralid was placed in the reactor, the COD values were further reduced from the expected COD values presumably due to the dilution effect.

Figure 4.13 indicates that the initial COD value increased as the clopyralid concentrations increased from 50 mg/L to 300 mg/L. However, the difference in the initial COD value did not quite match the COD contributed by every 25-50 mg/L clopyralid increment (i.e. 60 mg/L at the 50 mg/L level). Although other parameters affect the COD values such as the age of synthetic feed and dilution, the initial rise in COD values appeared to be mainly due to the increasing clopyralid concentration in the reactor.

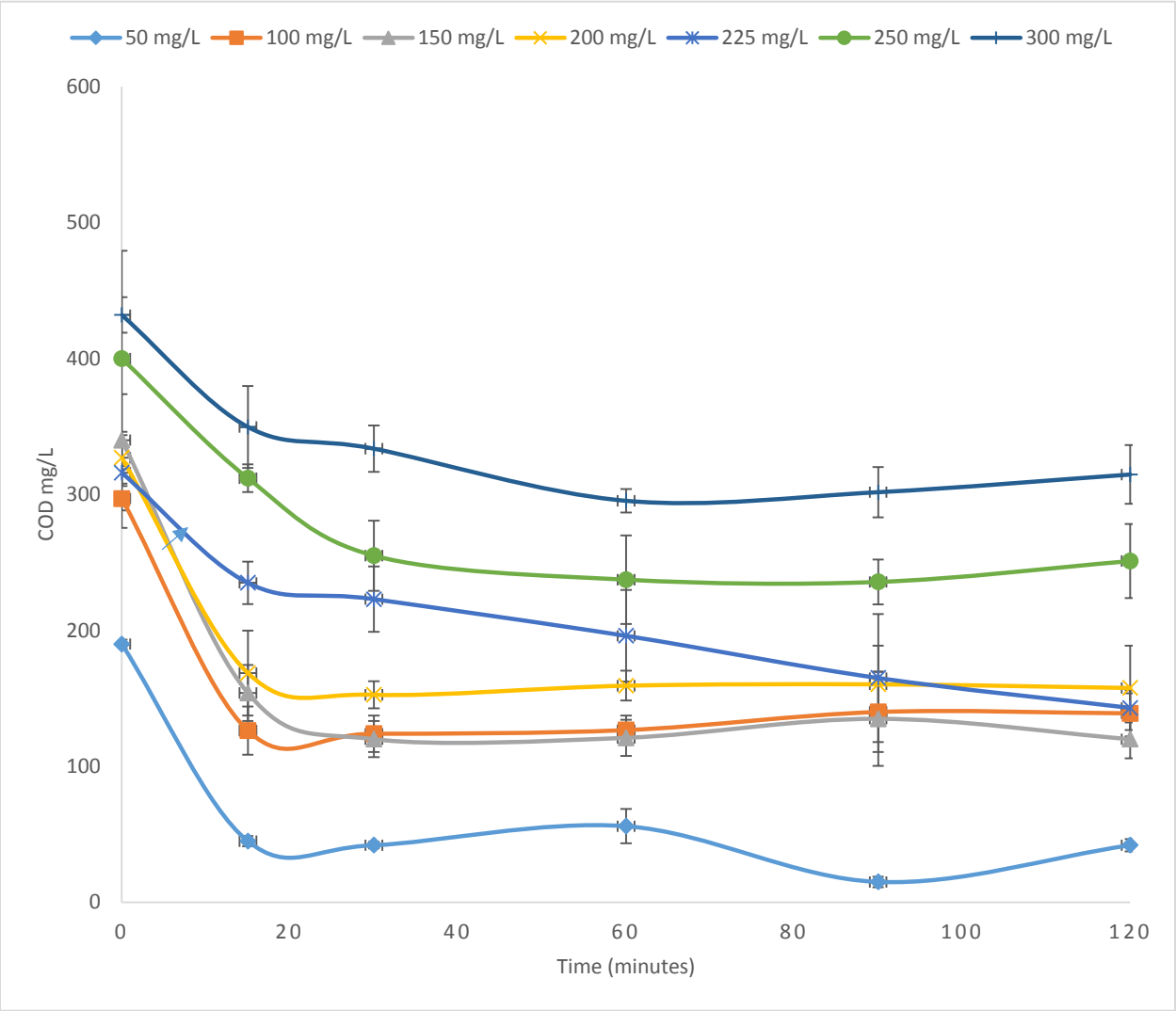


Figure 4. 11. COD degradation in first 120 minutes in batch reactors

Figure 4.13 shows that residual COD values increased as the clopyralid concentrations increased. This was unexpected since pure rather than commercial clopyralid was being used (i.e. there were no additives that could contribute to COD as was the case in the commercial formulation). As seen in Figure 4.13 the COD degradation for the first 120 minutes reduced from 85 % to 55 % with increasing initial clopyralid concentration from 50 mg/L to 200 mg/L; while on the other hand, for 225, 250 and 300 mg/L of clopyralid, the degradation dropped to 50 %, 30 %, and 25 %, respectively. Moreover, after 24 hours, the COD values increased slightly (data not presented) (in comparison to the COD values at 120 minutes). Since, there were no additives contributing to the declining COD, a possible explanation may be that the clopyralid was not degraded completely

and; instead, it was being converted into some other intermediate compounds (extracellular soluble microbial products (SMPs)).

This could have been also happening in the previous SBRs results; however, the contribution to the COD from the SMPs may have been swamped by the COD associated with the commercial additives. Alternatively, given that the SBRs were run under acclimated conditions, it is possible that any SMPs generated were eventually degraded.

Finally, one other reason could be the toxic effect of clopyralid on microbial communities present in activated sludge, which can create the biomass lyses (Jung et al., 2004; Divyalakshmi et al., 2015). The biomass lyses may also contribute to residual COD. Toxicity effect of clopyralid was also studied on *V. fischeri* bacteria by Berberidou et al., (2016), which proposes that clopyralid induced 83% inhibition within 15 min of incubation.

To identify what was happening, further experiments were performed (Chapter 5) which included the measurement of intermediate compounds (also including the presence of SMPs), the toxic effect of clopyralid on biomass (by examining the oxygen uptake rates of microorganisms) and measuring other organic compounds or reduced inorganic species such as TOC, nitrates, nitrites, and ammonia.

4.3.3 Clopyralid degradation in the absence of acetate

Clopyralid degradation was studied again using batch test for different concentrations of clopyralid i.e. 50, 100, 150, 225, 250 and 300 mg/L. However, in this set of batch tests, the synthetic feed only consisted of clopyralid dissolved in deionized water, meaning the only source of carbon for the biomass was clopyralid, as per the requirement of objective three. The clopyralid degradation is shown in Figure 4.14. The graph reveals that the clopyralid suddenly dropped from an actual injected value in the first instance i.e. time zero and this drop was substantially larger in comparison to when the synthetic feed contained acetate. It was theorized that this large drop was caused by some carbon-deficient bacteria suddenly adsorbing as much of the new substrate as they could.

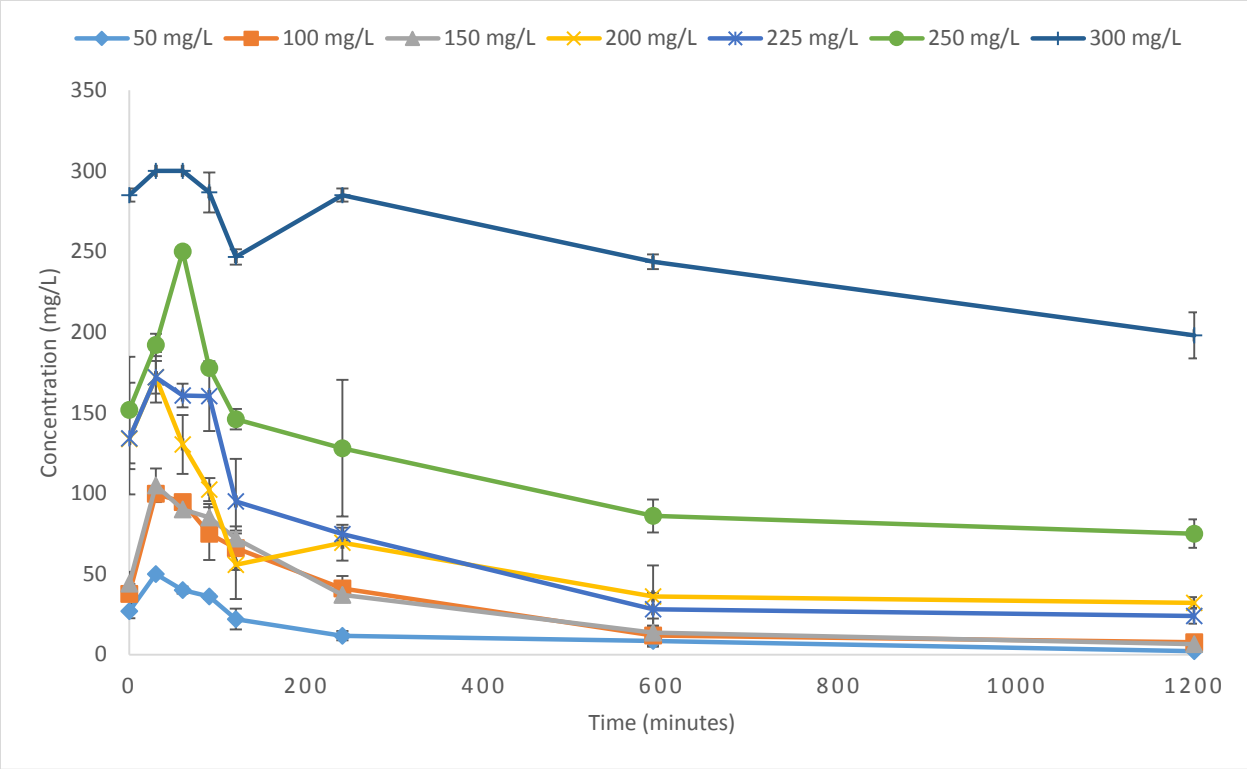


Figure 4. 12. Clopyralid degradation in the batch tests in the absence of any other carbon source

However, after about 30 minutes the initial value increased for all concentrations of clopyralid. This increase would presumably be because once the clopyralid was found to be toxic to the bacteria, they would release it again into the aqueous phase as they lysed. After about 60 minutes, the clopyralid started to degrade and it was assumed that during this lag time, the remaining bacteria adapted to a new environment consisting of a potentially toxic carbon source with no other readily-biodegradable carbon source such as acetate. It was observed that essentially full degradation was obtained after 24 h (data not presented) for the first three clopyralid concentrations i.e. 50, 100 and 150 mg/L; however, the degradation efficiency varied afterwards; 85 %, 90 %, 70 % and 35 % for clopyralid concentrations 200, 225, 250 and 300 mg/L, respectively. In contrast to the case, where acetate was part of the synthetic feed and essentially full degradation was achieved in the first 120 minutes, without acetate it took approximately 600 minutes to achieve maximum degradation in all case (including clopyralid concentrations).

4.3.4 COD degradation in the absence of acetate

The COD degradation for the conditions mentioned in Section 4.3.3, were also plotted and the results are shown in Figure 4.15. The graph reveals that the influent COD was quite high (450-800 mg/L) for different clopyralid concentrations, which again was completely unexpected because the batch reactor only had pure clopyralid in it and each 50 mg/L clopyralid concentration contributes about 60 mg/L in COD (as measured in the deionized water). A sensible explanation consistent with the previous paragraph appears to be that the addition of clopyralid immediately lysed some of the bacteria converting the end-products to SMPs contributing to a large influent COD. Later on (i.e. at times > 60 minutes) the high COD value could also be attributed to the formation of substrate by-products (which again are part of SMPs).

It was also noted in Figure 4.15 that the influent COD increased with the increasing concentration of clopyralid. However, the difference in the COD value does not vary exactly as per the clopyralid concentration; for example, COD values for 50, 100 and 300 mg/L of clopyralid concentration were 445, 519 and 779 mg/L, respectively. As per these values, the COD associated with biomass lysis contributed to 385, 339 and 419 mg/L for clopyralid concentrations of 50, 100 and 300 mg/L, respectively. It is possible that in each batch test the communities of particular micro-organisms can vary (batch tests were performed on different days). In such cases, it is suspected that some of the biomass affected by clopyralid are present in greater quantities than others (who are not affected by clopyralid). Due to this, as the clopyralid concentration increased from 50 to 150 mg/L, the COD release by biomass lysis decreased by comparison. However, the COD increased when 300 mg/L of clopyralid was injected, which might be because of the toxic effect of clopyralid to some bacteria.

In contrast, the effluent COD continued to build up with the increasing clopyralid concentration; possibly because of a lack of a healthy carbon source in the system, which means some micro-organisms started dying and releasing COD into the effluent in the system.

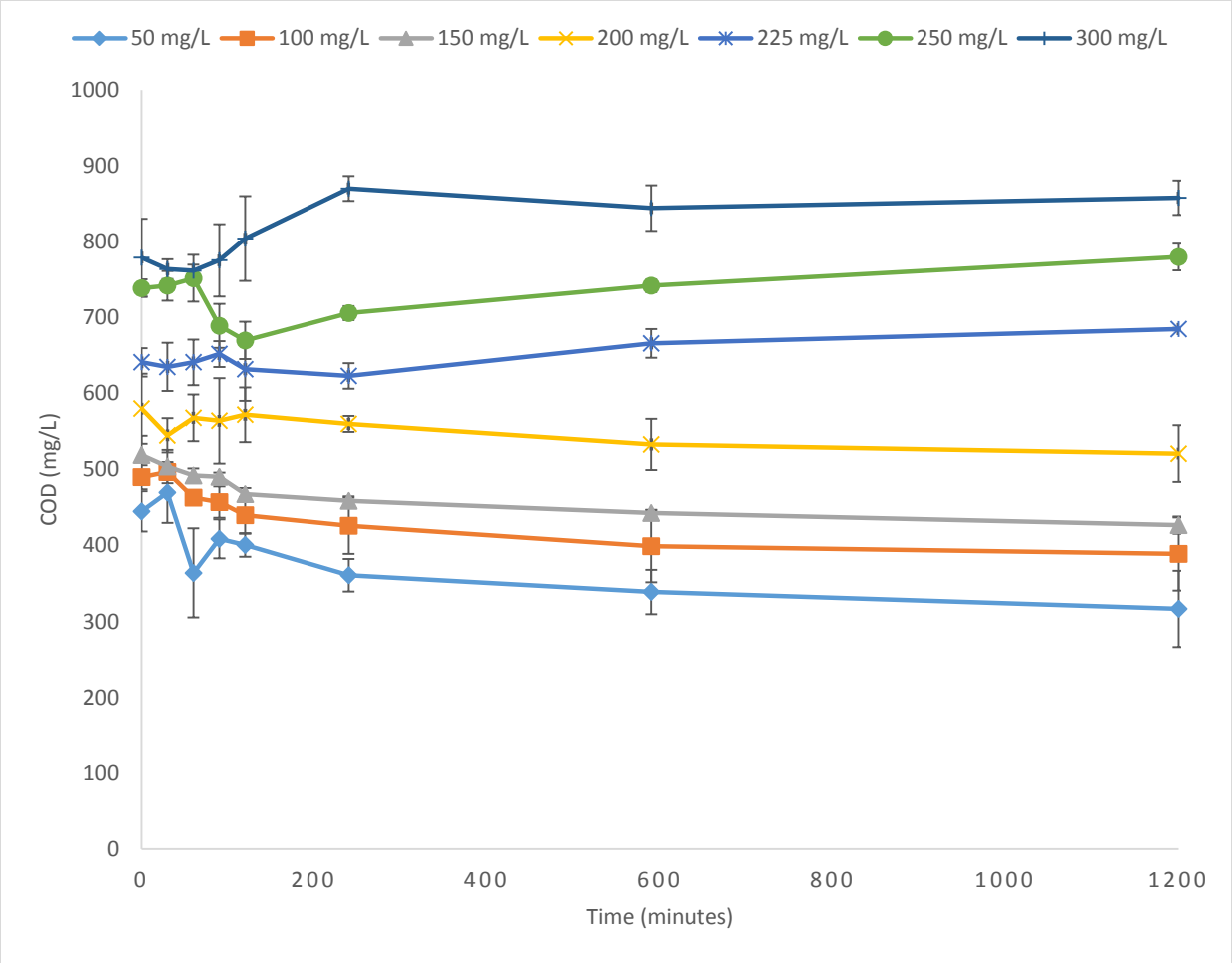


Figure 4. 13. COD degradation in the batch tests in the presence of clopyralid only and in the absence of any other carbon source

CHAPTER-5 CLOPYRALID DEGRADATION PATTERN AND MICROBIAL ACTIVITY IN THE REACTOR

5.1 Kinetic Modeling for Clopyralid Degradation (Phase IV)

5.1.1 Inhibition of substrate (clopyralid) degradation

For the substrate inhibition studies, the specific substrate utilization (i.e. consumption) rate r_s (day^{-1}) was calculated by using the following equation:

$$r_s = -\frac{dS}{dt\ X} \tag{5.1}$$

Where, X and S are the biomass and substrate (clopyralid) concentration in mg/L at time t (day).

Experimental data was collected for three conditions, however the specific utilization rate of the clopyralid was calculated for only the final two conditions as follows: The experimental conditions were (1) acetate-only additions to the batch reactor (2) clopyralid and acetate additions to the batch reactor as the source of carbon, and (3) clopyralid only additions to the batch reactor as the source of carbon.

In the first set of experiments (when only acetate was added to the reactor) the sole source of carbon was acetate and therefore it was the only compound that contributed to COD. Tests revealed that the COD was negligible after 24 h. This means the microorganisms were able to degrade acetate at a concentration of 425 mg/L. As such, the acetate was held at that concentration in the second set of experiments.

The results from the second and third experiments are shown in Figure 5.1 and indicate that a clopyralid concentration between 50 and 225 mg/L did not show any inhibitory effect on the biodegradation of clopyralid. However, when the clopyralid concentration was increased to 250 mg/L, the specific utilization rate dropped rapidly, approaching zero when the clopyralid concentration increased to 300 mg/L in both the cases. Brinton and Blewett (2004) observed similar trends for clopyralid degradation in the compost i.e. clopyralid degradation lower with high initial concentrations. This seems to indicate a toxicity effect of clopyralid on the biomass when the concentration gets too large. Moreover, this happens whether acetate is present or not; meaning

there is no apparent advantage given to the microbes in the presence of a rapidly-biodegradable substrate such as acetate, when the clopyralid concentration is high.

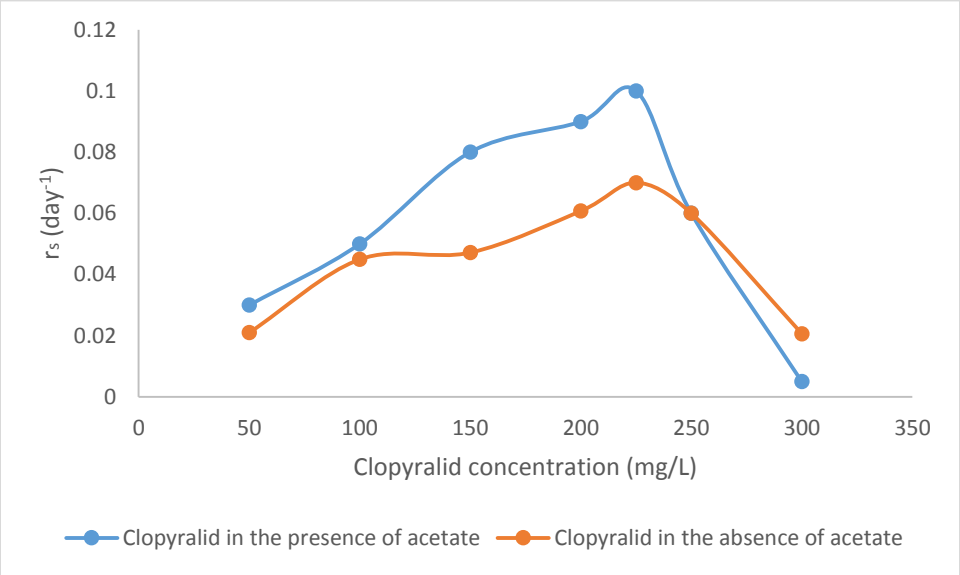


Figure 5. 1. Specific utilization rate of substrate clopyralid in the presence and absence of acetate in the batch reactor.

5.1.2 Mathematical modeling of the biodegradation of clopyralid

For mathematical modeling, different substrate consumption kinetic models which have been historically used to characterize substrate biodegradation kinetics were tested (Tazdait et al., 2014). Building on the literature review, the models considered were the Teissier (1970) and Aiba-Edwards (1968), Yano and Koga (1969), Luong (1985 & 1987), the Andrew (1968) and the Webb (1963) models. The coefficient of determination (R^2) was used to identify the kinetic model that could be best fitted to the experimental data. An R^2 value close to '1' indicates a good relationship between the dependent and independent variables of the equation and the Luong model (1985) had the highest R^2 value of 0.98 using the proprietary software (Polymath 6.1), with all other models having R^2 values of less than 0.7. It was noted from previous research (Mulchanadani et al., 1989), that the Luong model 1985 in equation (2.8) gives the best fit when the substrate consumption rate is stimulated at low concentrations and inhibited at high concentrations, which matches the experimental conditions of this research (Fig 5.1).

Figures 5.2 and 5.3 overlays the mathematical model on the experimental specific-clopyralid consumption rate data at different clopyralid concentrations in the presence and absence of acetate,

respectively. The values of the independent variables ($r_{s\ max}$, K_s and n) for the non-linear curve were determined by fitting the substrate inhibition data in equation (2.8). The values extracted were $r_{s\ max}$ = 0.22 mg/L/day and n = 7 for both the cases while, K_s = 250 mg/L and 450 mg/L, for Figures 5.2 and 5.3, respectively. Hence, this analysis clearly indicates that the clopyralid has an inhibitory effect, when the concentration is higher than about 250 mg/L.

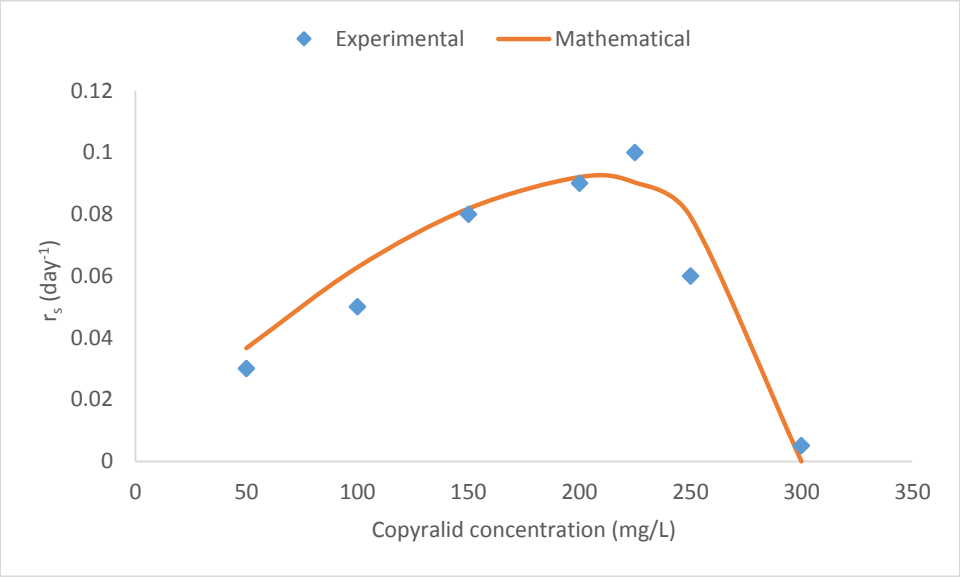


Figure 5. 2. Kinetic model for the clopyralid consumption rate in the presence of acetate

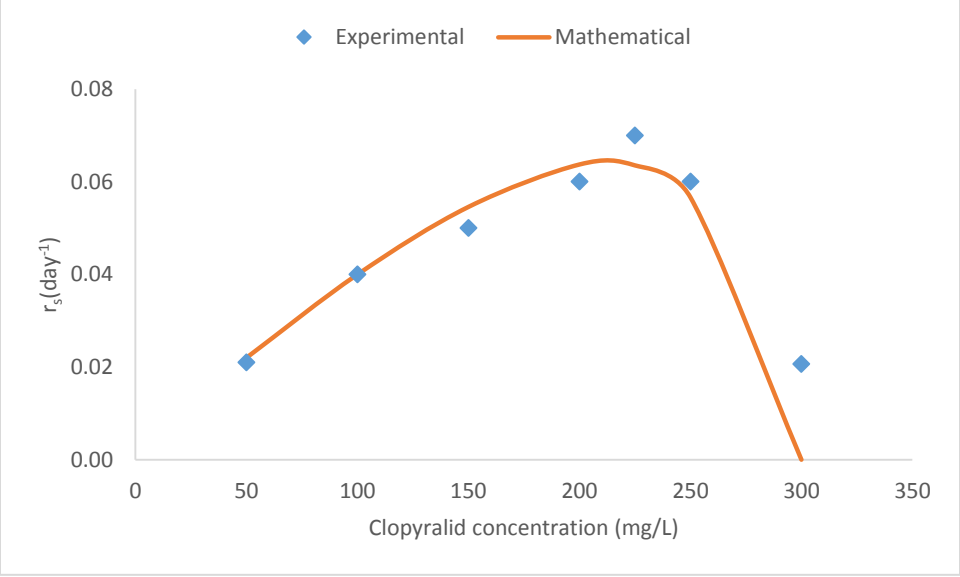


Figure 5. 3. Kinetic model for the clopyralid consumption rate in the absence of acetate

A search of the relevant literature revealed no other modelling studies carried out on the inhibition of clopyralid biodegradation in water, nor modelling of the degradation of compounds that have similar physic-chemical properties as clopyralid. However, Tazdait et al. (2013) using an Andrews (1968) model indicated very high K_s values (2318 mg/L) in their research on melathion degradation. Large values of K_s, suggest that the biomass undertaking the degradation had little affinity for degrading the pesticide. Another way of saying this is that the pesticide under consideration is a recalcitrant organic.

5.2 Analysis of Inorganics and Organic Compounds (Phase V)

To achieve the objective 5, this section provides the details of all inorganic as well organic species that contribute to the residual COD such as TOC, nitrate, nitrites, ammonia, transformation products and SMPs.

5.2.1 Analysis of total organic carbon (TOC)

TOC was carried out to investigate the proportion of organic compounds in the effluent COD. Examination of these compounds had only been done for two clopyralid concentrations i.e. 100 and 200 mg/L in the presence of acetate, as it was observed that the rate of degradation for clopyralid in the batch reactor was the maximum at a value of 100 mg/L of initial clopyralid concentration. However, the rate of degradation started to decrease after increasing the initial clopyralid concentration past 100 mg/L.

Track studies were done for TOC removal (as compared to COD removal) and the results for the first 120 minutes (as most clopyralid had been removed in the first 120 minutes) are shown in Figures 5.4 and 5.5. As seen after 120 minutes, COD and TOC were both still present and when the clopyralid concentration in the reactor were 100 and 200 mg/L, approximately 70% COD, 60% TOC and 35% COD, 30% TOC removal was achieved in 120 minutes, respectively. There was no significant degradation of COD and TOC observed in cases of higher clopyralid concentration, although clopyralid degradation was observed at 225 mg/L concentration. However, after 24 h (data not shown), in all cases the TOC and COD increased by around 30-50 %. This increase was likely due to biomass lyses (Jung et al., 2004; Divyalakshmi et al., 2015).

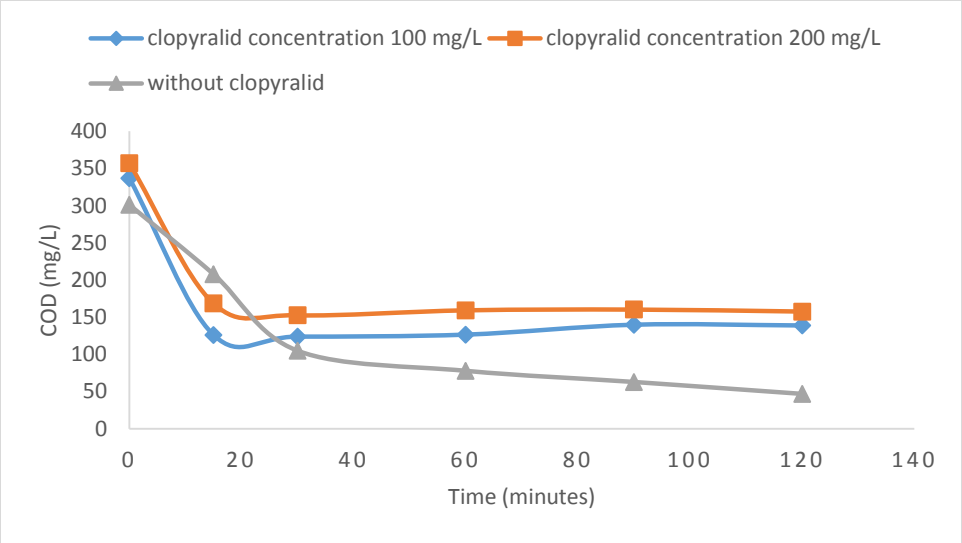


Figure 5. 4. COD removal in batch reactor for three different conditions; one with initial clopyralid concentration of 100 mg/L, second with 200 mg/L and final without clopyralid for first 120 minutes

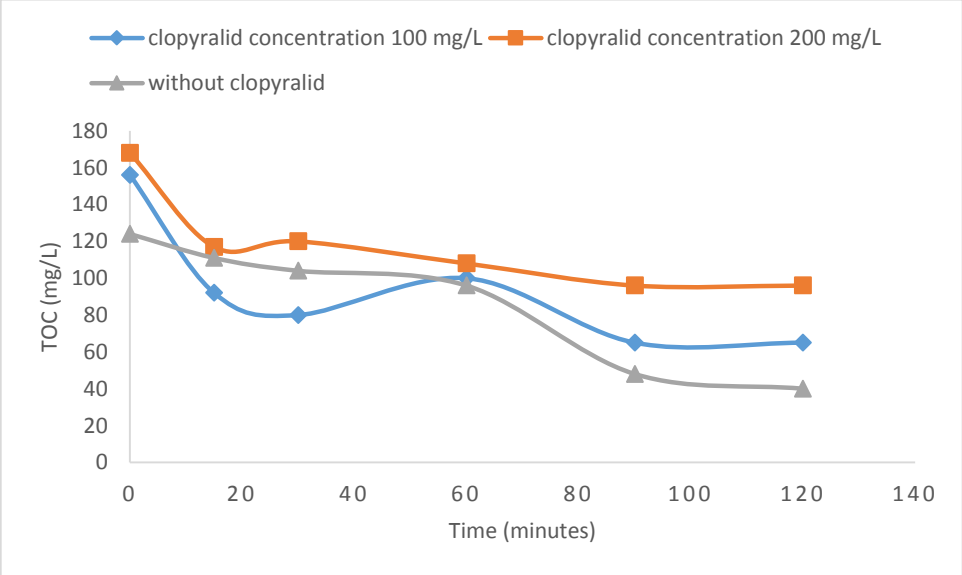


Figure 5. 5. TOC removal in batch reactor for three different conditions; one with initial clopyralid concentration of 100 mg/L, second with 200 mg/L and final without clopyralid for first 120 minutes

On the other hand, it can be estimated that approximately >85% of COD and >65% of TOC removal was achieved, when there was no clopyralid added in the batch reactor (i.e. only acetate). In contrast, COD degradation in the SBRs was reported at >95 % in Figure 4.9. This reduction

may be due to changing environmental conditions i.e. from a 20 L tank to a small 500 mL batch reactor.

5.2.2 Examination for transformation products

In parallel with the other tests mentioned above, possible transformation products formed during clopyralid degradation were also investigated using liquid chromatography and mass spectrometry (LC-MS). The absence or presence of transformation products reveals whether clopyralid undergoes complete degradation to carbon dioxide or water; or whether it is converted to some other complex by-products, which might contribute to residual COD or TOC.

The mass spectrum at the end of the 24 h batch tests associated with different conditions of clopyralid (along with the accompanying mass spectra formula reports from the MS) are shown in Figures 5.6 and 5.7. The mass spectrum report reveals the presence of different compounds by providing carbon formula along with their scores (also called intensity). The score does not provide the concentration of any compound, but instead confirms its absence or presence only. A compound with a score of 'zero' reflects impurity or contamination while a score of '100' reflects the purity of the compound.

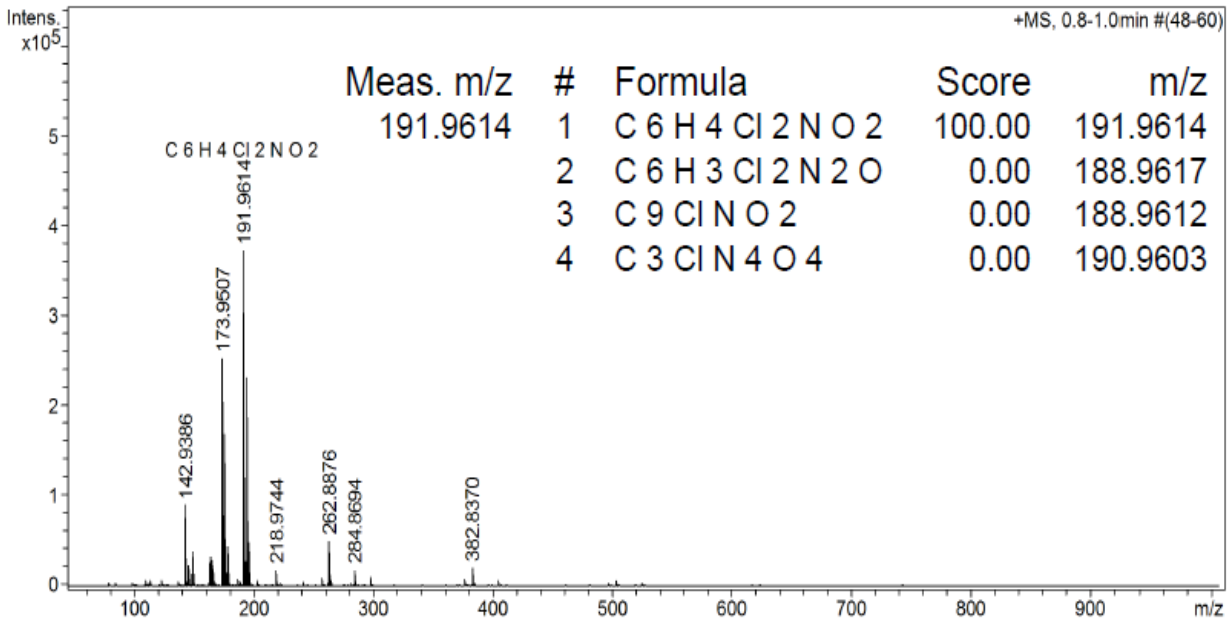


Figure 5. 6. Mass spectrum and mass spectrum formula report when only clopyralid at 100 mg/L is present in batch tests after 24 hours

It is observed from Figure 5.6 that when clopyralid is the only source of carbon in the batch reactor, after 24 h of treatment, clopyralid ($C_6H_4Cl_2NO_2$) is still present and the concentration was about 8-12 mg/L measured by a GC-ECD detector. The results suggest that the maximum amount of clopyralid was removed with no other complex products being formed, since all other potential compounds had a score of 'zero'. Notwithstanding the fact that no complex products were formed when clopyralid was used as the only carbon source, as discussed previously the residual COD was still very large (approximately 500 mg/L).

By way of contrast, it can be observed from Figure 5.7 that (at the end of 24 hr) some complex compounds appeared along with residual clopyralid. The measured concentration of clopyralid was around 1-2 mg/L, respectively; however, the transformation products were unable to be identified by using the method associated with the GC-ECD.

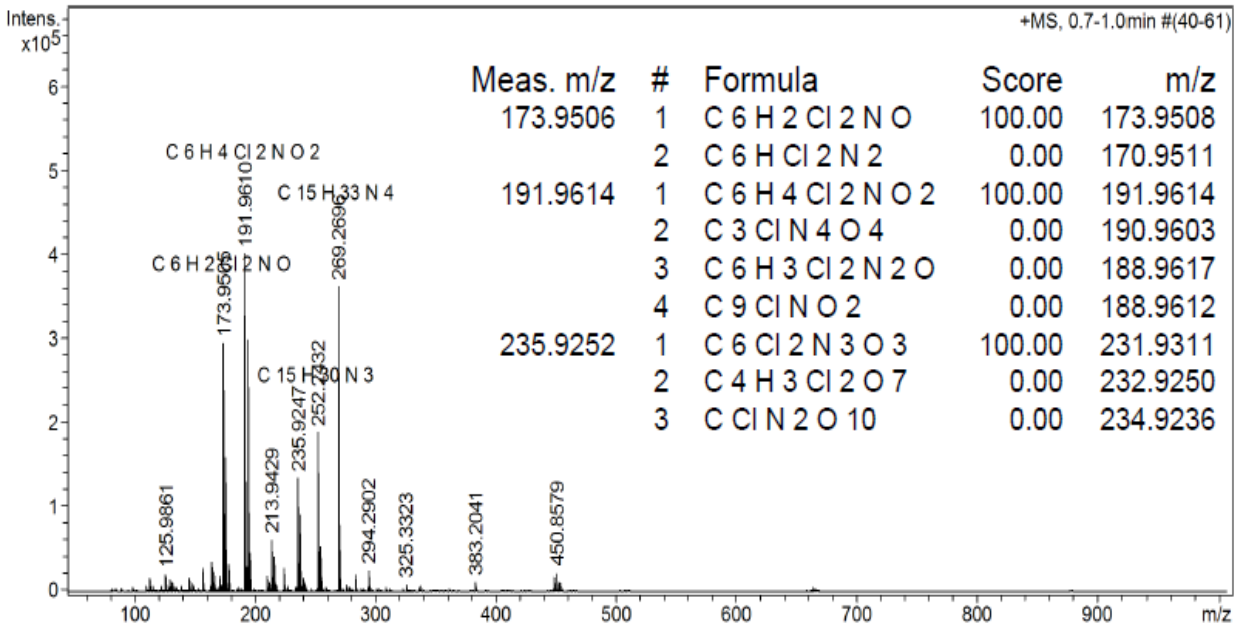


Figure 5. 7. Mass spectrum and mass spectrum formula report when clopyralid at 100 mg/L concentration and acetate both are present in batch tests after 24 hours

The results seem to indicate that when clopyralid and acetate are both added as source of carbon then complex compounds are formed by the interaction between acetate and clopyralid. Theoretically, these should contribute to residual COD; however, the residual COD was less than that observed with clopyralid only (i.e. the residual COD was around 130 mg/L as mentioned previously). These observations imply that clopyralid degrades well in both cases; however the

presence of an additional, readily biodegradable substrate enhances the degradation of clopyralid and somehow also acts to decrease the residual COD.

This implies that the residual COD most probably is derived from biomass lysis because of the general toxic effect of clopyralid on biomass. That is, when clopyralid is added as the main carbon source, the biomass has no option but to gain energy from toxic compounds which causes many bacteria to lyse and contribute to COD through extracellular soluble microbial products (SMPs). However, when both acetate and clopyralid are added together, then the biomass are able to adapt to the new environment by using the readily-degradable carbon source first, resulting in less overall residual COD.

5.2.3 Soluble microbial products (SMPs)

The presence or absence of SMPs was determined by using UV-Vis spectrophotometry, with the term SMP being defined as any group of organic compounds that are released into solution from substrate metabolism and biomass decay. As such, SMPs are compounds such as proteins, polysaccharides, amino acids, humic acids, antibiotics, extracellular enzymes and structural componenets of cells etc. (Azami et al., 201). The presence of SMPs also influences other parameters that cause high effluent COD (Barker and Stuckey, 1999). The main difficulty for SMPs is their identification because there are so many different types of compounds as indicated above (Barker and Stuckey, 1999) (i.e. for measuring each type of SMPs, there is one specific technique). On the other hand, by using different wavelengths via UV-Vis spectrophotometry, 5 to 6 compounds can often be detected via a presence or absence test method.

The absorbance obtained from UV-Vis spectrophotometry for different conditions is shown in Table 5.2. It can be noted that for all conditions, the overall absorbance increased in 24 h in comparison to the beginning of the test (i.e. 15 min).

Table 5. 1 Absorbance data for the 3 batch test conditions

Wavelength	**Acetate Only**		**Clopyralid and Acetate**		**Clopyralid Only**	
nm	***15 mins***	***24 hr***	***15 mins***	***24 hr***	***15 mins***	***24 hr***
230	0.015	0.05	0.035	0.076	0.043	0.066
254	0.018	0.046	0.043	0.078	0.051	0.062
280	0.066	0.064	0.083	0.138	0.078	0.088
490	0.005	0.006	0.001	0.02	0.001	0.038
595	0.0	0.002	0.0	0.012	0.002	0.015

The absorbance at 15 min for the condition when acetate was the sole carbon source was low for all compounds associated with the wavelengths of 230, 254, 280 and 490 nm reaching zero for proteins at 595 nm. The absorbance under the same conditions increased at 24 hr, but only for the PEG (230 nm) and the amino aci measured at 254 nm. This result suggests that when no clopyralid was added (even at the end of 24 h), no proteins and carbohydrates were formed, which is probably one reason that the residual COD for this condition was not large.

However, when clopyralid was added to the reactor (both with and without acetate), the absorbance change in the proteins and carbohydrates was most notable. In contrast, the change of absorbance in amino acids and PEG is not that dissimilar to the conditions when only acetate was present in the reactor. It seems reasonable to suggest therefore that overall, the increase in SMPs which corresponds to an increase in residual COD was because of the toxic effect of clopyralid on biomass causing some biomass to lyse thereby forming SMPs.

5.3 Microbial Activities in the Activated Sludge (Phase VI)

5.3.1 Oxygen uptake rates

The toxic effect of clopyralid can be discerned through the OUR of the microorganisms (activated sludge in this case) which have been exposed to clopyralid. In biological waste treatment facilities, the rate at which oxygen consumed by microorganisms indicates the biological activity of the system with a large value of OUR indicating increased biological activity and vice versa.

To evaluate the OUR, separate batch tests were run for different conditions as follows: a batch test in the presence of acetate only, a batch test in the presence of acetate as well as clopyralid at four different concentrations i.e. 50, 100, 200 and 300 mg/L; and a batch test in the presence of clopyralid only at two different concentrations i.e. 50 and 300 mg/L. For discerning the toxicity effect throughout the cycle of 24 h, the OUR was measured twice in each batch test i.e. at the beginning (15 min) and at the end (after 24 h). The results for the 15 min OUR are presented in Table 5.3.

Table 5. 2 OUR in the batch tests under different conditions

Condition		**OUR at 15 min (mg/L/hr)**	**OUR at 24 hr (mg/L/hr)**
1	Without Clopyralid (i.e. only acetate)	75	25
2	Acetate and Clopyralid (50 mg/L)	34	21
3	Acetate and Clopyralid (100 mg/L)	34	23
4	Acetate and Clopyralid (200 mg/L)	33	24
5	Acetate and Clopyralid (300 mg/L)	14	13
6	Clopyralid only (50 mg/L)	25	10
7	Clopyralid only (300 mg/L)	14	6

A comparison of the first 15 minutes of each condition in Table 5.3 indicates that the OUR dropped by approximately half from Condition 1 (i.e. batch test in the presence of acetate only) indicating that there was an immediate effect of the clopyralid. Further to this observation, this same effect was seen in the concentrations of clopyralid from 50 to 200 mg/L. However, the OUR dropped to a fairly low rate (i.e. one-fifth the original rate) when the clopyralid concentration reached a maximum level of 300 mg/L, where no removal of clopyralid was observed (as mentioned previously). From the above observations, it is suggested that there is an initial toxic effect of clopyralid on the biomass which kills some of the microorganisms that are more susceptible to the effects of clopyralid; however, a substantial portion of the biomass survives and actively accommodates the clopyralid without a further reduction in microbial activity until the clopyralid reaches a very high concentration i.e. 300 mg/L. At that point in time, the clopyralid concentration is so large that biological activity practically ceases, so that practically no clopyralid degradation occurs.

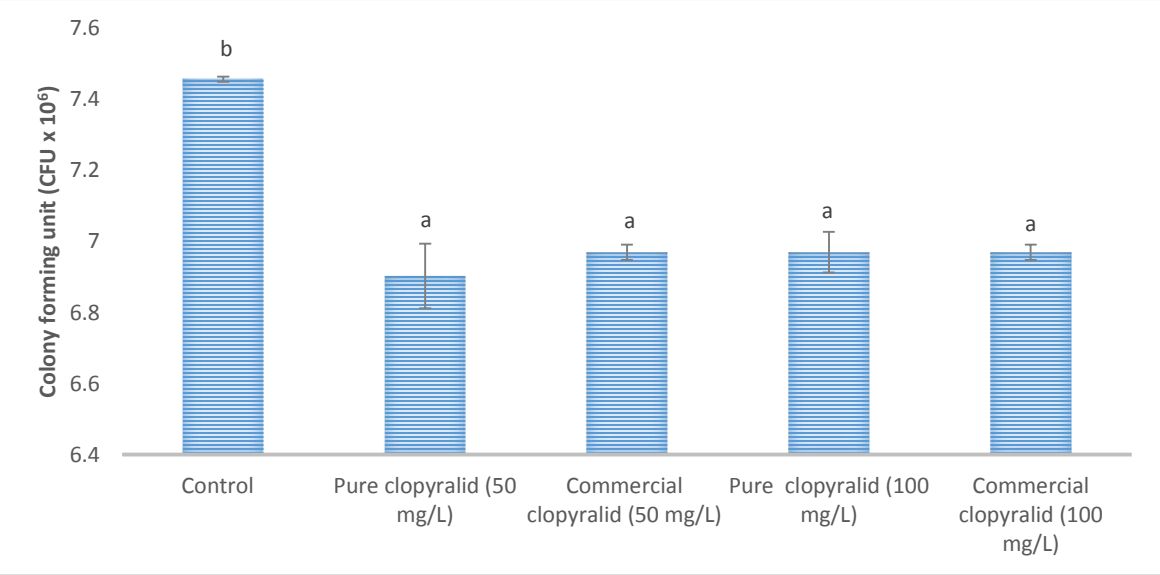
On the other hand, when clopyralid was the only source of carbon present in the batch reactor (at 50 mg/L), the OUR dropped to about one-third of Condition 1, while when the concentration of clopyralid reached 300 mg/L, the OUR dropped to about one-fifth the original rate. Based on the respective OUR values, it is clear of course that the biomass is healthier when having to metabolize acetate only (Condition 1) rather than clopyralid only (Condition 6). That is, acetate is a much more readily biodegradable substrate than clopyralid as shown by the much larger OUR and therefore it's presence may possibly directly alter the activity of biomass. Further evidence for this is shown when Conditions 2 & 6 are compared in that the presence of an easily degradable substrate allows for a higher activity of biomass as reflected by the larger OUR value (this may be a diauxic growth phenomenon). However, it is noted when Conditions 5 & 7 are compared, any beneficial effect of acetate is muted since the OURs are effectively the same.

The OUR was also measured at 24 h for all the different conditions and the pattern of results was similar to the 15 min case. In conclusion, the OUR data suggests a significant reduction in microbial activity as cells were lysed by the toxic effects of clopyralid. It seems reasonable therefore to suggest that some of the residual COD was caused by cell lysis products, therefore contributing to SMPs.

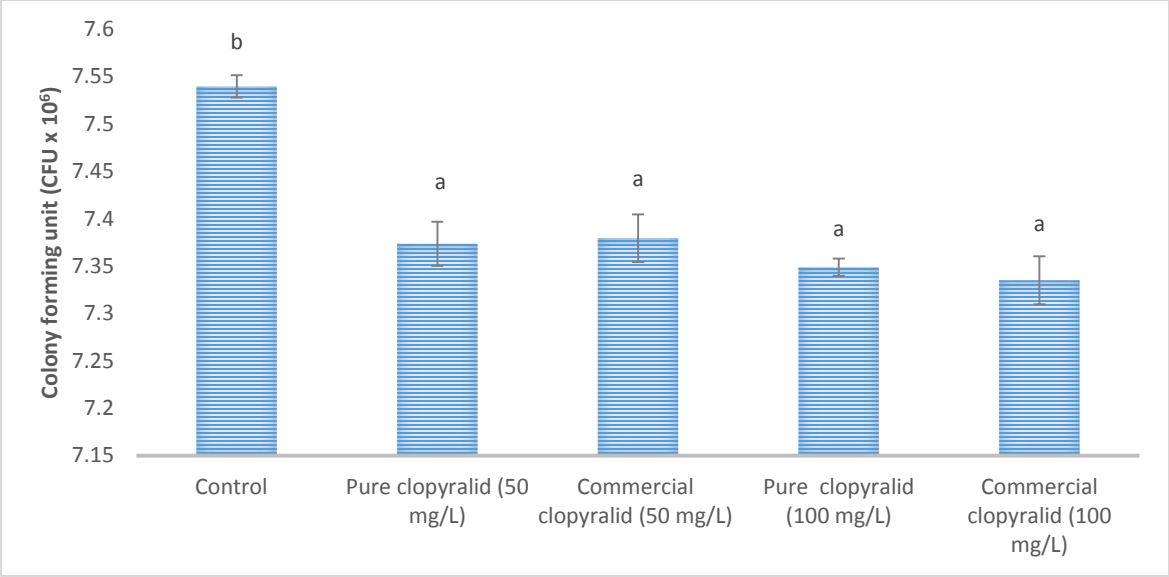
5.3.2 Bacterial and fungal identification and inhibition

To observe bacterial and fungal inhibition in the activated sludge, the NA and PDA media with and without clopyralid were used. In the first instance, both pure and commercial clopyralid were used at two different concentrations i.e. 50 and 100 mg/L. This allowed identification of any other recalcitrant compounds present in the formulation that could potentially cause microbial inhibition. The colonies formed were compared and counted as colony forming unit/ml which are shown in Figure 5.8.

The number of culturable colonies were reduced when clopyralid was added to the media regardless of whether commercial or pure clopyralid was used. This reflects the fact that microbial growth was inhibited in the presence of clopyralid. Furthermore, when commercial and pure clopyralid were compared, no large difference in the number of colonies between the commercial and pure clopyralid solutions was observed; neither was there a difference at the two different concentrations. Therefore, it seems to be reasonable to assume that there is no recalcitrant compound present in the clopyralid formulation that directly might inhibit culturable microbial growth. Thus for future experiments, the commercial formulation was used.



(a)



(b)

Figure 5. 8. Effect of different concentrations of pure or commercial clopyralid on microorganisms present in activated sludge (a) bacteria and (b) fungi.

In the subsequent experiment, the Petri plates were incubated again for another 48 h to see whether any other bacterial/fungal species would grow. This is because fungi are usually slow growers (Bosshard, 2011) and even some bacteria may take some time to grow on media depending on the environmental conditions (Schaechter, 2015). After two days, it was observed that seven bacterial and two fungal colonies appeared on the NA and PDA media, respectively. These bacteria and fungi were then isolated using the four flame streak and single spore method, respectively. The gene sequences were compared with the sequences in databases and their relationships were revealed. Some of the isolated bacteria which were identified using a PCR based molecular method are shown in Figure 5.9. The amplified fragments of 16S rRNA genes of bacteria and the ITS regions of fungi when analyzed by electrophoresis represent 1500 and 600 bp, respectively. All culturable isolates are listed in Tables 5.4 and 5.5. Furthermore, the Paper Disk method was used to identify any inhibition of these microorganisms.

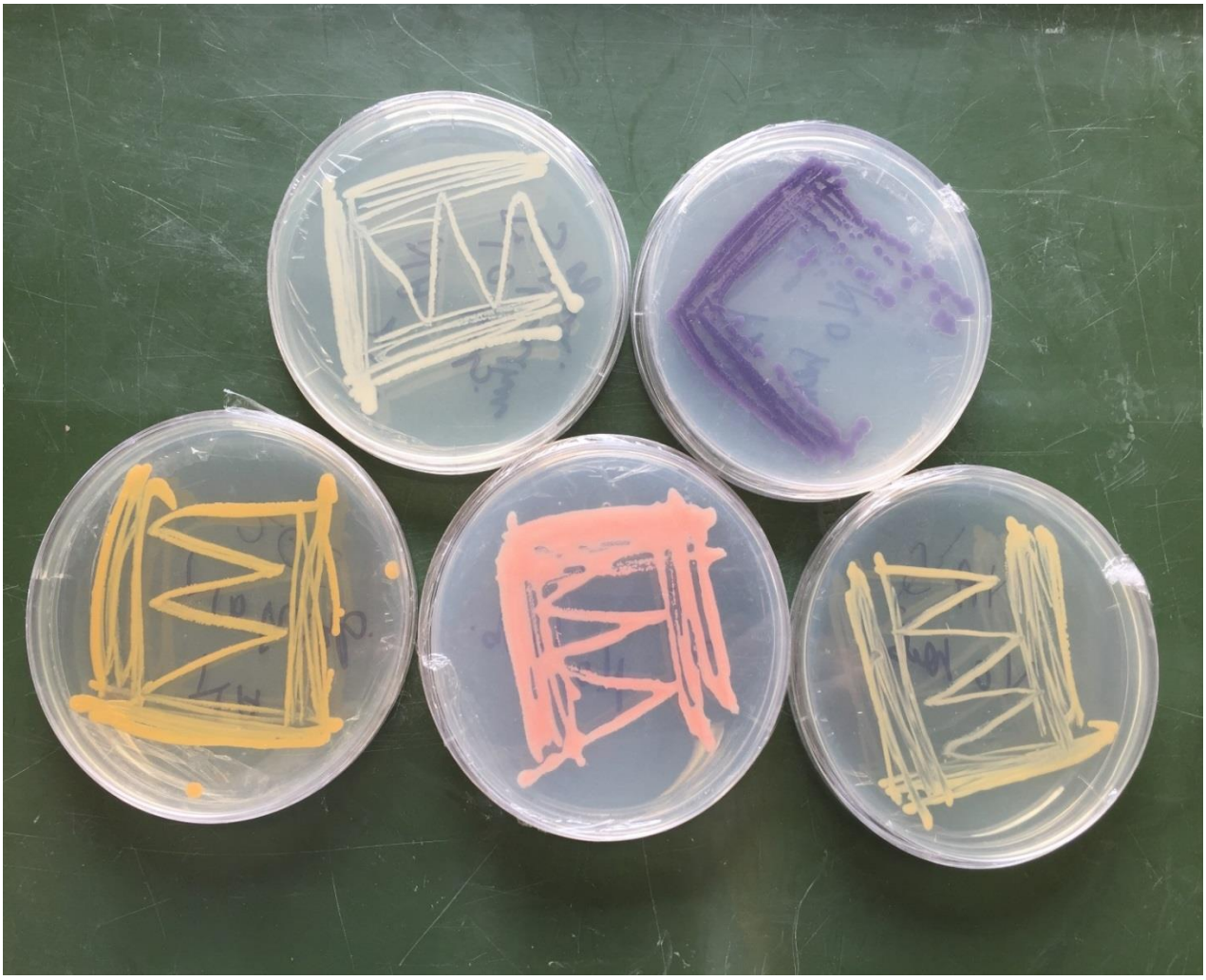


Figure 5. 9. Different bacteria isolated from wastewater

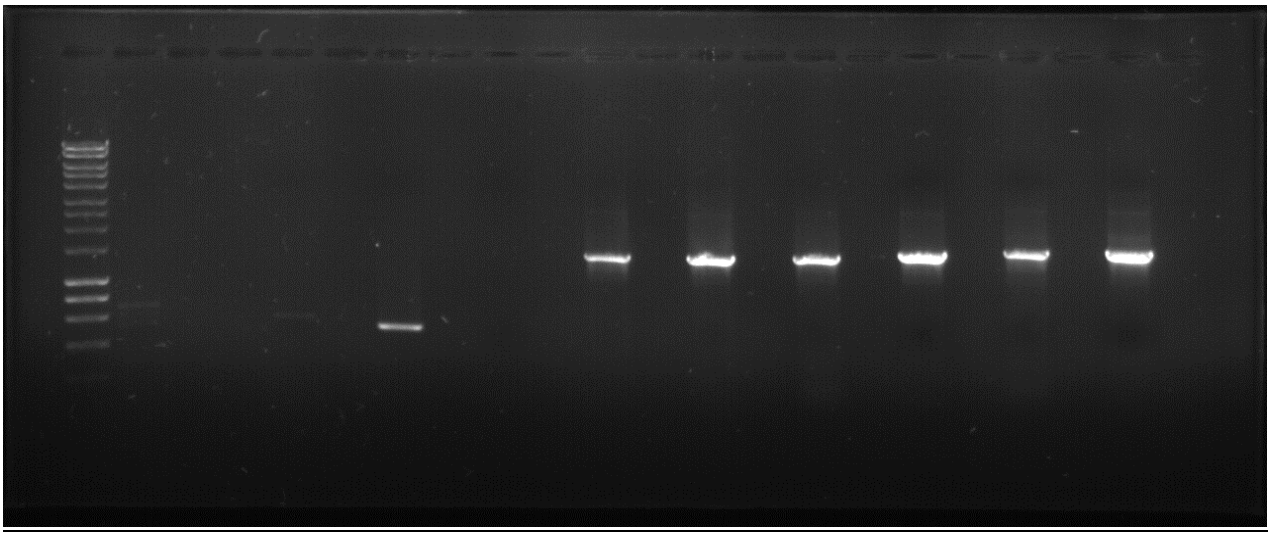


Figure 5. 10. Amplified fragments of 16S rRNA genes of bacteria and ITS regions of fungi analyzed by electrophoresis represent 1500 and 600 bp on a 1% agarose gel.

Paper disk method results

Clopyralid reduced the growth of microorganisms when applied on paper disks. The results shown in Tables 1 and 2.

Table 5. 3Bacterial Isolations and their growth Inhibition by Clopyralid

Bacterial isolates	**Scientific name**	**Similarity to database**	**Inhibition zone 'cm' by clopyralid formulation**		
			10 µL	20 µL	50 µL
PU12	*Janthinobacterium agaricidamnosum*	97%	0	0.8	1.9
P21	*Pseudomonas brenneri*	98%	0	0	1.2
YL18	*Chryseobacterium balustinum*	96.7%	0.08	1.1	1.4
Y26	*Chryseobacterium* sp.	99.47%	0	0	1.6
W30	*Enterobacter* sp.	96%	0	0	1.3
WT11	*Enterobacter Ludwigi*	99.64%	0.08	1	1.7

Table 5. 4 Fungal Isolations and their growth Inhibition by Clopyralid

Fungal isolates	**Scientific name**	**Similarity to database**	**Inhibition in 'cm' due to the presence of the clopyralid formulation**		
			100 µL	150 µL	200 µL
W260	*Trichosporon* sp.	99%	0	0	3.5
BC165	*Penicillium* sp.	93%	2	3.8	4.1

Chryseobacterium balustinum and *Enterobacter Ludwigi* were inhibited with 10 µL of clopyralid formulation and a greater growth inhibition was observed with higher amount of the herbicide. All other bacterial species were inhibited with 50 µL of clopyralid. In contrast, fungal growth was not affected by 50 µL (data not shown). Therefore, higher clopyralid formulation concentrations i.e. 100, 150 and 200 µL were applied. *Penicillium* sp. was inhibited at all clopyralid formulation concentrations tested while the *Trichocporon* sp. was only inhibited with 200 µL.

Incorporation of the herbicide into nutrient broth medium

The nutrient broth results are shown in Table 5.6 for bacteria exposed to low clopyralid concentrations.

Table 5. 5 Bacteria growth inhibition by different lower concentrations of clopyralid formulation

Bacterial isolates	**Control absorbance (nm)**	**Absorbance at different concentrations of Clopyralid (OD 600nm)**			
		20 µL	30 µL	40 µL	50 µL
PU12	1.53	1.34	1.38	1.29	1.04
P21	1.39	1.46	1.49	1.50	1.53
YL18	1.08	0.84	0.86	0.69	0.15
Y26	1.27	1.05	0.94	1.11	1.12
W30	1.25	0.18	0.270	0.10	0.13
WT11	1.62	1.66	1.56	1.59	1.54

Only two bacteria were inhibited with lower concentrations of the herbicide (Chryseobacterium and Enterobacter). Bacterium *Enterobacter* sp. was inhibited at the lowest clopyralid formulation concentration (20 µL) while the growth inhibition of *Chryseobacterium balustinum* was not significantly different between 20 and 30 µL of clopyralid. However, further inhibition occurred once the concentration increased past 30 µL. Therefore, this isolate (W30) was extremely sensitive to any concentration of clopyralid in comparison with *Chryseobacterium balustinum* which was more tolerant. However, those bacterial species that showed no growth inhibition were able to tolerate clopyralid concentrations at these low concentrations. In order to see whether the other bacteria would also experience inhibition, the experiment was repeated at higher concentrations of clopyralid. The results are shown in Table 5.7.

Table 5. 6 Absorbance of bacteria at different higher concentrations of clopyralid formulation

Bacterial isolates	**Control absorbance (nm)**	**Absorbance at different concentrations of Clopyralid Concentrations (nm)**			
		100 µL	150 µL	200 µL	250 µL
PU12	1.64	0.60	0.55	0.56	0.48
P21	1.17	1.15	0.95	0.31	0.33
Y26	1.05	0.06	0.01	0.01	0.01
WT11	1.34	1.39	1.13	1.201	1.15

At the higher concentrations of clopyralid, the growth of bacteria was inhibited in two cases as shown (*Janthinobacterium agaricidamnosum and Pseudomonas brenneri*) with varying degrees of inhibition depending upon the concentration. For *Chryseobacterium* sp., it appeared that growth ceased at a concentration 150 µL and above while for *Enterobacter Ludwigi*, there was no observable inhibition. For fungi, the experiment was repeated at even higher clopyralid formulation concentrations i.e. 200, 300 and 400 µL since using the Paper Disk Method there was no inhibition until after 150 µL of the clopyralid formulation. The results obtained are shown in Table 5.8. It can be observed that at all the concentrations studied, there was a cessation of fungal growth.

Table 5. 7 Absorbance of Fungi at different clopyralid formulation concentrations

Fungal isolates	Control absorbance (nm)	Absorbance at different concentrations of Clopyralid (nm)		
		200 µL	300 µL	400 µL
W260	0.51	0.01	0.02	0.01
BC165	1.83	0.02	0.01	0.02

From all of the above results, it can be clearly seen that bacteria and fungi responded differently depending upon both the species and concentrations of clopyralid. In mixed culture systems, it is very likely that some bacteria and fungi would be inhibited as soon as the herbicide was introduced, while other microbes would be much more tolerant to higher levels of concentration of herbicide.

As such, it can be estimated that all the isolated species showed the inhibition at higher concentrations (i.e. 1800 mg/L) of clopyralid, where eventually the concentration would reach high enough that the majority of microbes would experience inhibition, ultimately affecting the removal efficiency of the clopyralid. However, it can be evaluated from the results of OUR in Table 5.3, that in the presence of clopyralid, the 15 min respiration rate of biomass suddenly dropped to approximately half (in comparison to the reactor in which only non-toxic acetate was present), suggesting that some of the biomass were inhibited even at the lowest concentration of clopyralid (i.e. 50 mg/L) added. Unfortunately, these microbes were hard to identify as they were incapable of being cultured on synthetic media. Around 99 % of the microbes present in the world were not able to be cultured (Shah, 2016), but the method used in this research was the only feasible and cost-effective technique to identify some microbes in activated sludge.

Moreover, the above tests were not able to distinguish between death and mere suppression of growth, thus bactericidal/fungicidal and bacteriostatic/fungistatic tests for cultured/isolated microbes were conducted.

Bactericidal/fungicidal and bacteriostatic/fungistatic effect of clopyralid

All microorganisms grew after treating with the herbicide and washing three times with water suggesting that the herbicide is a bacteriostat/fungistat rather than bactericide/fungicide. Some compounds are bacteristat/fungistat in lower concentrations but at higher concentrations could kill the microorganism. We tested with 200 microlitre and found that the microorganisms did not die with this concentration.

The growth of all the bacteria as well as fungi were stopped, this implies that there was only bacteriostatic and fungistatic effect of clopyralid. As it was observed that by using 200 µL of clopyralid, the growth was stopped and with removing the clopyralid, microbes were again started growing suggesting that the compound in this amount is creating bacteriostatic and fungistatic effect and no bactericidal and fungicidal effect.

CHAPTER-6 – CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

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

1. From the preliminary phase (Phase I), it was concluded that the performance and stability of the aerobic sequencing batch reactors were achieved successfully, obtaining stable biomass in terms of total suspended solid concentration values i.e. 3500 ± 500 mg/L, consistent effluent COD removal i.e. ≥ 95% and stable ORP and DO ranges from 50 mV to 250 mV and 1.5 to 4.5 mg/L, respectively.
2. During the abiotic removal tests for clopyralid, it was concluded that active biomass can degrade most of the clopyralid present in the system; however, 10 – 15 % of the clopyralid may have adsorbed on the biomass without being degraded completely.
3. During the acclimatization period (Phase II), a commercial formulation of clopyralid at 50 mg/L concentration was continuously fed to the SBR system. The first sign of clopyralid degradation appeared on the first day; however, it was observed that 98% of clopyralid degraded on day 5. During track studies for clopyralid degradation on fifth day, it was observed that during the first two hours of operation most of the clopyralid (90%) was degraded. However, despite the excellent degradation of clopyralid, the COD concentrations in the influent as well in the effluent were very large and not equal to the clopyralid generated COD values (i.e. 60 mg/L of COD for 50 mg/L of clopyralid concentration). It was suspected that the large COD in the influent and effluent was due to the presence of other compounds in the commercial formulation of clopyralid such as monoethanolamine salt, isopropyl alcohol and polyglycol, which are reported as major additives in the formulation of commercial clopyralid.
4. Pure clopyralid was used in batch tests (Phase III) and the results revealed that in the presence of a secondary carbon source (i.e. acetate), the maximum amount (90 %) of clopyralid degraded during the first 2 h of a 24 h cycle for all different concentrations i.e. 50, 100, 150, 200 and 225 mg/L while complete degradation (equivalent to 98 %) occurred in 24 h. However, for 250 mg/L of clopyralid concentration, the degradation efficiency was reduced to 75 % in 24 h while for 300 mg/L of clopyralid concentration, no degradation appeared. When COD

track studies were done, the influent COD increase was approximately equal to the clopyralid concentration but the effluent COD was unexpectedly again very large. It was concluded that instead of being completely degraded from the system, clopyralid was converting to some other intermediate compounds. As well there was a toxic effect on biomass resulting in biomass lysis.

5. During the batch test (in the absence of acetate), clopyralid degradation began after 60 min and again it took 24 h to degrade the clopyralid completely for 50, 100, 150, 200 and 225 mg/L concentrations. For 250 mg/L, the degradation efficiency decreased to 70% in 24 h while no degradation appeared for 300 mg/L. However, the effluent COD values were significantly large for all concentrations. It was concluded that the large COD values were because of biomass lysis, because the influent also had large COD values but had no degradation occurred and there was no contribution of intermediate compounds. Also, only clopyralid was there to contribute to the COD and not acetate.
6. During the kinetic modelling phase (phase IV), it was concluded that clopyralid concentrations between 50 and 225 mg/L did not have any inhibitory effect on biodegradation, as the complete degradation of clopyralid was observed. However, for 250 mg/L, the clopyralid utilization rate dropped rapidly approaching zero when the clopyralid concentration increased to 300 mg/L. This seemed to indicate a toxicity effect of clopyralid on the biomass when the concentration got too high. Luong proposed a mathematical model in 1985 for these conditions i.e. when the substrate consumption rate is stimulated at low concentrations and inhibited at high concentrations and due to this Luong Model (1985) was selected, which gave the best fit for the experimental data with R^2 values around 0.98.
7. During Phase V, track studies were conducted to measure TOC, TKN and other transformation products for influent and effluent. SMPs were also measured at 15 min and at 24 h in batch tests. There were no significant change in TKN values in the 24 h cycle of batch tests, which implies that the large residual COD was not because of TKN. Furthermore, since no other transformation products appeared at different time intervals, they did not contribute to residual COD or TOC. However, it was found that SMPs, proteins and carbohydrates (resulting from lysis of microbes) increased significantly after 24 h, which can contribute to both residual COD and TOC.

8. Finally in the microbial inhibition phase (Phase VI), inhibitory assays and OUR were conducted to observe biomass performance in the system, against different concentrations of clopyralid. In the inhibitory tests for culturable microorganisms, it was demonstrated that the growth of all the isolates was inhibited at high concentrations (i.e. 1800 mg/L) of clopyralid, ultimately affecting the removal efficiency of the clopyralid. However, from the results of OUR, in the presence of 50 mg/L clopyralid, after 15 mins, the respiration rate of biomass suddenly dropped to approximately half (in comparison to the reactor in which only acetate was present), which means some of the biomass which cannot be cultured were inhibited even at the lowest introduced concentration of clopyralid (i.e. 50 mg/L). The OUR data suggest a significant reduction in microbial activity as cells were lysed by the toxic effects of clopyralid. It seems reasonable therefore to suggest that some of the residual COD was caused by cell lysis products, therefore contributing to SMPs. Moreover, according to the isolated microbial results, it was concluded that both bacteria and fungi showed inhibition in their growth rather than death at higher levels of clopyralid.
9.

6.2 Recommendations

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

1. The aerobic SBR was found to be an effective technology to treat various recalcitrant compounds including pesticides. However, further research could be carried out to treat the commercial clopyralid (as well as the COD associated with it) in the SBRs by identifying the concentration of major additives present in the formulation, as well by optimizing operational parameters such as SRT, HRT, temperature and pH.
2. One could measure the potential to degrade the clopyralid under anoxic or anaerobic conditions.
3. One could identify the complete microbial structure of activated sludge present in SBRs which includes culturable and non-cultutable microorganisms by using new generation sequencing technology.

4. One could identify all the SMPs that includes polysaccharides, antibiotics, extracellular enzymes, amino acids, humic acids and structural components of cells by using ion-exchange chromatography, GC-MS and HPLC
5. One could develop a kinetic model for microbial inhibition against different concentration of clopyralid. Depending upon the complexity of the recalcitrant compounds, different mathematical models can be used such as Michalis Menten, Haldane and Andrew etc.
6. One could test the biodegradation capability of some other structurally similar chlorinated herbicides such as triclopyr and picloram.

REFERENCES

Ahmad, R., James, T., Rahman, A., & T Holland, P. (2003). Dissipation of the Herbicide Clopyralid in an Allophanic Soil: Laboratory and Field Studies. *Journal of Environmental Science and Health. Part. B, Pesticides, Food Contaminants, and Agricultural Wastes*, *38*, 683–695. https://doi.org/10.1081/PFC-120025553

Agrawal, A., Pandey, R. S., & Sharma, B. (2010). Water Pollution with Special Reference to Pesticide Contamination in India. *Journal of Water Resource and Protection*, *02*(05), 432–448. https://doi.org/10.4236/jwarp.2010.25050

Aiba, S., Shoda, M., & Nagatani, M. (1968). Kinetics of product inhibition in alcohol fermentation. *Biotechnology and Bioengineering*, *67*(6), 671–690. https://doi.org/10.1002/(SICI)1097-0290(20000320)67:6<671::AID-BIT6>3.0.CO;2-W

Akmehmet Balcıoğlu, I., & Ötker, M. (2003). Treatment of pharmaceutical wastewater containing antibiotics by O3 and O3/H2O2 processes. *Chemosphere*, *50*(1), 85–95. https://doi.org/10.1016/S0045-6535(02)00534-9

Allister M., P. J., Rao Bhamidimarri, S. M., Chong, R., & Manderson, G. J. (1991). Biological Treatment of a Landfill Leachate Containing Phenoxy Herbicides and Chlorophenols. *Water Science and Technology; London*, *23*(1–3), 413–418.

Alizadeh, H., Kandula, D. R. W., Hampton, J. G., Stewart, A., Leung, D. W. M., Edwards, Y., & Smith, C. (2017). Urease producing microorganisms under dairy pasture management in soils across New Zealand. *Geoderma Regional*, *11*, 78–85. https://doi.org/10.1016/j.geodrs.2017.10.003

APHA, AWWA, WEF. (2005). Standard Methods for the Examination of Water and Wastewater, twenty first edition. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA., 2-59.

Ascon-Cabrera, M., & Lebeault, J.-M. (1993). Selection of Xenobiotic-Degrading Microorganisms in a Biphasic Aqueous-Organic System. *Applied and environment microbiology, 59*, 8.

Aslan, S., & Türkman, A. (2005). Combined removal of pesticides and nitrates in drinking waters using biodenitrification and sand filter system. *Process Biochemistry, 40*(1), 417-424. doi: 10.1016/j.procbio.2004.01.030.

Azami H., Sarrafzadeh M. H., & Mehrnia M. R. (2012). Soluble microbial products (SMPs) release in activated sludge systems: a review. *Iranian Journal of Environmental Health Science & Engineering*, *9*(1), pg.30.

Barbot, E., Seyssiecq, I., Roche, N., & Marrot, B. (2010). Inhibition of activated sludge respiration by sodium azide addition: Effect on rheology and oxygen transfer. *Chemical Engineering Journal*, *163*(3), 230–235. https://doi.org/10.1016/j.cej.2010.07.050

Barker D. J. & Stuckey D. C. (1999). A review of soluble microbial products (SMP) in wastewater treatment systems. *Water Research*, *33*(14), pp.3063–3082.

Berberidou, C., Kitsiou, V., Karahanidou, S., Lambropoulou, D. A., Kouras, A., Kosma, C. I., Poulios, I. (2016). Photocatalytic degradation of the herbicide clopyralid: kinetics, degradation pathways and ecotoxicity evaluation: Photocatalytic degradation of clopyralid: kinetics, degradation pathways and ecotoxicity. *Journal of Chemical Technology & Biotechnology*, *91*(9), 2510–2518. https://doi.org/10.1002/jctb.4848

Berberidou, C., Kitsiou, V., Lambropoulou, D. A., Antoniadis, A., Ntonou, E., Zalidis, G. C., & Poulios, I. (2017). Evaluation of an alternative method for wastewater treatment containing pesticides using solar photocatalytic oxidation and constructed wetlands. *Journal of Environmental Management*, *195*, 133–139. https://doi.org/10.1016/j.jenvman.2016.06.010

Brazil, S. (2008). New Zealand Official Yearbook 2008. Retrieved April 3, 2018, from https://www3.stats.govt.nz/New_Zealand_Official_Yearbooks/2008/NZOYB_2008.html

Brinton, W.F., & Blewett, T.C. (2004) Presence, Fate, and Effects of Clopyralid and Other Anthropogenic Residues in Green Waste Composting. Paper 4A-21, in: A.R. Gavaskar and

A.S.C. Chen, Remediation of Chlorinated and Recalcitrant Compounds—2004. *Proceedings of the Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compounds (Monterey, CA; May 2004)*. ISBN 1-57477-145-0, published by Battelle Press, Columbus, OH, www.battelle.org/bookstore.

Bosshard P.P. (2011). *Incubation of fungal cultures: how long is long enough?* - PubMed - NCBI. Retrieved December 14, 2018, from https://www.ncbi.nlm.nih.gov/pubmed/21605185

Boussahel, R., Bouland, S., Moussaoui, K. M., Baudu, M., & Montiel, A. (2002). Determination of chlorinated pesticides in water by SPME/GC. *Water Research*, *36*(7), 1909-1911. doi: http://dx.doi.org/10.1016/S0043-1354(01)00372-4.

Bovey, R. W., & Richardson, C. W. (1991). Dissipation of Clopyralid and Picloram in Soil and Seep Flow in the Blacklands of Texas. *Journal of Environment Quality*, *20*(3), 528. https://doi.org/10.2134/jeq1991.00472425002000030005x

Buyuksonmez, F., Rynk, R., Hess, T. F., & Bechinski, E. (2000). Occurrence, Degradation and Fate of Pesticides During Composting. *Compost Science & Utilization*, *8*(1), 61.

Cao, Y., Zhang, C., Rong, H., Zheng, G., & Zhao, L. (2017). The effect of dissolved oxygen concentration (DO) on oxygen diffusion and bacterial community structure in moving bed sequencing batch reactor (MBSBR). *Water Research*, *108*, 86–94. https://doi.org/10.1016/j.watres.2016.10.063

Celis, E., Elefsiniotis, P., & Singhal, N. (2008). Biodegradation of agricultural herbicides in sequencing batch reactors under aerobic or anaerobic conditions. *Water Research*, *42*(12), 3218-3224. doi: 10.1016/j.watres.2008.04.008.

ChemsafetyPro. (2018). *n-Octanol/Water Partition Coefficient (Kow/logKow)*. Retrieved December 24, 2018, from https://www.chemsafetypro.com/Topics/CRA/n_Octanol_Water_Partition_Coefficient_Kow.htm

Chong, N., & Chen, Y. (2007). Activated sludge treatment of a xenobiotic with or without a biogenic substrate during start-up and shocks. *Bioresource Technology*, *98*(18), 3611-3616. doi: 10.1016/j.biortech.2006.11.031.

Chouhan, D., Bello-Mendoza, R., & Wareham, D. G. (2017). MCPA biodegradation in an anoxic sequencing batch reactor (SBR). *International Journal of Environmental Science and Technology*, *14*(2), 365–374. https://doi.org/10.1007/s13762-016-1152-7

Chun, J., Lee, J.-H., Jung, Y., & Kim, M. (2007). EzTaxon: A web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *International Journal of Systematic and Evolutionary Microbiology*, *57*(10), 2259–2261. https://doi.org/10.1099/ijs.0.64915-0

Ciucanu I, Popa PS, Agotici V, & Dehelean G. (2014). Droplets-based microextraction assisted SPME for GC-MS analysis of polar compounds such as clopyralid in water. *Analytical Methods, 6.* 6571–6.

Close, M. E., & Humphries, B. (2016). The 2014 national survey of pesticides in groundwater in New Zealand, 16. *Journal of Hydrology (NZ), 55*(2), 73-88 2016.

Cox, L., Walker, A. Hermosin, M.C. & Cornejo, J. (1998). Measurement and simulation of the movement of thiazafluron, clopyralid and metamitron in soil columns. *Weed Research, 36,* 419–429.

Cox, N., Pantazis, D. A., Neese, F., & Lubitz, W. (2013). Biological Water Oxidation. *Accounts of Chemical Research*, *46*(7), 1588–1596. https://doi.org/10.1021/ar3003249

Davies, C. (2008). Home-grown veg ruined by toxic herbicide | Environment | The Guardian. Retrieved January 24, 2019, from https://www.theguardian.com/environment/2008/jun/29/food.agriculture

Divyalakshmi, P., Murugan, D., Sivarajan, M., Sivasamy, A., Saravanan, P., & Rai, C. L. (2015). In situ disruption approach on aerobic sludge biomass for excess sludge reduction in

tannery effluent treatment plant. *Chemical Engineering Journal*, *276*, 130–136. https://doi.org/10.1016/j.cej.2015.04.085

Dow AgroSciences. (1998). *Clopyralid a North American Technical Profile*. Dow AgroSciences.

DowElanco. 1997. *Clopyralid, a North American technical profile*. Indianapolis, IN.

Ebbing, D. D. and Gammon, S. D. (2009). *General chemistry*. Boston, Mass.: Houghton Mifflin Co. ISBN 0-618-73879-7.

Edwards, V.H. (1970). The Influence of High Substrate Concentrations on Microbial Kinetics. *Biotechnology and Bioengineering*, *12*, 679 - 712, 1970.

European Food Safety Authority (EFSA). (2006). Conclusion regarding the peer review of the pesticide risk assessment of the active substance clopyralid: Conclusion regarding the peer review of the pesticide risk assessment of the active substance clopyralid. *EFSA Journal*, *4*(1), 50r. https://doi.org/10.2903/j.efsa.2006.50r

Fauci, M., Bezdicek, D. F., Caldwell, D., & Finch, R. (2002). Development of Plant Bioassay to Detect Herbicide Contamination of Compost at or Below Practical Analytical Detection Limits. *Bulletin of Environmental Contamination and Toxicology; New York*, *68*(1), 79–85. http://dx.doi.org.ezproxy.canterbury.ac.nz/10.1007/s00128-001-0222-8

Fongsatitkul, P., Wareham, D. G., & Elefsiniotis, P. (2008). Treatment of four industrial wastewaters by sequencing batch reactors: evaluation of COD, TKN and TP removal. *Environmental Technology, 29*(11), 1257-1264. http://doi: 10.1080/09593330802308978.

Fontmorin, J.-M., Fourcade, F., Geneste, F., Floner, D., Huguet, S., & Amrane, A. (2013). Combined process for 2,4-Dichlorophenoxyacetic acid treatment—Coupling of an electrochemical system with a biological treatment. *Biochemical Engineering Journal*, *70*, 17–22. https://doi.org/10.1016/j.bej.2012.09.015

Gilbert, D. E. J., Barth, J., Favoino, E., & Rynk, D. R. (2010). An investigation of clopyralid and aminopyralid in composting systems. Project OAV031-002. *WRAP Material Change for a Better Environment*, 8-42.

HACH (2003). *Water Analysis Hand Book, 4th Edition, Revision 2*. Hach Company, Loveland Colorado, USA.

Haskell, D. E. (2003). California cracks down on clopyralid in compost. *Proceedings of the California Weed Science Society*, *55*, 163-166. Retrieved April 19, 2018, from http://gr2tq4rz9x.search.serialssolutions.com/?ctx_ver=Z39.88-2004&ctx_enc=info%3Aofi%2Fenc%3AUTF-8&rfr_id=info%3Asid%2Fsummon.serialssolutions.com&rft_val_fmt=info%3Aofi%2Ffmt%3Akev%3Amtx%3Ajournal&rft.genre=article&rft.atitle=California+cracks+down+on+clopyralid+in+compost&rft.jtitle=Pesticide+%26+Toxic+Chemical+News&rft.date=2003-04-14&rft.pub=Agra+Informa%2C+Inc&rft.issn=0146-0501&rft.volume=31&rft.issue=25&rft.spage=17&rft.externalDBID=IAO&rft.externalDocID=100071647&paramdict=en-NZ

Helling, C. S., P. C. Kearney, and M. Alexander. (1971). Behavior of pesticides in soil. *Advanced Agronomy Journal*, *23*, 147-240.

Hennequin, C., Abachin, E., Symoens, F., Lavarde, V., Reboux, G., Nolard, N., Berche, P. (1999). Identification of Fusarium species involved in human infections by 28S rRNA gene sequencing. *Journal Clinical Microbiology*, *37*, 3586–3589.

Horticulture New Zealand. (2014). *Fresh facts; plant and food research*. Retrieved from http://www.freshfacts.co.nz/files/fresh-facts-2014.pdf

Hutching B. (2006). *New Zealand Official Yearbook 2006. Statistics New Zealand*. p. 359. Retrieved April 19, 2018, from https://www3.stats.govt.nz/New_Zealand_Official_Yearbooks/2006/NZOYB_2006.html

Jayaraj, R., Megha, P., & Sreedev, P. (2016). Review Article. Organochlorine pesticides, their toxic effects on living organisms and their fate in the environment. *Interdisciplinary Toxicology*, *9*(3–4), 90–100. https://doi.org/10.1515/intox-2016-0012

Jozsef, P., & Krizsten, H. (2011). The impact of crop protection on agricultural production. *Studies in Agricultural Economics No., 113*, 47-66. Retrieved from https://ageconsearch.umn.edu/record/102401/files/Studies%20113-03.pdf

Kim, D.-J., Lim, Y., Cho, D., & Rhee, I. H. (2010). Biodegradation of monoethanolamine in aerobic and anoxic conditions. *Korean Journal of Chemical Engineering*, *27*(5), 1521–1526. https://doi.org/10.1007/s11814-010-0285-5

Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H., Won, S., Chun, J. (2012). Introducing EzTaxon: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *International Journal Systematic and Evolutionary Microbiology*, *62*, 716–721.

Kornboonraks T. & Lee S.H. (2009). Factors affecting the performance of membrane bioreactor for piggery wastewater treatment. *Bio-resoures Technology*, *100*, 2926–2932.

Kunacheva C. & Stuckey D. C. (2014). Analytical methods for soluble microbial products (SMP) and extracellular polymers (ECP) in wastewater treatment systems: A review. *Water Research*, *61*, 1–18.

Leitch, C., and P. Fagg. 1985. Clopyralid herbicide residues in streamwater after aerial spraying of a *Pinus radiata* plantation. *New Zealand Journal for Science*, *15*(2),195-206.

Lettinga, G. (1995). Anaerobic digestion and wastewater treatment systems. *Antonie van Leeuwenhoek*, *67*(1), 3–28. https://doi.org/10.1007/BF00872193

Linkfield, T. G. (1989). Characterization of the Acclimation Period before Anaerobic Dehalogenation of Halobenzoatest. *Applied and environment microbiology*, *55*, 6.

Lipson, D.A., Schmidt, S.K., 2004. Seasonal changes in an alpine soil bacterial community in the Colorado Rocky Mountains. *Application of Environmental Microbiology, 40*, 2867–2879.

Lubytė, J.D., Danutė, J., Antanaitis, S. and Antanaitis, A. (2007) Decomposition of chlopyralid [sic], tribenuron-methyl and pendimethalin in different organic substrates. *Ekologija, 53* (4), 77–83.

Luong, J.H.J. (1987). Generalization of Monod Kinetics for Analysis of Growth Data with Substrate Inhibition. *Biotechnology and Bioengineering*, *29*, 242 - 248.

Lv T., Zhang Y., Zhang L., Carvalho P.N., Arias C.A., Brix H. (2016) Removal of the pesticides imazalil and tebuconazole in saturated constructed wetland microcosms. *Water Research*, *91*, 126-136.

Madoni P, Davol D, Gibin G. (2000). Survey of filamentous microorganisms from bulking and foaming activated-sludge plants in Italy. *Water Research*, *34*(6), 1767–1772.

Mahmood, I., Imadi, S. R., Shazadi, K., Gul, A., & Hakeem, K. R. (2016). Effects of Pesticides on Environment. In K. R. Hakeem, M. S. Akhtar, & S. N. A. Abdullah. *Plant, Soil and Microbes Cham: Springer International Publishing*, 253–269. https://doi.org/10.1007/978-3-319-27455-3_13

Manea, L., Eklo, O. M., & Stenrod, M. (2017). *Economic importance and environmental impact of pesticides; a review of the literature*.

Mangat, S. S., & Elefsiniotis, P. (1999). Biodegradation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in sequencing batch reactors. *Water Research*, *33*(3), 861–867. https://doi.org/10.1016/S0043-1354(98)00259-0

Manktelow D, Stevens P, Walker J, Gurnsey S, Park N, Zabkiewicz J, Teulon D., & Rahman A. (2005). NZ-pesticide-trends.pdf-*Trends in Pesticide Use in New Zealand:2004*. Report to the Ministry for the Environment. Retrieved from https://dioxinnz.com/Spray-NZ-Hist/PDF/nz-pesticide-trends.pdf

Marsolek, M. D., Kirisits, M. J., & Rittmann, B. E. (2007). Biodegradation of 2,4,5-trichlorophenol by aerobic microbial communities: biorecalcitrance, inhibition, and adaptation. *Biodegradation; Dordrecht*, *18*(3), 351–358. http://dx.doi.org.ezproxy.canterbury.ac.nz/10.1007/s10532-006-9069-3

Michel, J., and Doohan. (2003). *Clopyralid and Other Pesticides in Composts*. Ohio State University Extension Factsheet.

Miltner, E., Bary, A. and Cogger, C. (2003). Clopyralid and Compost: Formulation and Mowing Effects on Herbicide Content of Grass Clippings. *Compost Science & Utilization*, *11*(4), p. 289-299.

Ministry of Health. (2017). *Guidelines for Drinking-water Quality Management for New Zealand.* Retrieved April 19, 2018, from https://www.health.govt.nz/publication/guidelines-drinking-water-quality-management-new-zealand

Mohlenhoff, P., Muller, L., Gorbushina, A.A., Petersen, K. (2001). Molecular approach to the characterisation of fungal communities: methods for DNA extraction, PCR amplification and DGGE analysis of painted art objects. *FEMS Microbiology Letters*, *195*, 169–173.

Moody Oliver. (2013). *"Weedkiller banned in US is getting into compost and killing garden vegetables"*. The Times. London.

Mrema, E. J., Rubino, F. M., Brambilla, G., Moretto, A., Tsatsakis, A. M., & Colosio, C. (2013). Persistent organochlorinated pesticides and mechanisms of their toxicity. *Toxicology*, *307*, 74–88. https://doi.org/10.1016/j.tox.2012.11.015

Mulchandani, A., Luong, J. H. T., & Groom, C. (1989). Substrate inhibition kinetics for microbial growth and synthesis of poly-fl-hydroxybutyric acid by Alcaligenes eutrophus ATCC 17697. *Applied Microbiol Biotechno*, *30*, 11-17.

Muñoz, R., & Guieysse, B. (2006). Algal-bacterial processes for the treatment of hazardous contaminants: a review. *Water Research*, *40*(15), 2799–2815. https://doi.org/10.1016/j.watres.2006.06.011

New Zealand Environment Risk Management. (2007). Retrieved November 28, 2018, from https://www.odt.co.nz/lifestyle/home-garden/reminder-not-spray-lawns-clopyralid

Nguyen, L.M., Buttner, M.P., Cruz, P., Smith, S.D., Robleto, E.A., (2011). Effects of elevated atmospheric CO_2 on rhizosphere soil microbial communities in a Mojave Desert ecosystem. *Journal of Arid Environments*, *75*, 917–925.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., & Hens, L. (2016). Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture. *Frontiers in Public Health*, *4*. https://doi.org/10.3389/fpubh.2016.00148

Nyholm, N., Jacobsen, B. N., Pedersen, B. M., Poulsen, O., Damborg, A., & Schultz, B. (1992). Removal of organic micropollutants at PPB levels in laboratory activated sludge reactors under various operating conditions: biodegradation. *Water Research*, *26*(3), 339–353. https://doi.org/10.1016/0043-1354(92)90031-X

Oerke, E.-C., & Dehne, H.-W. (2004). Safeguarding production—losses in major crops and the role of crop protection. *Crop Protection*, *23*(4), 275–285. https://doi.org/10.1016/j.cropro.2003.10.001

Oller, I., Malato, S., & Sánchez-Pérez, J. A. (2011). Combination of Advanced Oxidation Processes and biological treatments for wastewater decontamination—A review. *Science of The Total Environment*, *409*(20), 4141–4166. https://doi.org/10.1016/j.scitotenv.2010.08.061

Ordaz-guillén, Y., Galíndez-mayer, C. J., Ruiz-ordaz, N., Juárez-ramírez, C., Santoyo-tepole, F., & Ramos-monroy, O. (2014). Evaluating the degradation of the herbicides picloram and 2,4-D in a compartmentalized reactive biobarrier with internal liquid recirculation. *Environmental Science and Pollution Research International; Heidelberg*, *21*(14), 8765–8773. http://dx.doi.org.ezproxy.canterbury.ac.nz/10.1007/s11356-014-2809-8

Oxidation-Reduction-Potential (ORP) Explained | Molecular Hydrogen Institute. (2018). Retrieved December 5, 2018, from http://www.molecularhydrogeninstitute.com/oxidation-reduction-potential-orp-explained

Ozone Solutions. (2018). ORP level ozone, ORP levels, ozone, oxidation reduction potential. Retrieved December 4, 2018, from http://www.ozoneapplications.com/info/orp.htm

Palmer, WE, Bromley, PT, and Brandenburg, RL. (2007). *Wildlife & pesticides - Peanuts*. North Carolina Cooperative Extension Service.

Pesticide Properties Database (2018). *clopyralid*. Retrieved November 30, 2018, from https://sitem.herts.ac.uk/aeru/ppdb/en/Reports/169.html

Pik, A. J., E. Peake, M. T. Strosher, and G. W. Hodgson. (1977). Fate of 3,6-dichloropicolinic acid soils. *Journal of Agriculture and Food Chemistry*, *25*(5),1054-1061.

Pimentel, D. (1995). Amounts of pesticides reaching target pests: Environmental impacts and ethics. *Journal of Agricultural and Environmental Ethics*, *8*(1), 17–29. https://doi.org/10.1007/BF02286399

Pohanish, R. P. (2014). *Sittig's Handbook of Pesticides and Agricultural Chemicals: Second Edition.*

Poltak R.F. (2005). *Sequencing Batch Reactor Design and Operation Considerations***.** New England Interstate Water Pollution Control Commission, Massachusetts

Pretty, J., & Bharucha, Z. P. (2015). Integrated Pest Management for Sustainable Intensification of Agriculture in Asia and Africa. *Insects*, *6*(1), 152–182. https://doi.org/10.3390/insects6010152

Pubchem. (2018). *Clopyralid*. Retrieved December 24, 2018, from https://pubchem.ncbi.nlm.nih.gov/compound/15553

Rajendran, D. S. (2018). *Environment and health aspects of pesticides use in Indian agriculture.*

Ramiro Villalvazo. (2010). *Eldorado National Forest (N.F.), Freds Fire reforestation :environmental impact statement.* Retrieved from http://hdl.handle.net/2027/ien.35556039330923

Rangaswami, G., Bagyaraj, D.J. (2005). *Agricultural Microbiology*. Second edition Prentice Hall of India Private Limited, New Delhi.

Recycled Organics Unit (2007). *Risk Assessment of Garden Maintenance Chemicals in Recycled Organics Products.* Second Edition. Recycled Organics Unit, internet publication: www.recycledorganics.com

Rolando, C., Todoroki, C., & Watt, M. (2015). *Minimising the environmental impact of forest weed management in New Zealand.*

Roser Max, & Ritchie Hannah. (2017). *Fertilizer and Pesticides*. Retrieved November 28, 2018, from https://ourworldindata.org/fertilizer-and-pesticides

Rott, E., Pittmann, T., Wasielewski, S., Kugele, A., & Minke, R. (2017). Detoxification of Pesticide-Containing Wastewater with FeIII, Activated Carbon and Fenton Reagent and Its Control Using Three Standardized Bacterial Inhibition Tests. *Water*, *9*(12), 969. https://doi.org/10.3390/w9120969

Runes H.B., Jenkins J.J., Moore J.A., Bottomley P.J., B.D. (2003). Wilson Treatment of atrazine in nursery irrigation runoff by a constructed wetland. *Water Research*, *37*, 539-550

Sabumon, P. C. (2008). Development of a novel process for anoxic ammonia removal with sulphidogenesis. *Process Biochemistry*, *43*(9), 984-991. doi: http://dx.doi.org/10.1016/j.procbio.2008.05.004.

Sagasta, J. M., Zadeh, S. M., & Turral, H. (2017). Water pollution from agriculture: a global review - Executive summary. *Food and Agriculture Organization of the United Nations Rome, 2017 and the International Water Management Institute on Behalf of the Water Land and Ecosystems Research Program Colombo*, 35.

Sahu, O., & Chaudhari, P. (2013). Review on Chemical treatment of Industrial Waste Water. *Journal of Applied Sciences and Environmental Management*, *17*(2). https://doi.org/10.4314/jasem.v17i2.8

Santacruz, G., Bandala, E. R., & Torres, L. G. (2005). Chlorinated pesticides (2,4-D and DDT) biodegradation at high concentrations using immobilized Pseudomonas fluorescens. *Journal of Environmental Science and Health. Part. B, Pesticides, Food Contaminants, and Agricultural Wastes*, *40*(4), 571–583. https://doi.org/10.1081/PFC-200061545

Schaechter, M. (2015). A brief history of bacterial growth physiology. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.00289

Shah, M. P. (2016). Microbial Community Structure of Activated Sludge As Investigated With DGGE. *Journal of Advanced Research in Biotechnology*, *1*(1), 1–7. https://doi.org/10.15226/2475-4714/1/1/00111

Shahpoury, P., Hageman, K. J., Matthaei, C. D., & Magbanua, F. S. (2013). Chlorinated pesticides in stream sediments from organic, integrated and conventional farms. *Environmental Pollution*, *181*, 219–225. https://doi.org/10.1016/j.envpol.2013.06.035

Shawaqfeh, A. T. (2010). Removal of pesticides from water using anaerobic-aerobic biological treatment. *Chinese Journal of Chemical Engineering*, *18*(4), 672-680.

Shchegolkova, N. M., Krasnov, G. S., Belova, A. A., Dmitriev, A. A., Kharitonov, S. L., Klimina, K. M., & Kudryavtseva, A. V. (2016). Microbial Community Structure of Activated Sludge in Treatment Plants with Different Wastewater Compositions. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00090

Smith, A.E. and Aubin, A.J. (1989). Persistence Studies with the Herbicide Clopyralid in Prairie Soils at Different Temperatures. *Bulletin of Environmental Contamination and Toxicology*, *42*, 670-675

Solanki, H.A., Chitnis, R.D., Bhavsar, H.A. (2012). Physico-chemical and bacterial analysis of Sabarmati river in Ahmedabad. *Life Science Leaflets*, *2*, 70–82.

Spagni, A., Lavagnolo, M. C., Scarpa, C., Vendrame, P., Rizzo, A., & Luccarini, L. (2007). Nitrogen removal optimization in a sequencing batch reactor treating sanitary landfill leachate. *Journal of Environmental Science and Health, Part A*, *42*(6), 757–765. https://doi.org/10.1080/10934520701304435

Statistics New Zealand. (2014). *The Significance of Dairy to the New Zealand economy.* Retrieved April 19, 2018, from http://nzdotstat.stats.govt.nz/wbos/Index.aspx#

Tazdaït, D., Abdi, N., Grib, H., Lounici, H., Pauss, A., & Mameri, N. (2013). Comparison of different models of substrate inhibition in aerobic batch biodegradation of malathion. *Turkish Journal of Engineering & Environmental Sciences*, *37*(3), 221–230. https://doi.org/10.3906/muh-1211-7

The New Zealand Drinking-Water Process. (2018). Retrieved November 27, 2018, from http://www.drinkingwater.esr.cri.nz/general/nzprocesses.asp

Tomco Patrick L., Duddleston Khrystyne N., Schultz Emily Jo, Hagedorn Birgit, Stevenson Timothy J., & Seefeldt Steven S. (2016). Field degradation of aminopyralid and clopyralid and microbial community response to application in Alaskan soils. *Environmental Toxicology and Chemistry*, *35*(2), 485–493. https://doi.org/10.1002/etc.3222

Tobajas, M., Verdugo, V., Polo, A. M., Rodriguez, J. J., & Mohedano, A. F. (2016). Assessment of toxicity and biodegradability on activated sludge of priority and emerging pollutants. *Environmental Technology*, *37*(6), 713–721. https://doi.org/10.1080/09593330.2015.1079264

Toughill, K. (1999). *The summer the rivers died: Toxic runoff from potato farms is poisoning P.E.I., Toronto Star Atlantic Canada Bureau.*

Tu, M., Hurd, C., & Randall, J. M. (2001). *Weed Control Methods Handbook: Tools & Techniques for Use in Natural Areas*.

Tziotzios, G., Lyberatos, G., Pavlou, S. and Vayenas, D.V. (2008). Modelling of Biological Phenol Removal in Draw-Fill Reactors Using Suspended and Attached Growth Olive Pulp Bacteria. *International Journal of Biodeterioration and Biodegradation*, *61*, 142-50.

U.S. EPA (U.S. Environmental Protection Agency). (1990a). *Data Evaluation Report: Primary Eye Irritation in Rabbits: AGR 233252.* Reviewed by T.F. McMahon and Y.M. Ioannou. Health Effects Division. Washington, DC. November 9, 1990. MRID No. 416413-04.

U.S. EPA (U.S. Environmental Protection Agency). (1991). *3,6-Dichloro-pyridine carboxylic acid (Clopyralid): Review of a Rabbit Teratology Study Submitted by the Registrant.* Memo from T.F. McMahon, Health Effects Division to E. Wilson, Registration Division. Washington, DC. March 20, 1991. MRID No. 416498-01.

U.S. EPA (U.S. Environmental Protection Agency). (1996). *Exposure Factors Handbook.* Office of Research and Development, National Center for Environmental Assessment, U.S. EPA,

Washington, DC. EPA/600/P95/002Ba-c. Available from: NTIS: PB97-117683, 97-117691, PB97-117709.

U.S. EPA (United States Environmental Protection Agency). (1997a). *Hexachlorobenzene: IRIS Entry*. Last update 3/1/97. Retrieved from http://www.epa.gov/iris/subst/0374.htm

U.S. EPA (United States Environmental Protection Agency). (1997b). *Office of Pesticide Programs Reference Dose Tracking Report for Clopyralid, 02/19/97, page 10*. Copycourtesy of George Ghali, U.S. EPA/OPP.

U.S. EPA (U.S. Environmental Protection Agency). (1997c). *Toxicological oneliners: Clopyralid (Lontrel). Officeof Pesticide Programs.* Health Effects Division. Washington, DC. July8,1997.

U.S. EPA (United States Environmental Protection Agency). (1998). *DrinkingWater and Health Technical Fact Sheet on Hexachlorobenzene.* Last update ½7/98. http://www.epa.gov/OGWDW/dwh/t-soc/hcb.html.

Valdez, C. J., & Maradona, E. M. (2013). *Handbook of wastewater treatment: Biological methods, technology and environmental impact*.

Vandervoort, C., Zabic, M.J. Branham, D. and Lickfeld, W. (1997). Fate of Selected Herbicides on Turfgrass: Effect of Composting on Residues. *Bulletin of Environmental Contamination and Toxicology, 58,* 38-45.

Walsh, P.S., Metzger, D.A., Higuchi, R., 2013. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotech, 54,* 134–139.

Webb J. L. (1963). *Kinetics of Some Complex Enzyme Reaction Types*, Academic Press, New York.

Wiggins, B. A., Jones, S. H., & Alexander, M. (1987). Explanations for the acclimation period preceding the mineralization of organic chemicals in aquatic environments. *Applied and Environmental Microbiology, 53*(4), 791–796.

WSDOT. (2006). *Clopyralid- Roadside Vegetation Management Herbicide Fact Sheet*. Oregon State University and Intertox.

Yano, T. and Koga, S. (1969). Dynamic Behavior of the Chemostat Subject to Substrate Inhibition. *Biotechnology Bioengineering*, *11*, 139-153.

Yoo, H., Ahn, K. H., Lee, H. J., Lee, K.H., Kwak, Y.J., & Song, K.G. (1999). Nitrogen removal from synthetic wastewater by simultaneous nitrification and denitrification (SND) via nitrite in an intermittently-aerated reactor. *Water Research, 33*(1), 145-154.

Yu, T., & Bishop, P. L. (2001). Stratification and Oxidation-Reduction Potential Change in an Aerobic and Sulfate-Reducing Biofilm Studied Using Microelectrodes. *Water Environment Research, 73*(3), 368–373.

APPENDIX - I

(a) Raw Data:

Reactor-1 Total suspended solids in SBR for first 103 days before putting clopyralid

Time (days)	TSS (mg/L)
1	4660
2	4100
3	3460
4	3240
5	3400
6	2980
7	3400
8	3060
14	2760
22	2740
23	2840
24	2680
28	3420
32	3580
37	3520
40	3700
42	3100
49	4220
57	3700
63	3320
65	3080
67	3160
69	3300
73	3880

76	3900
79	4480
83	4080
86	3620
89	3880
92	3920
93	4000
96	4400
100	3920
103	5880

Reactor-2 Total suspended solids in SBR for first 103 days before putting clopyralid

Time (days)	TSS (mg/L)
1	4560
2	4050
3	3380
4	2840
5	2840
6	2680
7	2820
8	2940
14	2980
22	2560
23	2960
24	3060
28	3020
32	4101
37	3360
40	3040
42	3120
49	3660
57	3460
63	3040
65	4100
67	2760
69	3380
73	2920
76	3680
79	4060
83	4640
86	4860

89	3680
92	3080
93	5900
96	4380
100	4500
103	4760

Typical ORP values from one reactor

SBR Stages	Time (min)	ORP (mV)
Fill	1	115.07
Mix/Aerate	20	40.44
	40	37.92
	60	77.93
	80	107.44
	100	129.94
	120	150.46
	140	164.55
	160	176.87
	180	185.93
	200	194.55
	220	200.96
	240	207.16
	260	213.98
	280	221.39
	300	230.69
	320	245.86
	340	258.99
	360	264.40
	380	265.51
	400	266.06
	420	266.77
	440	267.43
	460	267.53
	480	267.50
	500	267.58
	520	267.04
	540	267.47

	560	267.15
	580	266.00
Settle	600	265.87
	620	194.49
	640	155.43
	660	129.43
	680	113.52
	700	104.04
Decant	720	105.26

Typical DO values from one reactor

SBR Stages	Time (min)	DO (mg/L)
Fill	1	0.01
Mix/Aerate	20	0.03
	40	0.00
	60	0.01
	80	0.07
	100	0.13
	120	0.27
	140	0.34
	160	0.37
	180	0.40
	200	0.44
	220	0.46
	240	0.48
	260	0.51
	280	0.52
	300	0.54
	320	0.57
	340	0.65
	360	0.72
	380	0.97
	400	1.50
	420	2.40
	440	3.60
	460	4.20
	480	4.50
	500	4.60
	520	4.72
	540	4.73

	560	4.74
	580	4.75
Settle	600	4.70
	620	2.30
	640	0.04
	660	0.05
	680	-0.03
	700	-0.01
Decant	720	-0.04

Reactor 1 COD track study in 3 different days (baseline)

Time (min)	COD (mg/L)			Average	Standard Deviation
0	300	310	292	301	7.36
30	80	100	108	96	11.77
60	65	85	78	76	8.28
130	39	76	33	49	19.01
240	35	71	47	51	14.96
370	16	55	37	36	15.93
590	15	22	28	22	5.31

Reactor 2 COD track study in 3 different days (baseline)

Time (min)	COD (mg/L)			Average	Standard Deviation
0	300	310	295	302	6.23
30	120	111	110	114	4.49
70	53	95	93	80	19.34
130	35	68	29	44	17.14
240	44	63	46	51	8.52
370	18	70	40	42	21.31
590	18	20	34	24	7.11

(b) Clopyralid Track Study in SBRs:

Time (min)	Clopyralid concentration (mg/L)			Average	Standard Deviation
0	48	44	49	47	2.16
15	30	30	25	28.33	2.36
30	10	9	9	9.33	0.47
60	4	6	10	6.67	2.49

90	2	3	1	2	0.82
120	0	2	1	1	0.82
450	0	0.5	1.5	0.67	0.62
590	0	0	0.8	0.27	0.38

Track studies for clopyralid degradation when initial concentration was 50 mg/L in SBR

Track studies for clopyralid degradation when initial concentration was 100 mg/L in SBR

Time (min)	Clopyralid concentration (mg/L)			Average	Standard Deviation
0	87	89	98	91.33	4.78
15	100	70	80	83.33	12.47
30	80	50	50	60	14.14
60	50	10	20	26.67	17.00
90	20	6	8	11.33	6.18
120	8	4	6	6	1.63
450	6	5	5	5.33	0.47
590	2	2	2	2	0.00

Track studies for COD removal when initial clopyralid concentration was 50 mg/L in SBR

Time (min)	COD (mg/L)			Average	Standard Deviation
0	500	480	485	488.33	8.50
30	289	270	274	277.67	8.18
60	253	220	214	229.00	17.15
120	193	200	189	194.00	4.55
240	152	170	154	158.67	8.06
370	180	164	150	164.67	12.26
590	153	148	164	155.00	6.68

Track studies for COD removal when initial clopyralid concentration was 100 mg/L in SBR

Time (min)	COD (mg/L)			Average	Standard Deviation
0	658	620	683	653.67	25.90
30	405	426	450	427.00	18.38
60	365	353	360	359.33	4.92

120	360	350	354	354.67	4.11
240	250	260	245	251.67	6.24
370	297	303	280	293.33	9.74
590	250	215	220	228.33	15.46

(c) Track Studies in Batch Tests

Track studies for clopyralid degradation in the presence of secondary carbon source at different initial concentrations in batch reactor

	Average Clopyralid Concentration for different initial concentrations (mg/L)						
Time (min)	50 mg/L	100 mg/L	150 mg/L	200 mg/L	225 mg/L	250 mg/L	300 mg/L
0	41	87	96	190	190	250	300
15	16	83	90	172	191	250	300
30	8	60	49	172	125	250	300
60	3	27	49	75	99	172	290
90	2	11	31	14	37	127	280
120	1	6	14	11	11	120	280
450	1	5	8	10	8	137	290
590	0	2	5	5	5	78	300
1200	0	5	3	2	6	65	180

Track studies for COD removal in the presence of secondary carbon source at different clopyralid concentrations in batch reactor

	Average COD for different clopyralid concentrations (mg/L)						
Time (min)	50 mg/L	100 mg/L	150 mg/L	200 mg/L	225 mg/L	250 mg/L	300 mg/L
0	190	297	340	327	316	400	432
15	45	126	154	169	235	312	350
30	42	124	120	153	223	255	334
60	56	127	121	159	196	237	295
90	15	140	135	160	165	236	302
120	42	139	120	158	143	251	315
450	21	128	111	183	158	265	342
590	40	141	114	207	145	248	336

1200	36	127	128	203	153	299	334

Track studies for clopyralid degradation in the absence of secondary carbon source at different initial concentrations in batch reactor

	Average Clopyralid Concentration for different initial concentrations (mg/L)						
Time (min)	50 mg/L	100 mg/L	150 mg/L	200 mg/L	225 mg/L	250 mg/L	300 mg/L
0	27	38	44	134	134	152	285
30	50	100	105	172	172	192	300
60	40	95	90	130	161	250	300
90	36	75	85	102	160	178	287
120	22	66	72	56	95	146	247
240	12	41	37	69	75	128	285
590	9	12	14	36	28	86	244
1200	2	8	6	32	24	75	198

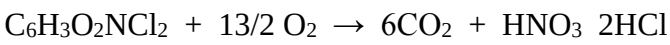
Track studies for COD removal in the absence of secondary carbon source at different clopyralid concentrations in batch reactor

	Average Clopyralid Concentration for different initial concentrations (mg/L)						
Time (min)	50 mg/L	100 mg/L	150 mg/L	200 mg/L	225 mg/L	250 mg/L	300 mg/L
0	445	490	519	580	641	739	779
30	470	497	504	545	635	742	764
60	364	463	492	568	641	752	762
90	409	457	491	564	652	689	776
120	401	440	468	572	632	670	804
240	361	426	459	560	623	706	871
590	339	399	443	533	666	742	845
1200	317	389	427	521	685	780	858

APPENDIX - II

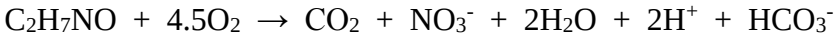
Equations used to Calculate the Theoretical COD for Clopyralid and Major Additives in Commercial Formulation

1. Clopyralid:



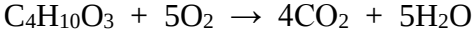
$$C_6H_3O_2NCl_2 + 13/2\ O_2 \rightarrow 6CO_2 + HNO_3\ 2HCl$$

2. Monoethanolamine:



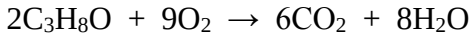
$$C_2H_7NO + 4.5O_2 \rightarrow CO_2 + NO_3^- + 2H_2O + 2H^+ + HCO_3^-$$

3. Polyethylene alcohol:



$$C_4H_{10}O_3 + 5O_2 \rightarrow 4CO_2 + 5H_2O$$

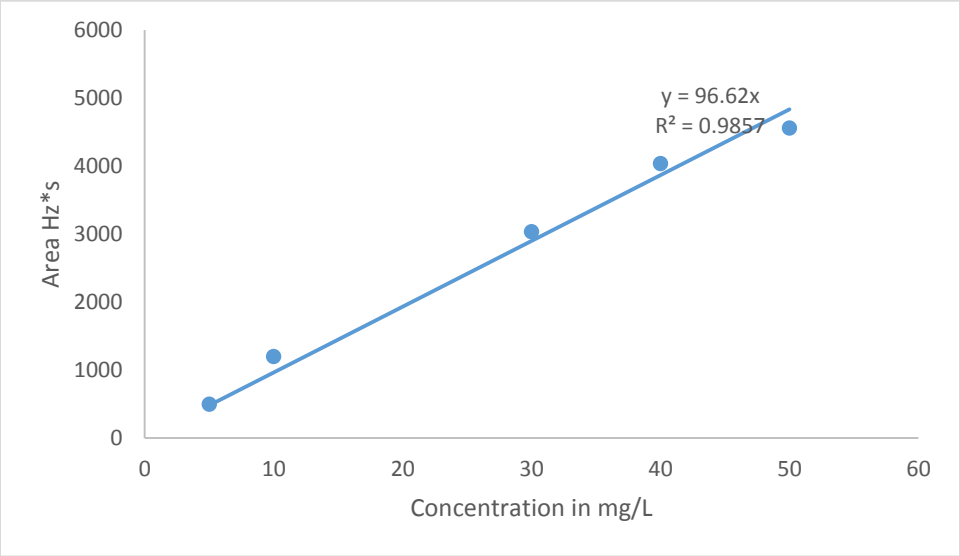
4. Isopropyl alcohol:



$$2C_3H_8O + 9O_2 \rightarrow 6CO_2 + 8H_2O$$

APPENDIX - III

Calibration Curve for Clopyralid using SPME-LPME-GC-ECD:



APPENDIX - IV

(a) Lab Experiment Photos

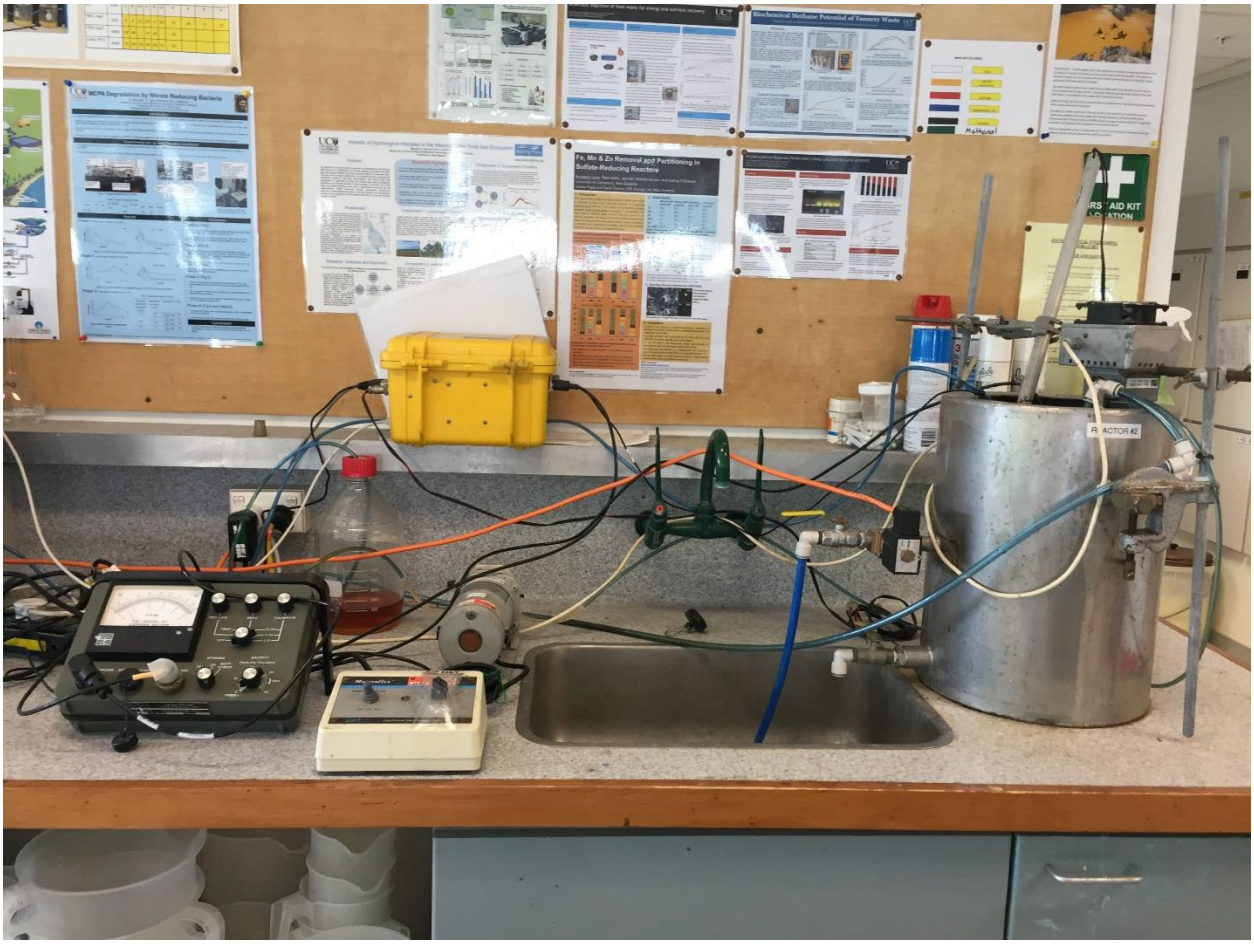


Figure AIII. 1. Experimental set-up for sequencing batch reactor in the environmental engineering lab

Figure AIII. 2. Experimental set-up for Batch Tests run in the environmental engineering lab

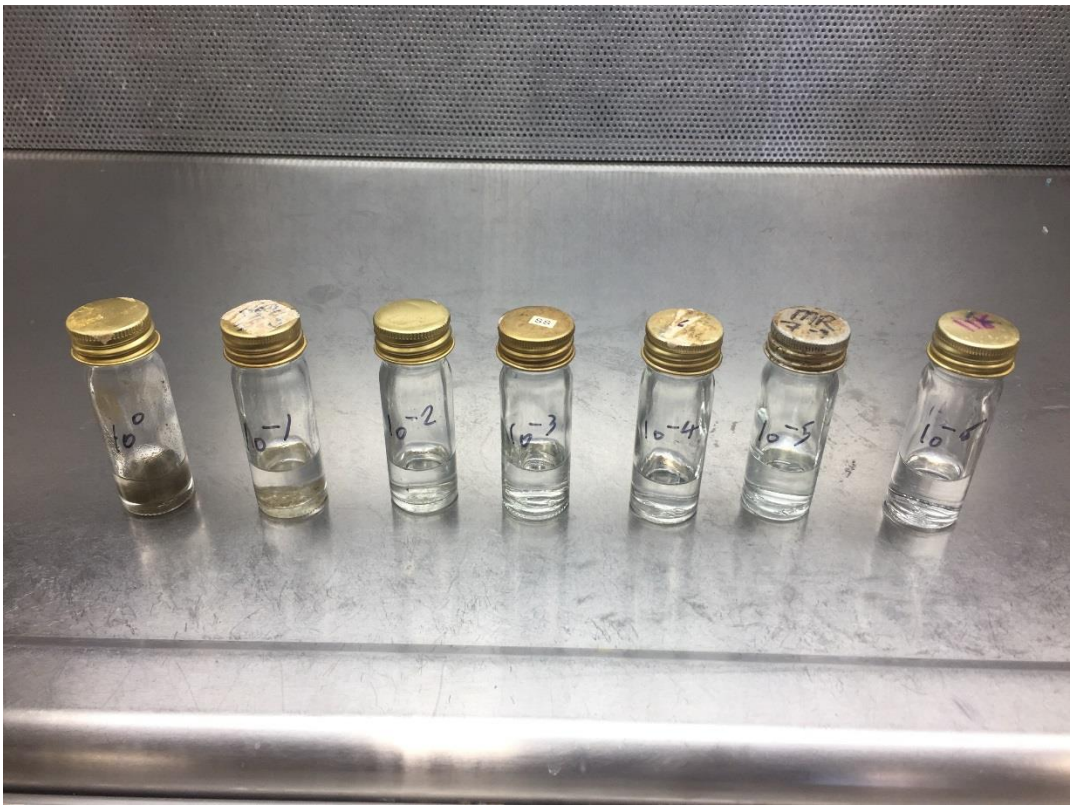


Figure AIII. 2. Serial dilution made in the microbiology lab Lincoln University for activated sludge

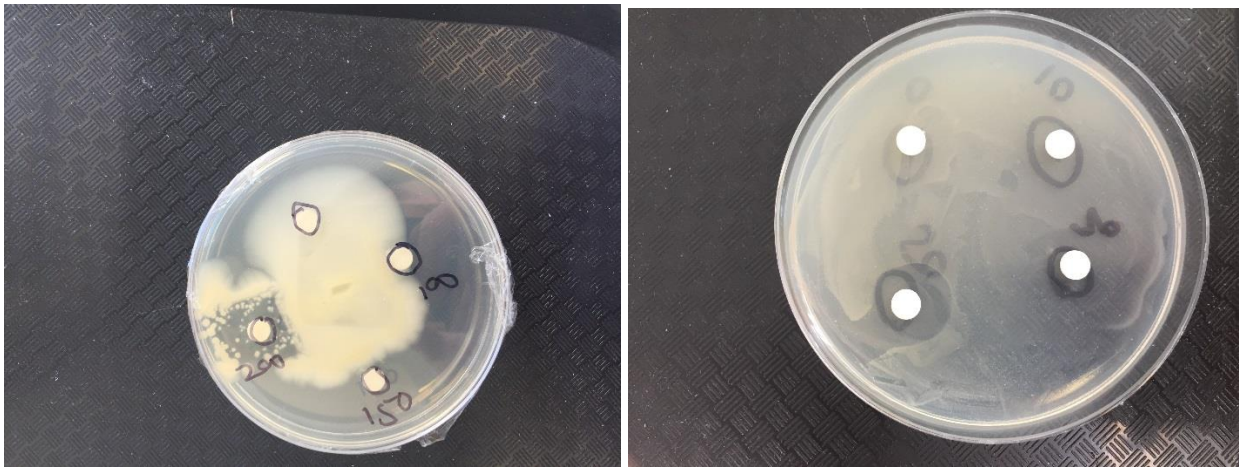


Figure AIII. 3. Results for paper disk inhibition test