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Degradation of Pesticides by the Ligninolytic Enzyme Laccase

– Optimisation of in vitro conditions, immobilisation and screening for natural mediators

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Abstract

Pesticides are widely used in many industries but the majority reaches non-target organisms or locations through point or diffuse sources. Understanding conditions for their degradation is therefore important. The degradation of glyphosate, its metabolite AMPA and isoproturon using the ligninolytic enzyme laccase was studied. Optimisation of in vitro conditions were tested with findings indicating that factors such as altering pH and the concentrations of both manganese and redox mediators can impact degradation giving insight into optimal conditions. A method of encapsulation was used showing it is possible to immobilise laccase suggesting a possibility of its suitability as a co-formulation agent in pesticide applications. The immobilised laccase was applied in a laboratory scale experiment to investigate degradation of glyphosate and AMPA in soil and sand. The findings showed an apparent ability of the encapsulated laccase to be liberated and have an effect on glyphosate degradation, although much work still remains in this area. In the final part of this project ligninolytic substrates were screened for natural and easily extractable mediators. Extracts were used to check enzymatic activity and degradation potential. Candidates that showed promising results included extracts from hemp and wheat.

***Keywords**: White rot fungi, Laccase, Bioprophylaxis, Diffuse sources, Co-formulation, Degradation, Mediators, Pesticides, Glyphosate, AMPA, Isoproturon, Enzyme encapsulation, Enzyme immobilisation, Natural mediators*

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1. Introduction

1.1 Background

In today's society many industries rely on the use of pesticides. Modern pesticides are believed to be much safer for the environment than classical products (Keum et al. 2009). Unsatisfactory management of pesticides has resulted in a high risk of contaminating the environment and precautions should be taken to limit their spread to non-target areas such as leaching to groundwater where traces of many pesticides are routinely found (Castillo et al. 2008, Önneby et al. 2009). It is estimated that as little as 5% of the pesticides used reach their target organism and that the remainder in addition to their transformation products are dispersed into the environment (Torres-Duarte et al. 2009). Developing novel solutions for this problem is therefore important.

1.2 Environmental protection from pesticide contamination

Preventative measures as part of a good management program is the first step in reducing pesticide contamination. The sources of this contamination can be broadly referred to as point sources and diffuse sources. Point sources are instances whereby a leak or spillage of a pesticide has resulted in contamination of the environment at a specific point, usually at a high concentration. Diffuse sources are losses of the pesticide into the environment after application through such routes as volatilisation, surface run-off and leaching. Bioremediation methods use microorganisms, fungi, green plants or their enzymes to remediate contaminated soils. Many of these methods have proven to be effective and simple to apply. Bacterial and fungal microorganisms are known to degrade organic pollutants metabolically and co-metabolically. Metabolic degradation being where the organism uses the contaminant as an energy source and co-metabolic degradation occurs in the presence of the organism but the contaminant is not used as an energy source (Ruis-Duenas and Martínez 2009). Bioprophylaxis is an area of contamination prevention that has been explored for both point and diffuse sources using the principles behind bioremediation. In the case of point source pollution the use of biobeds is a good example of how a system that is simple to install and maintain can significantly reduce contamination of the environment with pesticides. The biobed system was developed in Sweden as a means to curtail pollution of pesticides during application preparation and pesticide handling in an agricultural setting. The biobed is a system designed to collect, retain and degrade pesticides that have been spilled. The biobed is a 60 cm deep pit in the ground that has three components, the first is an impermeable clay layer at the bottom of the pit, the second is a biomix of substrates and the third is a grass layer on top. The substrates used in the biomix are designed to support lignin degrading fungi. A classic Swedish biobed contains a mix of straw, peat and soil.

The lignin degrading fungi produce ligninolytic enzymes to break down lignin in the straw to access their food source; these enzymes that are involved in the degradation of pesticides and perform this while the pesticide is contained in the biobed (Castillo et al. 2009, Pizzul et al. 2009). Diffuse source contamination is more complex and more difficult to control. Work in this area has been carried out and one concept being developed is co-formulation, i.e. to co-formulate the pesticide to be applied with an agent that will degrade it after it has carried out the desired task. In a paper by Önneby et al. 2009 pesticides were applied with pesticide degrading bacteria with the aim of preventing diffuse source contamination by degrading the remaining residues of pesticide after it has performed its task. The theories behind degrading pesticides with ligninolytic enzymes may also be applied in this area by investigating the use of these enzymes in degrading pesticides and their suitability in their future use in the area of bioprophylaxis.

1.3 Ligninolytic fungi: laccase and degradation

Ligninolytic fungi or lignin degrading fungi (such as white rot fungi) access their main carbon source ligninocellulose by breaking down the lignin that protects it. They do this by producing enzymes designed to co-metabolically degrade the complex recalcitrant structure of the lignin (Leonowicz et al. 2001). The enzymes are extracellular and nonspecific thus they have the ability to degrade a wide variety of organic substances that share features with lignin. Ligninolytic enzymes have many applications in many different industries from bio-bleaching of pulp in the paper industry to enzymatic modification of fibres and dye-bleaching in the textile and dye industries but most notably they are used for the bioremediation of contaminated soils and industrial wastewaters (Kotterman et al. 1998, Paszczynski and Crawford 1995, Wesenberg et al. 2003, Web Mycoenzyme 2009). Among the many other pollutants, ligninolytic enzymes are successful at breaking down the complex structures of many pesticides (Baldrian 2005, Castillo et al. 2009, Gianfreda and Rao 2004, Hofrichter 2002, Leonowicz et al 2001, Pizzul et al. 2009). These enzymes include phenol oxidases such as laccase and peroxidases such as lignin peroxidase (LiP) and manganese peroxidase (MnP); in this project the focus will be on the use of laccase. Laccase is a copper containing enzyme that has shown to be influenced by conditions such as pH and enzyme inhibiters (Stoilova et al. 2010). Laccase acts by oxidising a wide variety of aromatic and nonaromatic compounds which are used as hydrogen donors (Kunamneni et al. 2007, Stoilova et al. 2010). The usual pathway is oxidation of phenolic compounds to give phenoxy radicals and quinones. An extended range of compounds can also be oxidised in the presence of mediators. For example it has been shown that degradation of pesticides using laccase can be enhanced in the presence of redox mediators such as veratryl alcohol, ABTS and HBT (Kapich et al. 1999, Kunamneni et al. 2007). In literature the definition of a laccase mediator is a compound that strengthens a reaction because they have a lower molecular weight and a higher redox potential than laccase.

The reaction is driven with the oxidation of the mediator by laccase generating a strongly oxidised intermediate or radical which in turn oxidises the target compound (pesticide in this case) with a greater effect, this is commonly known as the laccase mediator system (LMS) (Camarero et al. 2005, Morozova et al. 2007). In this report the term mediator is used in a broader sense, in that a mediator is anything that enhances degradation of a pesticide using laccase.

1.4 Pesticides and their metabolites: glyphosate, AMPA and isoproturon

The pesticides that will be focussed on in this research project are glyphosate [N-(phosphonomethyl) glycine] and isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea]. Decomposition of a pesticide may occur photochemically, chemically or biologically. In the case of glyphosate and isoproturon the main degradation processes are carried out by microbial activity (Carlisle and Trevors 1987, Castillo et al. 2001, Torstensson 1985, Pizzul et al. 2009).

Isoproturon is a selective systemic herbicide designed to control annual grasses and weeds amongst cereal crops. It is applied directly to soil as a pre- and post-emergence control measure (Castillo et al. 2001, Von Viren-Lehr et al. 2001). It works as a photosynthetic electron transport inhibitor as such interfering with the way in which plants convert the sunlight to energy. It is commonly detected in water bodies such as groundwater, where it can be very persistent and can cause significant damage as it is very toxic to aquatic organisms (DEFRA website, Krueger 1997). This pesticide is applied directly to soil and therefore it is a good candidate for studying in this project.

Glyphosate is a non-systemic herbicide and is generally considered to have very low toxicity and low persistence in the environment (Carlisle and Trevors 1987, Torstensson 1985). Glyphosate is degraded by microorganisms using two pathways the most important of which results in the formation of the metabolite aminomethyl-phosphonic acid (AMPA) (Borggaard and Gimsing 2008, Torstensson 1985). AMPA is the main metabolite of glyphosate and will also be investigated as part of the project alongside glyphosate. AMPA is also biologically degradable but is degraded at a much slower rate than glyphosate. The half life of glyphosate can vary ranging from just hours to years. The initial degradation rate is high but this reduces considerably, this is usually attributed to its strong binding capacity to soils. Glyphosate adsorbs readily in soil explaining in part its low mobility but this adsorption is reversible. The mobility increases as pH increases and also in soils with a high phosphate level. AMPA is more mobile than glyphosate and also binds to sorption sites in soils. AMPA has a longer half life in the soil than glyphosate. It is generally agreed based on their immobility in soil that glyphosate and AMPA have little chance of leaching to groundwater in concentration that could cause problems; despite this there have been recorded cases of occurrence in groundwater (Kolpin et al. 2006, Danish EPA website).

Although it is well documented that glyphosate has a low toxicity level there are journals stating that due to the volumes used that there is a cause for concern for such reasons as adverse affects on non-target organisms, water quality and ecosystem function (Vera et al. 2010, Williams et al. 2000). Glyphosate is the most predominantly used pesticide in the world today and is used by professionals and amateurs alike for agricultural and urban use (Borggaard and Gimsing 2008). This coupled with the difficulties in degrading AMPA make it an interesting and important chemical to use in this project.

1.5 Aim, scope and hypotheses

The overall aim of this master thesis project was to study the degradation of glyphosate, its metabolite AMPA and isoproturon using the ligninolytic enzyme laccase. The study consisted of three projects; 1) optimisation of in vitro pesticide degradation by laccase, 2) laccase immobilisation and laboratory scale degradation of glyphosate and AMPA in soil and sand and 3) screening for natural mediators.

Project 1: Optimisation of pesticide degradation by laccase
The aim of the first project was to test different reaction conditions for degrading pesticides in vitro using laccase in order to identify the optimal conditions under which laccase degrades these compounds. During the experiments with glyphosate it was expected that AMPA would be produced. This factor was also considered in identifying the optimal conditions. The experiments included testing the stability of laccase under incubation conditions over time, the effects of different Mn^{2+} concentrations both on laccase and its ability to degrade pesticides and the effect of mediator concentration and buffer pH on the degradation of the compounds. This series of experiments was carried out in order to optimize the reaction conditions and to study the effect of different factors on pesticide degradation.

Project 2: Laccase immobilisation and laboratory scale degradation of glyphosate and AMPA in soil
In many studies involving in vitro studies the recorded behaviour and results attained are not always mirrored when tested in the field. Enzymes such as laccase are known to be inhibited by many factors such as certain ions (Kunamneni et al 2007). In a study by Kessler et al (2008) a bioencapsulation method for microorganisms was described. The same technique was used in the second project to encapsulate laccase in TiO_2 (titanium oxide) gel for application in degradation of glyphosate in soils at a laboratory scale.

Project 3: Screening for natural mediators
Artificial redox mediators such as ABTS are powerful reagents and can be costly. Natural mediators are often used as a more environmentally friendly method in many industries where laccase is used (Camarero et al 2007). A variety of natural substrates suitable or potentially suitable as a source of mediators were selected and screened.

The screening involved testing the substrates for laccase activity, effects on laccase activity and the degradation potential of glyphosate and AMPA formation.
The null hypothesis in all three projects is that no changes will be determined. The alternative hypothesis will be accepted in cases were changes can be identified.

2. Materials and Method

2.1 Laccase and chemicals

Laccase from *Trametes versicolor* was obtained from Sigma Aldrich (Steinhelm, Germany). The laccase was prepared into a solution using 0.5 mg/ml of water giving an approximate activity of 2 units/ml and stored at -18 ºC.

Glyphosate and AMPA were also supplied by Sigma Aldrich (Steinhelm, Germany), isoproturon by ChemService (Chester, UK). Tween 80 was supplied by Merck (Darmstadt, Germany). 3-(dimethylamino) benzoic acid (DMAB), 3-methyl-2-benzothiazolinone hydrazone (MBTH), 2,2,2-trifluoroethanol (TFE), trifluoroacetic anhydride (TFAA), 9-fluorenylmethylchloroformate (FMOC-CL) were supplied by Sigma Aldrich, D-7924 (Steinhelm, Germany). TiO2 sol ($Ti(OEt)_4$ in anhydrous EtOH and triethanolamine) was provided by Gulaim Seinseibaeva, Department of Chemistry, SLU.

2.2 Enzyme activity assay

Laccase activity in most experiments was measured by the MBTH-DMAB assay (Castillo et al., 1994). The assay is based on the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino) benzoic acid (DMAB). Laccase catalyses the formation of a deep purple compound with a peak absorbance at 590 nm in the presence of MBTH, DMAB and $MnSO_4$. A reaction mixture was prepared in a cuvette containing lactate succinate buffer 73.5 mM (pH 4.5), DMAB 0.99 mM, MBTH 0.07 mM, $MnSO_4$ 0.3 mM and 100 µl of the prepared laccase solution. The reaction was started with addition of the enzyme and absorbance at a wavelength of 590 nm was measured during 60 min at 30ºC using a Shimadzu UV 1800 spectrophotometer A polynomial regression was used to determine Δabs/min. Calculations of enzymatic activity (units/ml) were made using equation 1 where $0{,}053 \mu M^{-1}cm^{-1}$ is the extinction coefficient . One unit is defined as the amount of enzyme needed to form 1 µmole of product in 1 minute.

$$\frac{Units}{ml} = \frac{\Delta abs}{\text{minutes}} \times \frac{0.002}{0.053!} \times ml\ sample \qquad \text{Equation 1.}$$

The ABTS method was used to test for laccase activity in soil extracts, the principle of which is that laccase oxidises ABTS to a stable cation radical $ABTS^+$ that has a green colour measurable at 420 nm in the spectrophotometer at 25ºC. Cuvettes with 900 µl of peat soil extract and 100 µl of ABTS 30 mM are measured for 1 min. The Δabs/min is then calculated from the linear range of the curve and then used in equation 2 to determine the activity in U/ml. The extinction coefficient is $36000 M^{-1}.cm^{-1}$.

$$\frac{\text{Units}}{\text{ml}} = \frac{\Delta\text{abs}}{\text{minute}} * \frac{\text{Volume reaction mixture}(\mu\text{l}) * 1000}{36000 * 1 * \text{Volume enzyme solution } (\mu\text{l})} \qquad \text{Equation 2}$$

2.3 Project 1: Optimisation of pesticide degradation by laccase

2.3.1 Effect of incubation conditions over time and Mn^{2+} on laccase activity

The enzymatic activity of a laccase solution (0.5 mg/ml of water) after 72 h incubation at 35ºC and 150 rpm was measured. Samples were taken at time 0 and after 24 h, 48 h and 72 h to determine any effect over time. The samples were analysed using the MBTH-DMAB method (section 2.2.). This method was also used to measure the effect of Mn2+ replacing the $MnSO_4$ solution to a final concentration of 0.2, 0.5, 1 and 2 mM $MnSO_4$ solutions.

2.3.2 Effect of Mn^{2+} concentration, ABTS concentration and buffer pH on the degradation of glyphosate, AMPA and isoproturon

The experimental set up for these experiments was an in vitro preparation of a standard reaction mixture (1 ml) containing lactate succinate buffer, ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)), pesticide, $MnSO_4$ and laccase. All reactions had a replicate and were prepared in sterile 8 ml vials; the addition of laccase was left until the end thus starting the reaction. The vials were loosely capped and then incubated at 35 ºC on a shaking table set to 150 rpm. Samples were taken at 24 h and stored at -18 ºC until analysis.

Reaction mixtures

To test the effect of Mn^{2+} concentration replicates were made using $MnSO_4$ at concentrations of 20, 10, 5, 2 and 0 mM. Controls were prepared with no $MnSO_4$ and no laccase. The reaction mixture consisted of lactate succinate buffer 0.1 M (pH 6), ABTS 1 mM, glyphosate 10 ppm, laccase 0.15 U/ml and $MnSO_4$ at the various concentrations mentioned.

Effects of ABTS concentration were tested using the following reaction mixture lactate succinate buffer 0.1 M (pH 6), $MnSO_4$ 10 mM, glyphosate 10 ppm, laccase 0.15 U/ml and ABTS in concentrations of 1, 0.5, 0.1 and 0 mM. Controls were prepared with no ABTS and no laccase.

Effect of buffer pH was tested on glyphosate, AMPA and isoproturon using lactate succinate buffer 0.1 M at pH 4, pH 5, pH 6 and pH 7. An additional reagent was used in the reaction mixtures for the tests on glyphosate and AMPA (Tween 80).

The reaction mixture for the test on glyphosate degradation contained lactate succinate buffer 0.1 M (pH 4-pH 7), $MnSO_4$ 10 mM, glyphosate 10 ppm, ABTS 0.1 mM, Tween 80 1% w/v, laccase 0.15 U/ml. All the buffers were also used in reactions with no laccase added, which acted as the controls.

The reaction mixture for the test on AMPA degradation contained lactate succinate buffer 0.1 M (pH 4-pH 7), $MnSO_4$ 10 mM, AMPA 10 ppm , ABTS 0.1 mM, Tween 80 1% w/v and laccase 0.15 U/ml. In this case only the buffer at pH 6 was run without laccase as a control.

The reaction mixtures for the test on isoproturon degradation contained lactate succinate buffer 0.1 M (pH 4-pH 7), $MnSO_4$ 10 mM, isoproturon 5 ppm, ABTS 0.1 mM and laccase 0.15 U/ml. Controls contained no laccase and were run for all pH levels tested.

2.4 Project 2: Laccase encapsulation and degradation of glyphosate and AMPA in soil and sand

2.4.1 Laccase encapsulation

To encapsulate laccase a pure enzyme solution (10 ml, 2 U/ml) was mixed with 2.5 ml of TiO_2 sol ($Ti(OEt)_4$ in anhydrous EtOH and triethanolamine), and allowed to evaporate until the mixture was set into a gel (Kessler et al 2008, Kessler 2009). The gel was then cleaned by adding distilled water, shaking and centrifuging at 4500 rpm for 10 min. The supernatant was tested for laccase activity using the MBTH-DMAB method. This was repeated until the supernatant tested negative for laccase activity. To test laccase activity in the gel a portion was dissolved in 100 mM citrate buffer (pH 6) and the activity was checked using the MBTH-DMAB method. The gel was stored in the freezer at -18°C

2.4.2 Degradation of glyphosate using encapsulated laccase in soil

Laboratory scale assays were set up using sand and loamy soil to test glyphosate degradation using encapsulated laccase. The soils were autoclaved and weighed (20 g) into 50 ml Sarstedt tubes. Water holding capacity of the soils was determined to be approximately 100 µl/g soil and the treatments applied were based on this (approximately 2 ml/vial). The standard reaction mixture contained lactate succinate buffer 30 mM (pH 6), $MnSO_4$ 1.2 mM, glyphosate 10 ppm and ABTS 1 mM. Encapsulated laccase was dissolved in water to give a final activity of 0.23 U/ml and the laccase solution gave a final activity of 0.2 U/ml per vial in the treatments where they were used. The treatments for sand are outlined in Table 1 and for loamy soil in Table 2 (The experiment was run in duplicates).

Table 1. Treatment procedure for degradation of glyphosate using encapsulated laccase in sand. The reaction mixtures included $MnSO_4$ (1.2 mM), glyphosate (10 ppm) and ABTS (1 mM) in all vials. The reaction mixture contained either lactate succinate buffer or deionised water. *Laccase was applied before the reaction mixture. §Laccase was mixed with the reaction mixture immediately before application

	Encapsulated Laccase (0.23 U/ml)	Laccase solution (0.2 U/ml)	Lactate succinate buffer (30 mM (pH 6))	Deionised water
EcB*	+	-	+	-
EcW1*	+	-	-	+
EcW2§	+	-	-	+
EsB*	-	+	+	-
EsW*	-	+	-	+
Control	-	-	-	+

Table 2. Treatment procedure for degradation of glyphosate using encapsulated laccase in loamy soil. The full reaction mixture included lactate succinate buffer (30 mM (pH 6)), $MnSO_4$ (1.2 mM), glyphosate (10 ppm) and ABTS (1 mM). The stage at which glyphosate was applied was important.

	Encapsulated Laccase (0.23 U/ml)	Full reaction mixture	Glyphosate application
Ec1	+	+	Before incubation
Ec2	+	+	After incubation
control	-	+	Before incubation

The vials were then incubated at 30ºC for 48 h (sand) and 24 h (soil) before extraction.Glyphosate was extracted with 30 ml of 0.1 M NaOH. The tubes were placed on a shaker for 1½ h, centrifuged (15 min, 4000 rpm) and 6 ml of the supernatant transferred to 15 ml Sarstedt tubes. To precipitate any organic material the extract was adjusted to pH 2 with HCL 6 M and allowed to stand for 30 min. The samples were then centrifuged for 5 min at 3000 rpm. The supernatant was then analysed.

2.5 Project 3: Screening for natural mediators

2.5.1 Substrate selection and incubation

The ligninolytic materials chosen to screen for natural mediators were five types of peat residue (peat residue, H5-H6 grade peat, H7-H8 grade peat, commercial peat and H3 lower grade peat), three types of agricultural residue (oats, wheat and hemp), two types of animal bedding (straw and saw shavings) and a biomix from a biobed (wheat straw, peat and soil 50:25:25 % v). The materials were ground up in a pestle and mortar. The substrates were then placed in foil tins, kept moist, loosely covered with a clear film and incubated at room temperature. The agricultural residue and bedding was incubated for 90 days while the peat residues and biomix was incubated for 67 days. Dry weights and pH measurements were taken before incubation (time zero, t=0) and at the time of extraction (t=extraction).

2.5.2 Laccase activity in the ligninolytic materials

The laccase activity in ligninolytic materials can be measured using a number of methods. Two of these methods, the MBTH-DMAB method and the ABTS method (section 2.2), were tested using peat soils. Extractions were made by weighing the peat soil (5 g) out into 50 ml Sarstedt tubes. When preparing samples for the MBTH-DMAB method the extraction buffer used was lactate succinate 0.1 M (pH 6). For the ABTS method phosphate citrate buffer 100 mM (pH 4.5) was used. The appropriate buffer (20 ml) was then added to each tube. The samples were then vigorously shaken for 1 hr and centrifuged for 15 min at 4000 rpm. The supernatant (approximately 5 ml) was then filtered through a 0.45 µm filter ready to be analysed. The extract was then used in place of the buffer and the laccase in the cuvette mixture then measured in the spectrophotometer as normal. In both methods samples were also run with the addition of H_2O_2 to determine how much MnP was present in the extracts (MnP is usually H_2O_2 dependant).

The MBTH-DMAB method was used for testing the activity of the substrates chosen for screening. This was carried out before and after the incubation period. Extraction and analysis of the substrates were carried out accordingly. The extracts were then screened for any effect on laccase activity using the MBTH-DMAB method (adding laccase solution). The reaction mixture was prepared in a cuvette containing lactate succinate buffer 73.5 mM (pH 4.5), DMAB 0.99 mM, MBTH 0.07 mM, $MnSO_4$ 0.3 mM and 100 µl of the prepared laccase solution. Controls were run with and without the addition of $MnSO_4$. For the screening protocol a portion of each extract (100 µl) was also added to the reaction mixture, these were run with and without $MnSO_4$.

2.5.3 Degradation of glyphosate in the presence of substrate extracts

The extractions from the incubations tested for laccase activity in section 2.5.2 were used the following day for this experiment; they were stored in the fridge overnight. Vials were prepared using the same techniques used in section 2.3.2. The standard reaction mixture contained ABTS 0.1 mM, glyphosate 10 ppm, $MnSO_4$ 10 mM and laccase solution 0.15 U/ml. The treatments were run with and without laccase and the substrate extracts were used instead of a buffer; duplicates were run for each substrate. The controls were run with and without laccase and lactate succinate buffer 0.1 M (pH 6) was used instead of extract. The vials were incubated at 35°C and 150 rpm. Samples were taken at time 24 h into 10 mM ascorbic acid in methanol and analysed.

2.6 Pesticide analysis

2.6.1 Gas Chromatograph Mass Spectrophotometer Analysis (GC-MS)

The samples from all the experiments using glyphosate and AMPA in this project were analysed by GC-MS (with the exception of the sand study). A small amount of each sample (100 µl from in vitro experiments and 500 µl from the loamy study) was mixed with 100 µl of internal standard (containing 0.1 µg of both glyphosate and AMPA in 100 µl deionised water) in an 8 ml glass test tube. These were then allowed to evaporate fully at 50°C under gentle air. Derivitisation of the samples was carried out by adding 1 ml of TFE (2,2,2-trifluoroethanol) followed by 2 ml TFAA (trifluoroacetic anhydride) and incubating at 100°C with sealed lids on for 1 h. The samples were then evaporated completely and 1 ml ethylacetate was added. These were then sonicated for 1 min and transferred to HPLC vials and loaded on the machine for analysis. An Agilents Gas Chromatography system HP6890 with Mass Selective Detector 5973 was used using the Gly-Einy method. If the retention time, qualifying ions (molecular weights) and response (area on the graph giving the abundance corresponding to the concentration) values were in the correct range and the Q value (accuracy estimation from the machine in percentage) was high then the result was accepted and processed.

2.6.2 High-Performance Liquid Chromatography Analysis (HPLC): Glyphosate and AMPA

The samples from the sand study (in section 2.4.2) were analysed by HPLC. To prepare for glyphosate and AMPA analysis in the HPLC the pH was adjusted to between 7 and 9 in the samples. Derivitisation was then performed using FMOC-CL [9-fluorenylmethylchloroformate], a common method used for this purpose (Hanke et al. 2008). This was done by taking 1 ml of sample and first adding 0.3 ml borate buffer 0.125 M (pH 9) and 0.7 ml deionised water. The FMOC-CL solution in acetonitrile 0.5 mM (2 ml) was then added. The samples were mixed in 15 ml Sarstedt tubes and stood for 1 h before being washed three times in ethylacetate and transferred to HPLC vials and analysed using an Agilent HPLC 1100. External standards were used for quantification.

2.6.3 HPLC Analysis: Isoproturon

A portion of the samples (500 µl) from the isoproturon experiment (section 2.3.2.) were analysed using the same Agilent HPLC 1100 series as used before. The samples were transferred directly to HPLC vials and injected into the system, analysed and results obtained. Quantification was done using external standards.

3. Results

3.1 Project 1: Optimisation for enhancement of pesticide degradation by laccase

3.1.1 Laccase activity over time under incubation conditions

It is known that the enzymatic activity of laccase remains stable at -18˚C thus this is how they were stored during the course of this project. When laccase was used for the experiments the method often includes an incubation period. This experiment was designed to investigate the changes in activity of laccase during a 72 h incubation period at 35˚C. As seen in Figure 1 the laccase activity reduced over time.

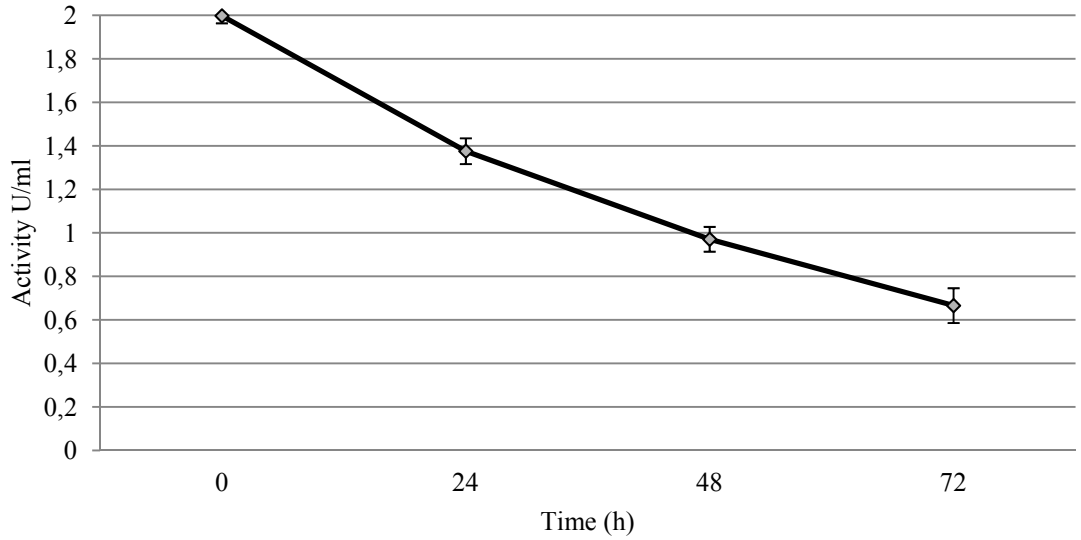

Figure 1. Laccase activity in units/ml over time (hours), values are means ±SD (n=2)

3.1.2 Effect of Mn concentration on laccase activity and on degradation of glyphosate by laccase

The effect of different concentrations of $MnSO_4$ on the activity of laccase was investigated. The concentrations of $MnSO_4$ tested had no effect on the activity the enzyme (Figure 2).

Although no effect was evident in the effect of manganese on the activity of laccase it was of interest to ascertain if $MnSO_4$ concentration had any effect on degradation of glyphosate and AMPA. The 24 h samples from the in vitro test were analysed (in all experiments the results given are for the 24 h samples unless stated to the contrary). It can be seen in Figure 3 that all glyphosate was degraded where $MnSO_4$ was added except at concentration 0.2 mM where a small portion of glyphosate remained. A small amount of glyphosate was detected in the laccase control (where no $MnSO_4$ was added) and that a large amount of glyphosate remained in the control where no laccase or $MnSO_4$ was added. In the results for this report it is assumed that all degraded glyphosate is converted to AMPA.
This assumption is based on experiments where after 4 hours there was a stoichiometric conversion from glyphosate to AMPA (Pizzul 2010, personal communication).

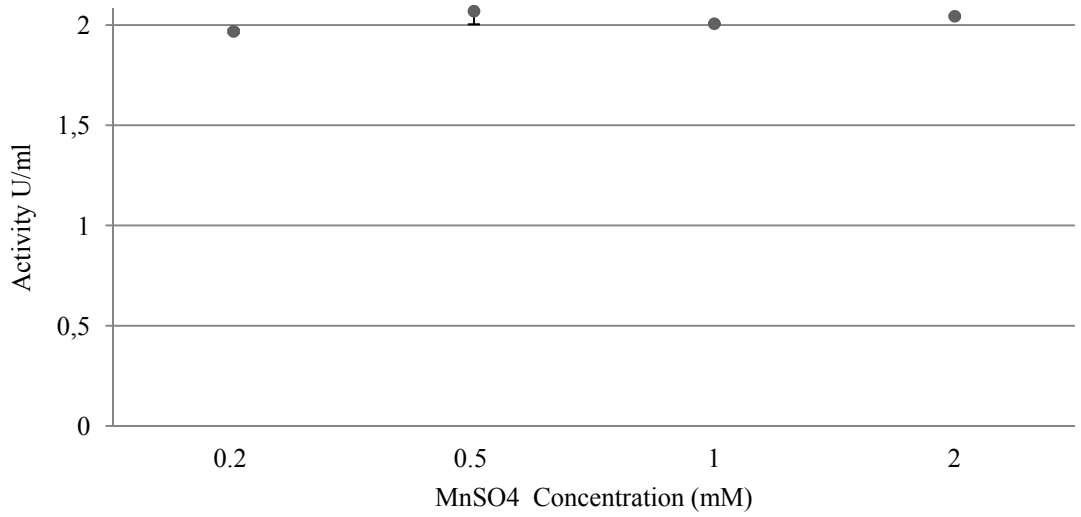

Figure 2. Effect on MnSO4 concentration on laccase activity, values are means ±SD (n=2)

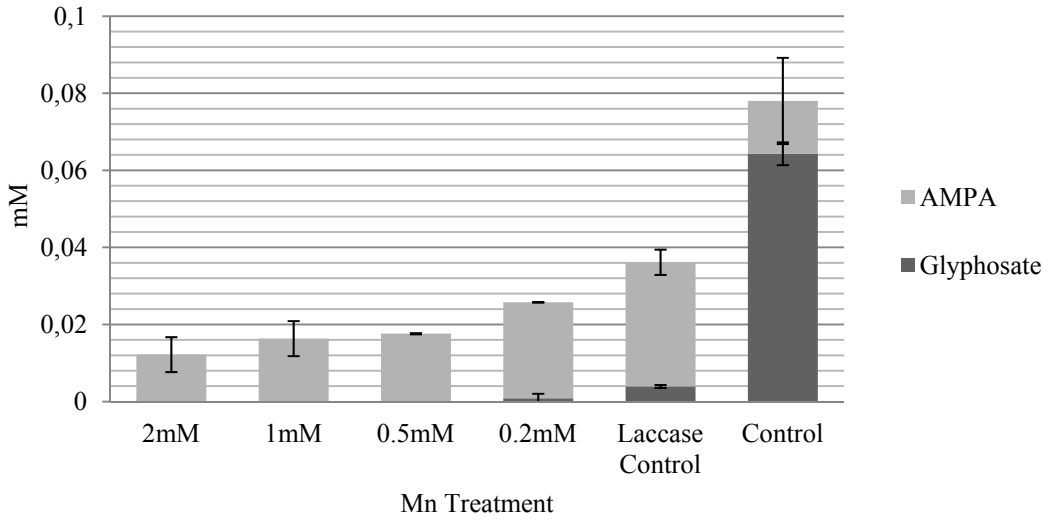

Figure 3. Effect on concentration of glyphosate and AMPA after 24 h incubation at 35ºC in the presence of laccase at different Mn^{2+} concentrations. The reaction mixture contained lactate succinate buffer, $MnSO_4$, ABTS, glyphosate and laccase. The laccase control included laccase but no $MnSO_4$ and the control contain neither laccase nor $MnSO_4$. Values are means ±SD (n=2).

It can be seen that the amount of AMPA detected was similar between all the concentrations of $MnSO_4$ with the lowest amounts being detected at the higher concentrations of $MnSO_4$. AMPA was also detected in the control, this was not expected and a high standard deviation was noted. In fact for all the results in this report (with the exception of those measured in the HPLC and reported in project 3) there was a background level of AMPA in the GC-MS, this probably accounts for the readings for AMPA in the controls, on saying this the same pattern will be seen just with an overestimated value for AMPA.

The concentration of $MnSO_4$ that was deemed optimal at this stage was 1 mM and as a result it was the concentration used in proceeding experiments.

3.1.3 Effect of ABTS concentration on the degradation of glyphosate by laccase

The results in Figure 4 show that where ABTS was used all the glyphosate disappeared. Glyphosate was reduced in the treatment with no ABTS but not to the same degree. The most AMPA was detected where the lowest concentration of ABTS were used and the least AMPA was detected in the highest concentration of ABTS suggesting that AMPA was degraded at the higher concentrations of ABTS. It appears the concentration of 1 mM ABTS has the optimal result in this case, however, the concentration used in the proceeding experiments was 0.1 mM ABTS. The reason for this will be outlined in the discussion.

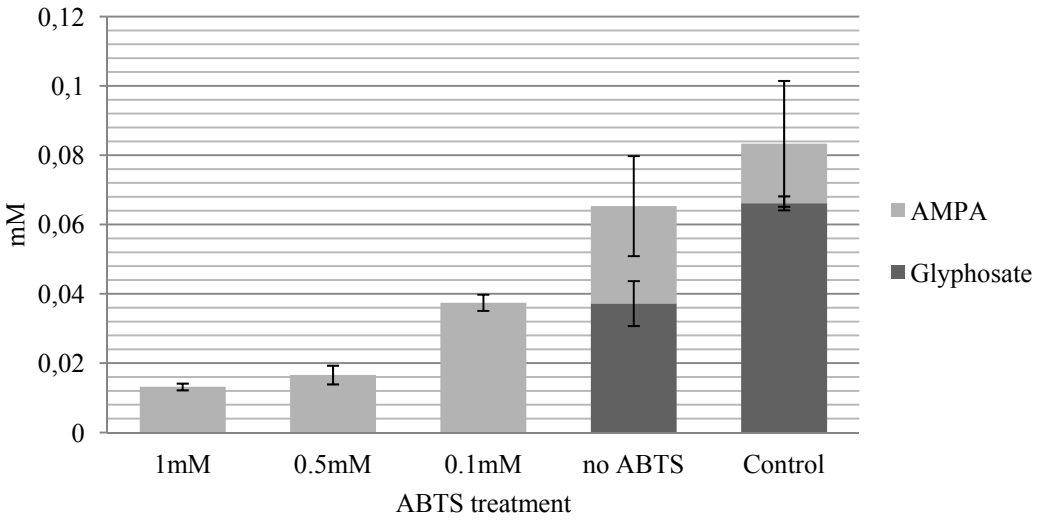

Figure 4. Effect on concentration of glyphosate and AMPA after 24 h incubation at 35ºC in the presence of laccase at different ABTS concentrations. The reaction mixture contained lactate succinate buffer, $MnSO_4$, ABTS, glyphosate and laccase. The laccase control contained laccase but no ABTS and the control contains no laccase. Values are means ±SD (n=2)

3.1.4 Effect of buffer pH on degradation of glyphosate, AMPA and isoproturon

The glyphosate was reduced to trace levels at pH 7, pH 6 and pH 5 in the treatments using laccase (Figure 5). The reduction of glyphosate was poor at pH 4. At pH 7 the least amount of AMPA was detected followed by pH 6 with the most being detected at pH 5. This suggests that AMPA degradation was occurring at the higher pH levels.

In the experiment using AMPA as the test substance (Figure 6) no reduction was seen in the treatments using buffer at pH 4 or pH 5. A reduction is seen at pH 6 and an even further reduction at pH 7 although the standard deviation was visibly higher. A similar pattern can be seen in Figure 7 where isoproturon was tested. There was complete degradation at pH 7 and only trace amounts remained after treatment using pH 6. The lowest reduction in isoproturon was noted at pH 5 and pH 4 (pH 4 showing the least reduction). The pH chosen for buffers used in proceeding experiments was pH 6.

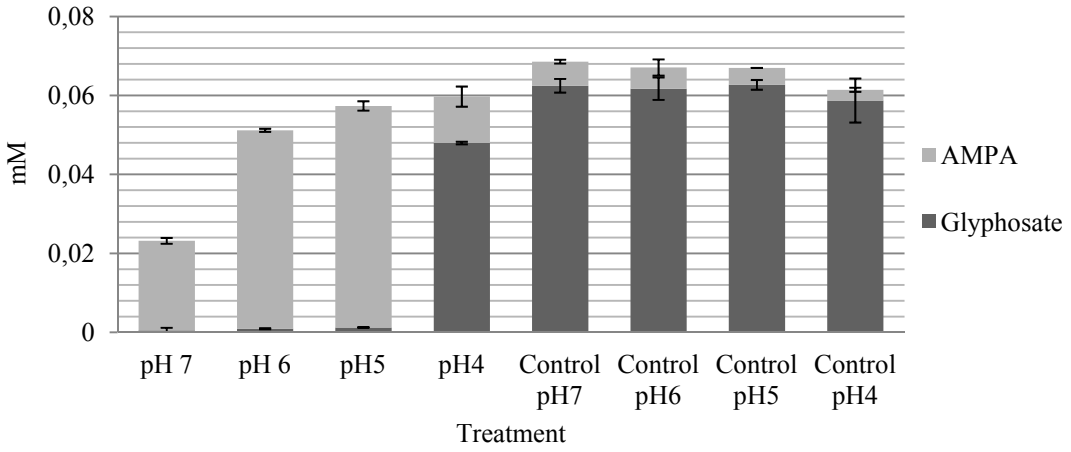

Figure 5. Effect on concentration of glyphosate and AMPA after 24 h incubation at 35ºC in the presence of laccase using different pH buffer. The reaction mixture contained lactate succinate buffer with different pH, $MnSO_4$, ABTS, glyphosate and laccase. The controls contain no laccase. Values are means ±SD (n=2)

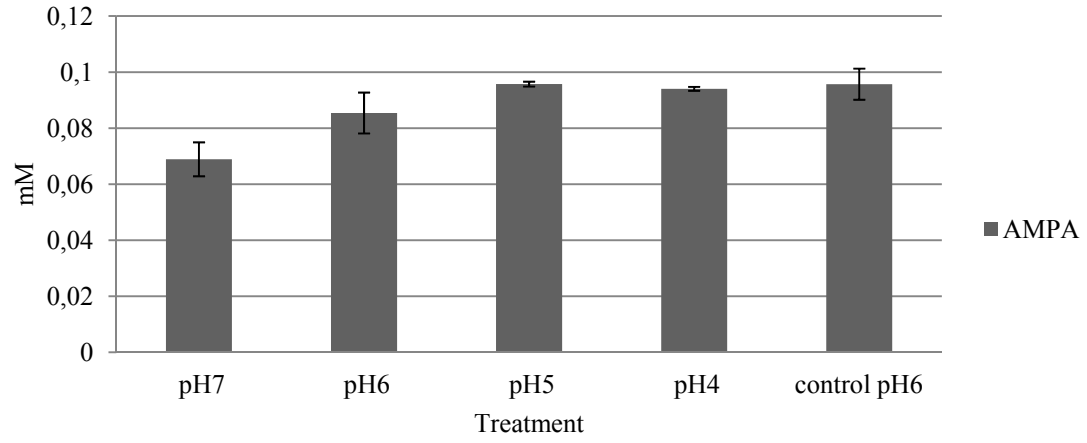

Figure 6. Effect on concentration of AMPA after 24 h incubation at 35ºC in the presence of laccase using different pH buffer. The reaction mixture contained lactate succinate buffer with different pH, $MnSO_4$, ABTS, AMPA and laccase. The control is in pH 6 buffer and contains no laccase. Values are means ±SD (n=2)

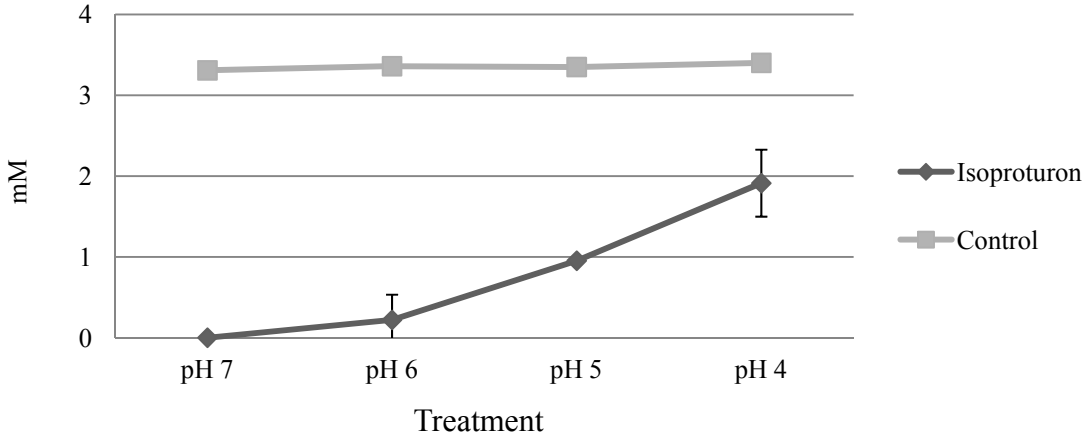

Figure 7. Effect on concentration of isoproturon after 24 h incubation at 35ºC in the presence of laccase using different pH buffer. The reaction mixture contained lactate succinate buffer with different pH, $MnSO_4$, ABTS, isoproturon and laccase. The controls contain no laccase. Values are means ±SD (n=2)

3.2 Project 2: Laccase encapsulation and degradation of glyphosate in soil and sand

3.2.1 Laccase immobilisation

The immobilisation of laccase using titanium oxide gel was successful in that it was possible to encapsulate laccase in the gel and then reactivate it. When the gel was washed there was enzyme activity measured in the supernatant (waste water) and therefore not all the laccase that was added to the gel was immobilised (the gel was washed until no activity was detected in the supernatant). The activity of the gel (dissolved) was measured (n=12) and the mean was calculated to be 20.5±5.05 SD units/gram (U/g) of gel.

3.2.2 Glyphosate degradation in sand and soil using encapsulated laccase

The effect of encapsulated laccase on glyphosate degradation was tested in a laboratory scale experiment using sand. The results are shown in Figure 8. The treatment WR acted as the control where no laccase was added and the result was supposed to indicate the original concentration of glyphosate added to the sand as no degradation was expected. The original concentration added was 0.5 ppm/g sand which converts to 2.96 μmoles/kg sand. The result in WR shows that not all the added glyphosate was detected; this is probably due to losses during extraction and derivatisation. As all samples were equally treated it is assumed that the results are comparable and WR will be referred back to as the 'original concentration'. When encapsulated laccase was added followed by the reaction mixture in buffer (EcB in Figure 8), there was a reduction in the amount of glyphosate detected, compared to WR. Similar values for glyphosate were recorded when encapsulated laccase was added followed by the reaction mixture in water (EcW1) or mixed with the reaction mixture in water (EcW2). In the treatments where pure laccase solution was added (EsB and EsW) the lowest amounts of glyphosate were detected. The amount of AMPA was highest in the treatments with encapsulated laccase (EcB, EcW1 and EcW2) compared to pure laccase (EsB and EsW). AMPA was also detected in the control (WR) suggesting degradation was occurring in the presence of the reaction mixture as no background level of AMPA was believed to be present in the HPLC.

The effect of encapsulated laccase on glyphosate degradation was also tested in a similar experiment using a loamy soil. Laccase in solution was not used as it is known inactivation occurs when pure solution is added to soil (Müller, 2009). The amounts of glyphosate and AMPA detected in the loamy soil after the addition of encapsulated laccase are depicted in Figure 9. A reduction in glyphosate content was found in the soil with encapsulated laccase (Ec1) compared to the control. The largest amount of glyphosate was detected in the treatment where glyphosate was added after the incubation period (Ec2). This treatment was included in order to detect possible glyphosate degradation during extraction where the conditions could favour laccase release from the gel. The control contained no laccase and the results showed a similar amount of glyphosate to that detected in Ec2 therefore suggesting little or no degradation during extraction

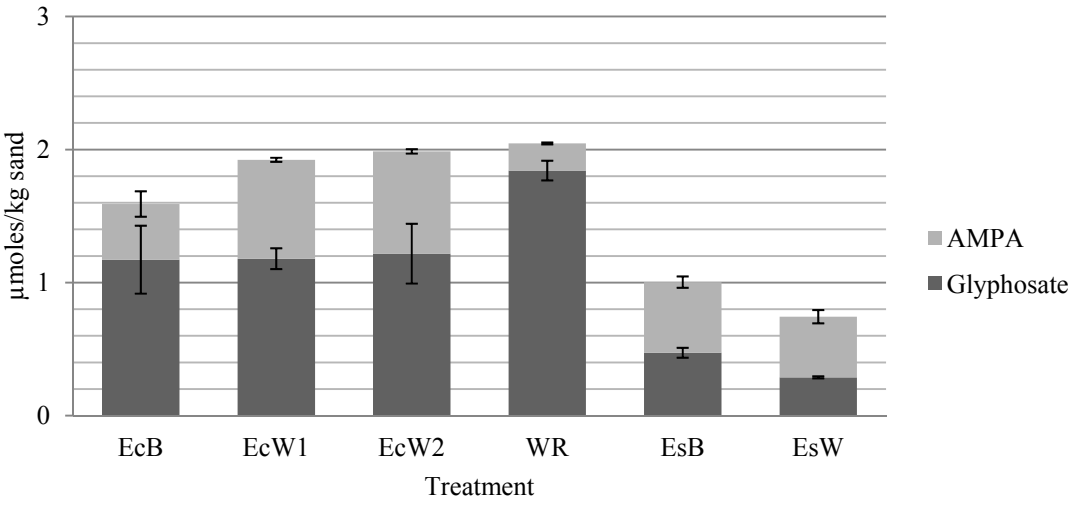

Figure 8. Effect on concentration of glyphosate and AMPA after 48 h incubation at 30ºC in the presence of encapsulated laccase in a laboratory based sand experiment. The reaction mixture contained lactate succinate buffer (B) or deionised water (W), $MnSO_4$, ABTS, glyphosate and encapsulated laccase (Ec) or pure laccase solution (Es). Laccase (Ec) was applied before the reaction mixture in EcW1 whereas it was mixed with the reaction mixture before application in EcW2. No laccase was added to the control (WR). Values are means ±SD (n=2).

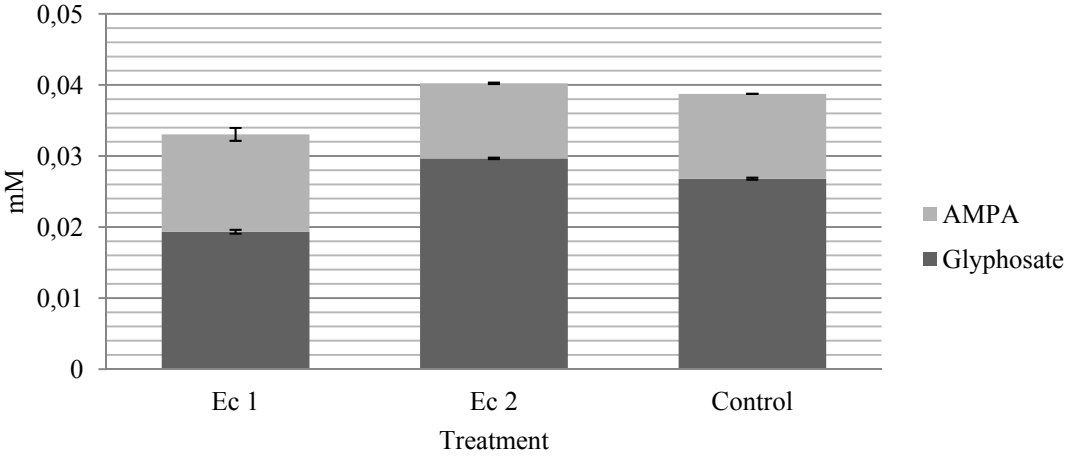

Figure 9. Effect of concentration of Glyphosate and AMPA after 24 h incubation at 35ºC in the presence of encapsulated laccase in a laboratory based soil experiment. The reaction mixture contained lactate succinate buffer, $MnSO_4$, ABTS, glyphosate and encapsulated laccase. Treatments were run with encapsulated laccase and glyphosate was added either before or after incubation. No laccase was added to the control. Values are means ±SD (n=2).

It was puzzling therefore that there was so much AMPA measured in the last two treatments. To identify the source of this AMPA another experiment was run verify where the AMPA was coming from, the results of which showed the AMPA was in the soil used. This accounts for the elevated levels of AMPA in the samples. In the samples where glyphosate is degraded there is more AMPA detected. As not all the glyphosate was degraded it is safe to say no AMPA was degraded. As for the encapsulated laccase, there does seem to be an effect with its use in the degradation of glyphosate in soils at a laboratory scale.

3.3 Project 3: Screening for natural mediators

3.3.1 Substrate characteristics

The pH of the ligninolytic materials was measured before incubation (t=0) and at the time of extraction (t=extraction) and the results are outlined in Figure 10. The substrates ranged from approximately pH 4 to pH 7. The commercial peat and Hemp 2 showed a reduction in pH after the incubation period while Straw, Wheat, Saw shavings and Hemp 1 showed an increase in pH. The remaining substrates showed little or no difference between the pH recorded before and after incubation. The incubations for Hemp 1 and Hemp 2 were originally planned to be duplicates but after the incubation they appeared very different from one another (one looked dark and the other bleached) and due to the extreme difference in pH they were treated separately.

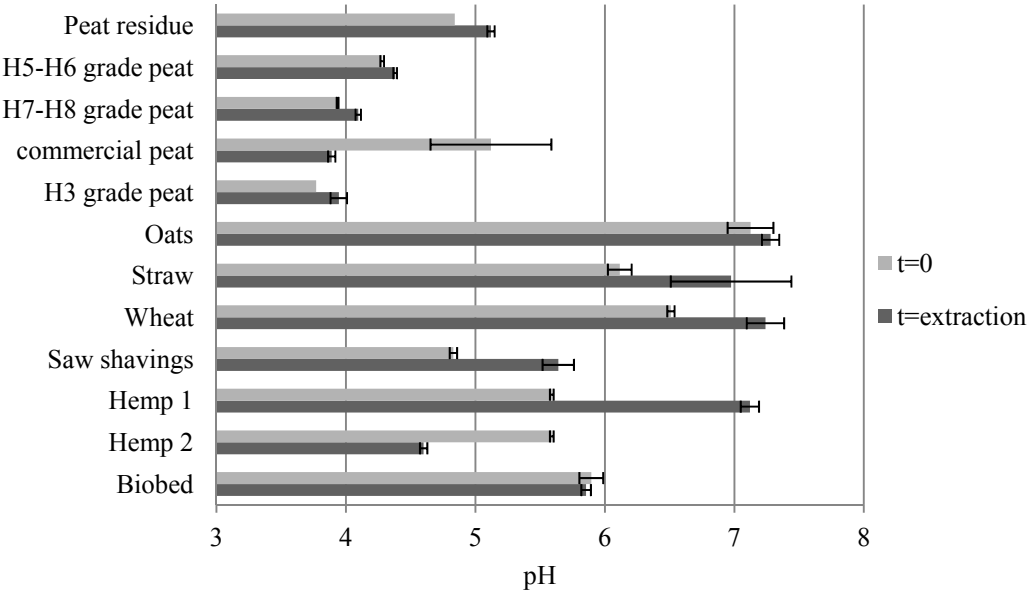

Figure 10. Substrates pH value before and after incubation (t=0 and t= Extraction). Values are means ±SD (n=2).

3.3.2 Laccase activity in soil and substrate extractions

Two different methods to measure enzymatic activity in soils were tested. In the first experiment the ABTS method was used. Five different peat soils were used but only one, commercial peat, registered activity, 0.6±0.06 mU/g soil. In the second experiment the ABTS method was compared the MBTH-DMAB method in two peat soils. The MBTH-DMAB method was run with and without H_2O_2, which is used to determine total phenoloxidase activity. Activity was measured using the MBTH-DMAB method whereas no activity was detected with the ABTS method. The activity was higher in the H5-H6 graded peat compared to the commercial peat (Figure 11). The samples containing H_2O_2 had much higher activity than without. The MBTH-DMAB method was chosen for the screening of natural mediators.

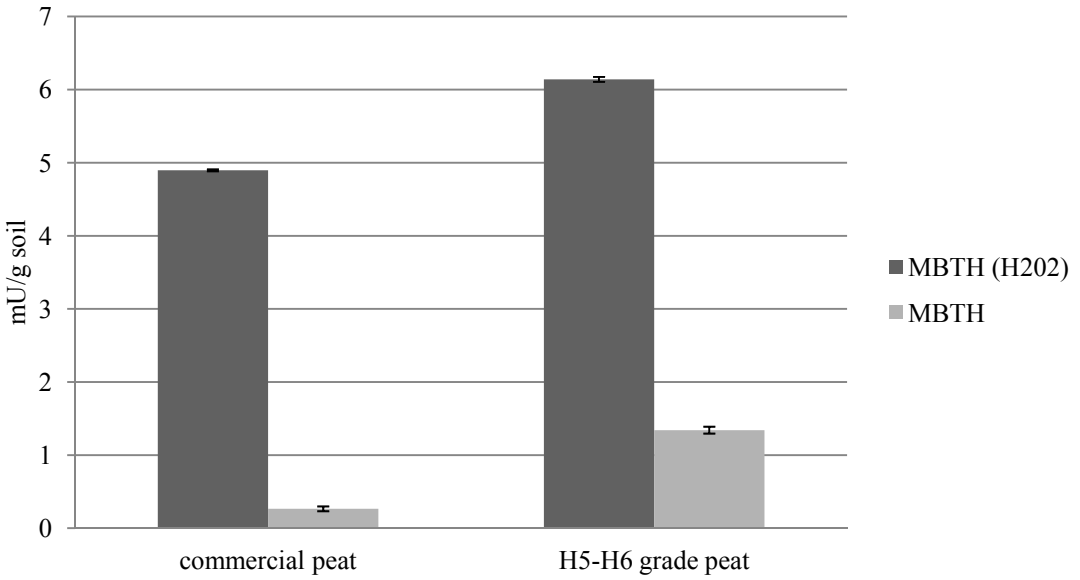

Figure 11. Laccase activity in peat soils using the MBTH-DMAB method both with and without H_2O_2. Values are means ±SD (n=2).

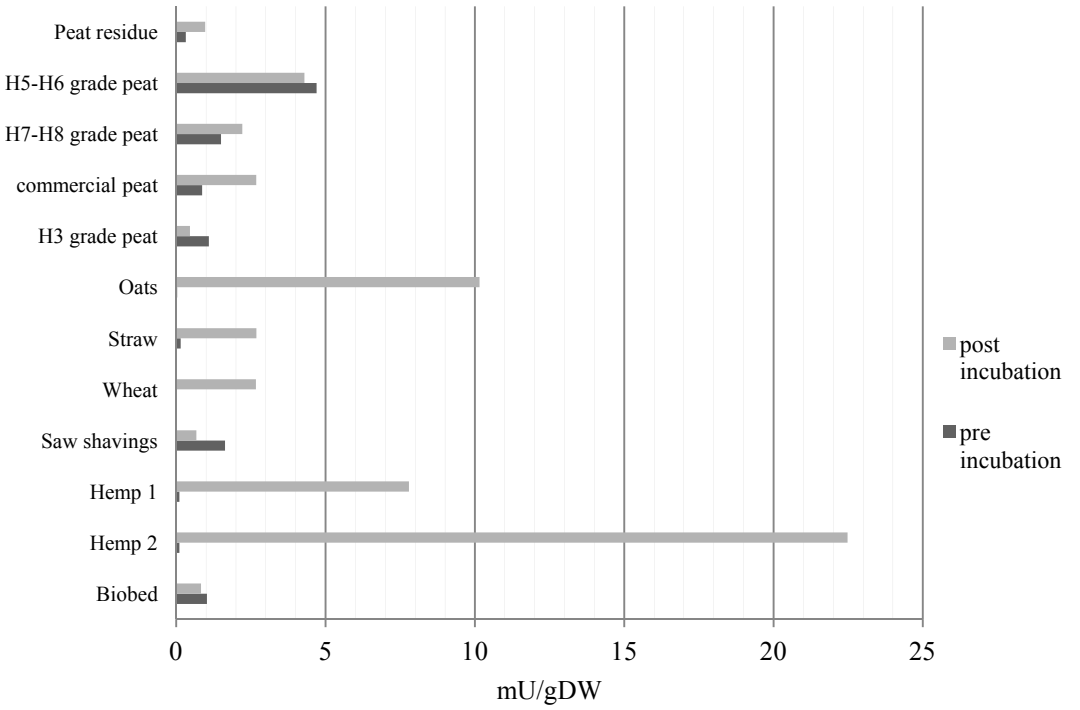

Figure 12. Activity in the substrate extracts pre and post incubation expressed in mU/gDW

Using the MBTH-DMAB method the laccase activity in the ligninolytic substrates was measured pre- and post-incubation (Figure 12). A minimal decrease was noted for H5-H6 and H3 grade peat, Saw shavings and Biobed post-incubation. The remainder of the substrates increased in activity after the incubation period, the most dramatic increases are visible for Oats, Hemp 1 and Hemp 2. In results where MnP was measured the highest recordings of activity were Hemp 2 followed by Oats. There was no activity recorded in Hemp 1. The remaining substrates had a similar pattern (Bergander 2010, personal communication)

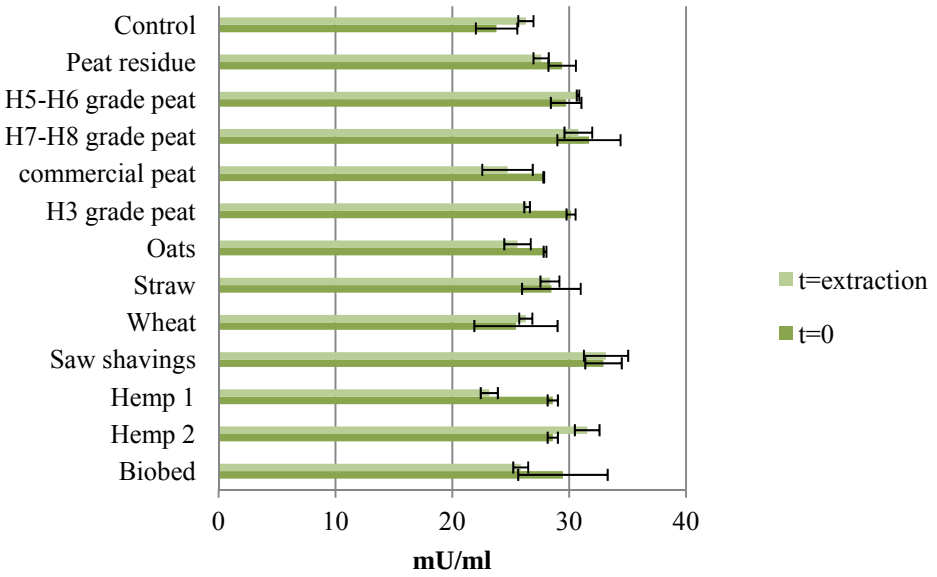

Figure 13a. Effect of substrate extracts pre (t=0) and after incubation (t= extraction) on laccase activity. Activity was measured by MBTH-DMAB method and is expressed in mU/ml. Values are means ±SD (n=2).

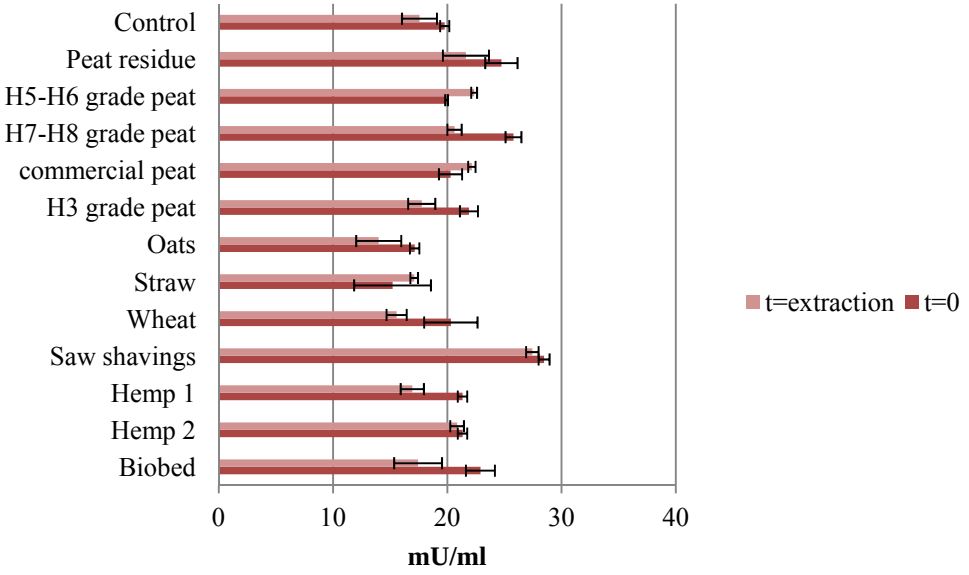

Figure 13b. Effect of substrate extracts pre (t=0) and after incubation (t= extraction) on laccase activity. Activity was measured by MBTH-DMAB method and is expressed in mU/ml. Values are means ±SD (n=2). No manganese was used in these samples. Values are means ±SD (n=2).

3.3.3. Screening for natural mediators in the ligninolytic materials: laccase activity

The extracts from the ligninolytic materials were tested on a laccase reaction in order to see if there was any enhancement in activity. The samples were run with (Figure 13a) and without $MnSO_4$ (Figure 13b) in the reaction mixture and the controls contained no extract.

The inclusion of $MnSO_4$ resulted in a higher activity and there appeared to be a positive effect on the activity with the use of the extracts (Figure 13a).

The H7-H8 graded peat and the saw shavings were the substrates that showed the greatest difference when compared to the control. When comparing the results for the extracts without $MnSO_4$ (figure 13b) the extracts for oats, straw and wheat showed a negative result, this may indicate a deficit in manganese in these substrates. In most cases there appeared to be very little difference when comparing the substrate extracts measured before and after incubation for each substance. The substrates that showed the greatest difference between the activity measured before and after incubation were H3 grade peat and hemp 1 (figure 13a). When $MnSO_4$ was not used (figure 13b) it was also found that hemp 1 showed the greatest difference in activity.

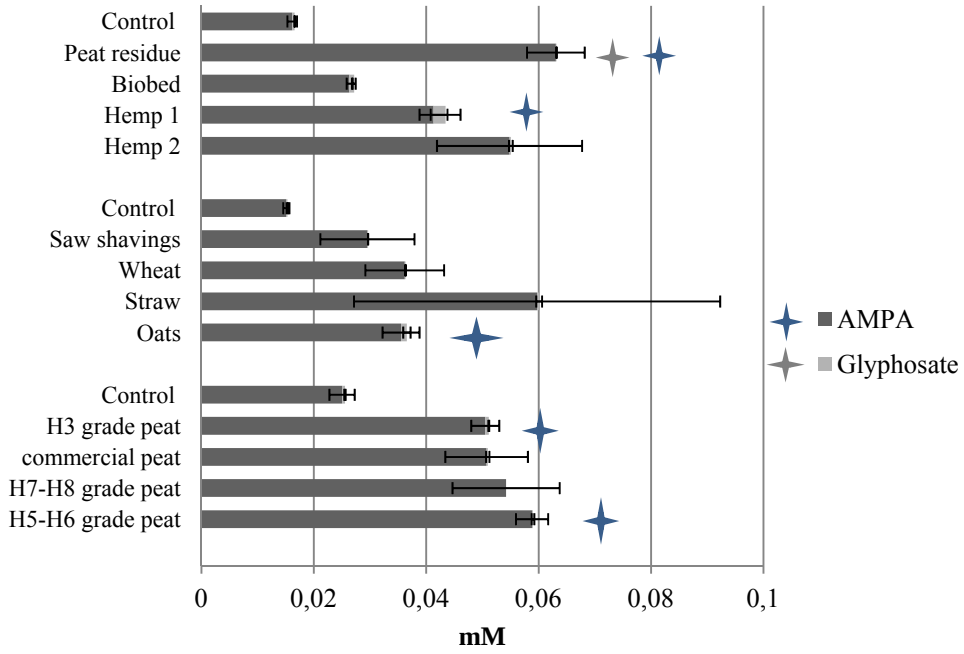

Figure 14. Effect on concentration of glyphosate and AMPA after 24 h incubation at 35ºC in the presence of laccase using different extracts from ligninolytic substrates to screen for natural mediators. The reaction mixture contained ligninolytic substrate extracts, $MnSO_4$, ABTS, glyphosate and laccase. The controls contain lactate succinate buffer and laccase. Values are means ±SD (n=2). Results that were significantly different from the control are marked with a colour coded star.

3.3.4. Degradation of glyphosate in the presence of ligninolytic substrate extracts

The effect of the extracts on glyphosate degradation by laccase was tested in *in vitro* experiments. For ease of display the result for the samples run with laccase solution are shown separately to the controls without laccase solution, Figures 14 and 15 respectively. The results for these are divided into three parts as they were carried out in three stages, each stage had its own control and the results of the screening are compared with their corresponding control, where the extract was replaced by buffer. It can be seen that no remaining glyphosate was detected in most cases when laccase was added, in the cases where it was detected the amount was minimal and the standard deviation was high (Figure 14). In all cases the AMPA detected appeared to exceed the amount detected in the control. Of these, peat residue, Hemp 1, Oats, H3 and H5-H6 graded peat were found to be significantly different ($p=0.05$) from the control when t-tests were performed. The substrate that showed the lowest amount was Biobed. The standard deviation was quite high in many of the samples but especially the straw.

The results for the samples tested without the addition of laccase solution are shown in Figure 15. It was not expected that AMPA would be produced in the controls as no laccase or extract was used. The results for H5-H6, Hemp 1 and hemp 2 appeared to show a reduction in the amount of glyphosate in comparison to the control. When t-tests were performed it was found that only Hemp 2 showed a significant difference (p=0.05). The amounts in oats and wheat are also lower but the standard deviation is high. The highest readings for AMPA were found in Hemp 1, Hemp 2 and commercial peat, the standard deviation in the commercial peat samples is very high.

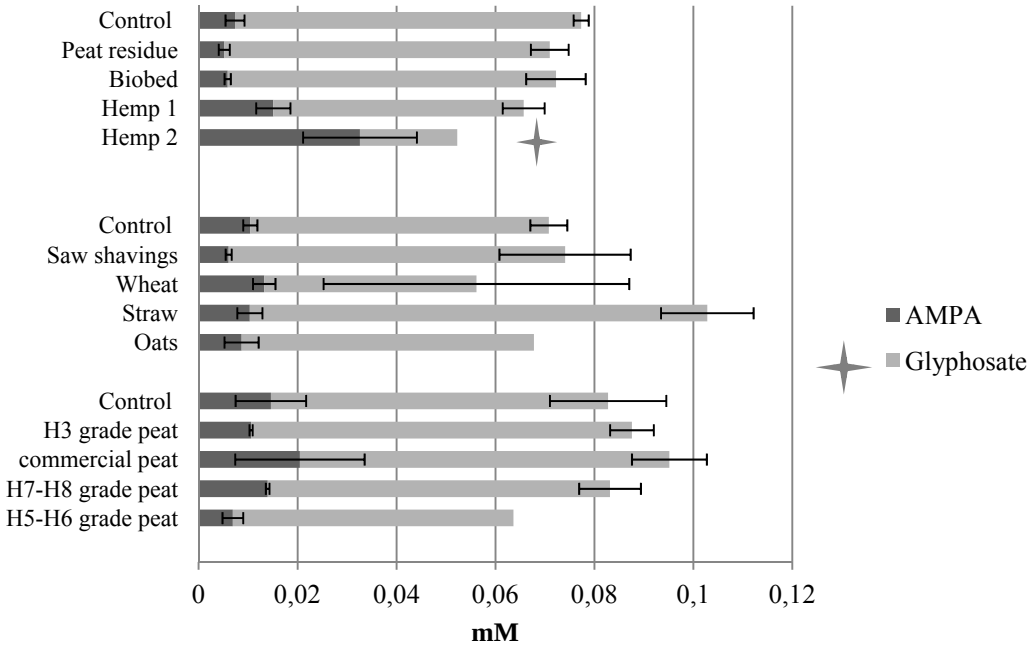

Figure 15. Effect on concentration of glyphosate and AMPA after 24 h incubation at 35ºC in the presence different extracts from ligninolytic substrates to screen for natural mediators. The reaction mixture contained ligninolytic substrate extracts, $MnSO_4$, ABTS and glyphosate. The controls contain lactate succinate buffer. Values are means ±SD (n=2). Results that were significantly different from the control are marked with a star.

4. Discussion

Ligninolytic fungi and their enzymes have been documented to degrade many xenobiotics including pesticides. In a paper by Castillo et al. (2001) it was found that isoproturon was degraded using the white rot fungi *Phanerochaete chrysosporium* and particular attention was paid to the role of manganese peroxidase and lignin peroxidase. It was found in this report that laccase can also degrade isoproturon in the presence of $MnSO_4$ and ABTS. The effect on degradation of glyphosate by these enzymes was investigated by Pizzul et al. (2009) where under in vitro conditions laccase was shown to degrade glyphosate to AMPA in the presence of $MnSO_4$, ABTS and Tween 80. The results in this report have show that degradation of glyphosate and possibly AMPA can be enhanced under certain conditions. An overall observation in all the experiments involving glyphosate degradation was that the controls showed AMPA production; there was a high standard deviation in most of these cases. It was not expected that AMPA would be produced in the controls. The assumption was that a background level in the GC-MS was registering and that no AMPA should have been produced (this does not explain the HPLC results for the sand experiment). This made interpreting results

for AMPA degradation problematic. As mentioned in the results it is assumed that all degraded glyphosate is converted to AMPA based on experiments by Pizzul (2010) and that no other metabolic pathway is in play. When considering these assumptions and in instances where glyphosate was reduced to trace and there are varying amounts of AMPA detected it can be assumed that AMPA was degraded in these samples. It could be better to rely on experiments using a pure AMPA solution rather that from transformations from glyphosate experiments, but it is also important to consider if the presence of glyphosate is affecting the degradation of AMPA in some way.

The findings in project 1 showed in a number of ways that the degradation of pesticides by laccase under in vitro conditions can be enhanced. Glyphosate can be degraded directly by manganese in the form of manganese oxide in an abiotic reaction. Manganese can also aid degradation of glyphosate by acting as a mediator, laccase is known to oxidise Mn2+ to Mn3+ which in turn acts upon glyphosate (Barrett and McBride 2005, Höfer and Schlosser 1999, Nowack and Stone 2003). For this reason it is important to include Mn in the optimisation process. It was found that glyphosate degradation using laccase was enhanced in reaction mixtures that contained $MnSO_4$ as not all glyphosate was degraded in the absence of $MnSO_4$ whereas it was in the treatments where different concentrations of MnSO4 were added, The presence of manganese also appeared to have an effect on AMPA, the amount detected varied between the concentrations with the least amount of AMPA detected at the highest concentration of $MnSO_4$. Considering that all treatments containing laccase and $MnSO_4$ had little or no residues of glyphosate remaining it was assumed that AMPA was being degraded where $MnSO_4$ concentrations were highest. Given time the lower concentrations may have had the same effect but within the scope of this project it was decided to use a higher concentration of $MnSO_4$ to optimise degradation in proceeding experiments. It was found that the activity of laccase was not influenced by different concentrations of $MnSO_4$ but the activity was influenced by its absence which is in agreement with the findings in the paper by Pizzul et al. (2009). In the ABTS experiment it resulted that the concentration played an important role in the optimisation of degradation conditions. It was found that glyphosate disappeared regardless of the three concentrations tested but the assumed degradation of AMPA was influenced with the least amounts detected using the highest concentration (1 mM in the reaction mixture). The highest concentration of 1 mM appears to be the optimal concentration to enhance degradation. By the time these results came in the decision had already been taken to use ABTS at a concentration of 0.1 mM in the experiments testing the effects of pH (an earlier experiment found no difference.see annex 1). In the interest of continuity it was decided to use this concentration throughout the research project. This decision was also beneficial from an economic perspective as ABTS is expensive.

For all the compounds tested it was found that there was greater degradation at pH 7 than the other pH levels tested. In soil glyphosate is more likely to be bioavailable at higher pH levels and therefore more susceptible to degradation. At lower pH levels glyphosate is more likely to be bound to surface sites (Borggaard and Gimsing 2008,). However, it must be considered that the best pH range for laccase activity is between pH 4 and pH 5 (Baldrian 2005, Pizzul 2010 personal communication). It was also recommended in a paper by Balakshin et al. (2001) that in order to avoid laccase deactivation experiments should be carried out at a pH close to neutral. This lead to some conflict when choosing which pH buffer would be most appropriate. It was decided that it is more important that glyphosate is in a state where it is degradable

rather than optimising conditions for laccase. It was thought that using pH 7 may be too harsh on the laccase and pH 6 gave the next best effect on degradation thus pH 6 was chosen as the best option. Project 1 was the only section in which isoproturon was tested. Laccase efficiently degraded isoproturon at the higher pH levels and gave promising results for degradation at the lower pH levels in the presence of ABTS and $MnSO_4$. Glyphosate was chosen for further investigation due to its prolific use worldwide. Another reason it was chosen was to see how its main metabolite AMPA reacts under different circumstances.

One of the main problems with using laccase and other enzymes for the degradation of pesticides is their unpredictability when applied to soils as purified free cell enzymes. An important factor limiting the degradation of any pesticide using laccase is its propensity to denature or become deactivated (Gianfreda and Rao 2004). These issues will have to be overcome if laccase is to be useful as a candidate for the co-formulation concept. Immobilisation methods are commonly used to increase stability of enzymes (Ahn et al. 2002). It will have to be immobilised in such a way that it can be stored together with the pesticide until application at which point it can be liberated to act on the pesticide before the pesticide becomes unavailable. In using the method to encapsulate them it is hoped that this problem can be overcome. It can be seen by the laccase activity measurements that in the experiment to encapsulate laccase it was possible to immobilize the enzyme and that the activity was retained in the gel. Not all the laccase added was immobilized. The activity was variable when measured suggesting laccase was not homogenously immobilised in the gel or possibly that the samples were not fully dissolved when the readings were taken. Despite this the activity within the gel was high and is a promising result for the use of laccase for degradation purposes. The method needs development so as to lessen the waste of laccase (portion that was not immobilised) and to have a more predictable distribution within the gel.

The effect on glyphosate and AMPA degradation using the encapsulated laccase was first tested on sand, a more inert material. This was an effort to simplify the system to get a clearer result (less variables than a more complex soil). Laccase in solution was also used for comparison, it was thought there was less chance of laccase deactivation due to the nature of sandy soil (less sorption capacity). The best results for glyphosate degradation were seen in the treatments using laccase in solution especially in the treatment where water was used in place of the buffer. The laccase in solution may have performed better as the encapsulated laccase may not have been as readily available to react during the incubation period. In addition to this the deviation was quite high in the samples for the treatments using the encapsulated laccase which may also be explained in part to the irregular activity readings. It must also be noted that the conditions used were optimal for the laccase in solution and perhaps different conditions are necessary to optimise the reaction conditions when using encapsulated laccase. There was a reduction in glyphosate all the same so it is believed that some of the laccase was released intact and acted upon the pesticides

Soil was then tested to see behavior on a more complex structure. Laccase in solution was not used as it is know that laccase deactivates upon contact with the soil (Gianfreda and Rao 2004). The strange thing about this result was that there was a considerable amount of AMPA detected. It was first thought that this may be an indication that there was a problem with the GC-MS machine but on further investigation it was found that the source of AMPA was the soil itself. AMPA as mentioned in the introduction has a

strong capacity to adsorb to surfaces in soil. It is assumed therefore that glyphosate must have been sprayed on or near where that soil before it was sampled. Experiments with soil can be very complex for these types of experiments, glyphosate is known to behave differently under different conditions such as pH, temperature and microbial activity. Sorption also plays a key role, it was found that after 24 hours up to 84% of glyphosate was adsorbed in a soil experiment (Shushkova et al. 2010). Targeting the glyphosate with laccase while it is still bioavailable is key for its degradation (Önneby et al. 2010). This experiment shows that there is a lot left to be understood about this method but the ability to encapsulate laccase and its apparent ability to be liberated to degrade glyphosate and is a good start in this process

Before embarking on the experiments to test the activity in the substrates used to screen for mediators an experiment was carried out to learn how to best measure laccase from substrates. In literature the most common method used for measuring laccase activity in substrates is the ABTS method and is recommended as the best method for this purpose (Li et al. 2008). This was tested with rather disappointing results as out of five peat samples only one soil gave a measurement. The soils used fell below the detection limit for the ABTS method which is between 1 and 20 mU/ml (Alcalde and Butler 2004). Laccase is also capable of catalyzing the oxidative coupling of MBTH and DMAB, which are usually considered to be specific substrates for manganese peroxidase (Jordaan and Leukes 2003). This time the ABTS method yielded no results at all for laccase activity. The MBTH-DMAB method seemed better as it gave a clearer response. H_2O_2 was used as it drives the reaction for any MnP; the activity measured without H_2O_2 this is deemed to be laccase (other enzymes may have been present). In all cases the amount of laccase activity is less prolific than MnP. As a result the MBTH-DMAB method was used for all further testing for laccase activity in substrates.

Mediators have been shown to be important in the degradation of pesticides by laccase. By screening ligninolytic materials for natural and easily extractable mediators could identify cheap and ecofriendly alternatives that can efficiently enhance the degradation of pesticides in the presence of laccase. The concept of screening for natural mediators in laccase based oxidative reactions has been reported in many studies. The motivation for these studies is often economical as mediators such as ABTS are expensive, finding natural alternatives that are easily extractable also provide also more environmentally friendly method for mediating these reaction. Natural mediators have been reported as laccase mediators for polycyclic aromatic hydrocarbons (PAHs) and a host of other recalcitrant xenobiotics (Camarero et al. 2005, Camarero et al. 2007, Campos et al. 2001, Itoh et al. 2000, Johannes and Majcherczyk 2000, Kang et al. 2002). Ligninolytic materials have been screened for the presence of natural laccase mediators for the decolourisation of recalcitrant dyes with positive results for compounds such as syringaldehyde (Camarero et al. 2005). As part of the screening process in this report ligninolytic materials were selected and incubated. In choosing ligninolytic substrates it was hoped to identify a possible source for a natural mediator. As biobeds are successful in the degradation of pesticides it is likely that natural mediators are present in the substrates used, a classic Swedish biobed contains straw, peat and soil. Straws are mainly considered to be responsible for microbial activity (especially ligninolytic fungi) and peat is mainly considered to maintain a low pH and moisture control as well as sorption capacity. The straws chosen for screening are typical of the straws used in biobeds, the hemp residue and saw shavings have similar characteristics to the straws and could be considered candidates for use in biobeds and thus for this screening

experiment. The five peat soils chosen are also typical examples of what is used in biobeds. In measurements for pH the results varied between pH 4 and pH 7.5. A general observation was that the peat soils had lower pH readings (pH 4-5) while the straw like materials were in the range of pH 5.5 to pH 7.5 with the exception of Hemp 2 which was closer to pH 4.5. As pH is so important in degradability of pesticides like glyphosate this variation highlights the importance for biobed composition, especially when substituting materials and conditions in different locations and different resources (Vischetti et al 2007, Coppola et al 2007).

The materials were tested for their laccase activity to distinguish if there was an effect of mediators or enzymatic activity during the screening process. Laccase activity in the substrates varied with some substrates (hemp and oats) showing higher activity than the other substrates while others had comparatively low laccase activity such as some of the straws and 'straw like' substrates and all the peat samples. When the substrate extracts were screened for an effect on the activity of laccase, slightly positive effects were seen in comparison to the control in most cases but especially in the Saw shavings and Hemp 2 samples. In the case of saw shavings this is not thought to be connected to the activity in the extract as it had a low enzymatic activity reading in comparison to the others and therefore there could be a mediator acting here. Hemp 2 on the other hand gave the highest reading for laccase activity so it is unclear if the positive effect came from a mediator or the elevated activity in the extract. In the absence of Mn there was a lower response across the board but in general positive effects were seen. There appeared to be negative effects on three of the substrates giving rise to the notion that something in the extract was inhibiting laccase activity. Another explanation could be that the substrates showing these effects contain low levels of Mn. It was thought that, according to their positive effect on the laccase activity, H5-H6, H7-H8, Saw shavings and Hemp 2 would be the best candidates when it came to the degradation of glyphosate.

The effect on concentration of glyphosate using the extracts in the presence of purified laccase solution was that glyphosate was degraded in all but one sample were a trace amount remained (Hemp 1). There was a clear inhibition of AMPA when compared to the control. In five of the samples there was a significant difference in AMPA production compared to the control. All the samples that were not significantly different had high standard deviations except for the Biobed extract, which also showed the lowest amount of AMPA produced. In this experiment none of the extracts screened therefore showed any potential sign of mediators. The most interesting results are for the samples run without the presence of purified laccase as Hemp 1, Hemp 2, Wheat and H5-H6 grade peat showed signs of degradation. The laccase activity was low in the cases of Wheat and H5-H6 leading to the conclusion that either another enzyme and/or mediators were responsible for degrading the glyphosate. Hemp 1and Hemp 2 had high recordings for laccase activity but the samples were so diluted it is possible that despite the high activity reading there was a mediator present. Out of these results only for Hemp 2 was significantly different from the control, on saying this due to the low sample size it is hard to say for sure whether these statistics can be trusted. The results for the t-tests were run through a post-hoc test in the statistical power program G*Power where no power was recorded for any of the results showing significance. With this said, this indicate promising results for the presence of mediators in some of the substances. To investigate this further it would have to be discovered as to why the replicates for Hemp turned out so different after the incubation. What were the

differences in microbial populations? What effect did this have on the substrate and what substances were produced? Answering these questions could lead to identifying a natural mediator for reactions of this nature using laccase

5. Conclusion

The buffer composition, pH and presence of mediators all influence the degradation of the pesticides by laccase allowing reaction mixtures to be optimised and the potential for natural mediator to be identified and utilised. The ability to encapsulating laccase was another way in which laccase proves itself as a good candidate for use in pesticide degradation methods, although much more work needs to be done to improve the consistency of this method.

The conditions investigated in this research project are a long way off for use in the field but the results indicate a positive move in that direction. Learning more about how to enhance the conditions for degradation of pesticides using laccase will help in the development of these methods for application purposes in the future. Industries where these methods could have a place are many. Within agriculture alone laccase could potentially be used in the area of bioprophylaxis as outlined in the introduction. Another area could be in hydroponic systems, these closed loop growing systems used for vegetable production are plagued with problems and many artificial chemicals are used to control the artificial environment that is created. Using an enzyme like laccase to break down pesticides used may prove useful. Other than the potential agricultural applications laccase could be used in breaking down pesticides used in urban environments, pesticides are notorious for leaching in these areas due to the high run off potential, developing a technique in degrading these pesticides before they find their way into water bodies would be very beneficial from an environmental stand point, laccase could play a role here.

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8. Appendix

Appendix 1:

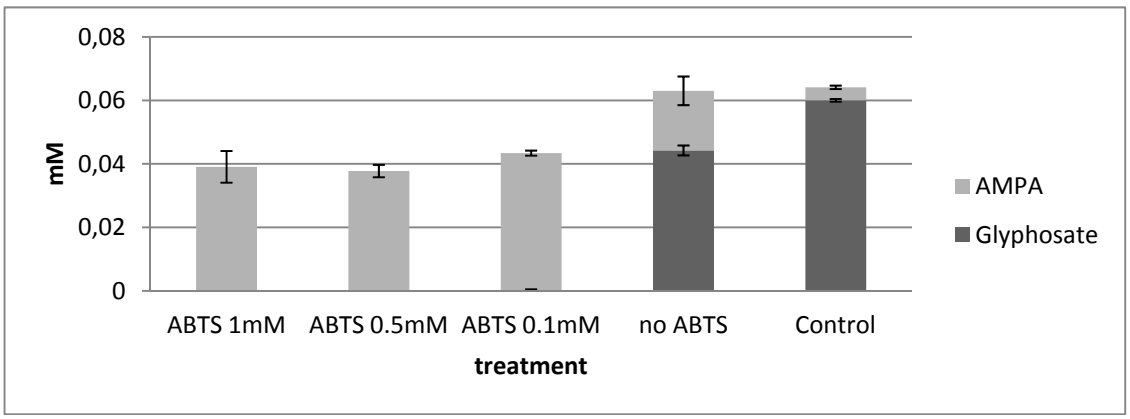

Figure 1. Effect of ABTS concentration on degradation of glyphosate and production of AMPA using laccase where samples were taken in methanol. Values are means ±SD (n=2).

On the first occasion the samples were taken into methanol, the results in Figure 1 show that the concentration of ABTS did not influence the results as in all cases no glyphosate was detected and a similar amount of AMPA was produced. Degradation of glyphosate was greatly affected if no ABTS was added.