The effect of conventional herbicides, used for the control of alien vegetation on microbial communities within freshwater biofilms

by
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Declaration

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Summary

Freshwater streams in South Africa are heavily permeated with invasive plant species. These invasive plant species pose a major threat to native vegetations by competing for available ecosystem resources. A common method used for the removal of these species, is herbicide application. The overuse of these chemicals and the surface runoff into the nearby rivers, has become problematic. The effect of these chemicals on the freshwater biofilms, which consists of various microbial communities, is, however, still unknown.

Microbial communities play an important role in the functioning of riparian ecosystems especially in biogeochemical cycling. Biofilms are mostly beneficial to their surrounding environment and the microbial communities change depending on the exposure to external factors. These factors are mostly abiotic factors and also include exposure to chemicals. These chemicals can either be harmful or beneficial to the microbial community. Some microbial species are able to break down these chemicals and utilise it as a nutrient source.

In this study, the effect of three conventional herbicides, Springbok, Garlon and Plenum, on freshwater biofilms was investigated. The Berg River in the Western Cape was chosen based on the fact that it flows through herbicide applied zones. An optimized herbicide detection method using liquid chromatography coupled to mass spectrometry (LC-MS) was developed and used to determine the current levels of herbicide active ingredients present in the river system.

A preliminary study was done to determine the effect of glyphosate on freshwater biofilms. This study showed that a significant microbial community shift occurs after the application of the herbicide. A physical disruption of the biofilm was also observed at the concentrations that were applied.

In a follow-up study, river water collected from the Berg River which was used to set up a laboratory-scale artificial river system. Flow cells were used to cultivate the biofilms, using the river water as the natural inoculum. The biofilms were allowed to establish for 10 days after which the herbicide and the active ingredient were applied. The flow cells were then used for fluorescent microscopy and DNA extractions. Garlon, Plenum and Springbok were found to decrease the biofilm biomass significantly ($p<0.05$) for all treatments analysed.

Finally, the DNA of the biofilm was extracted from the flow-cells using the standard DNA extraction protocol. The DNA was then used for PCR amplification with bacterial and fungal specific primers. Automated ribosomal intergenic spacer analysis (ARISA) primer sets were used. Significant shifts ($p<0.05$) were found for the herbicides, Springbok (glyphosate) and Plenum (fluroxypyr and picloram). The Shannon diversity index showed a decrease in diversity for both these treatments. This suggest that some species tend to dominate the biofilm because they can tolerate the environmental change.

The hypothesis for this study which states that conventional herbicides influence the growth of microbial communities within freshwater biofilms, is not rejected. This is supported by the significant ($p<0.05$) decrease in biofilm biomass that was seen after herbicide application and also a microbial community shift occurred after herbicide application.

Opsomming

Varswaterstrome in Suid-Afrika word swaar binnegeval deur indringerplant spesies. Hierdie indringerspesies vorm 'n groot bedreiging vir inheemse plantegroei en die beskikbaarheid van water. 'n Algemene metode wat gebruik word vir die verwydering van hierdie spesies is, onkruiddodertoediening. Die oorbenutting van hierdie chemikalieë en die afloop van die oppervlak in nabygeleë riviere, het probleme veroorsaak. Die effek van hierdie chemikalieë op die varswater biofilms, wat uit verskeie mikrobiese gemeenskappe bestaan, is egter nog onbekend.

Mikrobiese gemeenskappe speel 'n belangrike rol in die funksionering van oewer-ekostelsels in die biogeochemiese sirkulering. Biofilms is meestal voordelig vir hul omliggende omgewing, maar die mikrobiese gemeenskappe verander afhangend van die blootstelling aan eksterne faktore. Hierdie faktore is meestal abiotiese faktore, en sluit die blootstelling aan chemikalieë in. Hierdie chemikalieë kan beide skadelik of voordelig vir die mikrobiese gemeenskap wees. Sommige mikrobiese spesies breuk hierdie chemikalieë af en gebruik dit as ‘n voedingsbron.

Hierdie studie ondersoek die effek van drie konvensionele onkruiddoders, Springbok, Garlon en Plenum op varswater biofilms. Die Bergrivier in die Wes-Kaap is gekies omrede, dit deur gebiede vloei waar onkruiddoders toegedien word. 'n Geoptimaliseerde onkruiddoder opsporings metode met behulp van vloeistof chromatografie gekoppel aan massaspektrometrie (LC-MS) is ontwikkel en gebruik om die huidige vlakke van aktiewe bestanddele in die rivier te bepaal.

'n Voorlopige studie is uitgevoer om die effek van glifosaat op varswater biofilms te bepaal. Hierdie studie het getoon dat 'n beduidende mikrobiese gemeenskapsverskuiwing na die toediening van die onkruiddoder plaasvind, 'n fisiese ontwrigting van die biofilm is ook waargeneem teen dieselfde toegediende konsentrasie.

Na afloop van die ondersoek studie is rivierwater in die Bergrivier versamel wat gebruik is om 'n kunsmatige rivierstelsel in die laboratorium op te rig. Vloei selle is gebruik om die biofilms te kweek, met die rivierwater as die inokulum. Die biofilms is toegelaat om te groei vir tien dae voor die onkruiddoder en die aktiewe bestanddeel toegedien is. Die vloei selle was gebruik vir

fluoresserende mikroskopie en DNA ekstraksies. Garlon, Plenum en Springbok het bevind dat die biomassa aansienlik verminder het ($p < 0.05$) vir alle behandelings.

Laastens is die DNA van die biofilm uit die vloeiselle verkry deur gebruik van die standaard DNA-ekstraksie protokol. Die DNA was gebruik vir polimerase kettingreaksie (PKR) amplifikasie met bakteriese en swamspesifieke as inleiers. Geoutomatiseerde ribosomale intergeniese afstand ontleding (ARISA) inleier stelle is gebruik. Beduidende verskuiwings ($p < 0.05$) was gevind vir die onkruiddoders, Springbok (glifosaat) en Plenum (fluroxypyr en pikloram). Die Shannon diversiteitsindeks toon 'n afname in diversiteit vir hierdie behandelings, wat dui daarop dat sommige spesies geneig is om die biofilm te oorheers, omdat hulle die omgewingsverandering kan duld.

Die hipotese van die studie is dat algemene onkruiddoder toediening die mikrobiese gemeenskappe in vars water riviere beïnvloed is nie onaanvaar nie. Dit is gestaaf deur die resultate wat gevind is in die studie. Die resulte sluit in dat daar ‘n beduidende verskil was in biofilm biomassa na die toediening van onkruiddoders. Daar is ook gevind dat daar ‘n mikrobiese gemeenskap verskuiwing plaasvind nadat die onkruiddoder toegedien was.

Chapter 1

Literature review

Introduction

Riparian ecosystems in the Western Cape's fynbos biome provide unique ecological services that include riverbank stabilization, flood attenuation and water purification (Ruwanza et al., 2013). These services are essential for the health and functioning of the surrounding habitats. Most riverbanks of the middle and lower parts of catchments in the Western Cape are densely populated with invasive plant species. The control of invasive species has become a global challenge (Mcneely, 2000). These invasive alien plants (IAPs) spread rapidly from habitat to habitat, especially within river catchments, due to the linear and dynamic nature of the ecosystem. Invasion by and spread of alien plants mainly occurs through seed dispersal and human activity. One of the consequences of invasion is that space available for indigenous vegetation becomes limited, especially where it occurs close to continuous water sources like rivers (Theoharides & Dukes, 2007). Two major invasive plant species occur in the Western Cape of South Africa namely, *Acacia mearnsii* and *Eucalyptus camaldulensis.* These species, along with 200 other invasive plants, dominate most of the riverbanks and pose a major threat to the fynbos riparian ecosystems in the Western Cape. They also have a very high water consumption compared to indigenous trees (Chamier et al., 2012). Invading species monopolize water resources so effectively that native vegetation is almost completely excluded (Holmes et al., 2008).

Considering that only 12% of the land in South Africa can be used for crop cultivation (Agriculture, 2011), the removal of IAP's becomes vital (Ruwanza et al., 2013). Considerable energy and funds are expended by farmers and land managers to prevent and control invasive plants (Irvine et al., 2013). Two methods that are regarded as effective by the Working for Water (WfW) program for the control of invasive plants in South Africa are "slash and burn", which is an agriculture technique that involves the cutting and burning of plants, and the application of herbicides (Wigley, 1992). Herbicide application is one of the most effective ways to remove or inhibit growth of invasive plants (Irvine et al., 2013).

Working for Water

The WfW program has been established to reduce the invasion of alien plants in South Africa. The program was implemented in 1995 and is currently administered by the Department of Environmental Affairs. It is globally recognized for the outstanding effort it has made to clear invasive alien plants and restore hydrological ecosystems services (Görgens & Van Wilgen, 2004). The main objective for the program is to reduce the density of invasive alien plants and protect water resources and riparian ecosystems. They strive to achieve this through labor-intensive physical removal, mechanical and chemical control (Turpie & Blignaut, 2008).

Herbicides

Herbicide application is one of the most effective methods used by the WfW program for control of invasive plants. Herbicides are chemicals that interact with the biological pathways of certain plants to inhibit their growth. The goal of chemical control is to prevent and inhibit the growth of invasive plants to create land space so that restoration of native vegetation can take place or so that more crops can be planted (Richardson & van Wilgen, 2004). A few studies discussed the effect that herbicides have on individual microbial organisms but not on biofilms or whole communities (Haney, 2000; Zabaloy, 2008). The long-term application of glyphosate, a herbicide, can result in a microbial community shift and cause plant nutrition deficiencies and diseases (Dick et al., 2010). Dick et al. (2010), also used the fatty acid analysis of microorganisms to determine if microbial communities can adapt and tolerate glyphosate. They concluded that some microorganisms can use glyphosate as a nutrient source. Zabaloy et al. (2008), applied glyphosate concentrations well above the recommended rates and found that minimal changes to the soil biology and microbial diversity occurred. This is in contrast to some studies that showed significant effects, such as soil microbial community changes when glyphosate is applied to soil (Busse et al., 2001; Ratcliff et al., 2006). Zabaloy et al. (2008), concluded that field testing of long-term impacts is needed to verify the effects observed in their study and to validate the microcosms tested in the study. Molecular work such as sequencing would provide better insight to the community changes that occur during glyphosate exposure (Pollegioni et al., 2012).

The effects of the various herbicides applied to the field are still unknown. There is an urgent need to improve our understanding of the impact these chemicals have on riparian ecosystems

and the impacts on aquatic ecosystems is also relatively understudied. The effect on microbial communities and species recovery is yet to be investigated.

Soil health

Soil management is fundamental to all agricultural systems (Kibblewhite, 2008). Different herbicides could possibly affect the chemical and biological composition of soil. It is postulated that herbicides have a major influence on the soil microbial communities that are primarily responsible for nutrient cycling in the soil (Pose-Juan et al., 2017; Crouzet et al., 2016). Crouzet et al. (2016), showed that herbicides affect microbes that are responsible for nitrogen cycling in soil. When these microbes are suppressed, a decrease in the total nitrogen found in soil is seen. The effectiveness of the soil management systems needs to be investigated. This will help to manage soil health more effectively in the future (Nielsen & Winding, 2002).

Chemical cycles describe the pathway by which elements move through the biotic and abiotic parts of the earth. Major chemical cycles include nitrogen, oxygen, phosphorous, and sulfur cycles. Microbial communities are extremely sensitive to abiotic factors and form the basis of any ecosystem. Molecules form the basic building blocks for microbes to survive. Microbes are responsible for the primary degradation of the molecules mentioned above. When the microbial communities are disturbed, there could be a potential change in the biogeochemical cycle (Six et al., 2006). Soil health is linked to the microbial interactions that are responsible for different chemical cycles and can easily be disturbed by changing one or more of the microbial species in the soil or water (Nielsen & Winding, 2002). When microbial communities are impacted by chemicals such as herbicides, it could lead to a series of changes that will take place in the ecosystem (Nielsen & Winding, 2002).

Ahearn et al. (2005), showed that river water quality is controlled and directly affected by anthropogenic factors which change the chemistry in the river water. The chemistry is largely affected by interflow through organic rich soils and soils that are used for agricultural purposes. The nutrients in the soil along the riparian and buffer zones enters the river through rainfall and surface runoff. Therefore, when herbicides are sprayed on buffer zones, it affects the microbes responsible for placing nutrients in the soil which will lead to a decrease in nutrients that ends up in the river. The application of herbicides indirectly affects the quality of the river water streams that are running through affected areas (Turner & Rabalais, 2003).

Importance of microorganisms in a river ecosystem

Different forms of microbes exist in freshwater rivers. These forms include individual cells and multiple cells that aggregate (better cluster) together and can consist of a diversity of species. This form of microbial community is known as a biofilm and forms the major form of microbes in rivers.

Biofilms in freshwater consist of consortia of microorganisms such as algae, bacteria, cyanobacteria, fungi and protozoa that attach themselves to surfaces. These microorganisms are embedded in an extracellular matrix, which consists of polymeric substances (Sabater et al., 2007). Biofilms play an important role in the uptake of organic and inorganic nutrients in freshwater rivers and act as a filter for harsh chemicals (Cortes-Lorenzo et al., 2006). The community structure of the biofilms may vary depending on the environment they are found in or exposed to. Biofilms are used in industrial wastewaters for the removal of ammonia, phenol and other chemicals that become harmful in high concentrations (Cortes-Lorenzo et al., 2006). The biofilms that are used in wastewater treatment plants, usually are dominated by alpha and beta proteobacteria (Ivnitsky et al., 2007).

Freshwater biofilms are usually attached to rocks, stones, sand, leaves, wood etc. Most biofilms produce extracellular enzymes in the polymeric matrix that facilitate organic nutrient uptake (Sabater et al., 2007). These enzymes are responsible for the sorption of exogenous organic compounds in order for the accumulation of nutrients from their environment as well as the accumulation of enzymatic activities. The extracellular polymeric substance (EPS) matrix can be defined as a medium which allows for cooperation and communication among cells in microbial aggregates (Laspidou & Rittmann, 2002).

The influence of herbicides on biofilms may affect the activities and processes in which biofilms are involved (Sabater et al., 2007). However, the effects of herbicides on aquatic microbial diversity are relatively unknown and will be investigated in this study. Numerous invasive species have invaded riverine environments, and is being actively managed by chemical means by WfW personnel, implementing agencies, as well as agricultural landowners; it is therefore important to determine what the impact is of herbicides on biofilm composition and function (Tererai et al., 2013).

1) Overview

The invasion of alien plant species is a problem that is globally recognized, with different countries across the world managing their own unique set of invasive plant species (van Wilgen, 2000 ; Early et al., 2016 ; Callaway & Aschehoug, 2000). It is estimated that the annual global cost of control of invasive plant species equals about 5% of the world's economy. In South Africa, R6.5 billion is spent annually on invasive plant control in order to sustain ecosystem services (van Wilgen et al., 2012). One of the major programs involved in the clearing of invasive plants is WfW, an ecosystems restoration initiative started in 1995. The program is unique in its scope, as well as tying together ecosystems repair with social upliftment, and has been largely successful in clearing woody invasive species from some critical resource areas (van Wilgen & Wannenburgh, 2016). Invasive species that are faced by WfW are several species within the Australian genera *Acacia* and *Eucalyptus*. Some of the most invasive species involved are *Acacia mearnsii* and *Eucalyptus camaldulensis* (Figure 1). Both species are transformer species which will change and adapt their characteristics, form and condition depending on the extent of the ecosystem they are found in (Le Maitre et al., 2002).

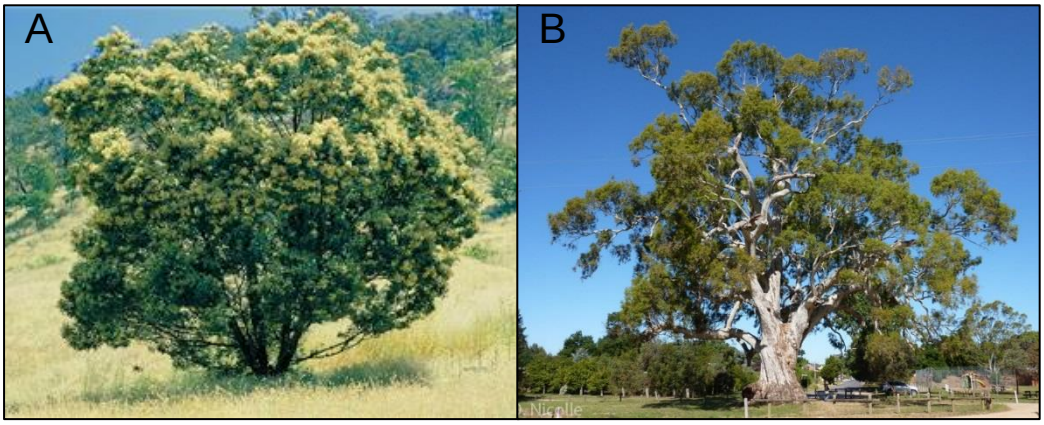

Figure 1: A) *Acacia mearnsii.* B) *Eucalyptus camaldulensis,* two of the most common invasive plant species along the riverbanks of the Western Cape in South Africa (Tian et al., 2000).

1.1) Invasive species

Le Maitre et al. (2002), estimated that alien plant species have invaded 10 million hectares of available ground surface in South Africa. The magnitude of this problem however, is larger than originally thought (Le Maitre et al., 2002). A preliminary classification was started by Nel et al. (2004), dividing invasive plants into two classes namely; major invaders and emerging

invaders. Nel et al. (2004), aimed to develop a management strategy for current programs that are assigned to clearing invasive plant species. The classification system provides a starting point for predictive modelling of major and emerging invaders.

Using this classification system, *Acacia mearnsii* and *Eucalyptus camaldulensis* falls under the very widespread and abundant category that invade both riparian and landscape areas across South Africa (Nel et al., 2004). The focus of clearing programs, therefore, shifted towards these two major invaders.

1.1.1) *Acacia mearnsii*

Australian *Acacia* species were initially introduced in South Africa for forestry, dune stabilization and to prevent riverbank erosion (Wilson et al., 2011). These species have a fast growth rate and prolific seed production which allow them to distribute quickly and over a wide area. These characteristics pre-adapted this species as an invader of disturbed environments, for example riverbanks in the fynbos biome, and pose a major threat to riparian fynbos ecosystems. Riparian areas are essentially the interface between terrestrial and aquatic systems (Naiman et al., 2004; Kaplan et al., 2012), and in the fynbos, are especially vulnerable to human disturbance (Holmes et al., 2008). A third of the total cost of alien plant clearing is spent on clearing *Acacia* species that dominate South African riverbanks. An estimated R354 million is spent towards labor by farmers, the government and land owners in the clearing processes. Detecting and preventing invasive species while the species population is relatively small will reduce the cost of its eradication (Kaplan et al., 2012). With limited funds available, it becomes important to prioritize efforts based on the environmental and economic risks involved, and such efforts often involve biological, chemical and mechanical control approaches (Foxcroft, 2001).

Acacia mearnsii (also known as Black Wattle) is a nitrogen fixing species that has the ability to accumulate long lasting seeds in the soil. It causes a number of problems along South African riverbanks. The spread of this species is hard to control and it is relatively resilient to chemical treatment (Wilson et al., 2011). It has a high-water demand and it is estimated that the annual cumulative consumption of water by *A. mearnsii* in South Africa is in the order of 300 million cubic meters. It grows extremely well in areas where water is in steady supply such as riverbanks. Other plants are out-competed due to the ability of *A. mearnsii* to form shady

thickets and large quantities of leaf litter. This allows this species to outgrow other indigenous vegetation along the riparian zones.

1.1.2) *Eucalyptus camaldulensis*

Eucalyptus camaldulensis is another species that dominates South African riverbanks (Wilson et al., 2011). *Eucalyptus camaldulensis* (also known as Red Gum) is known as an invasive species in South Africa and has the ability to reproduce as a seedling. *Eucalyptus camaldulensis* can tolerate drought and has the potential to grow rapidly (Ruwanza et al., 2013). The species colonizes water courses which make it one of the most successful species to grow near rivers. *Eucalyptus camaldulensis* relies on seasonal flooding or the presence of a high-water table. This plant has been recognized as an invasive species and a major problem species in South Africa (Tererai et al., 2013). In part due to the impacts of the species, a program was launched in 1995 called the WfW program. Masubele et al. (2009), describes *E. camaldulensis* as a habitat transformer species that will change their characteristic or nature of their environment.

1.2) WfW responsibilities and control methods

One of WfW's responsibilities is the clearing of *Acacia* and *Eucalyptus* species that pose a threat to the country's biological biodiversity and ecosystems (Tererai et al., 2013). These invasive species not only pose a threat to indigenous species but also to water security and the functioning of natural systems (Kaplan et al., 2012). Efforts made by the WfW program have been very successful, but optimization of their current methodologies used to control the invasive species is required. This will result in more effective clearing processes and minimize the costs involved in the program, as well as reduce the impact of the clearing methods on the environment, increase effectivity and enhance restoration efforts (Le Maitre et al., 2002). Currently, there are three methods that are used to prevent and control invasive alien plants (IAP's) in South Africa. The three methods involve biological control, chemical control and mechanical control.

Biocontrol is a used to control IAPs. The WfW program introduced parasites, pathogens and pests from natural habitats of invasive species in South Africa to control the rapid expansion of IAPs. The biological organisms are the IAP's natural enemy and effective biocontrol agents will reduce the invasive alien plant's competitive advantage. Biocontrol agents target specific plant organs and can be selective, depending on the plant's physiology (Wilson et al., 2011). Fungi

are often used as biocontrol agents (Kiss, 2003; Shah & Pell, 2003). Biocontrol agents go through very stringent initial screening, and are considered safe for the environment and should therefore not negatively affect other plants species in the applied area. The agent is thus only released when sufficient research has been done to determine if the control agent is host specific or not (Wilson et al., 2011). The effectiveness of the agent depends on the toxicity of the control agent that is being used and the ability of the host plant to resist said agent. In most cases a biocontrol agent will only suppress the growth of the IAPs and is not a good method for eradication (Brimner & Boland, 2003). Thus, alternative methods are used to completely eradicate IAPs where necessary (Turpie et al., 2008).

Mechanical clearing is an alternative method that is used in the program. It involves the cutting of IAPs, followed by leaving it intact on site, physically removing it, mulching and removing it, or burning the slash (Holmes et al., 2008). Physical removal techniques include “fell and remove” and “fell only”. The “fell and remove” technique is where the biomass is physically removed from the area. This technique is only used where it is practically feasible. The “fell and burn” technique is generally used in areas not easily accessible to large machines. The biomass is therefore, stacked or piled where it is then burned under controlled conditions without damaging the indigenous plants (Holmes et al., 2008). The agricultural technique which involves the cutting and burning of plants is often referred to as slash or pile burning (Kaplan et al., 2012). This is a form of shifting cultivation by cutting down IAPs and burning them. Slash and burn works reasonably well, but in some cases the soil under the piles becomes sterile to indigenous plants. Thus, an alternative method can be used which involves applying chemicals to the IAPs (Blossey et al., 1999).

Chemicals that are used to kill unwanted plants are known as herbicides. Depending on the purpose of application, herbicides are selective for certain plants. These chemicals interact with biological pathways of plants to inhibit their growth (Tiam et al., 2015). Various herbicides exist, each with a different mode of action, which are designed to kill target plants. Table 1 shows the four most used conventional herbicides for the control of *Acacia spp.* and *Eucalyptus* species. Herbicide application methods are very successful but may have a larger impact on the riparian ecosystems of rivers as well as in the river. In-depth research is needed to fully understand the impact of these chemicals on riparian ecosystems. Different active ingredients are used in herbicides and each active ingredient has a unique mechanism of action, and effect on the host plant (Table 1). Some herbicides are broad spectrum and will affect a wide range of plants,

while other herbicides are host specific and can be applied to the environment where it will only affect the target species.

Table 1: Examples of herbicides that are currently being used for control of *A. mearnsii* and *E. camaldulensis*. Recommended dosage is indicated according to Sharpe (2012) and Working for Water (2014).

Species	Tree size, treatment method	Herbicide concentration & Active ingredient	Application rate (L/ha or kg/ha)	Mode of action
Acacia mearnsii	Seedlings, foliar spray	Glyphosate (as ammonium salt) 680 g/kg	2.4	Shikimate pathway
		Triclopyr (as butoxy ethyl ester) 240 g/L	1.5	Limits nutrients
		Imazapyr 100 g/L	-	Inhibition of acetohydroxy acid synthase
		Picloram (as potassium salt) 240 g/L	3	Synthetic growth hormone
Eucalyptus camaldulensis	Young and Adult trees	Fluroxypyr 80 + Picloram 80 g/L	9	Auxin type response

1.3) Herbicides used by the WfW

1.3.1) Glyphosate

Glyphosate is a non-selective, foliar, systemic herbicide that is commercially used for the control of a wide range of annual and perennial grasses, broadleaf weeds and certain woody perennials. In South Africa, glyphosate is specifically used for the control of *A. mearnsii* (van Rooyen & van Rooyen, 2014). Glyphosate is also known as N-phosphonomethylglycine (Lima et al., 2014) and is applied to low cut stumps of *A. mearnsii*, above ground level. The recommended herbicide application rate for freshly cut stumps are sprayed to the point of run-off. It can also be applied to young trees up to one-meter high. Glyphosate interacts with a plant's ability to synthesize important amino acids (Pollegioni et al., 2011).

Aromatic amino acids, phenylalanine, tryptophan and tyrosine, are synthesized by a seven-step metabolic pathway known as the shikimate pathway (Tohge et al., 2013). This pathway is also used by fungi, bacteria and algae (Irvine et al., 2013). Phenylalanine and tryptophan are essential amino acids. Glyphosate acts as a substrate for the enzyme 5-enolpyruvoyl-shikimate-3-phosphate synthetase (EPSPS), and inhibits the activity of the enzyme EPSP (Figure 2). EPSP catalyzes the reaction between shikimate-3-phosphate and phosphoenolpyruvate to produce 5-enolpyruvylshikimate-3-phosphate. Disruption of the shikimate pathway leads to death of the plant.

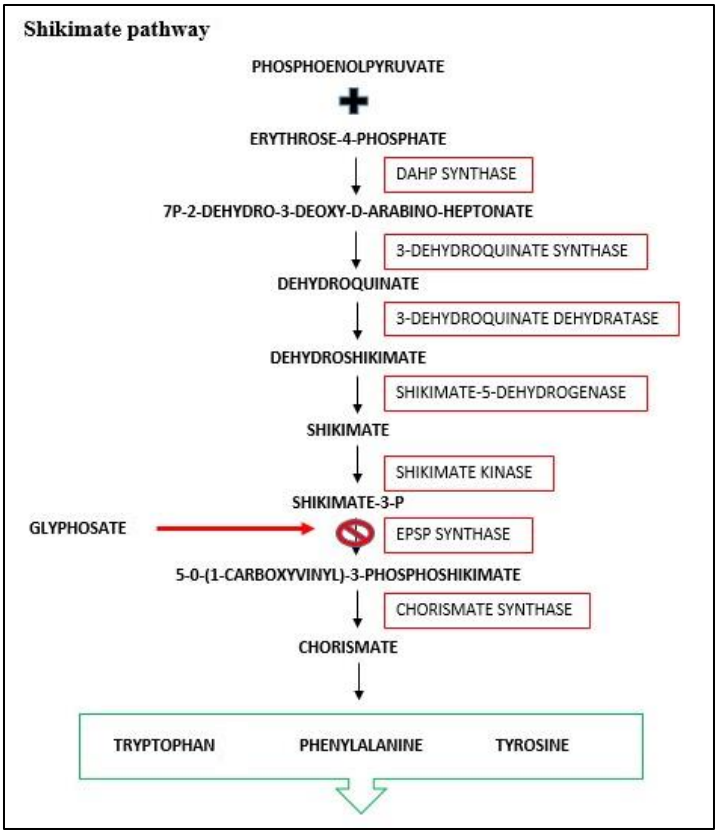

Figure 2: The shikimate pathway catalysis the conversion of phosphoenolpyruvate and erythrose-4-phoshate to produce chorismate by means of a 7-step enzymatic reaction. Chorismate is used to synthesize three aromatic amino acids, phenylalanine, tyrosine and tryptophan. Glyphosate interact with this pathway in step 6 (EPSP synthetase) by acting as a substrate for the EPSPS enzyme. Adapted from Roberts et al. (2002).

The role of glyphosate as a pollutant also needs to be considered as there is a lot of concern about possible health related risks to humans and animals (Lima et al., 2014). In previous

studies, the impact of glyphosate on soil and aquatic biomes has been investigated (Dick et al., 2010; Busse et al., 2001). In some cases the glyphosate enhanced the growth of microorganisms at certain concentrations (Lima et al., 2014). It was also found that continuous exposure of bacteria to low concentrations of glyphosate leads to an increase in aerobic growth (Lima et al., 2014). Glyphosate is taken up by plants through their foliage during growth, accumulates in plant tissues and can leach from roots into the soil. Rivers and fresh water streams can be contaminated by run-off following rainfalls and the overuse of glyphosate (Sanchís et al., 2012).

1.3.2) Auxin type herbicides

Auxins are a class of plant growth hormones and are essential for the plant to survive and grow. There are five naturally occurring auxins in plants which include indole-3-acetic acid, 4-chloroindole-3-acetic acid, phenylacetic acid, indole-3-butyric acid and indole-3-propionic acid. Indole-3-acetic acid (IAA) is the most abundant and is the most important in plant growth while the other four auxin hormones are only marginally important (Grossmann, 2010). Auxins can be harmful to the plant in high concentrations which will lead to plant death. This characteristic of auxins is an advantage in chemical control agents such as herbicides.

For over half a century, synthetic auxins have been used with a high degree of success. The most important chemical classes of synthetic auxins include pyridines. Triclopyr, fluroxypyr and picloram are all herbicides that falls under the pyridine based synthetic auxins. Synthetic auxin chemicals mimic the effects of high endogenous auxin concentrations (Grossmann et al., 2001).

A mode of action was proposed by Grossmann (2001), (Figure 3) which suggests that ethylene is involved in the regulation of leaf senescence and inhibits elongation growth. When ethylene is over expressed in a plant, it becomes toxic and will lead to plant death. A few studies have shown evidence that auxin type chemicals stimulate the over production of ethylene through the induction of indole acetic acid (ACC) synthase. When ethylene production is stimulated, it elicits abscisic acid (ABA) biosynthesis through increasing xanthophyll cleavage to xanthoxal which is a ABA precursor. When ABA is accumulated, it leads to the reduction of stomatal apertures and carbon dioxide assimilation. This chain reaction results in growth inhibition and tissue senescence. Grossmann et al. (2001), also showed that auxin type herbicides are responsible for the accumulation of hydrogen peroxide which contributes to the progression of tissue damage.

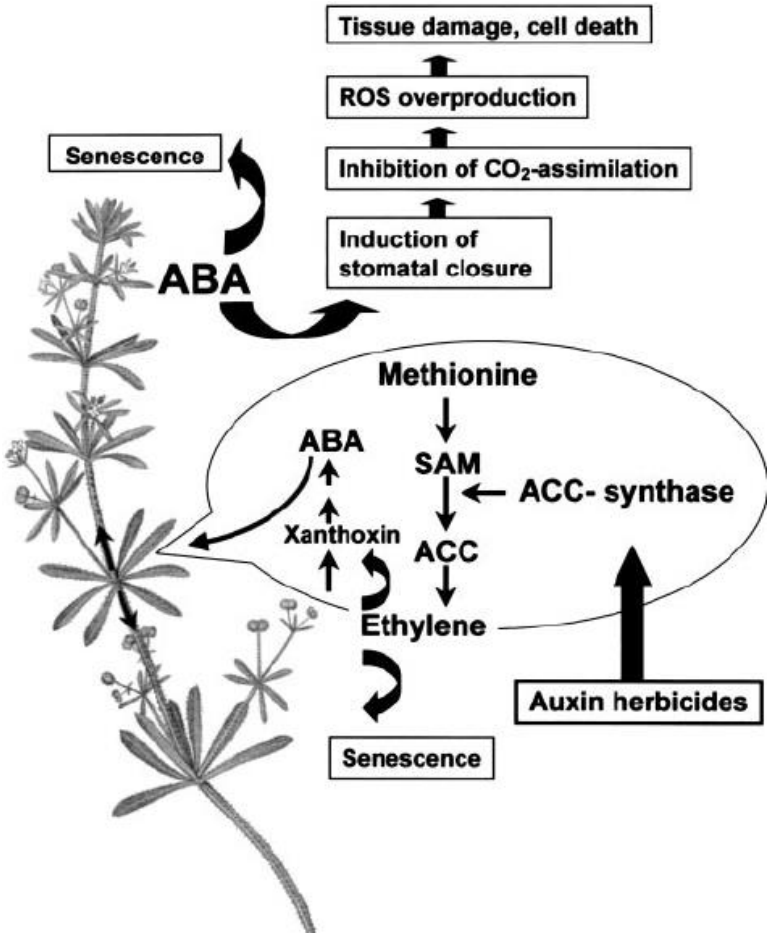

Figure 3 – The proposed mode of action of auxin herbicides on a plants' metabolic pathways (Grossmann et al., 2011).

1.3.3) Triclopyr

Triclopyr is a selective systemic herbicide that is used for the control of a wide range of woody and broadleaf plants (Souza-Alonso et al., 2015). It is less toxic than glyphosate and has little to no effect on grasses. Triclopyr can exist in two forms, namely trimethylamine salt and butoxyethyl ester. These compounds mimic the plant hormone auxin (ACC) and cause uncontrolled plant growth. The exact mode of action is not known but it is believed that the compound interacts with the RNA and DNA replication cycle which results in uncontrolled cell growth (Tu et al., 2001).

The amine or salt form is sold under the tradename Garlon 3A and the ester form is sold under Garlon 4. Microorganisms are primarily responsible for the degradation of these compounds in soil and the major metabolite that is formed after degradation is triclopyr acid (Souza-Alonso et al., 2015). Photolysis and hydrolysis could also contribute to the degradation of these two compounds. The half-life of this compound in soil ranges from 30 to 90 days and this is due to the weak bond it forms with soil particles. In water, the compound degrades rapidly.

1.3.4) Fluroxypyr and picloram

Fluroxypyr is a herbicide that is used for a wide range of broadleaf weeds and plants. It is produced commercially as an ester which is called starane. Starane degrades over time to flyroxypyr acid. Fluroxypyr acts as a synthetic form of auxin and interacts with the RNA and DNA of the plant which results in uncontrolled cell growth (Gunsolus et al., 1999).

Fluroxypyr is used in conjunction with picloram to enhance the efficacy of the herbicide against *Eucalyptus* species. Picloram on its own is a systemic herbicide which in general works for all woody plants but works best for *Acacia* species where it interferes with the growth hormones of the plant resulting in uncontrolled growth and will lead to plant death. This compound binds weakly to soil particles which means that it can easily percolate through the soil profile and end up in freshwater streams and nearby water sources (Halimah et al., 2004).

1.4) Surfactants and Adjuvants

Herbicides commonly use a chemical that enhances the performance of the herbicide which is referred to as adjuvants or surfactants (Moore et al., 2010). Adjuvants are chemicals that modify or change the mode of action in which the active ingredient enters the plant cells. The potency of the active ingredient is enhanced when used in combination with the adjuvant (Mesnage et al., 2013). In some cases more than one adjuvant can be added to the herbicide but this depends on the purpose of the herbicide (Ruwanza et al., 2013). Herbicides which are non-specific will contain more than one adjuvant compared to herbicides that are specific and designed especially for one plant species. Surfactants are often referred to as adjuvants. A surfactant is a type of adjuvant which reduces the surface tension of the external surface layers of water in which the herbicide is mixed (Moore et al., 2010; Paria & Khilar, 2004).

The toxicity of adjuvants to the environment is not well studied. Hundreds of chemicals can be used as an adjuvant in herbicides and pesticides, so it is difficult to determine the effects on the

environment of each adjuvant. Studies showed significant effects of some of the adjuvants that are used in herbicides. Wilkinson et al. (1997), showed that the commonly used adjuvant, nonylphenol is highly toxic to freshwater fish species that were tested in the study. Brausch and Smith (2007) tested polyethoxylated tallow-amine (POEA) which is a commonly used surfactant in "Roundup-Ready". POEA showed significant toxic effects on the macroinvertebrate *Thamnocepjalus platyurus* in all the recommended formulation ratios. Based on this and other studies, adjuvants can be potentially more toxic than the active ingredient itself.

Herbicides end up in the nearby rivers due to run off and the overuse of the chemicals, leading to contamination of the nearby rivers. Water contamination is a major concern in the Western Cape, especially in the Berg River (Fuggle & Rabie, 2009), which is why it is important to understand the impact of these chemicals on the environment. Contaminating the freshwater rivers may lead to ecosystem changes and may affect the microorganisms in aquatic environments (Dorigo et al., 2004).

1.5) The effect of herbicides on biofilms

A typical biofilm consists of an extracellular matrix which is mostly composed of water and a few extracellular polymeric substances (Flemming & Wingender, 2010). The EPS is primarily responsible for cell to cell and cell to surface interactions and forms the foundation of a biofilm. This improves the formation stability of the overall biofilm cells. The structural components of the EPS matrix are made of proteins, DNA, RNA, lipids, proteinaceous pili and a mixture of polysaccharides. The components of the EPS matrix play important roles in the uptake of organic and inorganic nutrients. Rendueles et al. (2013), highlighted the importance of exopolysaccharides in maintaining the stability of the biofilm.

Bryers and Ratner (2004) discussed the formation principle of a biofilm (Figure 4). Starting from single planktonic cells (step 1) followed by cell adsorption and cell to cell signaling (Step 2-5). Once the cells have reached a threshold value, they will start to replicate and grow. In the growth phase the cells will secrete polysaccharides which forms the matrix (Bryers & Ratner, 2004).

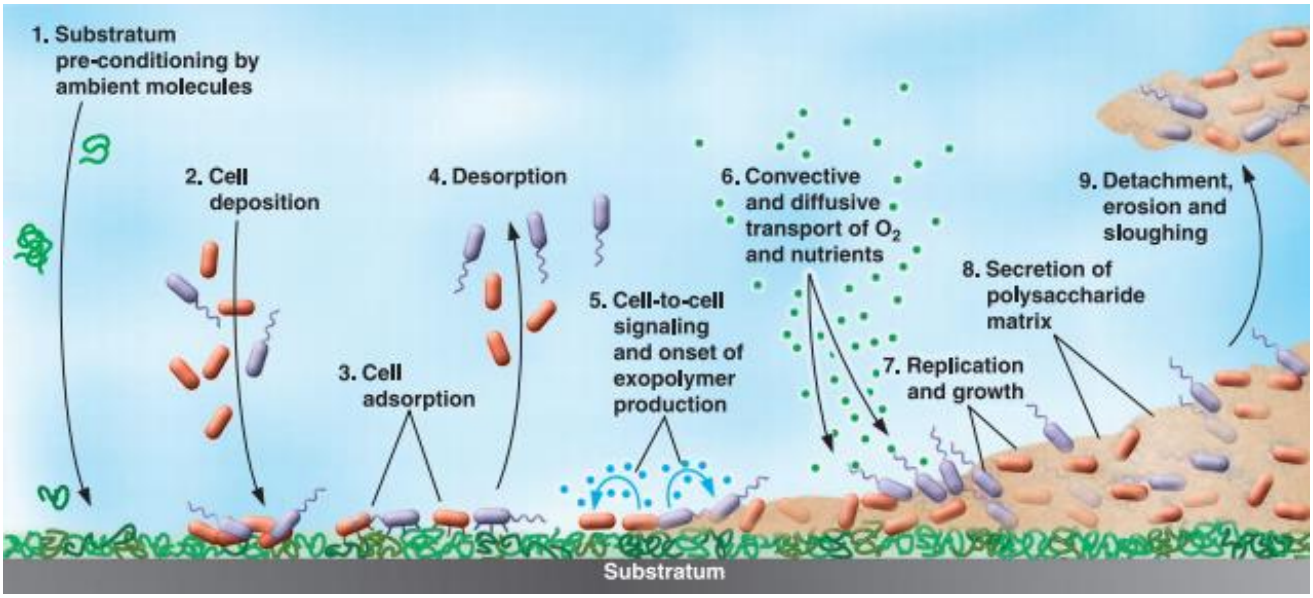

Figure 4 – The process and formation of biofilms by which planktonic cells attach themselves to surfaces (Bryers & Ratner, 2004).

Biofilms participate in fundamental processes such as biogeochemical cycling, energy flow and biodegradation in freshwater streams and rivers (Lawrence et al., 2004). The role of biofilms in water systems are the cycling of nutrients and the breakdown of large compounds into smaller compounds that can be utilized by plants and other living organisms (Battin et al., 2003). Without these microbial processes, many compounds cannot be utilized as nutrient sources. Biogeochemical processes typically include nitrogen, oxygen, phosphorous and carbon cycling. When biofilms are affected by chemicals such as herbicides, it directly affects nutrient cycling processes in the water streams (Battin et al., 2003).

1.6) The effect of surfactants and active ingredients on microorganisms

Microorganisms are unique, and some species can utilize harmful chemicals. Herbicides that contain the active ingredient glyphosate can be utilised by two bacterial species, *Strenotrophomonas maltophila* and *Providencia alcalifaciens,* that were isolated by Nourouzi et al. (2011). The bacterial cell first degrades glyphosate to amino methyl phosphonic acid (AMPA) that contains a phosphorous atom and mainly consist of carbon molecules. In the study it was observed that *S. maltophila* and *P. alcalifaciens* removed glyphosate without a significant lag phase (Nourouzi et al., 2011). Both glyphosate and its major metabolite can be

used as carbon and or phosphorous source. These findings in Nourouzi et al. (2011), are consistent with the findings of McAuliffe et al. (1990).

Some researchers suggest that extensive treatment of soil with herbicides can cause populations of beneficial microorganisms to decline. In some cases, the herbicide can enhance the growth of certain bacteria and completely inhibit the growth of others (Rathore & Nollet, 2012). Rathore and Nollet (2012) described the overuse of herbicides on soil as similar to the effect of the overuse of antibiotics on humans. This is especially critical as fungal and bacterial communities are mainly responsible for the quality of soil. This emphasizes the importance of microorganisms in the environment. Plants and other environmental factors depend on bacteria and fungi to transform atmospheric nitrogen into nitrate which they can utilize. Triclopyr, picloram and fluroxypyr inhibit microorganisms that are responsible for the transformation of ammonia into nitrate (Mahmood et al., 2016). Glyphosate inhibits the growth of free-living bacteria that are responsible for nitrogen fixing as well as those that form symbiotic root nodules.

The exposure of biofilms to these herbicides is a major concern for eco-toxicologists and needs to be investigated. In work done by Folmar et al. (1979), the toxicity of the actual active ingredient (glyphosate salt) versus the surfactant (polyoxyethelene amine – POEA) on freshwater invertebrates were investigated. In the study they concluded that the surfactant was more toxic to freshwater invertebrates than the technical grade glyphosate (Folmar et al., 1979).

Another study on glyphosate was done by Tsui and Chu (2003), where they tested the effect of technical grade glyphosate and its surfactants on seven different organisms consisting of bacteria, algae, protozoa and crustaceans. In this study, they have found that algae, which are photosynthetic, possess similar metabolic pathways found in plants, such as the aromatic amino acid synthesis pathway. Therefore, when glyphosate is applied in an ecosystem, it may affect algae, which is a non-target species. An important pathway that is found in plants and microorganisms, and which is responsible for the synthesis of aromatic amino acids is called the shikimate pathway as described in section 1.4.1. Algae were more susceptible to the herbicide salt compared to organisms that are non-photosynthetic (Tsui & Chu, 2003). Non-photosynthetic organisms such as bacteria, protozoa and crustaceans should be more resilient to the toxicity of glyphosate salts. However, POEA that was tested by Tsui and Chu (2003), showed a significant effect on all seven organisms that were tested.

In plants, glyphosate interrupts the shikimate pathway which leads to the reduction in protein synthesis. The plant will die off within 4-20 days (Mahendrakar et al., 2014). In humans the effect of glyphosate is less pronounced, due to the absence of the shikimate pathway. Glyphosate in mammals is involved with the uncoupling of oxidative phosphorylation. The mechanism of action for triclopyr, picloram and fluroxypyr in mammals is yet to be investigated.

Surfactants are designed to help the active ingredient penetrate the plant cell and to reduce the surface tension on the leaf. Surfactants may possibly act to help the active ingredient penetrate the EPS matrix. The disruption of the EPS matrix may be due to the ability of the surfactants to penetrate the matrix. When herbicides are overused it ends up in the river water due to rainfall. A dilution effect occurs in the river due to the large quantity of water that gets mixed with the sprayed herbicide, which makes the concentration of herbicide to which the biofilms are exposed to, very low. The biofilm may build up resistance to the herbicides they are exposed to in low concentrations. This may make them more resilient to change when exposed to a more concentrated form of herbicide.

The present study will provide insight into the effect of three different herbicides namely Springbok, Garlon and Plenum on biofilm communities in river water. The active ingredient for each of the respective herbicide will also be tested on its own, without the surfactants. Currently, clearing operations are taking place to remove the invasive species *A. mearnsii* and *E. camaldulensis* in the Berg River catchment, which makes this river ideal for sampling for this study. The aim is to evaluate the impact of recommended levels of herbicides used to control alien invasive plants on riparian soil microbial and biofilm diversity. Physical alteration of the biofilm structure will be investigated through confocal microscopy and the influence on the microbial communities will be investigated using molecular fingerprinting techniques to observe the microbial diversity change.

The hypothesis is that the herbicide application will result in a microbial community shift so that certain microorganisms will dominate the biofilm. Three project aims were identified which include the following: the first aim was to optimize the detection protocol for conventional herbicides used in clearing operations to help in conducting a herbicide survey of the Berg River. The second aim was to determine the effect of conventional herbicides on the structure of freshwater biofilms. The third aim was to determine the effect of conventional herbicides on the microbial communities of freshwater biofilms. These aims were used to test the hypothesis.

1.7) Pilot study

The aim of the pilot study was to perform an experiment which will provide insight into the effect of glyphosate on biofilm communities in riparian zones. The second aim of this study was to accurately qualify and quantify the presence of AMPA. Currently, clearing operations are taking place to remove the invasive species *A. mearnsii* and *E. camaldulensis* in the Berg River agricultural areas, and so this area is ideal are for sampling. The objective is to evaluate the impact of recommended levels of glyphosate used to control alien invasive plants on riparian soil microbial and biofilm diversity. The hypothesis was that the herbicide application will result in a microbial community shift so that certain microorganisms will dominate the community. A second river, the Eerste River that is not contaminated by glyphosate, was sampled to serve as a control. The Eerste River flows through the Jonkershoek Nature Reserve which is protected from most anthropogenic activities.

1.7.1) Pilot study findings

Glyphosate is used extensively by farmers and conservationists for alien invasive clearing. Two concentrations of glyphosate were used in the pilot study, the first concentration was a concentration which farmers use in the field (2.25 %) and the second concentration was a lower concentration of 1 %. The study showed that glyphosate has a significant effect on the microbial communities in fresh water ecosystems, and that AMPA was present in the river water, which suggests that some microorganisms are able to convert glyphosate to a less toxic metabolite. To validate this statement experimental design needs to be adapted.

A physical disruption or reduction in biofilm biomass could be seen after herbicide application therefore (Figure 5), future studies should look at imaging techniques that can be used to quantify the biomass. After herbicide application a significant ($p<0.05$) microbial community shift occurred as a result from the applied glyphosate herbicide (Table 2).

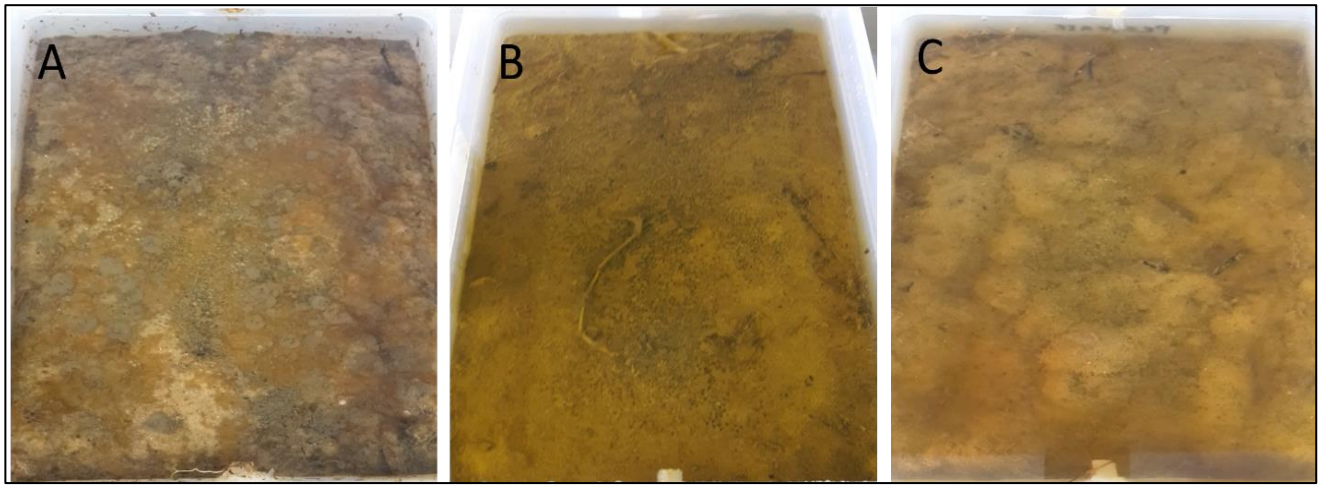

Figure 5: Physical disruption of the biofilms can be seen between the control (A) and treated samples (B & C). Synthetic river B and C were flushed with a 2.25% and a 1% glyphosate concentration respectively.

Table 2: The Eerste River R-values obtained from the ANOSIM comparison made between the control, recommended and under samples for both bacteria and fungi. All of the ANOSIM data showed significant differences ($p<0.05$), below is the r-values of the respective ANOSIM tests. Labels include recommended (2.25 % herbicide), under (1 % herbicide), control (0 % herbicide

R-value		**Fungi**	**Bacteria**
Berg River	Control x Recommended x Under	0.387	0.746
	Recommended x Under	0.170	0.502
	Control x Under	0.375	0.808
	Control x Recommended	0.464	0.770
R-value		**Fungi**	**Bacteria**
Eerste River	Control x Recommended x Under	0.446	0.895
	Recommended x Under	0.225	0.892
	Control x Under	0.476	0.934
	Control x Recommended	0.603	0.842

The results suggest that biofilms can contribute to the decontamination of glyphosate-contaminated rivers. The effects of glyphosate on non-target organisms and plants need to be elucidated in future studies. Future studies should identify the bacterial species that dominate in these biofilms which will lead to potential candidates that can be used for bioremediation of herbicide-based contaminants in fresh water rivers.

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Chapter 2

Optimisation of herbicide extraction methods using LC-MS and an ecological survey of a herbicide contaminated river

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Abstract

Due to the increasing agricultural and invasive clearing operations, this river has become prone to herbicide contamination. Therefore, an optimised herbicide detection method was needed for the monitoring of herbicide levels in the Berg river. A rapid and sensitive analytical method was developed for the detection of glyphosate, triclopyr, picloram and fluroxypyr using liquid chromatography coupled to mass spectrometry (LC-MS). In order to optimise the detection of the active ingredients of these herbicides, solid phase extraction (SPE) cartridges were used to extract the active ingredients from water sampled from the Berg river in the Western Cape, South Africa. The limit of detection for glyphosate was 1 ppb and for triclopyr, fluroxpyr and picloram it was 10 ppb. A survey over a period of nine months was conducted on this river. Significant high ($p<0.05$) levels of triclopyr was found over this period of time with a concentration of 0.0092 ppm measured for triclopyr. Glyphosate had the second highest concentration of 0.000216 ppm.

1) Introduction

The utilisation of herbicides begun in the early 20th century where these chemicals were used to control the invasion of alien invasive plant species (Ziska & Dukes, 2014). The idea behind the herbicides of today, is to synthesise chemicals that target very specific functions of a certain plant to disrupt their life cycle. However, the impacts on the surrounding environment must be considered. One can ask yourself what will happen to these chemicals once it has been applied and what impact does it have on other living organisms? The ideal active ingredient should have a short half-life to limit environmental exposure. To investigate what happens to these chemicals, a herbicide detection method is required. In the last 20 years, the use of liquid chromatography-mass spectrometry (LC-MS) advanced dramatically in terms of the sensitivity and specificity of detecting certain compound groups (Mira Petrovic, 2013).

Groundwater is the major source of water in many countries (Hoekstra & Chapagain, 2006). As described by Krystyna, (2011), the need to monitor herbicide residues in natural water sources is essential to obtain good water quality. LC-MS is used to monitor herbicide residues found in aqueous and solid environments and the contamination of these herbicides have been a concern for many years. The increased sensitivity and specificity of LC-MS has helped to detect these chemicals in both aqueous and solid environments. This analytical method is mainly used to detect low concentrations in water and soil environments (Mira Petrovic, 2005). Currently, one of the great challenges in environmental sampling is the evaluation and control of the risk associated with the presence of contaminants in the sample (Mira Petrovic, 2013). One solution to this challenge is to use better clean-up procedures and methods to get rid of unwanted chemicals and contaminants during monitoring.

Such a clean-up procedure involves the use of solid phase extraction (SPE) cartridges. Solid phase extraction is the most popular method used for environmental samples, (Krystyna, 2011). SPE cartridges are specifically designed to extract a target compound using a solid phase that consist of nanoparticles with functional groups that acts as an attachment point for the target compound. SPE cartridges use less organic solvents and do not need expensive glassware and special apparatus to perform the extraction. Target compounds can be extracted from a matrix and at the same time it provides a clean-up procedure for any contaminants that may be in a sample (Riley, 1996).

SPE cartridges vary in sorbent properties depending on the compound in question. The nature of the matrix, compound, concentration and type of chromatography involved are all factors that need to be considered when deciding which sorbent to use. Sample pre-treatment can improve the limit of detection (Krystyna, 2011). Different sorbents are used depending on the analyte. The interaction between the analyte and the sorbent can be reversible hydrophobic and/or anion exchange. Silica based sorbents and polymeric sorbents are the most commonly used sorbents available (Spivakov et al., 2006; Fontanals et al., 2007).

The most conventional herbicides used in and around the Western Cape are under the trade names Springbok 360SL, Garlon 4 and Plenum. Springbok contains the active ingredient glyphosate, Garlon 4 contains the active ingredient triclopyr and plenum contains two active ingredients namely fluroxypyr and picloram (Figure 1). These herbicides are used extensively in invasive clearings and weed control as described in literature.

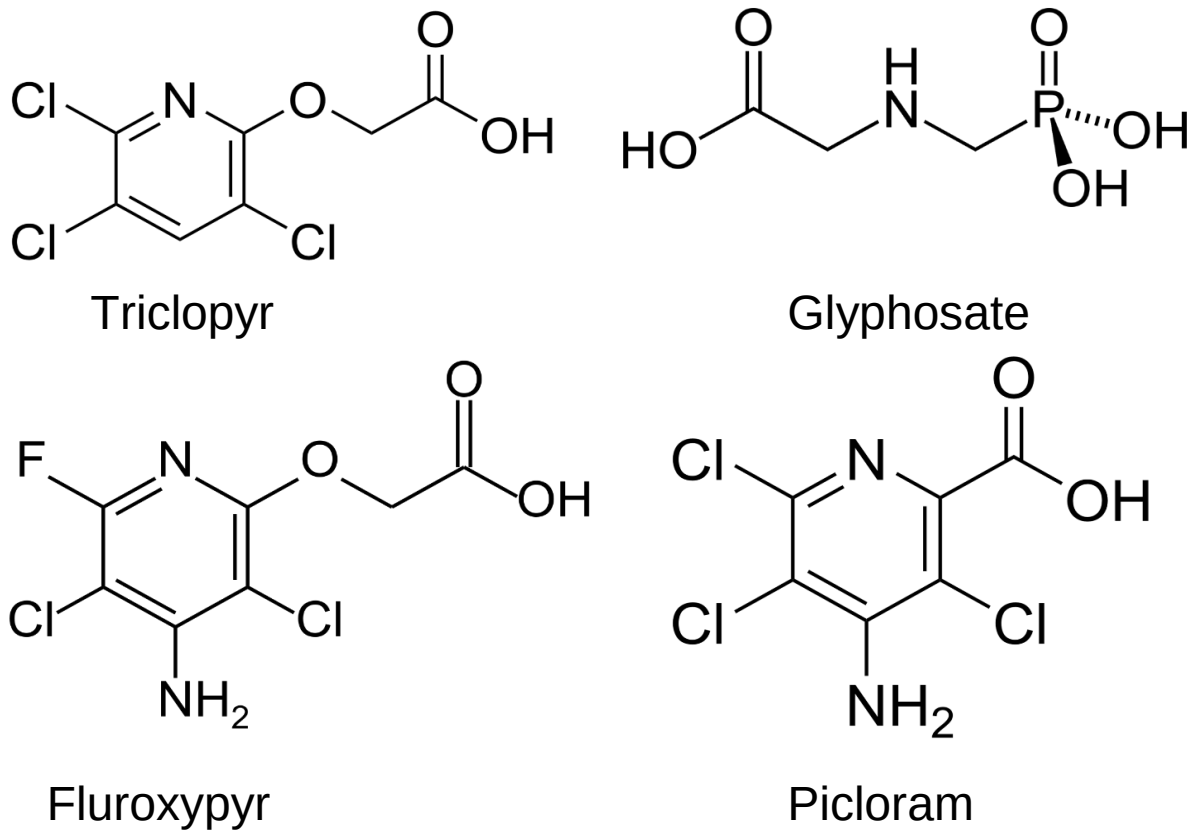

Figure 1 - Active ingredients used within Springbok (glyphosate), Garlon (triclopyr) and Plenum (fluroxypyr & picloram).

Thus, this chapter aims to develop a reliable and sensitive method for the simultaneous detection of these four active ingredients. Liquid chromatography coupled to mass spectrometry is a good detection method to fulfil the aim of this chapter (Krystyna, 2011). The concentration of herbicides in river water is diluted, thus a solid phase extraction is required to concentrate the sample in order to detect it with LC-MS. Different types of sorbent mechanisms are used for different target analytes. These mechanisms are explained below and describes the interaction with the target analyte.

1.1) Types of sorbents

A common solid phase extraction procedure involves five steps. The first is to condition the column, where the cartridge is equilibrated with a mid-polar to a non-polar solvent. This is to wet the exterior of the bonded phase. The sample is then washed with water to wet the silica surface. Then the second step is to load the sample onto the cartridge and as the sample is slowly passed through the sorbent or stationary phase, the polar or non-polar compounds interact with the sorbent, which will result in that specific type of compound being retained. For example, when using a polar sorbent, all the polar compounds in the sample will interact with the sorbent and the non-polar compounds will pass through. Step 3 and 4 involves the washing of the cartridge to get rid of all the impurities. A wash solvent typically consists of a non-polar solvent or buffer at a specific pH. The last step is to elute the analyte from the cartridge. The sample is eluted using a polar solvent.

1.1.1) Reversed phase packings

Reversed phase packings separate the analytes based on their polarity (Guzzetta, 2001). These types of material are usually derived from hydrocarbon chains. Hydrocarbons have the ability to retain analytes with medium to low polarity. When the analyte is retained in the hydrocarbon material it can then be eluted by using a non-polar solvent. When using a non-polar solvent to elute from a non-polar sorbent it will disrupt the interaction between the analyte and the sorbent, which releases the analyte from the column. Weakly polar to non-polar compounds will adsorb to the surface of the hydrocarbon (Supelco, 1998).

1.1.2) Ion exchange packings

The ion exchange packing can be ionic or anionic depending on the properties of the analyte that will be extracted. Ion exchange packing separates the analytes based on the electrostatic

properties of the compounds. The analyte will interact with groups on the sorbent that is either positively or negatively charged. This interaction will only occur when both the sample and stationary phase are at the same pH which causes the stationary phase and sample to be charged.

1.1.2.1) Cation exchange

Cation exchange retains analytes that are positively charged. Chemicals with positively charged functional groups include bases. A typical strong cation exchange sorbent contains aliphatic sulfonic acid groups which are negatively charged when in an aqueous solution and becomes charged when the pH is above 5 (Rhim et al., 2004). For example, to recover a strong base a weak cation exchanger will be used. The elution step involves a solvent that neutralises the ionic interaction between the sorbent and analyte (Summer & Miller, 1996).

1.1.2.2) Anion exchange

Anion exchange is exactly the opposite of cation exchange. Anion exchange packing is used to retain negatively charged anions such as acids. The stationary phase consists of functional groups that are positively charged. Sorbents with a strong anion exchange capability typically contain quaternary ammonium groups with a permanent positive charged state in an aqueous solution. An example of a weak anion exchanger are sorbents that contain amine groups. To elute analytes from the sorbent, a solvent that neutralises the charge of the analyte or sorbent is used (Ng et al., 2000).

1.2) Analyte properties

Glyphosate has a high degree of polarity and is soluble in water but insoluble in organic solvents. This property of glyphosate makes it difficult to separate from an aqueous solution and limits the extraction methods available (Goscinny & Hanot, 2001). Goscinny and Hanot concluded that glyphosate is difficult to analyse due to its physiochemical properties which complicates the extraction process. Other compounds such as sugars, amino acids and salts have a strong polarity and are retained and extracted along with glyphosate. These compounds may interfere with the analysis process. Thus, a solid phase extraction method is the most commonly used method for glyphosate and has the highest yield.

Triclopyr, fluroxypyr and picloram have the same physiochemical properties and they all contain a pyridine structure. Different side chains are attached to the pyridine backbone which gives each active ingredient an unique mode of action (Figure 1). The physicochemical structure of the three compounds give them a moderate to non-polar polarity. The difference in polarity between the active ingredients make simultaneous extraction difficult. A unique sorbent is required to retain the non-polar compounds and the polar glyphosate compound. A sorbent with a mixed mode and a weak anion exchange groups would work best for the extraction of the four compounds.

1.3) Survey of the Berg river

Most rivers in South Africa are heavily invaded by invasive alien plants. The Berg River is located in the Western Cape, South Africa and is a region which is rich in grapevines, alien invasive plants and agricultural activity. Large-scale management operations are underway to clear invasive plant species. Multiple herbicides are used for the clearing process next to the riverbank which makes the river prone to contamination with chemicals due to run-off and the overuse of herbicides (Figure 2).

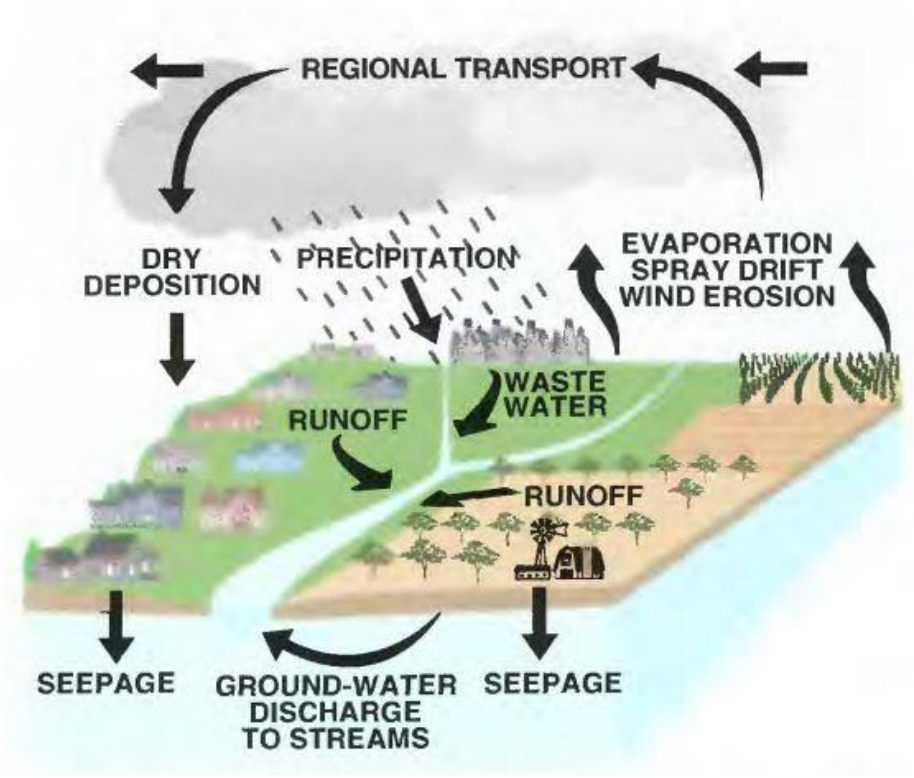

Figure 2 - Herbicide movement in a hydrologic cycle (Majewski & Capel, 1995)

This makes the Berg River ideal for sampling and for carrying out an ecological survey. The purpose of the survey is to examine the current state of the river and by monitoring the different herbicide concentrations in the Berg River. An ecological survey will identify good sampling sites for potential future studies that involves herbicides.

A rapid herbicide detection method needs to be developed in order to accurately detect and quantify glyphosate, triclopyr, picloram and fluroxypyr. This method will allow for the simultaneous detection of these herbicides. The developed method will then be used to continue with a herbicide survey of the Berg River that will run over a period of nine months.

2) Materials and Methods

2.1) Chemicals and equipment

Analytical grade glyphosate (1 ml x 1000 µg/ml), triclopyr (99%), fluroxypyr (99%) and picloram (99%) were obtained from Sigma-Aldrich (Germany). The internal standard, 2,4-Dichlorophenyl acetic acid, was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Individual stock solutions for each compound was prepared with a serial dilution ranging from 10 ppm – 0.001 ppm in ultrapure water. The serial dilution was spiked with 1 ppm internal standard and used to develop a method for the detection of these compounds. Two different LC-MS columns were used respectively, one for glyphosate alone and one for triclopyr, fluroxypyr and picloram. All standards and stock solutions were prepared a day before the analysis and kept at 4ºC.

2.2) Sampling site and sample collection

Sampling took place in the Berg River, at a site located close to Wellington in the Western Cape, South Africa (33°38'57.7"S 18°58'05.6"E). Pre-cleaned and sterile 500 ml Schott bottles were used to sample river water in three different depths, at the surface, middle and bottom of the river. The samples were kept in a refrigerator at 4ºC until further processing. The samples were collected in triplicate and from various points in the river system.

The samples were then filtered using coffee filter paper to get rid of the large debris in the water samples. A 0.45 µm filter was used to filter the river water samples further. The filtering step was added prior to the solid phase extraction step. This was done to prevent clogging up the solid phase extraction cartridges with unwanted debris and microbial cells.

2.3) Solid phase extraction optimisation

Several solid phase extraction cartridges were tested in this study. The solid phase extraction cartridges were chosen based on their properties to retain the four active ingredients found in Garlon, Plenum and Springbok. The first cartridge is the Supelclean ENVI–18 SPE cartridge (Sigma-Aldrich; Germany). The ENVI–18 cartridge retention mechanism is reversed-phase based and the sorbent consist of polymerically bonded, octadecyl with 17% carbon which is endcapped. This cartridge is used for the extraction of nonpolar to moderately polar herbicides from aqueous solutions such as river water (Chu & Metcalfe, 2007). The second SPE cartridge that was tested is the Chromabond Easy SPE cartridge (Macherey-Nagel; Germany). The Easy column is used to extract polar herbicides from water and consist of a polar modified polystyrene-divinylbenzene copolymer with a weak anion exchanger (Macherey-Nagel, 2017). The third SPE column that was tested is the Oasis MAX cartridge (Waters, South Africa). The MAX cartridge is used for the extraction of acids and moderate polar to polar compounds. The MAX cartridge consists of a mixed-mode, reversed phase and weak cation-exchange polymer (Waters, n.d.).

The MAX cartridge worked best for the simultaneous detection of the four active ingredients. An EPA guideline (EPA, 2008) was followed for the conditioning of the column. A 6cc/150mg cartridge was used to load 500 ml of river water onto the cartridge. The column was conditioned first by loading 2 ml methanol, 4 ml 0.5 M NaOH and 2 ml water. The column was then rinsed using 4 ml 0.5 M NaOH and 2 ml ultrapure water after which the sample was loaded using auto sampler tubes. The sample was then eluted using 4 ml of a 2 % formic acid in methanol mixture. The elution deviated from the EPA method due to the polarity of picloram, fluroxypyr and triclopyr. After the elution step the sample was evaporated using an analytical nitrogen evaporator, thereafter the sample was reconstituted in ultrapure water and sent for LC-MS analysis.

2.4) Liquid chromatography and chemical standards

Analytical grade glyphosate, triclopyr, fluroxypyr and picloram were obtained from Sigma-Aldrich (Germany). The standards were made up using ultrapure water in amber LC-MS vials. The standards were made using concentrations ranging from 10 ppm – 0,001 ppm. 2,4-Dichlorophenyl acetic acid was used as an internal standard. The samples and standard curve samples were spiked using a known concentration of 1 ppm. A Waters Xevo triple quadrupole

mass spectrometry system (CAF, Stellenbosch) was used for the analysis and was operated using a 0.3 ml/min flow rate. For glyphosate, a Thermo, Hypercarb (5 µm, 2.1 x 100 mm) column was used with two solvents, 1% acetic acid and methanol in 1 % acetic acid respectively. For picloram, triclopyr and fluroxyoyr a Waters BEH C18 (1.7 µm, 2.1 x 100 mm) column was used with two solvents, 0.1 % formic acid and acetonitrile respectively (Tables 1 & 2).

Table 1 - Production ions and precursor ions with their respective cone and collision energy for each active ingredient and DCPAA (Internal standard)

Analyte	Precursor ion *m/z*	Production *m/z*	Dwell (s)	Cone (V)	Collision energy(V)	Ionisation mode
DCPAA(IS)	203	159	0.016	5	10	ES-
	205	161	0.016	5	10	ES-
Glyphosate	170	60	0.161	15	20	ES-
	170	88	0.161	15	20	ES-
Triclopyr	254	196	0.016	25	10	ES-
	256	198	0.016	25	10	ES-
Fluroxypyr	255	181	0.016	30	25	ES+
	255	237	0.016	30	10	ES+
Picloram	241	195	0.016	25	25	ES+
	241	223	0.016	25	15	ES+

Table 2 – LC-MS run time and percentage gradient for solvents A (1% acetic acid) and B (methanol in 1 % acetic acid).

Time (min)	Flow (ml/min)	Solvent A %	Solvent B %
0.10	0.30	100	0
5.00	0.30	0	100
5.10	0.30	0	100
6.00	0.30	100	0
7.00	0.30	100	0

2.5) Herbicide survey

River water samples were collected in the Berg River in clean 500 ml Schott bottles. Samples were taken every two weeks for nine months from September 2016 to May 2017. These samples were send to CAF for LC-MS for analysis. The different herbicide concentrations were determined, and a survey graph was constructed where herbicide concentration over time was plotted. This survey aimed to provide a better insight into the current state of the Berg River. The survey provided a herbicide concentration baseline for the Berg River. All samples were done in triplicate to minimise sampling error.

2.6) Statistical analysis

Basic statistics were performed on the survey data to determine whether the data are normally distributed. If the data was normally distributed a two way-ANOVA test was done to determine any significant differences between and within the sampling groups. If the data were not normally distributed, a nonparametric Kruskal-Wallis ANOVA test was performed. Statistica 13.2 was used to perform the statistical tests, where the mean differences between groups were compared.

3) Results and Discussion

3.1) Solid phase extraction optimisation

Several attempts were made to determine the concentration of the active ingredients directly from the river water. These attempts failed due to the limit of detection of the LC-MS instrument. Solid phase extractions were considered and presented a solution to the quantification of the active ingredients. The simultaneous detection of glyphosate, fluroxypyr, picloram and triclopyr was optimised by using three different solid phase extraction cartridges (Figure 3). The recovery rates for each active ingredient was compared between the three solid phase extraction cartridges used. As seen in Figure 3, the solid phase extraction which yielded the best results are the MAX cartridge. The percentage recovery for the active ingredients using the MAX column were glyphosate – 53%, triclopyr – 97%, fluroxypyr – 77% and picloram – 96%. The MAX cartridge consists of a mixed mode polymeric sorbent with anion exchange groups. This sorbent has a mixed-mode which allows for the retention of moderate polar to polar compounds (Lavén et al., 2009).

The recovery rates for each of the active ingredients differed when they are compared to each other (Figure 3). The recovery rate of triclopyr was well retained in the sorbent while glyphosate had a low recovery rate. The SPE optimisation experiment showed that moderate to non-polar compounds were better retained in the sorbent. This is supported when triclopyr, fluroxypyr and picloram are compared to glyphosate which is a strong polar compound (Figure 3). Thus, the ratio between the polar and non-polar polymer in the sorbent may be favoring moderate to non-polar compounds above polar compounds (Li et al., 2016).

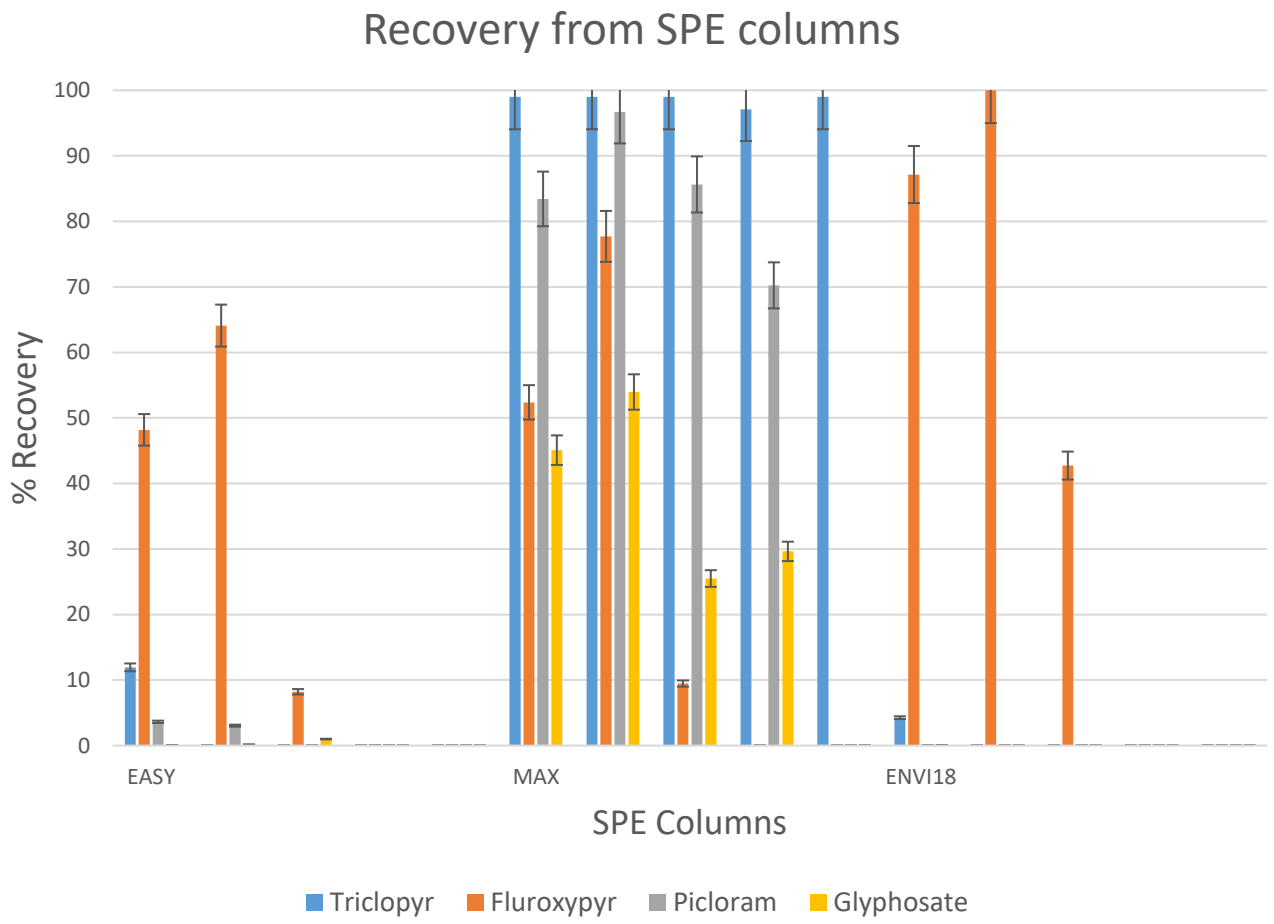

Figure 3 - Solid phase extraction (SPE) optimisation with the recovery rates for glyphosate, fluroxypyr, picloram and triclopyr. Error bars indicate standard deviation.

The recovery rates for the easy and envi-18 SPE cartridges were very low and only favoured the recovery of fluroxypyr (87%). These SPE cartridges were rejected due to the poor recovery

rates yielded. The Max cartridge was used to extract the active ingredients from the Berg river water in the survey that follows.

3.2) Herbicide survey data

The Berg River was sampled in triplicate to reduce sampling error in the experiment. Three different depths were sampled namely the bottom, middle and surface water. The samples were then concentrated by a x 500 factor using the SPE cartridge procedure. Thus, the dilution factor as well as the recovery rate percentages needed to be taken into account. There were no significant differences ($p>0.05$) in herbicide concentration found between the different depths sampled. However, significant differences between the different dates were found. Kruskal-Wallis ANOVA test was performed with the data, and yielded a significant difference ($p<0.05$) between samples. A nonmetric multiple comparison test was done to see where in the groups, the samples differed. Triclopyr showed a significant difference ($p<0.05$) in concentration compared to glyphosate, fluroxypyr and picloram. Overall, triclopyr was found to be the most abundant of the four active ingredients tested (Figure 4). Glyphosate was found in a lower concentration than triclopyr and peaked at 0.000216 ppm during September and October 2016. The concentration of glyphosate found is low compared to similar studies where glyphosate concentrations in surface waters ranged from 0.10-0.70 ppm (Peruzzo et al., 2008; Struger et al., 2008). In Struger et al. (2008), they found glyphosate concentrations as high as 17 ppm. These studies were performed in Argentina and Canada. respectively. Herbicide surveys in South African surface waters are not well studied. There is a lack of information regarding herbicide trace levels found in South African rivers. Therefore, it is difficult to compare the results obtained from this study to other similar studies in South Africa. However, the EPA standards were used as guidelines for this study. Glyphosate is still under the maximum contaminant limits (MCLs) for drinking water (EPA, 2008).

Method development for triclopyr, fluroxypyr and picloram were still under development during September – October 2016 which explains the absence of triclopyr, fluroxypyr and picloram peaks between these dates. The optimisation for these three active ingredients was successfully optimised in November 2016.

The highest active ingredient recorded was triclopyr which was in March 2017 at a concentration of 0.0542 ppm. This is a high concentration compared to fluroxypyr, picloram and glyphosate. Fluroxypyr and picloram were found in low concentrations, 0.00019 ppm and

0,0002 ppm, respectively (Figure 4). Clearing programs such as Working for Water and agricultural processes contribute to the levels of herbicide found in the Berg river.

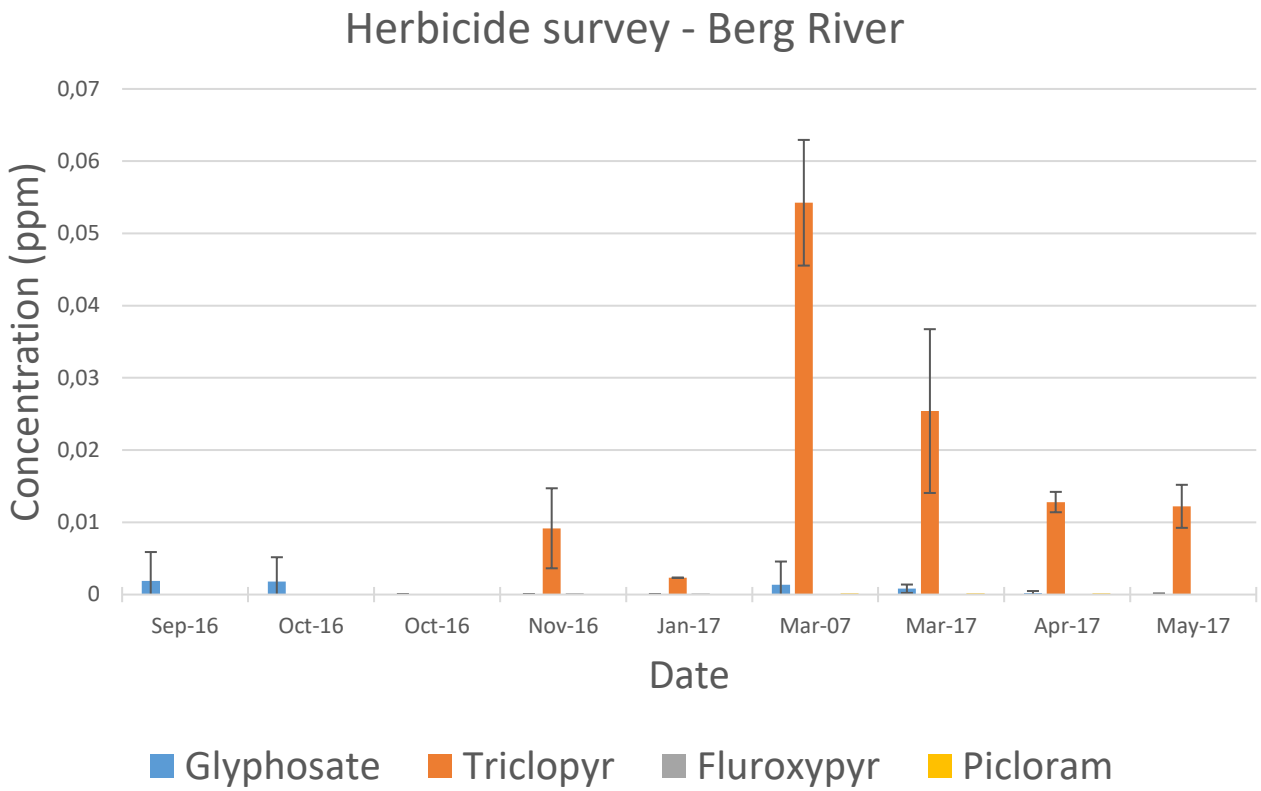

Figure 4 - Herbicide concentration survey of the Berg River over seven months. Error bars indicate standard deviation.

The high concentrations of triclopyr may possibly be an effect of climate conditions such as rainfall (Randall & Mulla, 2001). Randall and Mulla, (2001) explained how climate conditions influences the amount of nitrogen in surface waters. Rainfall will greatly increase the amount of herbicide found in freshwater rivers, as more herbicide can enter the river. The Berg River is dominated primarily by vineyards and farmers normally spray herbicides at the end of harvest season which falls between December and March. This may explain the spikes that can be seen for triclopyr. Glyphosate is known to adhere to soil particles and becomes less mobile in soil (Piccolo et al., 1994). Active ingredients such as triclopyr is more mobile through soil particles due to the nature of its chemical structure and functional groups.

4) Conclusion

Liquid chromatography coupled to mass spectrometry presented an excellent method for the rapid detection of herbicide levels in freshwater streams. All efforts were made to make the detection of these compounds as rapid as possible without the need for derivatization which can take days to perfect. The optimised LC-MS methods that was developed for the detection of the herbicides in this study, achieved great limit of detections for glyphosate (LOD = 0.1 ppb) and triclopyr, picloram, fluroxypyr (LOD = 1 ppb). The optimised methods for glyphosate, triclopyr, fluroxypyr and picloram can be used in future studies although method validation needs to be completed. Solid phase extraction cartridges are recommended for environmental water samples. Due to the large dilution effect a river can have on herbicides, a way of concentrating the sample is required such as solid phase extraction cartridges. Solid phase extraction cartridges not only place the target compound within the limit of detection of the LC-MS instrument but filters the sample from any debris and unwanted chemicals.

High levels of triclopyr were found in the Berg River by the survey conducted in the study and can possibly be as a result of climate conditions. A future study should include the weather conditions when sampling is taking place and for a longer period of time.

Clearing operations conducted by Working for Water and agricultural land owners could distribute clearing and herbicide application schedules. These schedules can then be used to see whether there is any correlation between the time they have sprayed the herbicide and the amount of herbicide measured in the river. The amount of herbicide entering the river can then be determined at time zero when the herbicide is applied. The active ingredient will then gradually enter the river stream and leach from the applied soil.

The results in the study yielded new questions for future studies and future monitoring of the freshwater streams in South Africa should be considered due to the implication it may have on other environmental processes.

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Chapter 3

The effect of herbicides on the structure of freshwater biofilms

Abstract

The Berg River flows through areas which are rich in crops, vineyards, invasive plant species and agricultural activity. These are also major land uses, all of which are activities where herbicides are applied regularly. The river is a major receiving body and becomes contaminated with herbicides due to rainfall runoff and the overuse of these chemicals. The effect of three conventional herbicides on freshwater biofilms were investigated in this study. The herbicides Garlon, Plenum and Springbok are used on a regular basis for the clearing of invasive plant species such as *Eucalyptus camaldulensis* and *Acacia mearnsii.* These herbicides contain the active ingredients, triclopyr, fluroxypyr and picloram, and glyphosate, respectively. Freshwater biofilms were cultured for 14 days in flow cells using the Berg River's water as inoculum. Confocal laser scanning microscopy (CLSM) was used to acquire three-dimensional images of the biofilm structure, after application of Garlon, Plenum and Springbok individually. The analysis of CLSM images with COMSTAT allowed for the quantification of biofilm parameters. Two treatments were used in this study which were the herbicide, along with the unknown surfactants, and a treatment with only the active ingredient of the herbicide. This was to investigate the effect of the surfactants on the biofilm structure. The physical structure of the biofilm and the total loss of biomass were investigated in this study. Garlon, Plenum and Springbok decreased the biomass significantly ($p<0.05$) for both the concentrations that were used, which was 1 ppm and 10 ppm respectively. In terms of the structure of the biofilm, no visible disruption was noted. Clearing operations should apply the herbicides with caution to the environment because these chemicals have a significant effect on freshwater microbial communities.

1) Introduction

Biofilms play an important role in maintaining the flow of nutrients in freshwater systems (Sternberg et al., 1999). Ecosystem functioning is dependent on the interactions between microbial communities. These interactions define the biosphere as a whole and defines the health of the ecosystem (Weathers et al., 2012; Torsvik & Ovreas, 2002; Kent & Triplett, 2002). Biofilms are responsible for important nutrient cycling processes in an ecosystem such as freshwater systems. Different soil microorganisms can partake in one or more cycles, depending on their metabolic versatility (Tate III, 1995). The breakdown and mineralising of compounds by biofilms will occur through a degradative process where proteins and other organic chemicals are degraded into amino acids by proteolytic organisms. It can thereafter be used as carbon or nitrogen sources for driving oxidation/reduction reactions which lead to cell growth.

At the end of these cycles, different metabolites and end products are released into the environment which is essential for the growth and persistence of native plant communities. These metabolites and end products are essential for maintaining soil quality and health (Naude, 2012).

Biofilms are mostly beneficial to their surrounding environment and the microbial communities change depending on the exposure to external factors. These factors are mostly abiotic factors and also include exposure to chemicals. These chemicals can either be harmful or beneficial to the microbial community. Some microbial species are able to break down these chemicals and utilise it as a nutrient source.

Herbicides are examples of chemicals that can affect biofilms. Herbicides are applied extensively along riverbanks to control the invasion of alien plant species such as *Acacia* species and *Eucalyptus* species. Due to rainfall and the overuse of herbicides, the fresh water recipient waters easily become contaminated with these chemicals. To investigate the effect of chemicals such as herbicides on biofilms, an *in vitro* method of cultivating biofilms is required.

Microbial biofilms consist of either planktonic or multiple sessile cells. They grow in an aggregated pattern and is structurally organised (O'Toole et al., 2000). These patterns vary between biofilms and is due to the nature of biofilm growth. Factors such as pH, temperature, flow rate and available nutrients affect the growth of biofilms (Sternberg et al., 1999). In freshwater rivers, microbes attach themselves to surfaces such as rocks, stones and debris in

the river. Once the planktonic cells are attached, cell-to-cell adhesion occurs due to attached cell multiplication where they form a biofilm community which are imbedded in an extracellular polymeric substance (EPS) matrix.

The most common way to grow and analyse the biofilm is by using flow cells (Sternberg & Tolker-Nielsen, 2006). Flow cells allow for the laminar flow of liquids, which is ideal for biofilm culturing under controlled hydrodynamic conditions. Different flow cell designs are used for different experiments, depending on the purpose of the experiment. The use of flow cells allows for the investigation of biofilm formation using direct microscopic techniques (Tolker-Nielsen & Sternberg, 2014) and furthermore allows for the addition of growth medium or change in growth medium at a certain point during the experiment.

One of the microscopic techniques used to study biofilms is confocal laser scanning microscopy (CLSM). CLSM is a widely used technique in the study of biofilms and is used to study the extracellular polymeric matrix which provides structure to the biofilm. In-depth detail can be seen by using this technique. CLSM works by using a laser at a specific wavelength to excite fluorescent dyes which are added to the sample and emits light which allows for closeup imaging of the structures in the specimen (Kihm, 2011). A stack of images can be created to form a three-dimensional image of the biofilm. This three-dimensional image can then be quantified using a software package called COMSTAT.

COMSTAT is an image analysis software package that is used for various calculations based on the three-dimensional image that is generated by CLSM (Beyenal et al., 2004). COMSTAT is commonly used for image-analysis and can calculate objective parameters such as average or maximum thickness and biovolume from the three-dimensional biofilm images. COMSTAT uses the variation of pixel intensities in a three-dimensional stack to compute the biofilm volume in a stack (Ma et al., 2006). The software uses binary thresholding to separate biomass from interstitial space or background. There are several limitations when the stack images that are created do not have adequate separation of grayscale intensities between biomass and the background (Larimer et al., 2016; Yang et al., 2001). However, regardless of these limitations, this software is an appropriate model to determine the biomass from three-dimensional images (Beyenal et al., 2004).

CLSM and COMSTAT will be used to study the biofilm structure and to quantify biofilm biomass. The goal of this experiment is to determine the effect of herbicides on the physical structure of

biofilms. A secondary goal is to quantify the biomass in the flow cell using COMSTAT, and to see whether there is an increase or decrease in biomass when exposed to herbicides at various concentrations.

2) Materials and Methods

2.1) Herbicide selection and application

Management operations in South Africa use different herbicides to deal with the invasive plant species. Three commonly used herbicides were utilised for this study. The three herbicides, Springbok 360 SL (active ingredient - glyphosate), Plenum (Active ingredients – Picloram and Fluroxypyr) and Garlon (active ingredient – Triclopyr), were used to spike the biofilm samples with different concentrations. The concentrations that were used to spike the biofilm samples were 1 ppm and 10 ppm. The 1 ppm concentration is the maximum contaminant level (MCL) of herbicides in freshwater streams according to European standards. The 10 ppm concentration was chosen to simulate a spillage of herbicide or a herbicide spike in the Berg River. All three herbicides were tested individually and the affects studied using confocal laser scanning microscopy. The different herbicide has their own unique formulation which is confidential and makes it impossible to test the surfactant alone. Therefore, the herbicide, containing both the active ingredient and surfactants, was tested and compared to the technical grade active ingredient. Then, each of the active ingredients were tested and studied separately. This is to determine whether the herbicide, which contains the active ingredient along with the surfactants or active ingredient alone, is responsible for the results that were obtained.

2.1.1) Glyphosate

The glyphosate containing herbicide selected for this study is Springbok 360 SL (Arysta LifeScience). It is a non-selective, foliar, systemic herbicide that is commercially used for the control of a wide range of annual and perennial grasses, broadleaf weeds and certain woody perennials. Springbok 360 SL is also specifically used for the control of *A. mearnsii* and *E. camaldulensis* (van Rooyen et al., 2014). The active ingredient in the herbicide is glyphosate (containing 360 g/l) as a glyphosate isopropyl amine salt (480 g/l). This specific herbicide is among the most commonly used herbicide types (Olckers et al., 2004). The selected herbicide

is applied in the field at 4.5 l/ha, which is the recommended dosage (Holmes et al., 2008). The Berg River, which flows through the areas treated with the herbicide, was sampled. River water was collected, taken to the laboratory and used to set up artificial river systems for experiments. The technical grade glyphosate was obtained from Arysta LifeScience and is a 95% purity chemical. Both the herbicide and active ingredient was tested at two different concentrations, namely 1 ppm and 10 ppm. The active ingredient was dissolved in 1 L of water to get the desired concentration and the same was done for the herbicide/active ingredient mixture.

2.1.2) Triclopyr

Garlon is the herbicide containing the active ingredient triclopyr, which is a foliar herbicide. Triclopyr is pyridine based, which limits the nutrients that are available to the target plant (Tu et al., 2001). Information obtained from local farmers and clearing operation managers, suggests that Garlon is a common herbicide that is used on a regular basis. Garlon contains triclopyr at a concentration of 480 g/l, along with surfactants. Technical grade triclopyr and the herbicide was obtained from Arysta LifeScience and has a purity of 97%. The technical grade triclopyr was also tested on its own. The active ingredient and herbicide were tested at concentrations of 1 ppm and 10 ppm.

2.1.3) Fluroxypyr and picloram

Fluroxypyr and picloram are most commonly found under the herbicide tradename Plenum. Picloram is most effective against *Acacia* species where it induces uncontrolled growth of the plant cells (Tu et al., 2001; Turpie et al., 2008). Picloram is a synthetic growth hormone and mimics the natural growth hormone, auxin. Fluroxypyr is also an auxin type response active ingredient which interrupts the growth of the plant. Fluroxypyr is specifically used to control *Eucalyptus* species (Tao & Yang, 2011). Both fluroxypyr and picloram are found in Plenum at an individual concentration of 80 g/l. Technical grade fluroxypyr and picloram were obtained from Arysta LifeScience and has a general purity of 97% and 96%, respectively. The herbicide and active ingredients were tested at two different concentrations, which were 1 ppm and 10 ppm.

2.2) Sampling site

Sampling took place in the Berg River, at sites located close to the town of Wellington in the Western Cape, South Africa (S $33^0$28.887' E $018^0$56.245'). This region is rich in agricultural activity that contribute to the herbicide contamination in the Berg River.

2.3) River water sampling

River water was collected in the fast-flowing part of the river during the summer. The water was collected in a sterile 30 L canister and transported to the laboratory where it was kept at 4^0C until further use.

2.4) Biofilm establishment

Flow cells were used to establish the biofilms. A unique flow cell design was used to be compatible with the microscope sample insert. The design used in this study is shown in Figure 1. This is a widely used tool for *in vitro* cultivation of biofilms and is ideal for microscopy to study the effects of herbicides on the biofilm structure (Song et al. 2014).

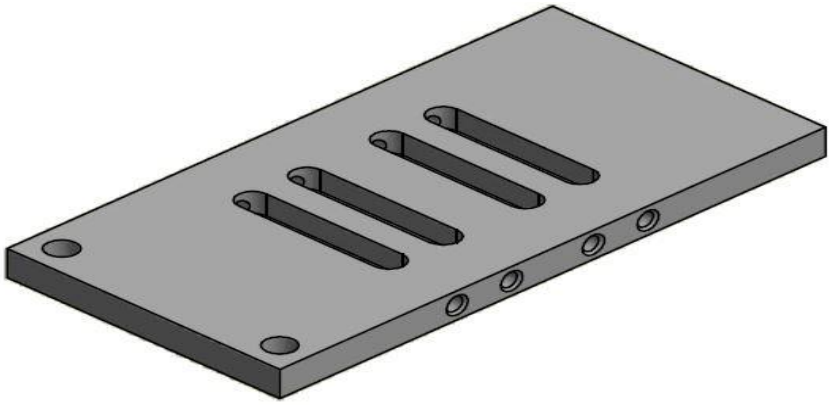

Figure 1 – A 4 channel flow cell design used for the cultivation of biofilms

Oxygen-permeable silicon tubing was used so that the biofilms can grow aerobically. The silicon tubing was used to connect the different parts of the setup for example to connect the inlet flask to the pump and the pump to the bubble trap etc. River water was pumped (6 ml/h) through the flow cell so that biofilms can form in the flow cell. The flow cell consists of a 100 X 50 mm Perspex block that is milled according to Figure 1. Staining of the biofilm for microscopy was done without influencing the biofilm in any way. A glass cover slip was glued over the open

channel, providing an attachment surface for the biofilm. Seven glass beads, with a diameter of 2.0 mm, were also added in the channel to enhance biofilm attachment. The complete setup of the experiment can be seen in Figure 2. The experimental setup was sterilised in between the experiment using a 20% sodium hypochlorite solution. New flow cells and bubble traps were build using clean cover slips and new syringes.

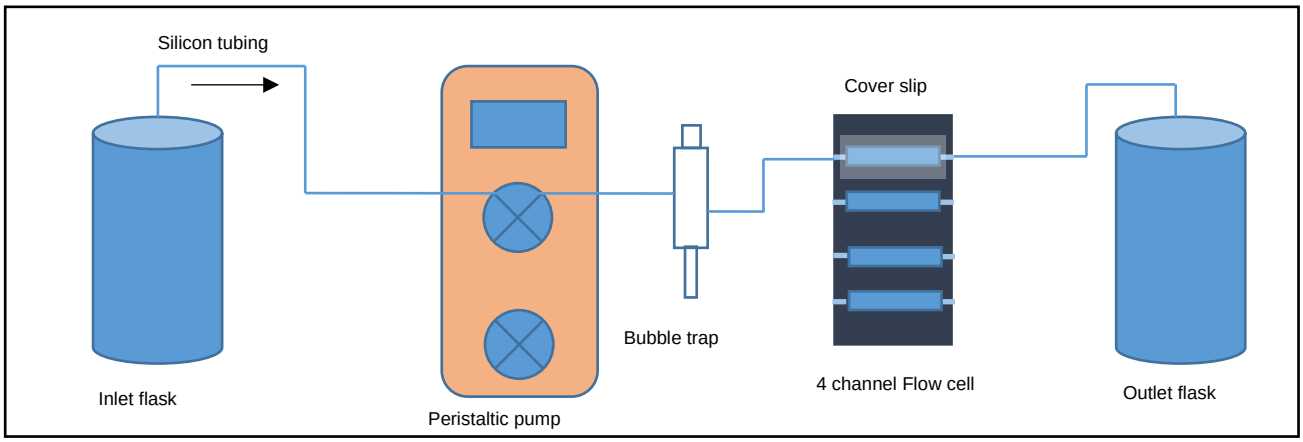

Figure 2 - Complete flow cell setup

2.5) Spiking samples with herbicide

Four flow cells were used per experiment and each flow cell with its four channels were assigned to a different treatment being either a control, active ingredient or herbicide. Table 1 shows the flow cell and channel allocation for each treatment.

Table 1 - Flow cells (A-D) with their respective channel allocation (1-4) which received the different treatments. (*) indicate the cells that were used for microscopy imaging.

Channels	Flow cells			
	1	2	3	4
A	Control	Herbicide *	Herbicide	Active ingredient
B	Control	Herbicide *	Herbicide	Active ingredient
C	Control *	Active ingredient *	Herbicide	Active ingredient
D	Control *	Active ingredient *	Herbicide	Active ingredient

The flow cells were allowed to run for 10 days before a treatment was applied. Ten days were allowed due to preliminary testing which indicated a steady state of biofilm formation. The

treatment was mixed with river water and the inflow was replaced with the treatment. On day 14, the channels marked with an asterisk (*) were stained and examined using fluorescent microscopy. Four days of exposure time at the respective concentrations were enough time for the herbicide effect to take place. A concentration of 1 ppm for both the herbicide and active ingredient was applied and the control channels received no treatment. After 14 days, the system was disinfected as described in Bester et al. (2013), and rebuilt using new glass beads and fresh river water was sampled and used to restart the experiment. The second concentration of 10 ppm was then used to spike the biofilms. This experiment was repeated twice for each of the three herbicides that were investigated in this study. The channels that are not marked with an asterisk were used for DNA extraction and community profiling (Chapter 4).

2.6) Biofilm staining

In order to study the biofilm structure using laser scanning microscopy, a dye needs to be added to the sample which fluoresces under a certain wavelength. The dye that was used in this study is a non-destructive dye that binds to the DNA of bacterial and fungal cells, called diamidino phenylindole (DAPI) stain (Molecular probes, Thermo Fisher). This dye gives a clear overall image of the biofilm structure (Donlan, 2002). The DAPI staining protocol that is described online on the Thermo Fisher website was followed (Thermo Fisher, n.d.). This protocol was adapted in terms of the concentration that was used. The concentration suggested in the protocol was a 300 nM (83.2 µg/l) DAPI stain solution. This concentration was too low to penetrate into the biofilm structure and was, therefore, increased to a final staining concentration of 5 µg/ml. The incubation time was also increased from 5 minutes to 30 minutes to give the stain time to penetrate the EPS matrix of the biofilm. 2 ml of the stain was injected into each channel using a sterile needle and syringe. The flow cells were then clamped off at the ends of each channel and used for microscopy imaging.

2.7) Fluorescent microscopy

Confocal scanning laser microscopy is a well-known technique that is used to study biofilms and their structure. CSLM was used to study the effects of Springbok 360 SL, Garlon 4 and Plenum on biofilms. A Carl Zeiss LSM 780 confocal microscope from the Central Analytical Facility (CAF) at Stellenbosch University was used. The biofilm formation was evaluated using a 100X (1.46 NA) oil immersion objective. A 405 nm laser was used for the excitation of the

DAPI stain, and a one way emission filter was used to observe the blue fluorescent cells. A Z stack was captured, each at a thickness of 3 µm. The images that were created were 8-bit 512 X 512 images, which were sufficient for the quantification of the biofilm biomass (Heydron, 2000). Four images per channel were taken to get an overall average of the biomass in the channel. Preliminary testing showed that four images per channel provided enough data for the quantification of the biomass in the channel. The biomass on the glass beads were captured, because no visible biofilm adhesion could be seen on the cover slip. Four z stack images were taken of each channel that is marked with an asterisk (*) in Table 1. The first image was on bead 1, which was the bead that was exposed first to the treatment. The second and third set of z stack images were on bead 3 and 5 respectively. This was to represent a mid-point in the channel. The last z stack was taken on bead 7, which was the last bead in the channel. This bead was also the last to be exposed to the herbicide.

2.8) Data analysis and statistics

The images that were created were converted to greyscale images using IrfanView, which is an image analysis software. The image names and file order were also rearranged and renamed to be compatible and in the correct format for the COMSTAT software, which is a script that is written in MATLAB 5.3. The images where then imported into MATLAB where it was analysed using the COMSTAT script. Statistica 13 was used to perform ANOVA testing, to test for if there were any significant differences between the biomass.

3) Results and Discussion

In an initial survey, a physical disruption of the biofilm structure occurred therefore, fluorescent microscopy focused on the structure of the biofilm, so in-depth image analysis was done. The disruption of the biofilm could be a result of the herbicide presence. The images that were captured using CLSM are shown in Figure 3 below. Figure 3 A, D and G are the controls for each of the three herbicides that were used. The middle column (B, E & H) are the images that received the herbicide treatment which contains the active ingredient along with the surfactants. The last column (C, F & I) contains the images that received the active ingredient alone.

From the images in Figure 3, it is visually clear that the control channels contain more biomass than the treated channels. The images that are created in Figure 3 is the maximum intensity projection of the representative images for each of the herbicides. Springbok, which contains glyphosate is shown in images A-C. The channel that received the herbicide (B) showed less effect (decrease in biomass) on the biofilm when compared to the channel that received only the active ingredient (C). Therefore, the surfactants used in Springbok possibly have a less severe effect than the active ingredient alone.

The second herbicide treatment, which was Garlon is shown in Figure 3 (D-F). The biomass in the herbicide channel (E) is significantly lower when compared to the active ingredient channel (F). The surfactants used in Garlon, likely, have a severe effect on the biofilm structure compared to the active ingredient.

The third herbicide, Plenum, is shown in the last row of Figure 3 (G-I). A decrease in visual biomass for the active ingredients, picloram and fluroxypyr, can be seen from these images. This suggests that the active ingredient alone has a more severe effect on the biofilm structure. Since plenum consists of two active ingredients, it can therefore affect the biofilm in more than one way. Both these active ingredients can act on the microbial communities and have different mode of actions against plants.

The observations made from Figure 3 are all based on visual findings. These observations correlate with the results obtained from the biomass quantification software, COMSTAT (Figure 4). The biomass is presented in cubic micrometer per square micrometer in the results obtained from the COMSTAT software. Figure 3 is a stacked image therefore; a single still image cannot purely represent the sample, but it allows to look at the quantitative biofilm biomass numbers.

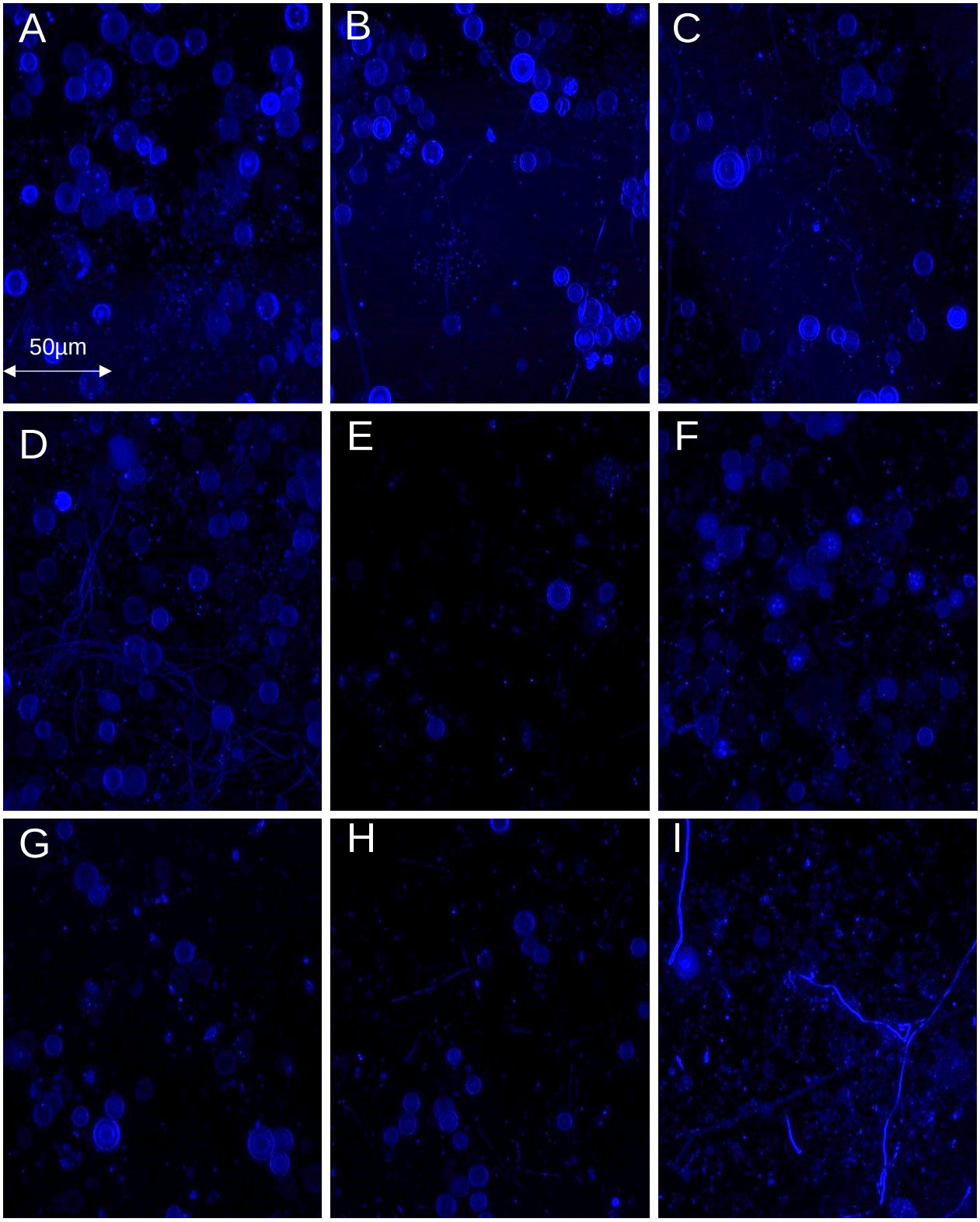

Figure 3 - Confocal microscopy images of the biofilms that were grown in flow cells and treated with 1ppm of the different herbicides. Images (A-C) represent the biofilms treated with Springbok, images (D-F) represent the biofilms treated with Garlon and images (G-I) represent the biofilms treated with Plenum. The first column contains the control images, the middle column contains the cells that were treated with herbicide and the last column contains the cells that were treated with the active ingredient.

Figure 4 represents the biomass for each of the herbicide treatments. From the results, it is clear that all of the herbicides and active ingredients that were applied had a significant effect on the biomass of each biofilm ($p<0.05$). The loss in biomass suggests that the chemicals used in this experiment is harmful to freshwater biofilms. From literature it is clear that the shikimate pathway, that is used for the synthesis of aromatic amino acids in plants and microorganisms, is affected by glyphosate which disrupts this pathway (Schönbrunn et al., 2001). In Souza-Alonso et al. (2015), the effects of triclopyr on soil microorganisms were found to be toxic. The exact mode of action of triclopyr on microorganisms is still vague and needs to be investigated. In some cases, the loss of biomass is greater for the active ingredient treatment compared to the herbicide, which contains surfactants and other chemicals. One of these cases can be seen for Garlon (Figure 4). The primary purpose of these chemicals is to enhance the penetration of the active ingredient and to reduce the surface tension of the target plant and it is clear that it can significantly affect biofilms.

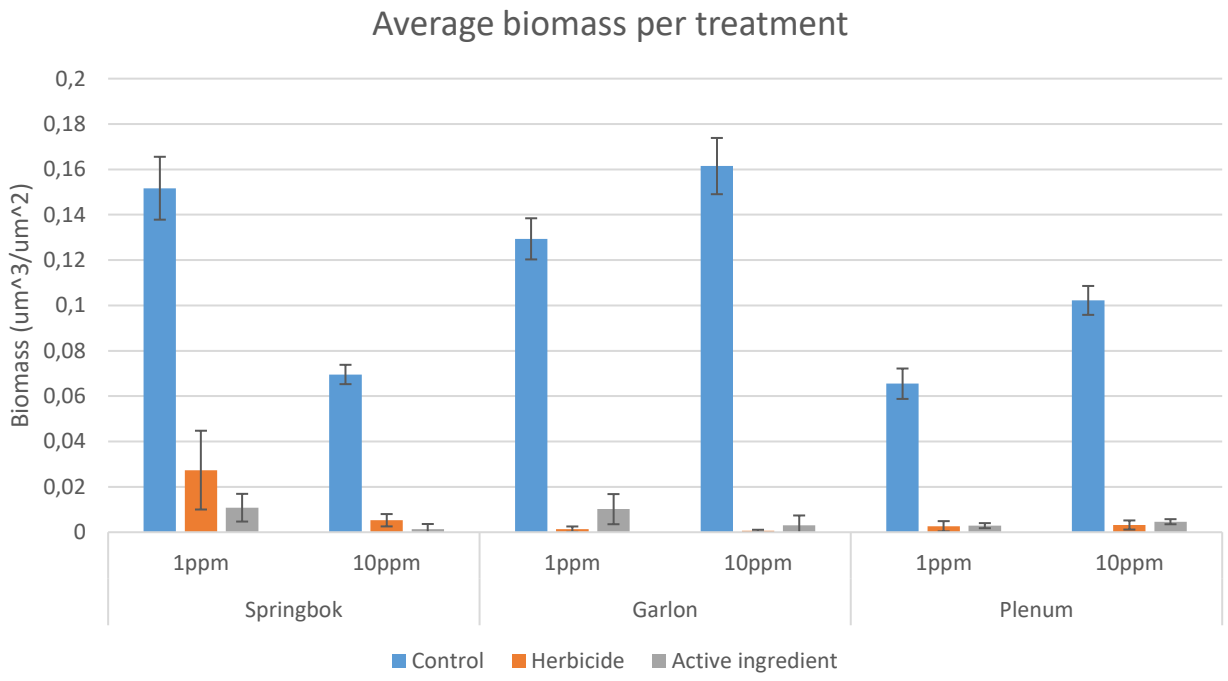

Figure 4 - A graph representing the biomass of the biofilms that were grown in the flow cells. The biomass is represented in $\mu m^3/\mu m^2$. The graph shows the average biomass for the control, herbicide and active ingredient channels. COMSTAT was used to calculate the biomass represented in the graph. Error bars indicate standard deviation.

There is a 45 % decrease overall in biomass when the concentration of the herbicide increased. This is as expected since the chemical is 10x stronger than the lowest concentration that were used. In each of the herbicides that were applied, the same trend can be seen. The herbicides, Garlon and Plenum showed a lower degree in biomass reduction, when the concentration of the herbicide was increased. Glyphosate had the opposite effect and showed a higher degree of loss in biomass, when the concentration was increased.

In Pesce et al. (2009), they found that when microbial communities are exposed to a short pulse of herbicide, the viability of the communities will vary between experiments, types of herbicide used and can be seasonally dependent. Thus, a three-day exposure to herbicide, such as this experiment, may be not enough time for the effect of the herbicide to take place.

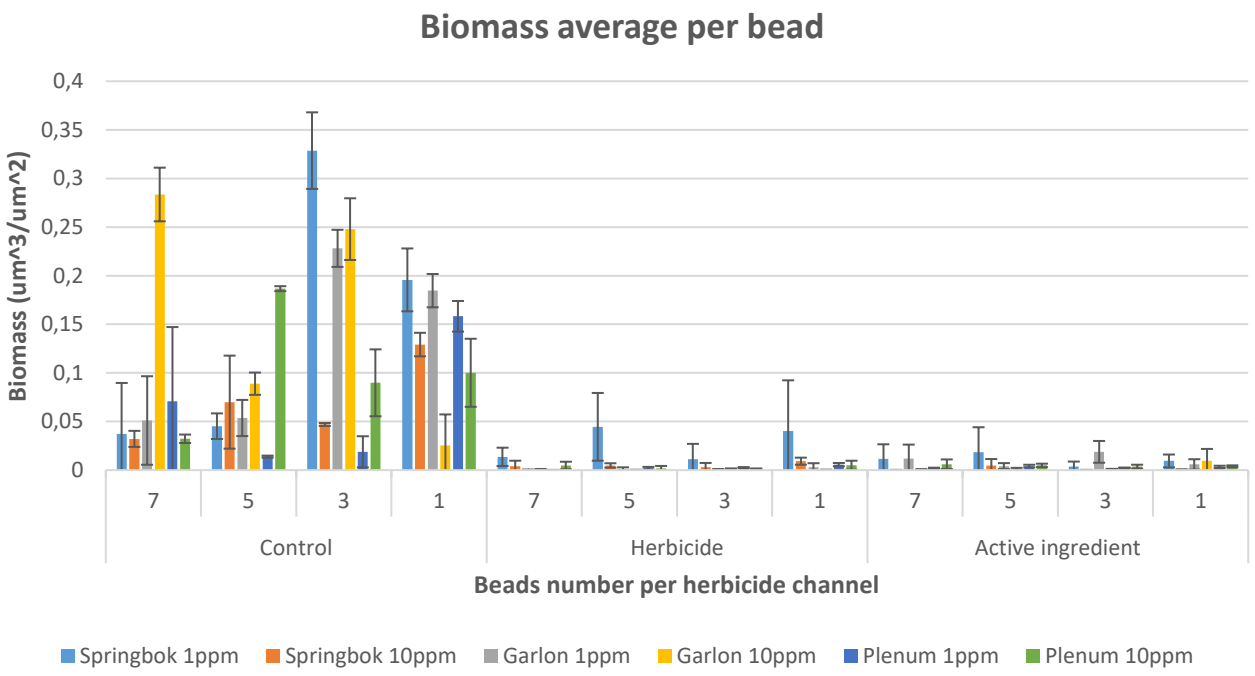

Figure 5 - The biomass per bead or stack of images is displayed. Four images per channel were taken during microscopy. The four positions in the channel where the images were taken is displayed on the graph. Error bars indicate standard deviation.

The biomass in Figure 4 represents the overall average of the four z stacks that were taken in a treatment channel. The individual biomass per bead is represented in Figure 5. This was to test whether there was a biomass gradient in the channel. The expectation from Figure 5 was

to see a decrease in biomass when comparing bead 1 to bead 7. However, no distinct pattern could be observed. The biomass on each bead differed due to the nature of biofilm formation. Some beads were covered in a thicker biofilm and some were covered with less biofilm. More biomass on the 1st and 3rd beads of the control channels, and less on the 5th and 7th beads. This can be expected since the biomass on the first few beads will have access to more nutrients and/or oxygen from inflowing water. However, a consistent decrease in biomass can be observed when comparing the treated samples to the controls. This decrease in biomass is most likely the result of the chemicals it was exposed to, since this was the only factor that changed in the system.

4) Conclusion

This study provided insight into the effect of various herbicides on freshwater biofilms. The cultivation technique served as an appropriate technique to be used for microscopy. Relatively fast biofilm formation had already started after one week of incubation, which suggest that the setup was ideal for biofilm cultivation. The flow cell design that was used in this study was more than suitable for this experiment and for microscopy. CLSM is a widely used technique across the globe and provided excellent insight in biofilm formation. In terms of the results that were obtained in the study, the loss in biomass is a result of herbicide exposure. The exposure of all three herbicides that were tested in this study, resulted in a decrease in biomass. For both concentrations, 1 ppm and 10 ppm, a significant decrease in biomass were observed. No distinct patterns were observed in terms of biomass, for biofilms that are first exposed to the herbicide, compared to the biofilms further downstream in the flow cell channel. The biomass that got detached from the glass beads could have been lysed by the herbicide chemicals and washed out through the flow cell.

The results obtained in this experiment alone is an indication to what extent herbicides influence biofilms. Further molecular experiments follow in the next chapter where the focus will be shifted from visual effects to a molecular insight. Future research is needed to identify the species that are able to tolerate the herbicide concentrations. In terms of the management of these herbicides, alternatives to the uses of herbicides should be investigated which will result in freshwater microbial communities that will not be affected.

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Chapter 4

The effect of herbicides on microbial communities of freshwater biofilms

Abstract

Invasive plant species such as *Eucalyptus* and *Acacia* have become a major concern for native crop vegetation. These species dominate the riverbanks of South Africa, especially in the Western Cape. An example of a river that is heavily invaded by these species is the Berg River, which flows through zones where herbicides are used to control the invasive species. The use of herbicides and other chemicals pose a problem to the environment and health of ecosystems. In this study, the effect of Springbok, Garlon and Plenum along with their surfactants, on freshwater biofilms were investigated. The active ingredients of these herbicides were also tested which include glyphosate, triclopyr and a combination of fluroxypyr and picloram, respectively for each herbicide treatment. The aim of this study was to investigate the effect of these chemicals on the bacterial and fungal communities which are found in freshwater biofilms. A community fingerprinting technique called ARISA, was used to determine the community profile of both fungi and bacteria respectively, which were then used to determine any significant profile shifts after the herbicide treatments. Significant shifts ($p<0.05$) were found for the herbicides, Springbok (glyphosate) and Plenum (fluroxypyr and picloram). The Shannon diversity index was also determined and showed a decrease in diversity for these treatments. This suggest that some species tend to dominate the biofilm because they can tolerate the environmental change. This study provided meaningful insight into the effects of these herbicides on freshwater biofilm communities and will provide motivation for the design of environmental friendly chemicals.

1) Introduction

All of the herbicides mentioned in the previous chapter may affect microbial ecosystems in some way if they are used excessively. Whether these herbicides influence microbial species in a positive or negative way, remains unclear and unexplored. Research into this will provide some understanding towards the problem. A starting point will be to look at the impact these chemicals have on the soil microbes, which are present in the soil of the riparian zones. These chemicals may affect the physiology and biochemistry of soil microbes (Yang et al., 2000). Studies on widely used herbicides have shown that these chemicals influence soil nutrient levels and have made alterations to soil microbial activity and diversity (Weidenhamer & Callaway, 2010). Microbial communities ensure several key ecological processes in soil such as nutrient cycles and other chemical cycles. When microbial communities in an ecosystem are changed, it will lead to alterations of soil health and will have an impact on soil fertility and sustainable agricultural productivity (Crouzet et al., 2010). Challenges such as invasive plants and various other challenges will be faced by the global agriculture industry. Agriculture must produce more food to feed a growing world population, which demand a more diverse diet (Cgiar Consortium, 2012). It is therefore essential to understand the impact of herbicides on the environment. This will help improve herbicides in the future to be more target specific and environmental friendly. Non-target organisms are constantly affected by these chemicals and this include biofilm communities that grow in freshwater rivers.

1.1) Biofilms

Various types of microbial communities occur in rivers, such as sessile and planktonic types. These types consist of individual microorganisms or groups of microorganisms, known as biofilms. Biofilms in freshwater consist of a consortium of microorganisms such as algae, bacteria, cyanobacteria, fungi, phytoplankton and protozoa that attach themselves to surfaces (Neu & Lawrence, 1997). Freshwater biofilms are usually attached to rocks, stones, sand, leaves, wood etc. These microorganisms are embedded in an extracellular matrix, which consists of polymeric substances (Romanı, 2009). Many microorganisms play a fundamental role in aquatic ecosystems. They decompose dead material, releasing nutrients stored in organic tissue. Most biofilms produce extracellular enzymes in the polymeric matrix that facilitate organic nutrient uptake (Romanı, 2009). Biofilms play an important role in the uptake of organic and inorganic nutrients in freshwater rivers and act as a filter for harsh chemicals

and, biofilms are used in industrial wastewaters for the removal of ammonia, phenol and other chemicals that become harmful in high concentrations (Cortes-Lorenzo et al., 2006). Biofilms mainly consist of bacterial and fungal cells and the different types are explained below.

1.1.1) Bacteria

Bacteria makes up a large fraction of living organisms in aquatic systems (Gasol & Duarte, 2000). Bacteria are not as restricted to their metabolic capabilities when they are compared to fungi and other microorganisms (Jeong et al., 2000). They consist of autotrophic and heterotrophic bacteria. Heterotrophic bacteria play an important role in the decomposition of organic matter and the cycling of nutrients in aquatic ecosystems. Autotrophic bacteria are often characterised as algae, because they are primary nutrient producers in aquatic systems, the same as true algae (Moriarty, 1997). The Cyanobacteria is another phylum in the Bacteria domain. These bacteria obtain their energy through photosynthesis and are also known as the blue-green algae.

1.1.2) Fungi

Fungi that occur in aquatic systems are single celled or hyphal. Hyphomycetes are most abundant in aquatic ecosystems and are classified as heterotrophic. They obtain their nutrients by secreting exoenzymes into the environment which will break down complex compounds so that they can be absorbed by the fungi. Heterotrophic fungi play an important role in the decomposition of dead plant material, due to their unique ability to break down cellulose and lignin which make up the structure of plants.

The effect of herbicides on microbial communities is still unclear. A few studies have discussed the effect that herbicides have on individual organisms but not on biofilms or communities (Zabaloy et al., 2008; Haney et al., 2009). The long-term application of herbicides may result in a microbial community shift and cause plant nutrition deficiencies and diseases (Dick et al., 2010). Dick et al. (2010), also used the fatty acids produced by some microorganisms to determine if microbial communities can adapt to tolerate some of the active ingredients in the herbicide. Zabaloy et al. (2008), applied glyphosate concentrations well above the recommended rates and found that minimal changes to the soil biology and microbial diversity occurred.

Biofilms usually consist of numerous different species of both fungi and bacteria and can be identified, up to species level, using next generation sequencing methods and techniques (Flemming & Wingender., 2010). ARISA, which is a molecular fingerprinting technique, is used to rapidly profile the community structure, and serves as an ideal technique to study the effect of herbicides on biofilm communities (Danovaro et al., 2006). This technique does not identify the individual species that is present in the sample, but it will provide an insight on a molecular level of the community structure (Ramette, 2009).

1.2) Automated Ribosomal Intergenic Spacer Analysis (ARISA)

A common technique that is used to determine the community fingerprint of both bacteria and fungi is called ARISA (Fisher & Triplett, 1999; Ranjard et al., 2001). This is a molecular method commonly used to study populations of environmental samples (Slabbert et al., 2008). This technique delivers reproducible results for both fungal and bacterial communities. ARISA-PCR uses fluorescently labelled primers that can be detected by a DNA automated system such as a ABI genetic analyser. This technique is effective and a rapid way for determining the effect of a certain factor, such as herbicides, on a community structure.

In this study two different concentrations of triclopyr, glyphosate, fluroxypyr and picloram will be applied to biofilms that are cultured in the laboratory, using flow cells. The aim of this study is to look at the effect of these herbicides on the overall microbial community structure within the biofilm. ARISA will be used to determine a profile of the fungal and bacterial communities. The treated samples will be compared to the controls to see if there is a significant shift in the community structure of the biofilm after they are exposed to the herbicides.

2) Materials and Methods

2.1) River water sampling

River water was collected in the Berg River at a site located near a small town called Wellington in the Western Cape, South Africa (S $33^0$28.887' E $018^0$56.245'). The river water was collected in sterile 30 L canisters in the fast-flowing water of the river. The water sample was then transported to the laboratory where it was kept at 4ºC until further use.

2.2) Biofilm establishment

Flow cells were used to establish the biofilms in the laboratory. The river water that was collected was used as the inoculum for the flow cells. This is to allow for the formation of biofilms in the flow cell channel. The flow cell design and setup is the same as described in the previous chapter (Chapter 3).

2.3) Flow cell setup and treatments

As discussed in Chapter 3 (Section 2.5) the flow cells were allowed to run for ten days before a treatment was administered. On day 14 the DNA of the biofilms were extracted from the selected channels that were not used for microscopy. For each herbicide, two concentrations were used for the technical grade active ingredient of the herbicide as well as for the herbicide itself. The concentration of the herbicide and active ingredient was 1 ppm and 10 ppm separately. The two concentrations were tested at different times due to the availability of the equipment.

2.4) Biofilm DNA extraction

The DNA extractions were performed on three flow cells, each containing four channels. One flow cell was used for the control and the other two for the treatment of the herbicide and active ingredient, respectively. A standard Zymo Research soil DNA isolation kit was used to extract the DNA from the biofilm (Zymo Research, California, USA). The protocol for the extraction was adapted from the standard protocol since the biofilm needed to be lysed inside the flow cell. Lysis solution was injected into the flow cell using a sterile syringe and needle. The flow cell was then loaded onto a bead basher vortex and allowed to shake for five minutes. The samples were then added to the lysis tube which is supplied by Zymo in the soil DNA extraction kit. Additional lysis solution was added to bring the total lysis volume to 750 µl. The rest of the extraction was followed according to the protocol supplied. After the DNA extraction, the presence of DNA was checked on a 1% agarose gel stained with ethidium bromide.

2.5) ARISA community fingerprinting

ARISA was used to determine the shifts within the microbial community of the flow cells. The protocol for the technique is followed as described in Slabbert et al. (2008). This technique

makes use of highly conserved genes in prokaryotic and eukaryotic DNA to produce fingerprints. Between the genes is a hyper variable internal transcribed spacer (ITS) which is a non-coding region. This gene region for bacterial and fungal communities differ, therefore different primer sets are required for bacteria and fungi. Bacterial and fungal communities were analysed separately.

Fungal ARISA PCR reactions were carried out using the fungal-specific primers ITS4 and FAM (carboxy-fluorescein)-labelled ITS5 (Slabbert et al., 2008). The primer sets and sequences can be seen in Table 1. The reaction mixture consisted of 0.5 µl of the purified genomic DNA extracted from the biofilms in the flow cells, 500 nM of each primer and 5 µl of 2X KapaTaq Readymix in a total volume of 10 µl. PCR reactions were performed using a GeneAmp PCR system 9700 (Applied Biosystems, USA). The PCR program consists of a denaturing step of a 4 min cycle at 95 °C followed by 36 cycles of 94 °C, for 30 s, 56 °C for 45 s and 72 °C for 70 s. The reaction completes with a final step at 72 °C for 5 min and is followed by a cooling and holding step at 4 °C as described in Slabbert et al. (2008).

Table 1 - ARISA primer sequences for bacteria and fungi respectively

Community	Primer name	Primer sequence
Fungal primer set	ITS4	TCCTCCGCTTATTGATATGC
	ITS5 – FAM labelled	GGAAGTAAAAGTCTAACAAGG
Bacterial primer set	ITSf – FAM labelled	GTCGTAACAAGGTAGCCGTA
	ITSReub	GCCAAGGCATCCACC

To determine the bacterial community fingerprint, the Eubacterial specific primers ITSReub and ITSf-FAM [carboxy-fluorescein] were used. (Slabbert et al., 2008). The specific primer sequences can be seen in Table 1. The reactions were amplified using a GeneAmp PCR system 9700 (Applied Biosystems, USA). The ARISA reaction mixture contained 0.5 µl of purified genomic DNA that was extracted from the biofilms in the flow cells. Each primer had a concentration of 500 nM and 5 µl 2X KapaTaq Readymix were added to the mixture. The reaction mixture had a total volume of 10 µl.

PCR products were separated on a 1% agarose gel stained with ethidium bromide and visualised using ultra violet light to confirm that the reactions were successful. The PCR

products were then analysed using an ABI 3010xl Genetic analyser to obtain an electropherogram of the different fragment lengths and fluorescent intensities. The products were run with a Lizz 1200 size standard, which ranged from 100-1200 bp. The ARISA data was analysed using Genemapper 4.1 software, and converted to fluorescence data and expressed on an electropherogram where the peaks represent fragments of different sizes. Fragment sizes larger than 0.5 % of the total fluorescence were excluded for analysis, this is to minimise the total background noise in the sample (Slabbert et al., 2008).

2.6) Data analysis

Statistical tests were done on the data generated through ARISA analysis. The microbial diversity was calculated using the relative abundance of operational taxonomic units (OTUs). The Whittaker similarity index between each of the samples was calculated using Statistica 13 (Lande, 1996). This index is a measure of the relative abundance of OTUs that uses a numerical measure of the absence and presence of OTUs which was then used to construct a distance matrix representing the community structure. The distance matrix was graphically represented using non-metrical multidimensional scaling (nMDS) to detect meaningful groupings or clusters. A Scree-test was performed to determine the number of dimensions used for every nMDS analyses.

R-3.2.2 is a program that contains software packages for statistical computations and graphics which was used to determine the statistical significance of similarities between and within communities. Analysis of similarity (ANOSIM) was used to analyse the communities for similarities. ANOSIM is part of a R statistical package “vegan”. The vegan package was used in R to perform ANOSIM and nMDS analyses (Oksanen et al., 2015).

2.7) Alpha diversity test

The alpha diversity is a way of measuring the diversity of a specific group of organisms within a specific area (Slabbert et al., 2008). The Shannon index is used to describe alpha diversity in an experiment and is a popular index that is used in ecology studies. The Shannon index accounts for both the evenness and abundance of the species that are found in the sample. A higher index number represents a larger diversity compared to a lower index number which represents a lower diversity sample. The formula below shows the calculation for the Shannon’s

diversity index where S is the number of species (richness), P_i the proportion of S made up with the *i*th species and E_H is the equitability or evenness of the sample (Pielou. 1966).

$$H = -\sum_{i=1}^{S} P_i \ln P_i$$

2.8) Beta diversity test

The difference of species composition over space and time can be defined as the beta diversity. A comparison between two or more sites is needed in order to calculate the beta diversity. The Whittaker index is one of the many indices that can be used to calculate the beta diversity. This index proves to be ideal for the analysis of ARISA data because the relative abundance of the species present in the sample is given by the fluorescent intensities (Slabbert et al., 2010). The Whittaker indices are used to create a dissimilarity matrix which is then used to plot the beta diversity on nMDS graphs. The formula below shows the calculation for the Whittaker index. If both sites contain the same number of species C_S^T and β_W will equal 0 and if no species is shared it will equal 1 (Diserud, 2007)

$$C_S^T = \frac{T - \beta_W}{T - 1}$$

3) Results and Discussion

3.1) Overall OTU counts

The OTU's were determined for both the fungal and bacterial groups which are represented in Figures 1 and 2. The OTU's are a measure or quantification of how many closely related organisms are in a sample. For both the fungal and bacterial communities, between 7 to 47 OTU's were recorded for the samples which is an indication of how diverse the samples are. Biofilms are known to consist of a more diverse microbial community in a natural environment and ecosystem (Davey & O'Toole, 2000). The highest bacterial OTU count was obtained from the 1 ppm glyphosate active ingredient channel (Figure 1). This channel yielded a higher overall OTU count than for the control channels and may be due to the growth of the biofilm which varies between channels. More microbial cells may enter one channel compared to another, which makes it impossible to regulate the growth of the biofilm so that all the flow cell channels are identical in biofilm growth.

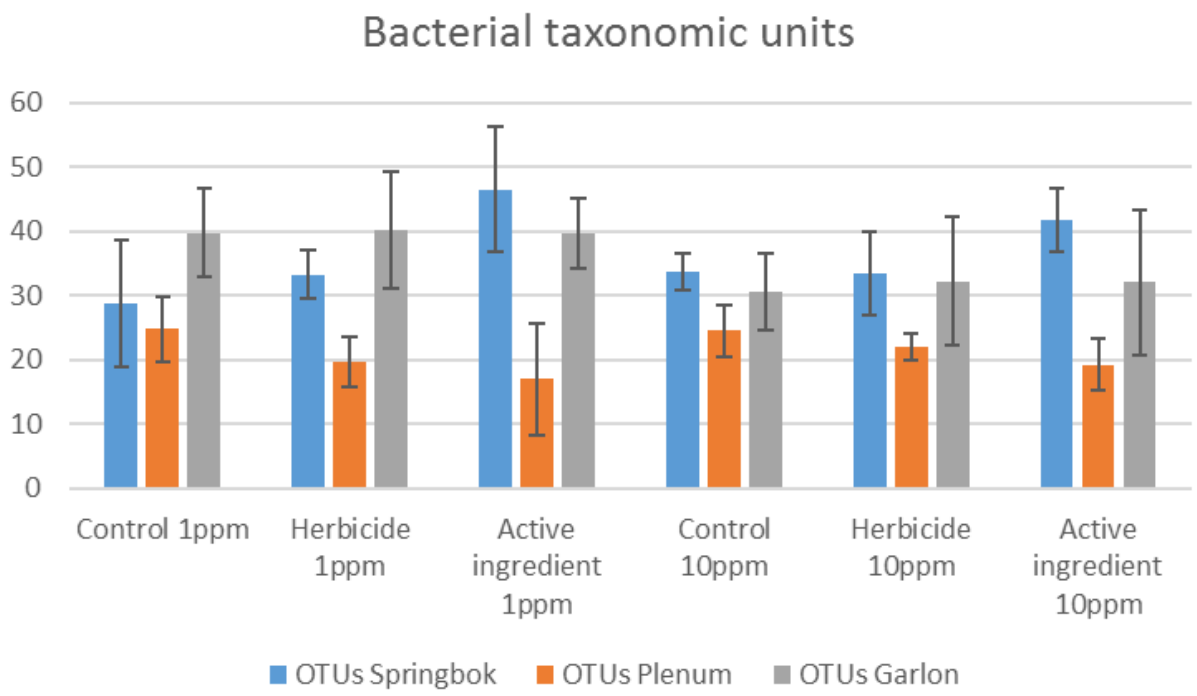

Figure 1 - The total number of OTU's observed for the bacterial communities. The OTU's were determined from the ARISA data. Error bars indicate standard deviation.

The significance of the total OTU counts is shown in Table 2. This table shows the ANOVA results for the total number of OTU's and the Shannon diversity index for each of the herbicide

treatments. A significant (p=0.0405) difference was found in the overall OTU count of bacteria, when comparing the channels that received the Springbok herbicide with the Plenum and Garlon treated channels.

Table 2 -ANOVA test results for the total number of OTU counts obtained from each treatment. Samples containing a (*) are significantly different.

ANOVA		P-value		
		Springbok	Plenum	Garlon
Bacteria	Shannon index	0.9084	0.0151*	0.1111
	OTUs	0.0405*	0.4257	0.3405
Fungi	Shannon index	0.0711	0.0060 *	0.0887
	OTUs	0.0065 *	0.0878	0.0584

When comparing the OTU's for the fungal communities, the highest OTU count is observed for the glyphosate active ingredient at 10 ppm and the lowest OTU count was observed in the 1 ppm Springbok channel (Figure 2). This may be due to the toxic effect of the surfactants that are used in the herbicide mixture. ANOVA tests were performed on the number of OTU's for both the fungal and bacterial communities to determine the statistical significance of the data.

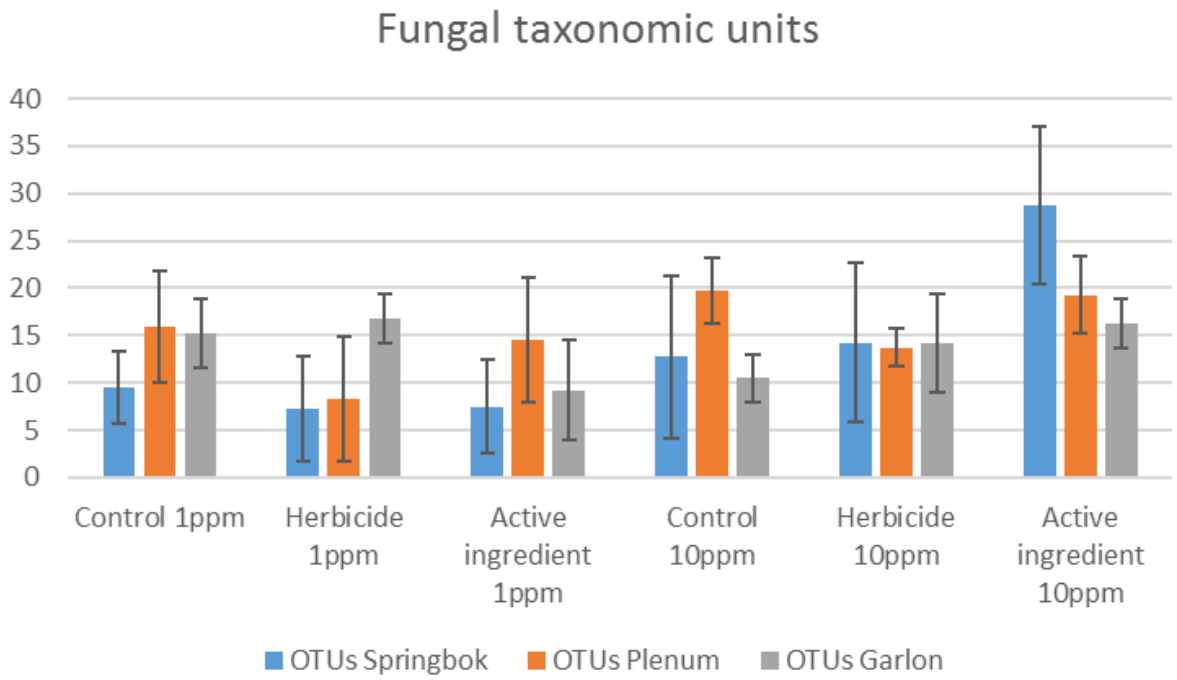

Figure 2 - The total number of OTU's observed for the fungal communities. The OTU's were determined from ARISA. Error bars indicate standard deviation.

A significant difference (p=0.0065) was found when comparing the overall fungal OTU count of Springbok to the channels that received the Garlon and Plenum treatment.

The data explained above will be visualised on nMDS plots below, for each treatment. The nMDS plots allow for a better understanding and interpretation of the results. Additional statistical tests are performed to determine the significance of the clusters that are observed on the nMDS plots. This will define the beta diversity of the samples.

3.2) Beta diversity

3.2.1) Glyphosate results - nMDS

ANOSIM was performed on the nMDS data to test for similarity between and within sample groups (Table 3). The significance of the test is indicated by the P-value, and the R-value represents the degree of significance. For example, a lower R-value means that the factor has a small effect on the variable. In ANOSIM the R-value will provide insight to what extent the factor affects the variable. The bacteria ARISA results for the glyphosate and Springbok treatment are shown in Figure 3 on an nMDS plot. A clear clustering between the two concentrations used can be seen on the plot. This is expected, because the two experiments were done two weeks apart from each other and fresh river water was sampled, therefore the two concentrations differ significantly from each other. This is also supported by the ANOSIM values that were obtained in Table 3. The symbols that are filled represent the samples that were used during the 1 ppm experiment and the non-filled symbols represent the 10 ppm experiment. The samples that received the lower concentration of herbicide and active ingredient, tend to group closer together and are significantly different (p=0.001), when compared to the samples that received the higher concentration. This is expected since the two concentrations were tested at different times and the lower concentration has a less significant effect when compared to the higher concentration.

Table 3 -ANOSIM test results from all the nMDS plots used for each of the herbicide treatments. Samples that contain a (*) are significantly different.

ANOSIM		Springbok			Plenum			Garlon		
		1ppm	10ppm	ALL	1ppm	10ppm	ALL	1ppm	10ppm	ALL
Bacteria	R - value	0.359	0.254	0.598	0.109	0.072	0.592	0.245	0.007	0.007
	P - value	0.060	0.049 *	0.001 *	0.174	0.211	0.001 *	0.076	0.419	0.419
Fungi	R - value	0.245	0.419	0.331	0.035	0.048	0.112	0.454	0.113	0.317
	P - value	0.01 *	0.056	0.001 *	0.546	0.424	0.051	0.062 *	0.179	0.001 *

For both the 1ppm and 10ppm experiment in figure 3, no definite clustering can be seen within the treatments of each concentration. There is no clustering of the treatment channels versus the control groups. A more rapid spreading of the data points can be seen in the experiment that received the 10ppm concentration of herbicide and active ingredient. The treatment samples and the controls form separate clusters which are statistically different from each other ($p=0.049$; $r=0.254$). Therefore, no significant shift can be detected between the active ingredient and the herbicide treatments.

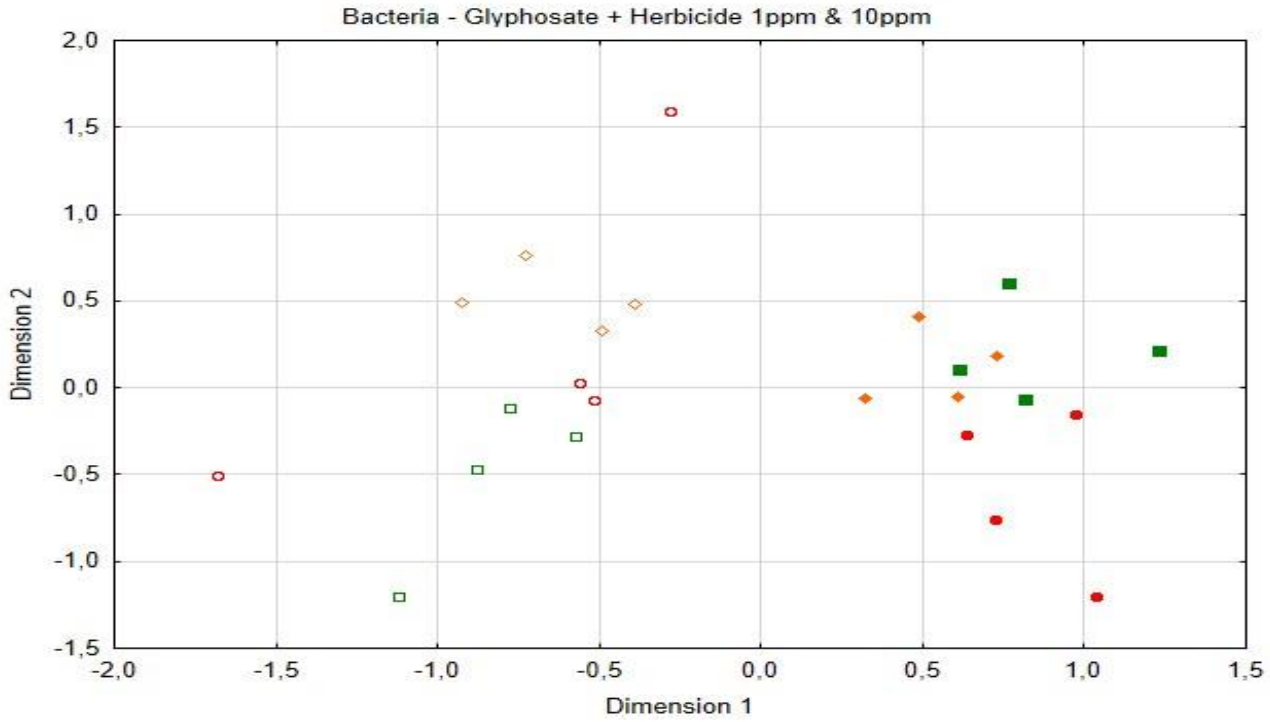

Figure 3 - Non-metric multidimensional scaling graph for the bacterial communities, which shows the distance between the two different concentrations of glyphosate and Springbok used. The solid filled symbols are the data points for the 1 ppm concentration and the non-filled symbols are the data points for the 10 ppm concentration. The Green symbols represent the control groups, the red symbols represent the herbicide treatment and the orange symbols represents the active ingredient data points.

The fungal communities that received the Springbok and glyphosate treatments showed little to no clustering (Figure 4). A significant (p=0.001) clustering between the two concentrations used, can be seen in figure 4 (below). Another significant clustering between the treated samples of the 1ppm experiment and the control groups is noted (p=0.01 ; r=0.245). In this case the treated samples group together apart from the control samples. Again, as for the bacterial communities, it can be concluded that the active ingredient, glyphosate, and the herbicide had the same effect on the biofilm community. In terms of the higher (10 ppm) concentration, no significant differences can be seen between the treated and control groups.

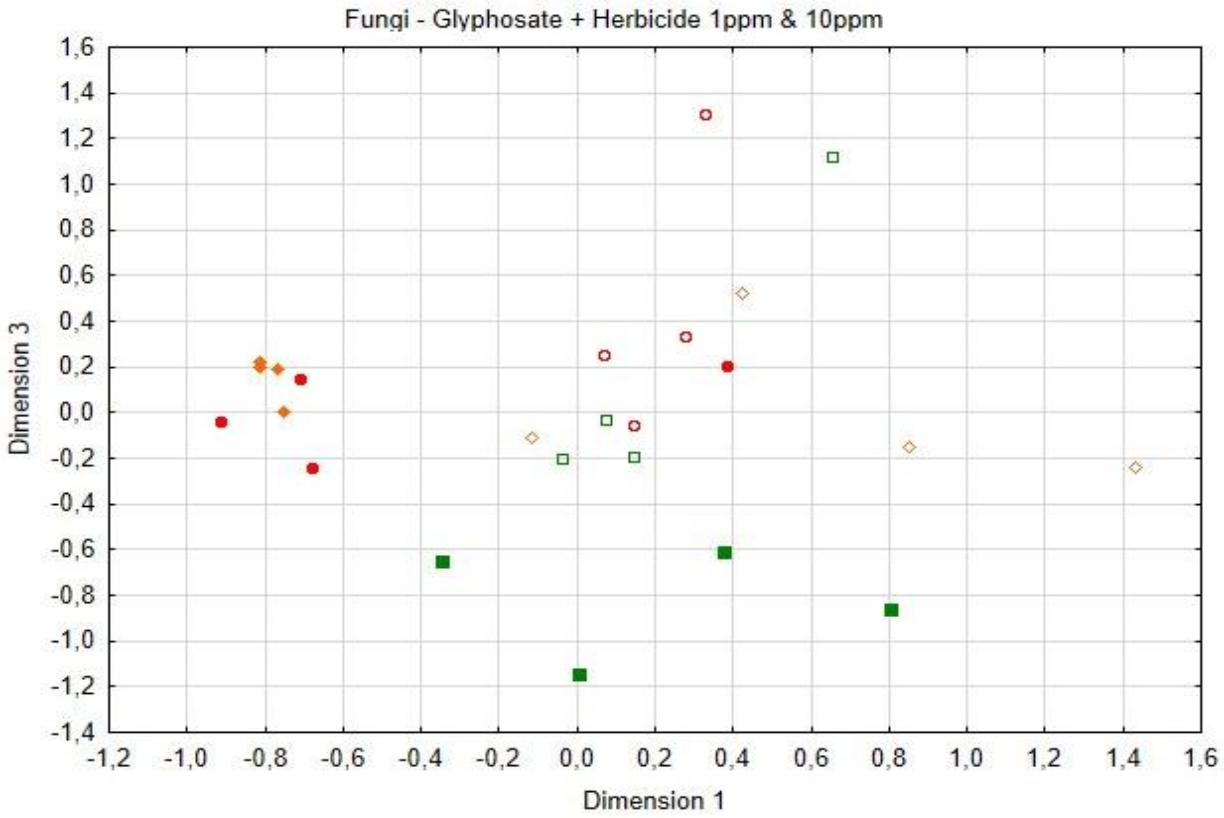

Figure 4 - Non-metric multidimensional scaling graph for the fungal communities, which shows the distance between the two different concentrations of glyphosate and Springbok used. The solid filled symbols are the data points for the 1 ppm concentration and the non-filled symbols are the data points for the 10 ppm concentration. The Green symbols represent the control groups, the red symbols represent the herbicide treatment and the orange symbols represents the active ingredient data points.

3.2.2) Plenum results - nMDS

When looking at the bacterial communities that received Plenum and its active ingredients, a significant shift between the two concentrations that were used can be seen (Figure 5). In terms of the ANOSIM values obtained, no significant differences within each concentration were found ($p=0.174$ and $p=0.211$) for 1ppm and 10ppm, respectively. The samples that received 10ppm concentration, showed a more scattered pattern which suggests that the concentration influences the bacterial communities. In the lower concentration, less scattering is observed (Figure 5). A possible reason for the scattering of the data points may be due to the combination of two active ingredients that are used in the herbicide. This allows for a further diverse mode of action which can affect more target and non-target species and organisms. The effectiveness

of the herbicide increases dramatically as explained in Esqueda et al. (2005), where they have investigated the effectiveness of the mixture picloram and fluroxypyr on perennial weeds.

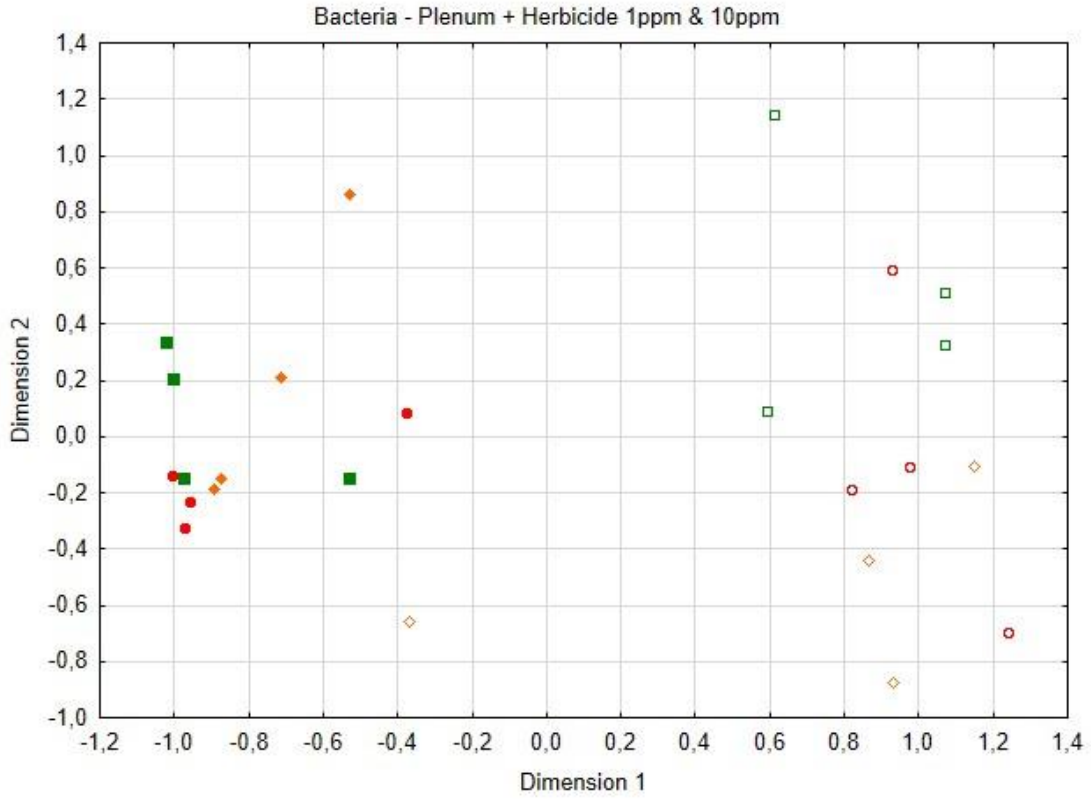

Figure 5 - Non-metric multidimensional scaling graph for the bacterial communities, which shows the distance between the two different concentrations of picloram + fluroxypyr and Plenum used. The solid filled symbols are the data points for the 1 ppm concentration and the non-filled symbols are the data points for the 10 ppm concentration. The Green symbols represent the control groups, the red symbols represent the herbicide treatment and the orange symbols represents the active ingredient data points.

No distinct separation between the treated and control samples for the fungal communities can be seen (Figure 6), this may be since fungi tends to be more resilient to environmental change (Abdel-Mallek et al., 1994). Fungi and bacteria differ significantly from one another in terms of cell wall and cell membrane content. These physiological differences could affect the ability of herbicides such as Plenum, to influence them. In Allison and Martiny (2008) the resistance and resilience of microbial communities are tested. They concluded that some microbial communities are more sensitive to environmental change than others. These less sensitive microorganisms include fungi that is found in a robust and polluted environment. The Berg River has been previously exposed to herbicides which make the resistance factor a possibility.

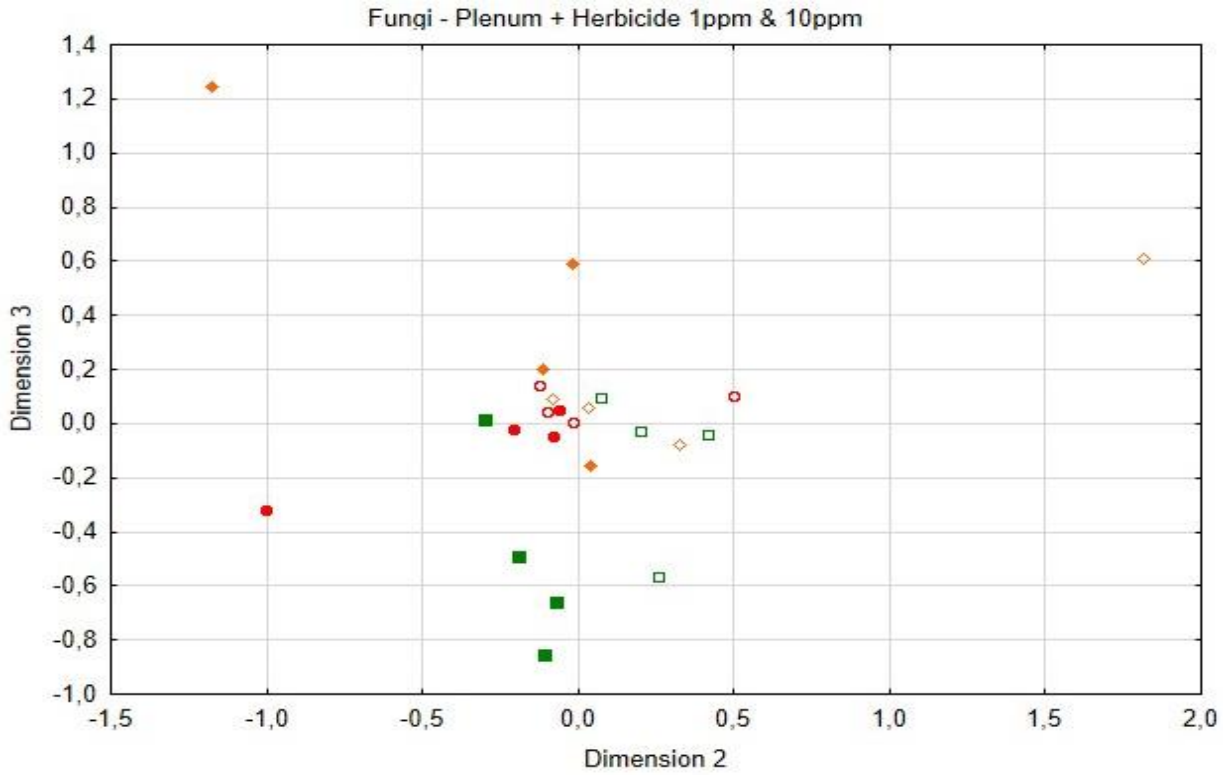

Figure 6 - Non-metric multidimensional scaling graph for the fungal communities, which shows the distance between the two different concentrations of picloram + fluroxypyr and Plenum used. The solid filled symbols are the data points for the 1 ppm concentration and the non-filled symbols are the data points for the 10 ppm concentration. The Green symbols represent the control groups, the red symbols represent the herbicide treatment and the orange symbols represents the active ingredient data points.

3.2.3) Garlon results – nMDS

Figure 7 represents the data point for the flow cell channels that received Garlon and the active ingredient, triclopyr. No distinct pattern or clustering is observed, which suggests that the active ingredient, triclopyr, and the herbicide, Garlon, have little to no effect on the bacterial community. This is supported by the ANOSIM analysis which yielded no significant p-values ($p<0.05$).

Netherland and Getsinger, (1992), investigated the efficacy of triclopyr and the required exposure time threshold. Under controlled conditions they have found that certain combinations of concentration and exposure time met the excellent control threshold. A lower concentration of herbicide can be lethal to the plant when the exposure time is longer. Thus, a longer exposure time is necessary for lower concentrations when compared to a higher concentration which needs a shorter exposure time to be fatal. These combinations include 0.25 mg/l for 72 hours and 1 mg/l for 36 hours. However, the concentrations used in this study were 1 mg/l and 10 mg/l for 24 hours. The herbicide in Netherland and Getsinger (1992) was sprayed directly onto the target plant while the herbicide in this chapter was made up in water and flushed through the herbicide channels. Herbicide degradation within the 24 hours of exposure is a possibility since the active ingredient, triclopyr, becomes unstable and tend to break down in water. The effectiveness of the herbicide decreases after a certain time, which allows the biofilm to recover.

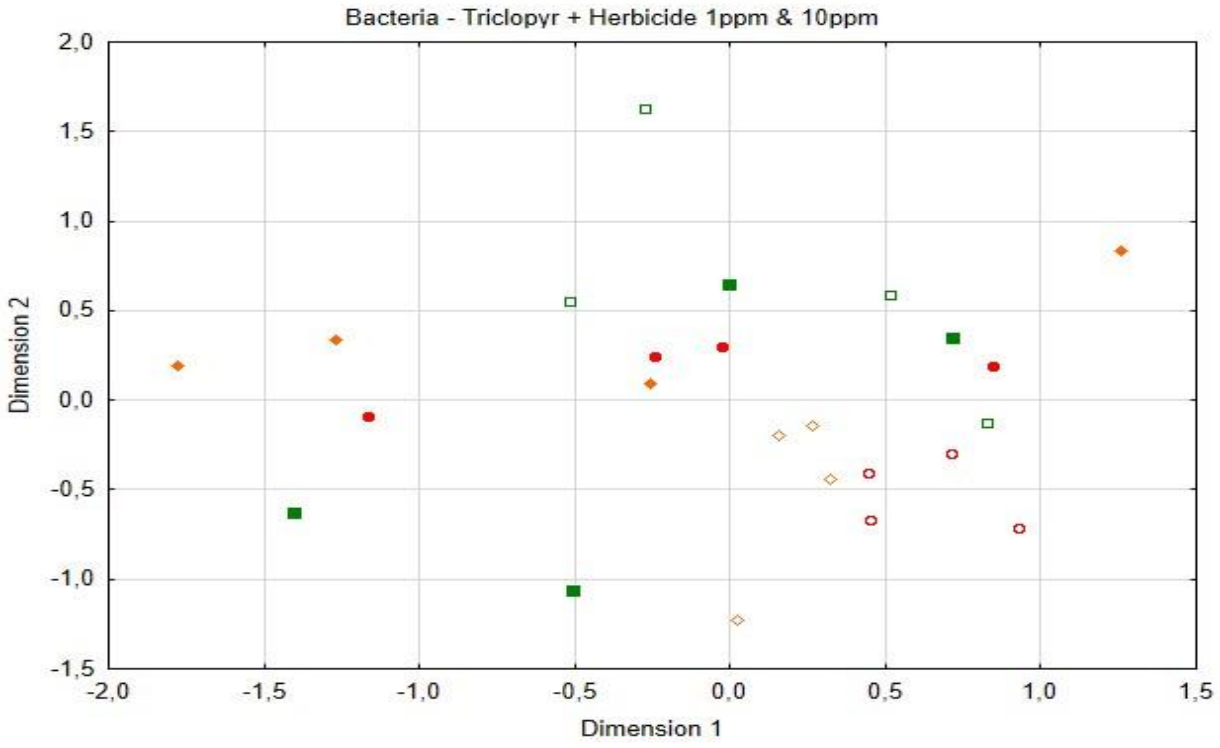

Figure 7 - Non-metric multidimensional scaling graph for the bacterial communities, which shows the distance between the two different concentrations of triclopyr and Garlon used. The solid filled symbols are the data points for the 1 ppm concentration and the non-filled symbols are the data points for the 10 ppm concentration. The Green symbols represent the control groups, the red symbols represent the herbicide treatment and the orange symbols represents the active ingredient data points.

The fungal data points that received Garlon and the active ingredient triclopyr, are represented by Figure 8. The same trend is followed as seen on the nMDS plot that represents the bacterial communities (Figure 7). Little to no effect of the treatments compared to the controls can be seen in this figure. In terms of the ANOSIM tests, the only significant difference is between the two concentrations, which is as expected (p=0.001 and r=0.317). Chakravarty and Sidhu, (1987), have found that Garlon and its active ingredient, triclopyr, become more toxic at concentrations above 10 ppm. Significant (p=0.05) results were obtained when applying Garlon on fungi. This may be due to the ability of fungi to tolerate the herbicide exposure below a concentration of 10 ppm. Above 10 ppm the herbicide becomes toxic to the organisms.

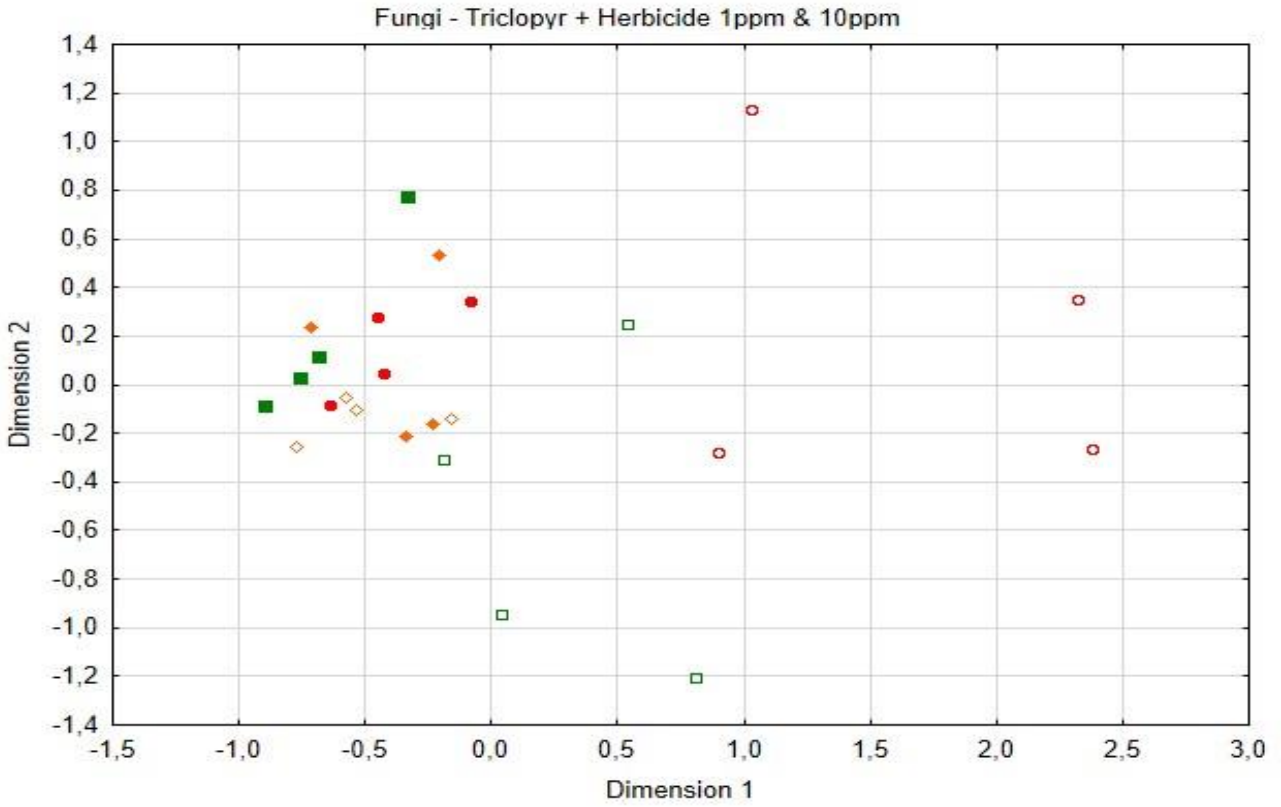

Figure 8 - Non-metric multidimensional scaling graph for the fungal communities, which shows the distance between the two different concentrations of triclopyr and Garlon used. The solid filled symbols are the data points for the 1 ppm concentration and the non-filled symbols are the data points for the 10 ppm concentration. The Green symbols represent the control groups, the red symbols represent the herbicide treatment and the orange symbols represents the active ingredient data points.

3.3) Alpha diversity – Shannon diversity index

The alpha diversity of the total bacterial communities was calculated and is represented in Figure 9 (below). The Shannon diversity index shows how diverse the species are in the sample. The higher the Shannon diversity index, the higher the diversity of the sample. The lowest diversity can be seen in the samples that received the herbicide, Plenum. This is supported by the ANOVA test (p=0.151). This suggests that the herbicide, Plenum, certainly affects the bacterial community of freshwater biofilms. In some cases, the diversity of the bacterial communities increased when a herbicide was applied compared to the control groups. This may be due to the ability of the organisms that are able to use the herbicide compound as a nutrient source. For example, in the case of glyphosate a slight increase in diversity can be seen when comparing both the concentrations with their respective control groups. The alpha diversity cannot be compared to the beta diversity since they are two different indices, thus no correlation between the two can be drawn.

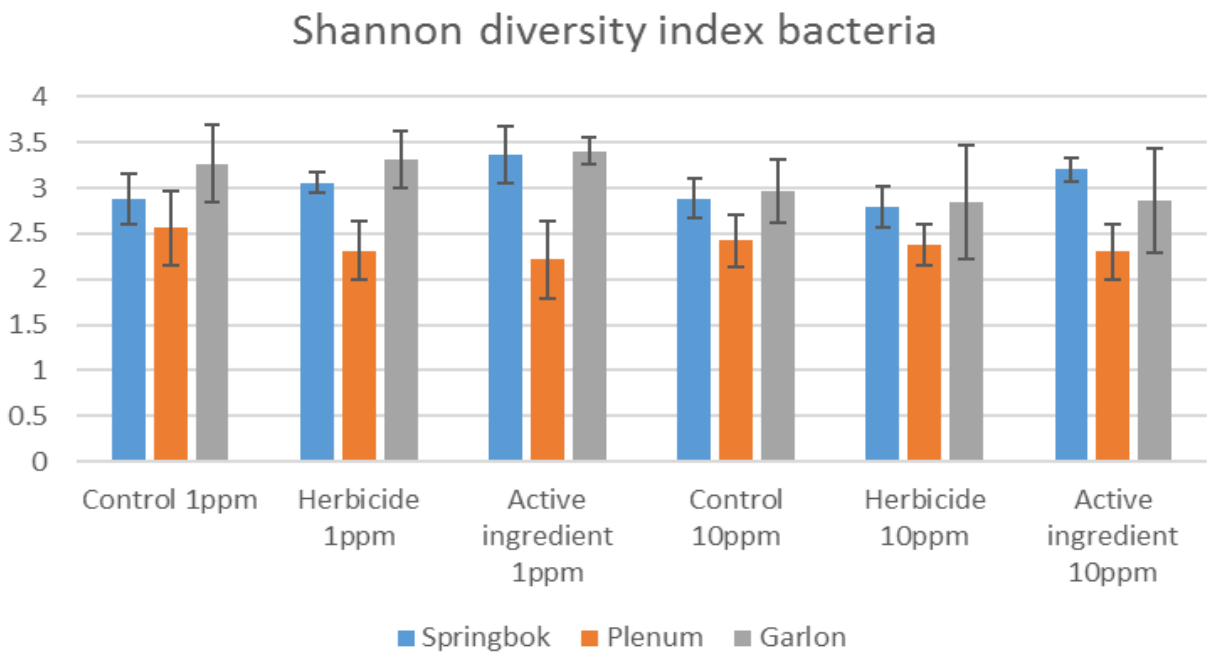

Figure 9 – Shannon diversity index was performed on the bacterial samples of all the treatments in order to determine the alpha diversity. Error bars indicate standard deviation.

The Shannon diversity index for the fungal communities was calculated and is represented in Figure 10. No significant differences can be seen in Figure 10, except for the herbicide, Plenum, which had the lowest diversity in all the samples which received the herbicide. This is supported by the ANOVA test (p=0.006). However, the decrease in diversity cannot be linked back to the nMDS fungal plot for Plenum. A community shift does not necessarily mean that there is a decrease or an increase in diversity. The Shannon diversity index shows whether there is a possibility that some species may dominate in the sample or are more abundant than other species. Overall the highest diversity of microorganisms is observed for 1 ppm Garlon, this may be due to the natural growth of the biofilm in that specific channel.

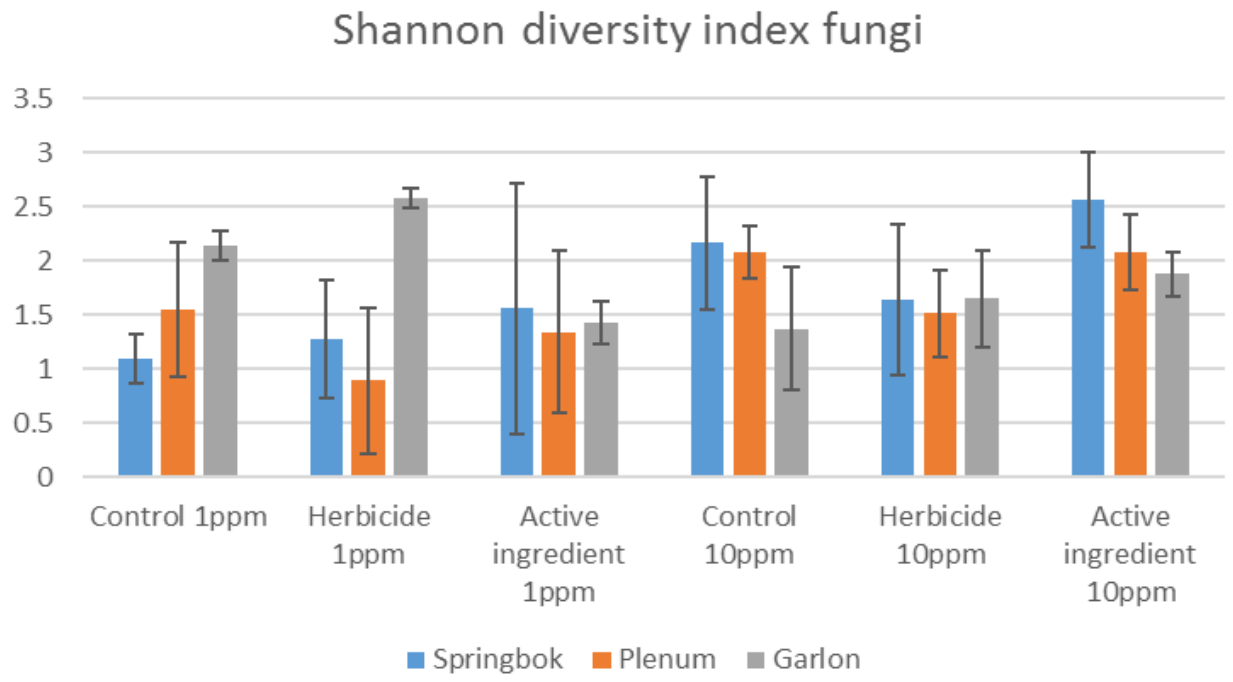

Figure 10 – Shannon diversity index was performed on the fungal samples of all the treatments in order to determine the alpha diversity. Error bars indicate standard deviation.

4) Conclusion

In this study, the overall alpha and beta diversity indices of freshwater biofilms were determined after the application of three conventional herbicides. These indices are not comparable to each other, but both provide meaningful insights into the effect of these herbicides on biofilms and

how it changes the community profile. The alpha diversity index showed how the total number of OTU's varied between samples and the different herbicides. This is an indication of how many species or organisms are suppressed when the herbicide is applied.

In some cases, the total number of OTU's increased after herbicide application which may be due to the nature of the biofilm growth. This suggest that these organisms are resistant to these herbicides and have been previously exposed to them. In terms of the total number of OTU's obtained for the bacterial communities, the lowest number of OTU's were observed for the herbicide, Plenum. This may be due to the dual active ingredients that are present in the herbicide, which allows for a more broader mode of action which allows the herbicide to disrupt metabolic pathways of plants and organisms in more than one way.

In terms of the total number of OTU's for the fungal community profile, the lowest OTU's were observed for the herbicide, Springbok, which contains the active ingredient, glyphosate. Glyphosate is known to disrupt the shikimate pathway that is present in both bacteria and fungi. The decrease in OTU's may be due to the metabolic pathway that is disrupted by this chemical, therefore less species or OTU's are observed for this sample.

For the beta diversity tests, the most significant clustering is observed for the bacterial communities of the Springbok and Plenum treatments. Distinct clustering between both concentrations is noted, this is due to the different times the samples were taken and when the experiments were set up. The Garlon treatments showed little to no significant community shifts. The degradation of the active ingredient, triclopyr, may be a factor that contribute to the efficacy of the compound since it is made up in water and left for 24 hours.

This study provided meaningful insight into the effect of three conventional herbicides that are used excessively in the Western Cape. A suitable indication of the toxicity of these herbicides have been found in the results that have been obtained. Some herbicide treatments result in a less scattered community structure which indicates that it is a less toxic chemical. Future studies should include a more diverse concentration range as well as more samples. The exposure time should also be considered since this can affect the results that will be obtained in future studies.

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Chapter 5

Conclusion and future research

The aim of this study was to investigate the effect of conventional herbicides on freshwater biofilms and how it impacts the community profile of the biofilm. To fulfil this aim, confocal microscopy and PCR techniques were used to study the biofilm structure and community profile respectively. This project presented methods and techniques that can be used to study biofilms in depth and used to detect four conventional herbicide active ingredients in rivers. An optimised method for detecting herbicides in any river water has been developed with great limits of detection and quantification. High levels of triclopyr were found in the Berg River by the survey conducted in the study and can possibly be as a result of the application schedules. During the testing period, more triclopyr could have been sprayed close to the riverbanks of the Berg River therefore adding to the amount of herbicide that end up in the river due to runoff. A possible reason for triclopyr spikes being detected may be that farmers apply herbicides to vineyards at the end of harvest season which falls between December and March.

The microscopy technique that was used in this study, presented a rapid and accurate quantification measure of the biomass in the flow cell, by using a simple non-destructive stain (DAPI). The COMSTAT image quantification software that was used, was appropriate for the quantification of the biofilms. A clear correlation between the herbicide treatments and controls can be seen when looking at the biomass data.

ARISA delivered reproducible and accurate community profiles for both fungi and bacteria. The ARISA data showed that fewer operational taxonomic units were observed for fungi when compared to bacteria. This may be due to the nature of a biofilm where bacteria tend to dominate an environmental biofilm. ARISA along with the biofilm quantification technique revealed the significant effect of the herbicide, Springbok, when compared to the controls. In terms of the surfactants and adjuvants that are used with the active ingredients, little to no effect can be seen when comparing the different data sets that are presented in this study.

Future studies should test the known surfactant and adjuvant mixture on freshwater biofilms. Weather conditions when sampling also play an important role and should be noted when

sampling is taking place and should be done over a longer period of time. This would help to explain the results found in this study from another point of view. Rainfall will influence the amount of herbicide entering the nearby rivers, thus this can be taken into account when investigating the herbicide levels in freshwater streams. Clearing operations, such as the Working for Water, and agricultural land owners can supply their clearing and herbicide application schedules which will lead to a better understanding of how many herbicides are sprayed close to the rivers Herbicide application schedules could not be obtained from the clearing programs and agricultural landowners.

Organisms that have been previously identified to tolerate glyphosate includes bacterial genera that can use glyphosate as a carbon source. Two bacterial genera have been identified which are *Strenotrophomonas* and *Providenciai.* The fungal species can use glyphosate as a phosphorous source and these genera include *Aspergillus, Fusarium, Verticillium, Acremonium* and *Scopulariopsis.*

This study showed that the effect of conventional herbicides is severe on the microbial communities that occur in fresh water rivers. There is a significant reduction in biofilm biomass as well as a shift in the overall species richness and evenness. The herbicide concentrations (1 ppm and 10 ppm) that were used, to spike the flow cells, in this study was higher than the actual concentrations measured in the Berg River survey. Therefore, the data gathered can be potentially used to simulate the worst-case scenario if a herbicide spill occurs in the river. The EPA states that herbicide levels >1 ppm have been measured in freshwater rivers in America. However, the herbicide concentrations (<1 ppm) found in the Berg River survey, South Africa can therefore be regarded as potential safe and non-toxic to aquatic organisms when compared to other fresh water streams overseas.

Future studies should look at staining live versus dead cells, but since two separate stain pairs are used for prokaryotic and eukaryotic cells, it remains a challenge to stain a multi species biofilm. Next generation sequencing can be done, to identify the groups of functional species in the biofilm that are able to tolerate the herbicides and possibly utilise the active ingredients as a nutrient source. Microbes that are able to utilise these active ingredients will result in metabolites that will be released into the environment, therefore, methods for detection and quantifying these metabolites should be looked at. This will give an indication of the microorganisms' activity towards the breakdown of the active ingredients in freshwater rivers.

This will lead to possible microorganisms that can be used for bioremediation of herbicide spills. The results in the study yielded new questions for future studies and future monitoring of the freshwater streams in not only the Western Cape but across the globe, should be considered due to the implication it may have on the environmental processes.