How do Pesticides Impact Soil Microbial Structure and Functioning?

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List of Abbreviations

°C: Degrees Celsius

%: Percentage

1,3-D: 1,3-Dichloropropene

2D: 2-dimensional

2,4-D: 2,4-Dichlorophenoxyacetic acid

2,4-DCP: 2,4-Dichlorophenol

2,4,5-T: 2,4,5-Trichlorophenoxyacetic acid

6-FAM: 6-carboxyfluoroscein

^{13}C: Radioactive carbon-13

^{14}C: Radioactive carbon-14

$^{14}CO_2$: Radioactive ^{14}C carbon dioxide

^{15}N: Radioactive nitrogen-15

ADNT: 2-amino4,6-dinitrotoulene

ANOSIM: Analysis of similarity

ANOVA: Analysis of variance

ARDRA: Amplified ribosomal DNA restriction analysis

ATP: Adenosine triphosphate

Avo: Avogadros number

bp: Base pairs

Bq: Becquerel

BSA: Bovine serum albumin

C: Carbon

cDNA: Complementary DNA

$C_2H_4O_2$: Acetic acid

CH_3CHO: Acetaldehyde

CH_3COCOO^-: Pyruvate

$CHCl_3$: Chloroform

CO_2: Carbon dioxide

cfu: Colony forming unit

Cr(VI): Hexavalent chromium

CTAB: Cetyl trimethylammonium bromide

Cu: Copper

DANT: 2,4-diamino-6-nitrotoluene

DBCP: Dibromochloropropane

DETP: Diethylthiophosphoric acid

df: Degrees of freedom

DGGE: Denaturing gradient gel electrophoresis

DNA: Deoxyribonucleic acid

DNT: Dinitrotoluene

dNTP: Deoxyribonucleotide triphosphate

DOM: Dissolved organic matter

dpm: Degradations per minute

DT_{25}: Time for 25% degradation to occur

DT_{50}: Time for 50% degradation to occur

EDTA: Ethylenediaminetetraacetic acid

EU: European Union

FEPA: Food and Environmental Protection Act.

FERA: The Food and Environment Research Agency

$FeSO_4$: Iron sulphate

FISH: Fluorescent *in situ* hybridisation

g: Grams

H': Shannon diversity value

HPLC: High performance liquid chromatography

ITS: Internal transcribed spacer

KCl: Potassium chloride

K_2HPO_4: Dipotassium phosphate

KH_2PO_4: Monopotassium phosphate

KNO_3: Potassium nitrate

kPa: Kilo Pascals

K_2SO_4: Potassium sulphate

LB: Lysogeny broth

lbs: Pounds

LiP: Lignin peroxidase

LME: Lignin-modifying enzymes

LSD: Least significant difference

M: Molar

MBS: Minimal basal salts

MBSA: Minimal basal salts agar

MCPA: 2-methyl-4-chlorophenoxyacetic acid

MIC: Minimum Inhibitory Concentration

µg: Micrograms

$MgSO_4 \cdot 7H_2O$: Magnesium sulphate heptahydrate

$MnSO_4 \cdot 4H_2O$: Manganese sulphate tetrahydrate

µl: Microlitres

µM: Micromolar

ml: Millilitres

mol: moles

MRL: Maximum residue level

mRNA: Messenger RNA

MSE: Mean standard error

MW: Molecular weight

N: Nitrogen

NaCl: Sodium chloride

NaOH: Sodium hydroxide

NBDO: Nitrobenzene diooxygenase

NH_4NO_3: Ammonium nitrate

$(NH_4)_2SO_4$: Ammonium sulphate

Nin-N: Ninhydrin-reactive nitrogen

nm: Nanometres

NMDS: Non-metric multidimensional scaling

NP*n*EO: Nonylphenol ethoxylate

NA: Nutrient agar

NT: Nitrotoluene

OD: Optical density

OECD: Organisation for Economic Co-operation and Development

PAH: Poly-aromatic hydrocarbon

PCB: Polychlorinated biphenyl

PCP: Pentachlorophenol

PCR: Polymerase chain reaction

PHMB: Polyhexamethylene biguanide

Pic: Chloropicrin

PLFA: Phospholipid fatty acid

PNCB: Pentachloronitrobenzene

PNP: *Para*-nitrophenol

ppm: Parts per million

Q_o: Ubiquinone

qPCR: Quantitative PCR

rDNA: Ribosomal DNA

RDP: Ribosome database project

RNA: Ribonucleic acid

rRNA: Ribosomal RNA

RT-PCR: Reverse transcriptase PCR

SIMPER: Similarity percentage

SIP: Stable isotope probing

SOC: Super optimal broth + catabolite repression

SMB: Soil microbial biomass

TAT: 2,4,6-triaminotoluene

TCA: Tricarboxylic acid

TCP: 3,5,6-trichloro-2-pyridinol

TE: Tris + EDTA

TGGE: Temperature gradient gel electrophoresis

THF: Tetrahydrofuran

TINV: Inverse *t* value

TNT: Trinitrotoluene

TPF: 2,3,5-triphenyl formazan

TRF: Terminal restriction fragment

T-RFLP: Terminal-restriction fragment length polymorphisms

TTC: 2,3,5-triphenyltetrazolium chloride

UK: United Kingdom

USA: United States of America

UV: Ultra-violet

v/v: Volume/Volume concentration

X-GAL: Bromo-chloro-indolyl-galactopyranoside

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Declaration

I declare that the work presented in this thesis was conducted by me under the direct supervision of Doctor Gary D. Bending and Professor Kirk T. Semple, with the exception of those instances where the contribution of others has been specifically acknowledged. None of the work presented here has previously been submitted for any other degree.

Christopher Carl Howell

Abstract

Pesticides are used worldwide and exhibit a plethora of different modes of action against a wide spectrum of organisms. Therefore, before they can be marketed they have to be tested against certain standardised regulations. These include the Food and Environment Protection Act 1985 (FEPA), the Control of Pesticides Regulations 1986 (COPR), and more recently the European Council Directive 1991 91/414 and the Plant Protection Products Regulations 1991 (PPPR). However, the current tests used to determine pesticide impacts on microorganisms as detailed by the OECD focus on only broad-scale analytical methods that may mask more subtle effects that may still be ecologically significant. Therefore, this project aimed to determine the effects of a widely-used model pesticide, azoxystrobin on both target and non-target microbial communities across different trophic levels.

The techniques used to perform this included broad- (soil microbial biomass and soil dehydrogenase activity) and fine-scale (T-RFLP, cloning/sequencing, and qPCR) analytical methods. The results of these analyses showed that the application of azoxystrobin had a significant, concentration-dependent impact on soil dehydrogenase activity whilst biomass was unaffected. The molecular analyses showed that azoxystrobin significantly impacted fungal community structure, diversity and gene copy number. Additionally, pesticide application significantly altered nematode community structure and general eukaryotic diversity. Soil and liquid culture enrichments showed that azoxystrobin degradation can be enhanced following repeated applications and enabled the isolation of two degrader organisms with sequence homologies to a *Cupriavidus* sp. and a *Rhodanobacter* sp. Further work showed that sequential enrichments with azoxystrobin also conferred cross-enhanced degradative abilities for three other strobilurin fungicides: pyraclostrobin, kresoxim methyl and trifloxystrobin. The work performed in this thesis served to illustrate how the current OECD test procedures may benefit from the incorporation of finer-scale molecular methods into its tests, as well as how difficult the task can be to produce compounds that persist in the environment long enough to perform their required function, but do not have significant deleterious impacts on non-target organisms when present.

Chapter 1 Introduction

1.1 The Importance of Pesticide use in Current Agricultural Processes

1.1.1 Worldwide Use

The use of pesticides to prevent or treat crop diseases has been an essential part of agricultural practices world-wide for nearly a century. The Food and Environment Protection Act (1985) (FEPA) defined a pesticide as "any substance, preparation or organism prepared or used, among other uses, to protect plants or wood, or other plant products from harmful organisms; to regulate the growth of plants; to give protection against harmful creatures; or to render such creatures harmless" (Health and Safety Executive, 2011a). Pesticides are generally classified based on the organism type that they are used to combat (e.g. insecticide) and/or the chemical structure of the compound (e.g. organophosphate).

In 1983, the total value of pesticides used worldwide totalled US$ 20.5 billion (Figure 1.1). By 1998, this value had increased to US$ 34.2 billion. The majority of the pesticides used were in North America and Western Europe, with the least being in Africa and the Middle East (Yudelman *et al.*, 1998). Over more recent years worldwide spending on pesticides has levelled-out, with the total value of pesticides used in the years 2000 and 2001 being recorded as US$ 32.8 billion and US$ 31.8 billion, respectively (Kiely *et al.*, 2004).

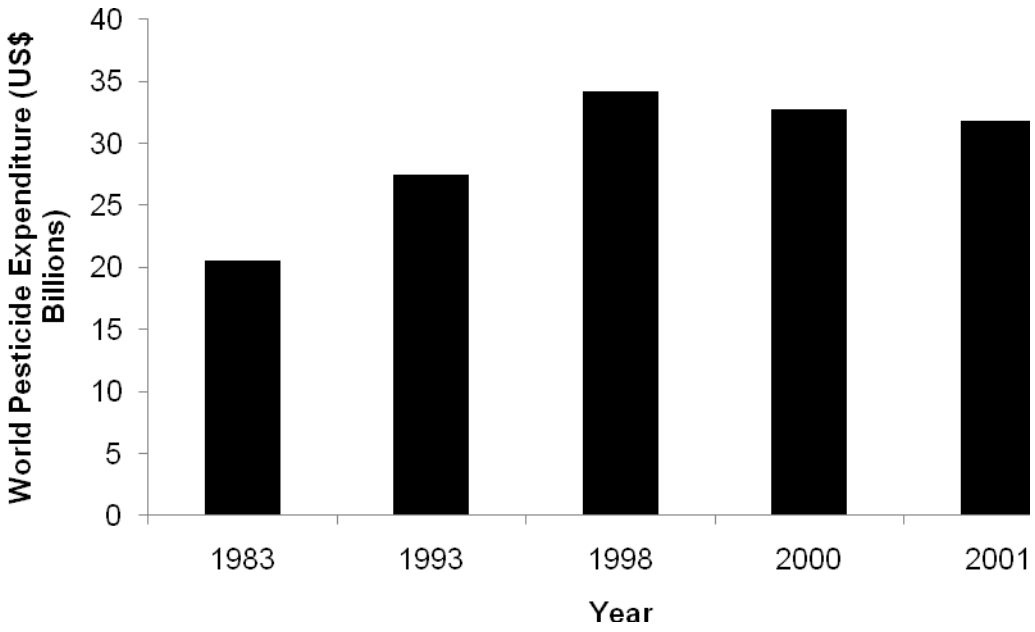


Figure 1.1 World pesticide expenditure from 1983 to 2001. From: Yudelman *et al.* (1998); Kiely *et al.* (2004)

In the years 2000 and 2001, herbicides were the most widely used and represented 44% of the total pesticide use. This compared with 28% for insecticides, 19% for fungicides, and 9% for other pesticide types. This equated to 1.9 billion lbs of herbicides, 1.4 billion lbs of insecticides, 516 million lbs of fungicides, and 1.5 billion lbs of other pesticides (Kiely *et al.*, 2004).

1.1.2 UK Pesticide Use

In the UK, the total area treated with pesticides increased from 44.9 million hectares in 1990 to over 71 million hectares in 2009 (FERA, 2009) (Figure 1.2). However, the development of compounds (such as the sulfonylurea herbicides) that are more active at lower concentrations has meant that within this period the total weight of pesticides applied in the UK decreased from 75.8 million lbs in 1990 to 44.5 million lbs in 2009 (FERA, 2009).

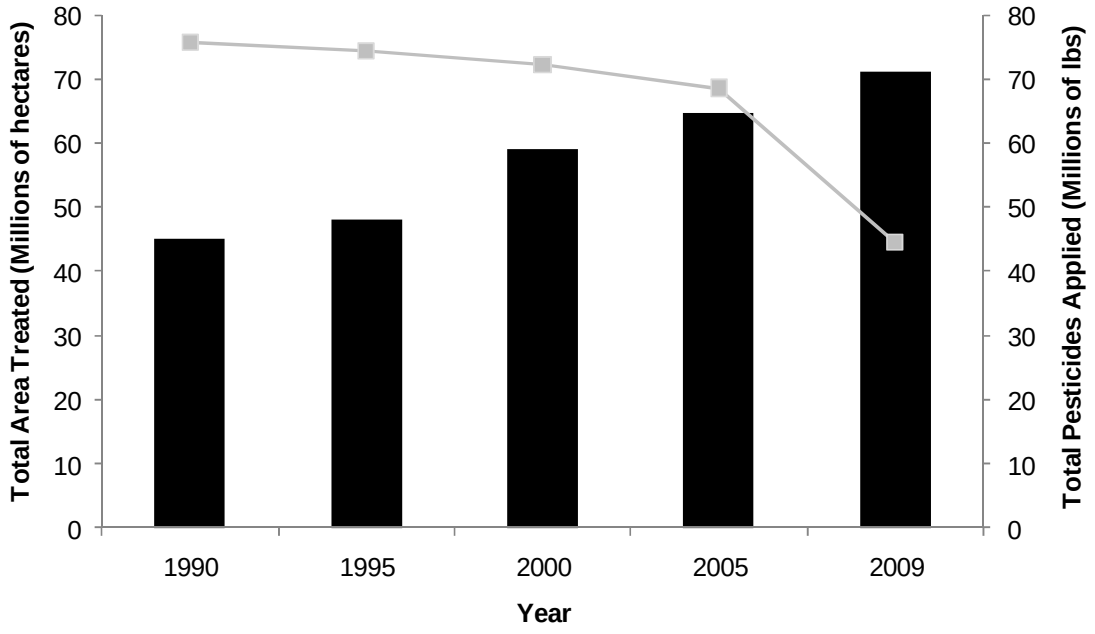


Figure 1.2 Total pesticides applied and total area treated in the UK between 1990 and 2009. From: FERA (2009). ■ Total Area Treated ■ Total Weight of Pesticides Applied

In 2009, the majority of the pesticides applied were either herbicides or fungicides which represented 43 and 30% of all applications, respectively. Of the remaining 27%, growth regulators accounted for 16% with insecticides and molluscicides representing 2% each. The remaining 7% was made up of "other" pesticides (FERA, 2009).

1.2 The Fate of Pesticides in Natural Ecosystems

Due to the biologically active nature of pesticides, there has been much research into the fate of pesticides in the natural environment and the factors that affect pesticide removal. Figure 1.3 illustrates these factors and indicates how they can be inter-linked. The processes responsible for the fate of pesticides in the environment can involve interactions with living organisms (biotic factors) such as degradation and uptake by plants, or with the natural environment (abiotic factors) such as photochemical degradation or volatilisation.

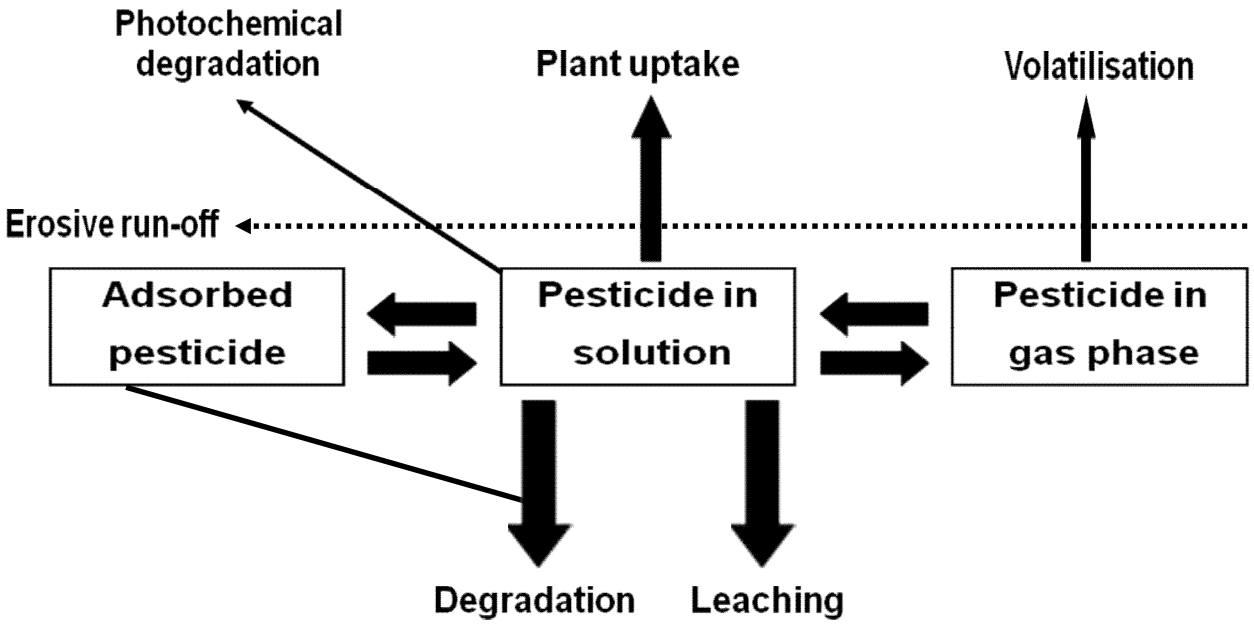


Figure 1.3 A conceptual diagram of the factors that determine the behaviour and fate of pesticides in natural environments.

1.2.1 Abiotic Factors

1.2.1.1 Photodegradation

One of the major abiotic degradation processes is photodegradation. Photodegradation has been defined as "the photochemical transformation of a molecule into lower molecular weight fragments, usually in an oxidation process" (Verhoeven, 1996). Burrows *et al.* (2002) reviewed the photodegradation of pesticides and reviewed its potential importance in the remediation of pesticides. The authors noted that many pesticides are resistant to photodegradation. The main reason for this is that many pesticides only absorb short wavelength UV radiation, which represents only a small percentage of the total UV radiation reaching the surface of the earth. Some of the photodegradation processes that have been observed include direct photolysis, photosensitized photodegradation and photolysis in heterogeneous media (lab-produced liquid media) (Burrows *et al.*, 2002). Photosensitized photodegradation refers to the indirect degradation of pesticides resulting from interactions with reactive intermediates produced by the photochemical reactions of other compounds in the environment, such as dissolved organic matter (DOM) or nitrates (Lam *et al.*, 2005; Ramezani, 2008).

The extent to which pesticides can be photodegraded can vary markedly between different compounds. Tsogas *et al.* (2006) noted that the insecticide active ingredient Carbaryl was relatively rapidly photodegraded in water samples (distilled water, tap water, and lake water) with half lives of between 12.4 and 13.1 hours. In addition, research by Ramezani (2008) showed that the imidazolinone herbicides imazapyr, imazethapyr and imazaquin were degraded significantly quicker under light conditions than in the dark. For imazapyr, the recorded half lives were 6.5 months and 1.8 days under dark and light conditions, respectively. This compared with 9.2 months, and 9.8 days for imazethapyr, and 9.6 months and 9.1 days for imazaquin. Eyheraguibel *et al.* (2009) described that the herbicides bentazone, clopyralid, and triclopyr are less affected by photodegradation in water. Triclopyr is the most quickly degraded with a recorded half life of between 12 and 31 hours. In comparison, bentazone has a recorded half-life of between 65 and 96 hours. Clopyralid has been found to be more persistent with a half life in water of 261 days. However, the substrate to which a pesticide is applied can also affect its susceptibility for photodegradation. The half life of bentazon in soils under UV irradiation has been recorded as greater than 940 hours, with little or no photodegradation recorded for clopyralid or triclopyr in soil substrates (Eyheraguibel *et al.*, 2009). In contrast, some pesticides can be rapidly photodegraded even when absorbed to solid substrates. Research by Tajeddine *et al.* (2010) showed that the organophosphate insecticide, fenamiphos had half lives under irradiation of only 40 and 75 minutes when applied to the substrates montmorillonite and kaolin, respectively.

1.2.1.2 Binding to Soils

Another abiotic factor that can affect the degradation of pesticides in natural ecosystems is the binding of pesticides to soils and other natural matrices (Gevao *et al.*, 2000). In soils, organic matter is thought to be the key element involved in these sorption processes. Pesticides can be bound to soil organic matter by a number of different processes. These include sorption resulting from hydrogen or hydrophobic bonding, electrostatic interactions such as ion or ligand exchange and charge transfer. Additionally there may be covalent bonding of a compound to the soil matrix (Bollag *et al.*, 1992).

Spark and Swift (2002) studied how the sorption of four pesticides (atrazine, 2,4-D, isoproturon, and paraquat) to soils was affected by soil composition and dissolved organic matter content. The dissolved organic matter was found to only have a minor effect on pesticide sorption whereas the solid organic fraction and soil clay content had much stronger effects. Pesticide sorption was found to be generally positively correlated with increased solid organic matter and/or clay content. Other factors that can affect the binding of pesticides to soils can include: the concentration of the compound applied, the number of times a compound is applied in a single area, and the mode of application of a compound (Gevao *et al.*, 2000).

1.2.2 Biotic Factors

Biodegradation also plays an integral role in the fate of pesticides within natural ecosystems. This biodegradation can be growth-linked or co-metabolic. Figure 1.4 illustrates the possible relationship between compound concentration, and bacterial population growth. Growth-linked biodegradation occurs when an organism breaks-down a compound whilst using it as a source of, for example, carbon and/or nitrogen (Figure 1.4a). This results in the proliferation of degrader organisms over the period that the compound is present in the environment. In contrast, co-metabolic degradation generally involves the breakdown of a compound by an organism using non-specific enzymes such as mono- and dioxygenases (Landa *et al.*, 1994). In this situation the compounds are not directly used as nutrient sources and are therefore the processes are not growth-linked and are characterised by very slow degradation of the chemical (Figure 1.4b) (Alexander, 1981).

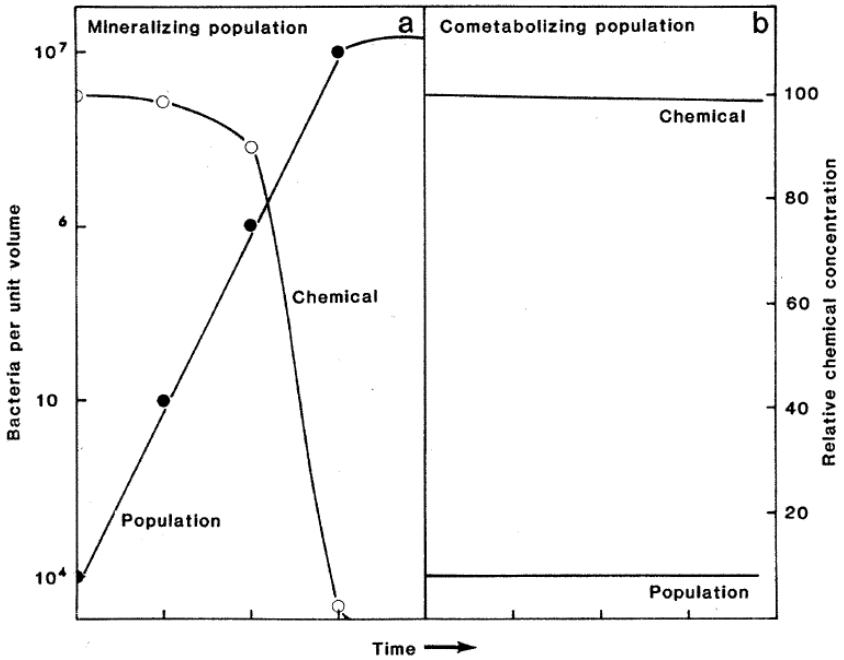


Figure 1.4 A conceptual diagram of chemical concentration/bacterial growth relationships under a) mineralizing and b) cometabolizing conditions. From: Alexander (1981).

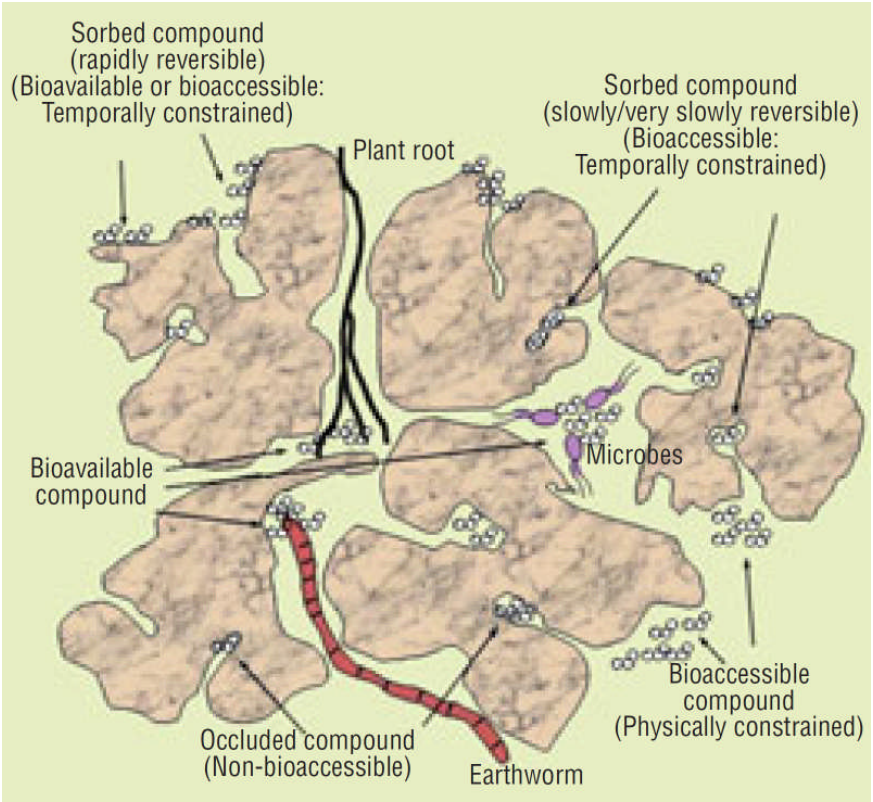


Figure 1.5 A conceptual diagram showing how pesticide interactions with the soil matrix can alter compound bioavailability/bioaccessibility. From Semple *et al.* (2004).

Pesticide degradation can either be incomplete and result in the formation of secondary metabolites, or complete where degradation results in the production of CO_2 and water (mineralisation) (Miller, 1996). Complete mineralisation of compounds is preferable as compound secondary metabolites can sometimes also be toxic to the environment (Madigan *et al.*, 2003).

Bioavailability and biodegradation are intrinsically linked with abiotic factors such as compound sorption to soils. Anderson *et al.* (1999) defined bioavailability as "a measure of the potential of a chemical for entry into biological receptors. It is specific to the receptor, the route of entry, time of exposure, and the matrix containing the contaminant''. However, it has been noted in the scientific literature that it is difficult to produce a single definition for bioavailability (Semple *et al.*, 2004). A related concept to bioavailability is bioaccessibility. Semple *et al.* (2004) defined a bioaccessible compound as one which is "available to cross an organism's cellular membrane from the environment, if the organism has access to the chemical". The binding of pesticides to the soil matrix is known to reduce bioavailability and bioaccessibility, and therefore the potential biodegradation of the compound (Figure 1.5). This sorption to the soil matrix can be light or moderate which would allow a compound to be made bioavailable again over time. In contrast, a pesticide could also become strongly sorbed or occluded into soil pores which would render the compound non-bioavailable/non-bioaccessible. However, there has been much debate as to whether this binding is beneficial or detrimental to the environment. For example, it may be detrimental for the environment that a pesticide is sorbed to the soil and therefore becomes more persistent, but this may be counter-balanced by the fact that the organisms are less exposed to the toxic effects of the compound (Gavrilescu, 2005). Karpouzas and Walker (2000) noted how degradation of the nematicide, ethoprophos, by the bacterium *Pseudomonas putida* strain epI was slower in soils with a higher organic matter content. This was thought to be due to the increased sorption/decreased bioavailability of the compound within these soils. Ahmad *et al.* (2004) showed that even weak sorption can reduce the bioavailability of compound (in this case carbaryl) over an extended time period (> 12 years). Chirnside *et al.* (2009) also noted that the degradation rate of atrazine and alachlor in soils decreased

with time. However, some compounds such as glyphosate have been found to be still microbially degraded even when sorbed to the soil (Schnürer *et al.*, 2006).

1.3 Environmental Concerns About Pesticide Use

Due to the high amounts of pesticides applied worldwide each year, and the potential for them to persist in the environment, significant levels of research have been carried out to determine the possible deleterious effects of this persistence. These potential effects can include leaching into drinking water and other freshwater systems, bioaccumulation, and impacts on non-target species.

1.3.1 Leaching into Drinking Water and Freshwater Habitats

One of the major concerns is that pesticides may leach from soil environments into freshwater systems, and potentially even into drinking water sources. Stoate *et al.* (2001) noted how pesticides can enter watercourses through direct leaching from soils or in association with eroded soil or sediment. Additionally, they can also enter through drains, storm sewers and other man-made routes (Gavrilescu, 2005). These can not only deleteriously affect the quality of the water; they also cause an additional financial burden resulting from the need for additional purification. The leaching of herbicides into freshwater environments has been of particular concern due to the potential impacts that the compounds may have on aquatic plants. Peterson *et al.* (1994) studied the impacts of herbicides on duckweed (*Lemna minor*) when applied at environmentally-realistic concentrations. The findings of this work can be seen in Table 1.1. The results showed that the impacts of herbicides on duckweed growth could vary from 0 to 100% inhibition depending upon the compound applied.

Table 1.1 The percentage growth inhibition of duckweed (*Lemna minor*) by different herbicide compounds. Data from: Peterson *et al.* (1994).

Herbicide	% Growth Inhibition
Atrazine	95
Cyanazine	100
Hexazinone	100
Metribuzin	100
Simazine	100
Chlorsulfuron	86
Ethametsulfuron-methyl	33
Metsulfuron-methyl	63
Triasulfuron	91
2,4-D	34
MCPA	42
Picloram	10
Triclopyr	23
Bromoxynil	0
Diquat	100
Acrolein	73
Glyphosate	0
Imazethapyr	46
Metolachlor	81
Tebuthiuron	100

Furthermore, pesticides can also leach rapidly downwards and possibly reach the levels of the natural groundwater, depending on the chemical composition of the compound and the structure of a particular soil. The capacity of a pesticide to leach from a soil can be considered in terms of its soil/water (Kd) and soil organic carbon/water (Koc) partitioning coefficients. The Kd value refers to the ratio between the concentration of a chemical sorbed to a soil matrix and the dissolved concentration when the soil is at equilibrium. The Koc value is produced by normalising the Kd value against the total organic carbon content of a soil. The higher the Koc value of a pesticide, the lower mobility the compound has within a soil (Kanazawa, 1989).

This may be exacerbated when high levels of rainfall occur on land that has only recently been amended with pesticides (Arias-Estévez *et al.*, 2008). Bucheli *et al.*, (1998) also noted that high concentrations of pesticides present in roof run-off and rainwater flows also had the potential to reach and potentially contaminate groundwater systems. The authors recorded maximum pesticide

concentrations following single rain events of 903, 191, and 106 ng L^{-1} for atrazine, alachlor and R-dichlorprop, respectively. More recently, Fava *et al.* (2010) described how, from a total of 43 pesticide compounds tested, 12 were found to be present in ground- and surface water samples at concentrations higher than the 0.1 µg L^{-1} maximum levels stated by the EU directive 98/83/EC. The highest concentration recorded was 1.02 µg L^{-1} for triazine.

1.3.2 Bioaccumulation

Bioaccumulation of pesticides is another potential environmental health risk. The term bioaccumulation refers to the ability of contaminants such as pesticides to be stored and concentrated within plant and animal tissues. This could potentially be followed by biomagnification of these compounds. This refers to an increase in the concentration of a compound as it is transported up to higher and higher natural trophic levels as a result of food web interactions (Gerba, 1996). For example, pollutants present in a watercourse can bioaccumulate in fish tissues. Then, if a higher animal such as an otter (or a human) consumes several of these fish, then they will be exposed to, and ingest even higher concentrations of these compounds. Beyer *et al.* (1996) recorded that a number of organochlorine compounds could bio-accumulate in the liver and muscle cells of caged cod and flounder following three months of exposure, although no phenotypic effects of the contamination were observed over this period. This was supported by Coat *et al.* (2011) who noted that organochlorine pesticides could bioaccumulate across the food web of a tropical river system, with particularly high levels recorded in juvenile fish and freshwater shrimps. Findings such as these are of particular concern as bioaccumulated compounds such as these have previously been implicated as causative agents in the production of a range of symptoms such as liver fibrosis and/or necrosis, leukocyte infiltration and an absence of some macrophages in Brazilian trahira freshwater fish (Miranda *et al.*, 2008).

Wang and Needham (2007) described how the presence of chemicals in the environment could transfer first to animal fats, and then potentially to a woman's breast milk which could be passed on

further to a child following breast-feeding. Pesticide bioaccumulation in humans could also result in severe long term health problems. Govett *et al.* (2011) analysed the bioaccumulation of organochlorine pesticides in the adipose tissues of seven patients with head and neck cancers. The authors recorded that all seven patients had elevated levels of pesticides in their adipose tissues, although they acknowledged that further work is required to confirm that there was a causative link (Govett *et al.*, 2011).

1.3.3 Non-Target Pesticide Effects.

The effects of pesticide bioaccumulation on living organisms can be considered as one example of an indirect non-target effect. However, there have been many other recorded examples of pesticides exerting both direct and indirect impacts on non-target species across different trophic levels. These non-target species can range from higher vertebrate animals (including humans), to insects, to microorganisms within soils.

1.3.3.1 Higher Vertebrates

One particular area of concern is the potential capacity of pesticides to act as endocrine disruptors in humans. In 2001, the Commission of the European Communities identified 45 pesticide compounds as having known or potential endocrine disrupting effects on humans, although McKinlay *et al.* (2008) admitted that the number of pesticides currently in use that may have such effects remains unknown. In other vertebrate animals, Eason *et al.* (2001) stated that there had been a number of recorded incidents of direct and indirect toxic impacts of the rodenticide brodifacoum on non-target species in New Zealand. These included a number of native birds and introduced game animals. As with humans, pesticide use has also been correlated with impacts on the endocrine systems of other vertebrates, such as amphibians (Bernanke and Köhler, 2009). A study by Davidson (2004) found links between pesticide application and population decline for the California red- legged frog (*Rana aurora draytonii*), foothill yellow-legged frog (*R. boylii*), Cascades frog (*R. cascadae*), and the

mountain yellow-legged frog (*R. muscosa*), particularly following the application of cholinesterase-inhibiting insecticides such as carbamates and organophosphates (Davidson, 2004).

1.3.3.2 Insects and Other Macroinvertebrates

Pesticides can also have wide ranging impacts on non-target insects and other macroinvertebrates. Desneux *et al.* (2004) described how compounds could potentially affect every aspect of a macroinvertebrate's life cycle from biochemical and neurological impacts, to behavioural impacts such as feeding and learning abilities. Peveling *et al.* (1999) recorded that application of the compounds fenitrothion, fenitrothion-esfenvalerate and triflumuron to combat locusts in Madagascar also reduced the populations of non-target organisms such as ants and springtails by over 75% for a period of one season. Research by Canty *et al.* (2007) showed that at an environmentally realistic concentration (0.1 mg L^{-1}), the organophosphate pesticide azamethiphos significantly altered the cell viability, phagocytic index and acetylcholinesterase activity of the non-target blue mussel (*Mytilus edulis*) after only 24 hours. In contrast, the feeding habits of the mussels were not affected.

1.3.3.3 Microorganisms

Pesticides can also significantly affect non-target microbial species. Kumar *et al.* (2008) observed that the organochlorine insecticide endosulfan reduced the growth, carbohydrate content, and photosynthetic pigment production of three cyanobacterial species (*Aulosira fertilissima*, *Anabaena variabilis* and *Nostoc muscorum*). Ekelund (1999) recorded that the fungicide fenpropimorph significantly reduced the population of bactivorous protozoa, and this was built upon by Ekelund *et al.* (2000) who recorded similar results following the application of a different fungicide, propiconazole. A study by Piotrowska-Seget *et al.* (2008) observed that repeated applications of the fungicide captan also significantly reduced culturable bacteria numbers as well as decreasing overall community acid and alkaline phosphatase activities. More recently, Li *et al.* (2010) described how the herbicide acetochlor could significantly alter the fungal community structure and diversity in soil samples over a 60-day period. The pesticide first had a stimulatory effect on the community. This was followed by

a suppressive effect. Over the final few sampling points the communities in the amended treatments increasingly resembled those of the un-amended controls in terms of both structure and diversity. This aspect of pesticide impacts is discussed further in section 2.1.3.

1.4 Pesticide Regulation in the EU

1.4.1 General Overview

There are many regulations currently in place covering many aspects of pesticide use. In 1993 the EU produced the first set of regulations (Council Directive 91/414/EEC) which aimed to form a framework for considering the possible environmental effects of products and formulations used for plant protection. The directive also attempted to produce a list of compounds considered "safe" for widespread use. These regulations were built upon (and superseded by) regulation (EC) 1107/2009 in June 2011. For the first time Regulation (EC) 1107/2009 introduced the need to consider possible synergistic and/or cumulative effects of pesticide application, along with possible physical impacts on humans and animals such as endocrine disruption (Health and Safety Executive, 2011b).

In foods, maximum residue levels (MRLs) are set by the EU Commission using the guidelines set out in regulation EC 396/2005. An MRL refers to the maximum allowable level of a pesticide residue within a particular food or feed. By 2008, approximately 45,000 MRLs had been implemented (Health and Safety Executive, 2008). Currently, before a pesticide is marketed it has to comply with regulations set by the Organisation for Economic Co-operation and Development (OECD) based on a number of factors including the potential impacts of the compound on soil microorganisms.

1.4.2 Safety Testing of New Compounds

The OECD guidelines for chemical testing are divided into 5 sections. Section 1 consists of 22 different assays aimed at analysing the physical-chemical properties of a compound. Examples of these tests include melting/boiling point and water solubility assays along with the determination of possible interactions within the environment by monitoring adsorption/desorption kinetics and

hydrolysis under different pH conditions. There are 36 assays detailed in Section 2 which cover aspects involved with the possible effects of a pesticide on biological systems. These include the analysis of impacts on microorganisms (e.g. microbial carbon and nitrogen transformation) and on higher eukaryotic organisms (e.g. fish, earthworm and honeybee toxicity tests). The 19 tests of Section 3 cover the degradation of a pesticide under different environmental conditions e.g. aerobic, anaerobic, high/low light intensity. Section 4 contains 50 tests aimed at determining the possible health impacts of pesticides. These range from the analysis of genetic toxicity in mice, fruit flies, and yeast, to determining dermal, eye and oral toxicity. Finally, Section 5 covers "other" tests such as residue metabolism in crops and livestock (OECD, 2011).

1.4.3 Current Issues

Whilst the tests detailed above could give a broad indication of pesticide impacts on living organisms, they focus on either broad-scale analyses where the impacts on individual communities cannot be determined (e.g. microbial carbon and nitrogen mineralisation), or at the other extreme, they focus on the impacts on a single organism group (e.g. mice) in isolation from the rest of the natural environment. Therefore, they could mask more subtle (yet still ecologically significant) changes which may have knock-on effects in the longer term. An example of this would be if the application of a pesticide removes a particular species from the community. This could result in significant changes to the community structure on a finer-scale level, whilst its broader-scale features such as those determined using the OECD test could remain unaltered. Therefore, this has led to researchers developing a wide range of techniques for determining the impacts of pesticides and other xenobiotic compounds on microbial communities. These range from other broad-scale analyses such as biomass and dehydrogenase activity that have been in use for several decades, to more recently-developed techniques that utilise molecular methods to determine impacts on specific microbial community structure, diversity and function.

1.5 Research Methods for Determining Pesticide Impacts on Microbial Communities

Microorganisms play essential roles in all natural ecosystems worldwide. They form essential components of food webs as both producers and consumers, and are therefore play vital roles in the cycling of nutrients in, for example, soil and water systems. These functions can then be essential for the health and life cycles of plants and other higher organisms. Soil microbial functions are also a major benefit to humans. For example, soil mycorrhizal fungi can form symbiotic relationships with crop plants which can benefit the growth of the host plant by providing it with essential nutrients. This therefore means that microorganisms are also essential in the provision of "ecosystem goods" (such as crop plants) and "ecosystem services" (such as bioremediation) Costanza *et al.* (1997). A review by Costanza *et al.* (1997) estimated the average total value of worldwide ecosystem services to be in the region of US$ 33 trillion per year. As a result, over the last 50/60 years there has been an extensive amount of scientific research into the effects of pesticides on microorganisms. Specific areas of research have included the analysis of potential non-target impacts of pesticides on microbial communities, and the identification and characterisation of organisms involved in the degradation of pesticide compounds. This research has ranged from the broad-scale analysis of aspects such as biomass or specific enzyme function, to culture-dependent analyses, and more recently culture-independent molecular and sequencing methods.

1.5.1 Broad-Scale Methods

1.5.1.1 Biomass

For many years the soil microbial biomass (SMB) has been analysed as a potential indicator of whether the soil microbial community has been impacted by the addition of a particular compound (Mazzarino *et al.* 1993). The SMB represents a nutrient pool with a fast turnover rate compared to soil organic matter (SOM), and acts as a catalyst for microbial transformations (Bååth and Anderson, 2003) such as the degradation of pesticides or other complex compounds. One of the main methods

for extracting the soil microbial biomass involves the fumigation of the soil using chloroform ($CHCl_3$) which lyses the microbial cells (Brookes *et al.*, 1985). This enables an extraction to be performed using a solvent such as 0.5 M potassium sulphate (K_2SO_4) or 2 M potassium chloride (KCl) (Amato and Ladd, 1988). However, one potential experimental disadvantage of using K_2SO_4 for this method is that in certain soils a white $CaSO_4$ precipitate can form. This can then disrupt the biomass analysis which relies upon the spectrophotometric monitoring of the samples (Joergensen and Brookes, 1990). Fumigation-extraction methods for biomass analysis have been previously used in the analysis of pesticide impacts on microbial communities. However, these methods are very broad-spectrum and therefore cannot be used to monitor the biomass of specific organism groups, for example bacteria or fungi. Furthermore, the broad-spectrum nature of the assay also means that more subtle (yet still ecologically-significant) impacts may be masked, causing the production of false-negative results. A finer-scale method of soil biomass analysis is phospholipid fatty acid (PLFA) analysis. This method can overcome some of the problems associated with fumigation/extraction analysis as it can be used to specifically monitor bacterial and fungal biomass. PLFAs are bound to cell membranes and the patterns of these molecules are thought to be largely representative of specific organism groups (Frostegård and Bååth, 1994). However, PLFA analysis still has some negative points. One of the main issues is that it has been estimated that PLFA analysis can also measure the non-living/inactive biomass within an environment which can lead to an over-estimation of the active biomass within a sample (Frostegård and Bååth, 1994). Furthermore, Nielsen and Peterson (2000) noted that the analysis of microbial biomass by PLFA can be disrupted by the presence of, for example, high levels of fatty acids derived from humic acids present in a soil.

Chen *et al.* (2001) noted that soil microbial biomass values were lower in soils treated with the fungicides captan, chlorothalonil, or benomyl compared with un-amended control soils. Herbicides have also previously been shown to deleteriously affect the soil microbial biomass. Work by Perucci *et al.* (2000) showed that the herbicides rimsulfuron and imazethapyr decreased the soil microbial biomass when applied at concentrations 10-fold greater than the recommended field dose. In contrast,

research by Zhang *et al.* (2009a) showed that the insecticide cypermethrin increased the total and bacterial biomass in soils taken from the pepper plant phylosphere as determined by PLFA analysis.

1.5.1.2 Enzyme Assays

Another approach that is widely used to determine the broad-scale impacts of a pesticide on microbial communities is to monitor how the activities of enzyme groups are affected by the amendments. These methods can be used to monitor the overall activity of the soil microbial community, as opposed to monitoring the SMB (which may include non-active organisms) or analysing specific functions within the community.

Dehydrogenase activity is one of the major enzymatic processes studied. Dehydrogenases are intra-cellular enzymes that form an integral part of active microorganisms. They are involved in the oxidation of soil organic matter by transferring hydrogen from a donor substrate to an acceptor. Therefore, measuring this activity within a population provides a good, broad overview of the overall activity of the microbial community (Skujins, 1976). Lenhard (1956) produced the first widely used method for analysing soil dehydrogenase activity. This assay focuses on the production of the water-insoluble, red-coloured compound 2,3,5-triphenyl formazan (TPF), from the reduction of the colourless, water soluble 2,3,5-triphenyltetrazolium chloride (TTC) by the activity of dehydrogenases (Tabatabai, 1994). Other enzyme groups that have been previously used as part of pesticide impact analyses include phosphatases, nitrogenases, and ureases (Hussain *et al.*, 2009). Phosphatases are enzymes that act by catalyzing the hydrolysis of the anhydride ($(RC(O))_2O$) and ester (RCO_2R) components of phosphoric acid (Eivazi and Tabatabai, 1977). Nitrogenase enzymes catalyze reactions involved in nitrogen fixation (Zehr *et al.*, 2003), whilst ureases catalyze the hydrolysis of urea which results in the production of ammonia and carbon dioxide (Petitt *et al.*, 1976).

Perucci *et al.* (2000) recorded that amendments with rimsulfuron or imazethapyr significantly affected soil alkaline phosphatase activity, whilst Sannino and Gianfreda (2001) noted that glyphosate

decreased phosphatase activity in 22 different soils by between 5 and 98%. A 1 ppm application of the organochlorine insecticide endosulfan has been found to decrease soil nitrogenase activity by up to 8 times over a 14-day period (Kalyani *et al.*, 2010). This contrasts with the effects of the herbicide monosulfuron which enhanced nitrogenase activity in three nitrogen-fixing cyanobacterial species (Shen and Luo, 2011). The fungicide metalaxyl reduced soil urease activity gradually over a 30-day experimental period (Sukul, 2006) whereas chlorimuron-ethyl and furadan amendments increased urease activity by up to 21% in meadow soils (Yang *et al.*, 2007). One problem with the use of enzyme assays such as these is that on some occasions they can give contradictory indications of pesticide impacts. For example, in the work of Sukul (2006) although metalaxyl had a gradual deleterious effect on soil urease activity, dehydrogenase and phosphatase activities increased initially before eventually decreasing. Furthermore, Trasar-Cepeda *et al.* (2000) noted how urease and dehydrogenase activities can vary significantly in response to pesticide application, compared to un-amended controls. As a result, these enzyme assays should only be used in conjunction with other analyses in order to get a fuller indication of pesticide impacts.

1.5.1.3 Culture-Dependent Analyses

Culture-dependent analyses offer another potential approach to determining pesticide impacts on (particularly non-target) microbial communities. Majchrowicz and Poprawski (1993) noted that nine fungicides each had varying levels of impacts on the growth of 8 entomopathogenic fungal species (*Beauveria bassiana, Conidiobolus coronatus, C. thromboides, Metarhizium anisopliae, Paecilomyces farinosus, P. fumosoroseus, Scopulariopsis brevicaulis and Verticillium lecanii*). Culture-dependent approaches were also used by Ekelund (1999) and Ekelund *et al.* (2000) to determine that the fungicides fenpropimorph and propiconazole had significant deleterious effects on bacterivorous, but not fungivorous, protozoa. Fenpropimorph has also been found to inhibit fungal hyphal growth during the first 10 days post-application (Thirup *et al.*, 2001). Additionally, the authors found that bacterial growth was inhibited at 17 days post-application and stimulated 56 days after amendment. Adetutu *et al.* (2008) showed that the impacts of the broad-spectrum fungicide

azoxystrobin on culturable fungi varied with the concentrations of compound applied. Culture-dependent methods have the big advantage of being able to determine whether pesticides can have direct impacts on non-target organisms. However, these approaches also suffer from the problem that only a small percentage of the total microorganisms within the environment are currently culturable. Janssen *et al.* (2002) noted that currently it is considered that less than 5% of the total soil bacterial community are culturable. This therefore makes it impossible to determine how impacts on culturable microorganisms relate to the structure and functioning of wider natural ecosystems.

1.5.2 Molecular Methods

In order to try and combat the problems associated with culture-dependent techniques, it has become common practice in research to also perform culture-independent molecular analyses as well in order to determine how a particular compound is affecting the **structure** or **function** of soil microbial communities. Two particular areas that are becoming of increasing interest are the possible impacts of pesticides on non-target microbial species, and the isolation and identification of organisms capable of degrading different pesticide compounds. Some of the methods used to carry out these culture-independent analyses include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphisms (T-RFLP), single-strand conformation polymorphisms (SSCP), and stable isotope probing (SIP).

1.5.2.1 Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE)

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are two methods that have been previously used to monitor the non-target impacts of pesticides on microbial community structure. These methods involve the separation of PCR products based on their sequence. For DGGE, the PCR products are first loaded into a gel containing an increasing concentration of a denaturant. As the samples move through the gel the DNA (or cDNA) will begin to melt at different distances down the gel depending on its base composition (Muyzer *et*

al., 1996). Differences in the fragment denaturation distances occur due to factors such as the number of G-C bonds (which are triple bonds) compared with A-T bonds (which are double bonds) within a sequence. TGGE acts in the same way as DGGE but uses a linear temperature gradient to separate the different fragments (Muyzer and Smalla, 1998) instead of a denaturant gradient. The resulting gel will show a series of DNA bands representing different sequences which can give an indication of the structure and diversity of a particular population. The main advantages of using DGGE and TGGE approaches are that changes in the microbial structure can be easily observed by changes in the gel banding patterns, and following this the bands can be excised and used for cloning and sequence analysis. However, it has been observed that a single band can be representative of multiple microbial species, and therefore they should only be used as general indicators of microbial community structure (Jousset *et al.*, 2010).

An example of the use of DGGE to monitor the impacts of pesticides on natural ecosystems can be seen in the work of Seghers *et al.* (2003) who observed that soils with 20-year histories of atrazine and metalochlor application had significant differences in bacterial community structure compared with un-amended control soils. Further work by Saeki and Toyota (2004) found that the sulfonyurea herbicide, bensulfuron-methyl, significantly affected the DGGE gel patterns obtained for paddy field soil and surface water bacterial communities. Valle *et al.* (2006) were able to detect shifts in soil bacterial community structure resulting from the application of another sulfonyurea herbicide, azimsulfuron. More recently, Wang *et al.* (2010b) used DGGE to determine that two more sulfonylurea herbicides chlorsulfuron and imazosulfuron significantly affected the bacterial community structures of two agricultural soils during a 1-month experimental period.

1.5.2.2 Terminal Restriction Fragment Length Polymorphisms (T-RFLP)

Terminal restriction fragment length polymorphisms (T-RFLP) analysis has also been used to determine the impacts of pesticides on non-target microbial community structures. This technique involves the restriction digestion of fluorescently-labelled PCR products to produce labelled terminal

restriction fragments (TRFs) of different lengths. The fragments are then separated out based on size using either capillary or gel electrophoresis methods, and then detected using an automated analyser. The results of this analysis can then be displayed as a chromatogram (Osborn *et al.*, 2000) with the size of the fragment (in base pairs) along the *x* axis and the intensity of the label (representing the prevalence of a particular fragment size in the community) along the *y* axis. A test assay by Rousseaux *et al.* (2003) showed that T-RFLP analysis could illustrate the changes in soil bacterial community structure following the application of the herbicide, 4,6-dinitroorthocresol. Zhang *et al.* (2008) showed that the insecticide cypermethrin significantly altered the structure of the bacterial community within cucumber phyllosphere soils. T-RFLP analysis showed that the TRFs at 58, 62, 89, 99, 119, 195, 239, 311, 340, and 473 bp increased in intensity following amendment whereas the TRFs at 44, 51, 96, 223, 306, and 338 bp were not present in treatments containing cypermethrin. T-RFLP analysis suffers from the same major drawbacks as DGGE and TGGE. Each TRF can be representative of multiple different organisms, and different strains of the same microbial species can produce different TRF sizes.

1.5.2.3 Single-Strand Conformation Polymorphisms (SSCP)

Single-Strand Conformation Polymorphisms (SSCP) analysis was originally developed to monitor mutations within human genes, but has since been adapted for use as a less labour-intensive alternative to DGGE or TGGE to monitor soil microbial community structures. SSCP, like TGGE and DGGE is a gel electrophoresis-based method which separates different single stranded DNA based on the natural secondary structures they form under non-denaturing conditions, which is determined by the by the nucleotide sequence of the fragment (Schwieger and Tebbe, 1998). However, currently SSCP has been used more to identify specific target organisms that show a resistance to pesticides, or to identify pest organisms on specific plants. One possible reason for this is that there can be high levels of DNA reannealing particularly using samples containing high DNA concentrations (Selvakumar *et al.*, 1997) and heteroduplex DNA fragments may be formed by different PCR fragments with similar sequences (Lee *et al.*, 1996).

1.5.2.4 Stable Isotope Probing (SIP)

Unlike the previous methods described which have been widely used to determine non-target pesticide impacts on microbial community **structure**, stable isotope probing (SIP) can be used to monitor the effects of pesticide application on specific microbial **functions** in the environment, or more commonly, to monitor pesticide biodegradation and/or fate within environmental systems. The process involves the application of a compound with a stable isotope label (such as ^{13}C) attached. Samples are then taken to monitor the incorporation of the labelled compound into the DNA or rRNA of the microbial community which acts as an indicator of microbial activity (Lueders *et al.*, 2004). Density gradient cell centrifugation methods can then be use to separate the labelled and un-labelled genetic material. This material is then usually used for further analyses such as gene cloning and/or sequencing (Dumont and Murrell, 2005).

Cupples and Sims (2007) used ^{13}C-based SIP in conjunction with T-RFLP to determine that bacteria from the β-Proteobacteria were the predominant members of the soil community involved in the degradation of the pesticide 2,4-D. Lerch *et al.* (2011) built upon this by using ^{13}C-based SIP to determine that the bacterium *Cupriavidus necator* JMP134 preferentially degraded the 2,4-D when in the presence of the compound plus ^{13}C-labelled glucose. Additionally, the presence of labelled glucose in the environment was found to have little impact on the utilisation of ^{13}C-2,4-D compared with soils containing no glucose. Research by O'Malley *et al.* (2007) used ^{15}N SIP to analyse the degradation of the biocide polyhexamethylene biguanide (PHMB) by two enriched microbial consortia. The authors were able to monitor the degradation of PHMB and concluded that species from the genera *Sphingomonas* and *Azospirillum* sp. were required for the consortia to grow.

1.5.2.5 Next-Generation Sequencing

Culture –independent methods, coupled with cloning and sequencing techniques have provided extensive information to researchers about how microbial communities (particularly non-target organisms) can be affected by the application of pesticides. However, over recent years the

development of "next-generation" sequencing techniques has further increased the ability of scientists to monitor the dynamics of overall and active microbial communities under a range of conditions.The processes used in these methods can involve PCR amplification of extracted DNA, cDNA production from extracted rRNA and mRNA (Urich *et al.*, 2008) followed by direct pyrosequencing, or even direct pyrosequencing from extracted bulk mRNA (Gifford *et al.*, 2011). The latter of these two approaches presents a highly desirable option for the future as it eliminates the inherent biases associated with any PCR-dependent method. However, although these techniques are now becoming more commonly-used for characterising distinct microbial communities, they are still expensive to perform compared to e.g. T-RFLP plus cloning and sequencing, and as result there is currently little or no published data regarding the use of them to determine pesticide impacts on defined ecosystems.

1.6 The Strobilurin Fungicides

1.6.1 Introduction

The strobilurin fungicides represent one of the most important groups of pesticides currently in use worldwide. The first strobilurin compounds, azoxystrobin and kresoxim methyl, were released in 1996. In 1999, sales of strobilurins had totalled US$620 million worldwide. By 2002, a further six compounds (metominostrobin, trifloxystrobin, picoxystrobin, pyraclostrobin, famoxadone, and fenamidone) had been released (Bartlett *et al.*, 2002). Since then, other notable releases include fluoxastrobin and dimoxystrobin in 2003 (Suty-Heinze *et al.* 2004; Balba, 2007) and orysastrobin in 2006 (van Ravenzwaay *et al.*, 2007). The chemical structures of the strobilurin fungicides are based on those of natural products produced by wood-degrading basidiomycete fungi such as *Oudemansiella mucida* and *Strobilurus tenacellus* and their anti-fungal activities can be either fungicidal or fungistatic. The compounds act by binding to the ubiquinone (Qo) site of cytochrome b which forms part of the cytochrome bc_1 complex in the fungal mitochondrial membrane. This binding disrupts the transfer of electrons from the cytochrome b portion of the complex, to the cytochrome c_1 portion, which subsequently prevents the fungal mitochondria from producing ATP for the cell (Bartlett *et al.*, 2002).

1.6.2 Azoxystrobin, Pyraclostrobin, Trifloxystrobin & Kresoxim Methyl

Azoxystrobin has been a mainstay of worldwide agriculture since its introduction in 1996 and by 1999 it had become the bestselling fungicide in the world with sales of approximately US$415 million (Bartlett *et al.*, 2002). Bartlett *et al.* (2002) further emphasised its importance by stating that by 2002 the compound was registered for use in 84 different crops, and in over 400 crop/disease systems across 72 countries world-wide. The compound can be applied both pre- and post-emergence (depending on the crop system), normally as a spray formulation. In 2009, azoxystrobin was applied to 1.25 million hectares of land in the UK alone (FERA, 2009). In the UK azoxystrobin is also licensed for use in 2-compound combinations with 4 other fungicides (chlorothalonil, cyproconazole, fenpropimorph, and difenconazole) (British Crop Protection Council, 2008). Pyraclostrobin, trifloxystrobin and kresoxim methyl are all currently licensed for use as broad-spectrum fungicides in the UK. In 2009, 1.5 million hectares of land were treated with pyraclostrobin in the UK. This compares with 519,000 and 154,000 hectares for trifloxystrobin and kresoxim methyl, respectively (FERA, 2009). The structures of azoxystrobin, pyraclostrobin, trifloxystrobin and kresoxim methyl can be seen in Figure 1.6 and their characteristics in the environment can be seen in Table 1.2.

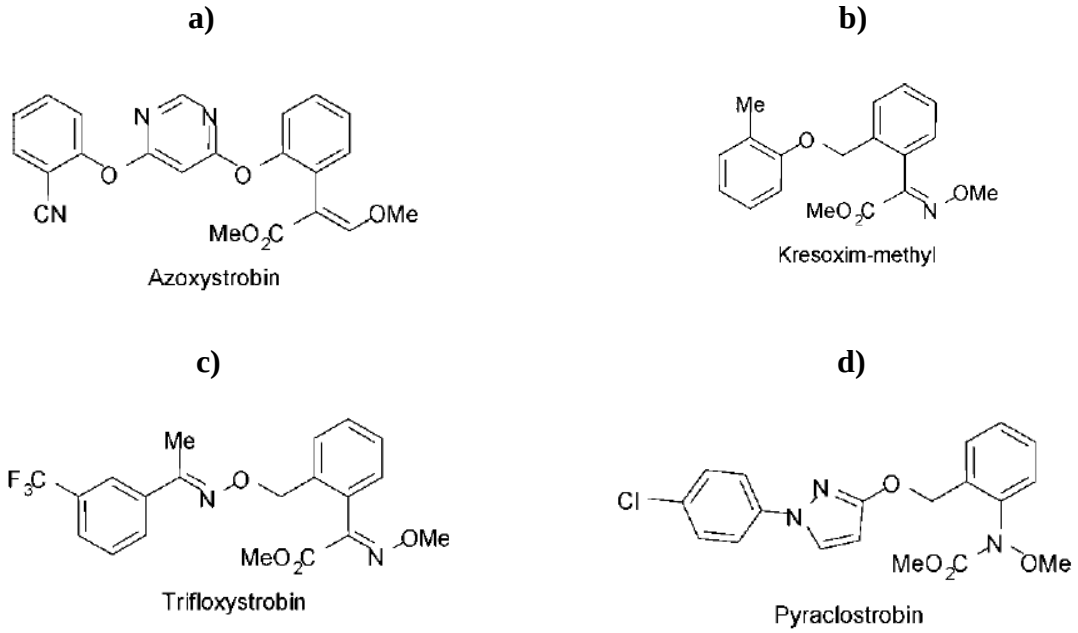


Figure 1.6. The chemical structures of a) Azoxystrobin b) Kresoxim-methyl c) Trifloxystrobin and d) Pyraclostrobin. From: Bartlett *et al.* (2002).

Table 1.2 The environmental characteristics of azoxystrobin, pyraclostrobin, trifloxystrobin and kresoxim methyl. From: British Crop Protection Council (2009)

Fungicide	DT_{50} in Soil (Aerobic) (Days)	Solubility in H_2O (20°C) (mg L^{-1})	K_{oc} Value	Mobility Rating	Kd (ml g^{-1})
Azoxystrobin	28	6.0	300 – 1690	Low – Non-mobile	1.5 – 23.0
Pyraclostrobin	8 – 55	1.9	6000 – 16000	Non-mobile	160
Trifloxystrobin	4.2 – 9.5	0.61	1642 - 3745	Low – Non-mobile	0.042
Kresoxim Methyl	< 1	2.0	219 - 372	Mobile	-

All of the pesticides described in Table 1.2 are considered to have low non-target toxicity to terrestrial vertebrates. However, toxicity tests have also shown that they are highly toxic to the model fish species *Oncorhynchus mykiss* (rainbow trout). The recorded EC_{50} values for this organism are 470, 190, 6 and 14 ppb for azoxystrobin, kresoxim methyl, pyraclostrobin, and trifloxystrobin, respectively (British Crop Protection Council, 2009).

Current official information regarding the impacts of azoxystrobin on living organisms remains largely restricted to official government agency tests. Furthermore, the vast majority of other research has focussed on the purely fungicidal properties of the compound and/or the development of fungal resistance. Bending *et al.* (2007) recorded that azoxystrobin application at normal field rates resulted in a short-term (≈ 2 month) decrease in soil community dehydrogenase activity. The authors also observed that a DGGE band representing sequences with high sequence homologies to the flagellated protozoa *Paraflabellula hoguae* were absent from azoxystrobin-treated samples. Further work by Adetutu *et al.* (2008) showed that azoxystrobin application could alter the structure of fungal communities from 21 days post-application, with differences still being observed after 84 days.

There have been indications that pyraclostrobin can have toxic effects on non-target freshwater eukaryotes such as freshwater mussels (Bringolf *et al.*, 2007), unicellular algae such as *Pseudokirchneriella subcapitata*, and the aquatic invertebrate *Daphnia magna* (Ochoa-Acuña *et al.*, 2009). However, at the present time no information exists about whether pyraclostrobin application can have significant impacts on non-target microbial communities. As with azoxystrobin and pyraclostrobin, the vast majority of current research involving kresoxim methyl has focussed on either its efficacy against defined pathogens under specific conditions, or on the development of fungal resistance to the compound. However, culture-dependent analysis by Walter *et al.* (2007) did show that the compound could have deleterious effects on the growth of some culturable bacteria (*Xanthomonas*- and *Pseudomonas*-like species), fungi (*Penicillium* sp.), and yeasts (*Cystofilobasidium capitatum*, *Aureobasidium pallutans*, *Phialophora*-like species). At the present time there is no readily available data from research articles available of the possible impacts of trifloxystrobin on non-target organisms.

1.6.3 Other Strobilurin Compounds

There are also many other strobilurin compounds that have either been in use for several years but only in specific countries, or that are currently being developed and have not yet obtained the necessary licenses. For example enestroburin has been licensed for use as a broad spectrum fungicide in China since 2002 but, although patents for its usage have also been obtained by some countries in America and Europe (Wang *et al.*, 2010a), by the end of 2009 it was still not in use in the UK (FERA, 2009). Picoxystrobin is also a broad-spectrum fungicide. When it was first released in the year 2000 it was the first broad spectrum fungicide to encompass both xylem systemicity and vapour activity which enhanced its distribution and, as a result, its spectrum of activity (Godwin *et al.*, 2000). There is currently no research data available that investigated whether picoxystrobin can have significant impacts on non-target organisms. Dimoxystrobin, however, has been previously found to significantly decrease soil urease and dehydrogenase activities over a 56-day period (Jastrzębska and Kucharski, 2007). Some strobilurin fungicides are used on more specific crop/disease systems. For example,

metominostrobin and orysastrobin are both solely registered for use in Japan for combating sheath blight and blast infections of rice plants (Bayer Crop Science, 2010; Stammler *et al.*, 2007). There is no information readily available about whether metominostrobin may have deleterious impacts on non-target organisms, but van Ravenzwaay *et al.* (2007) noted that orysastrobin was capable of producing a reversible increase in tumour incidences in the duodenum of rats and mice.

1.7 Aims and Objectives of the Project

The broad aim of work described in this thesis was to determine how the application of pesticides affects the structure and function of soil microbial communities across different trophic levels using the strobilurin fungicide, azoxystrobin as a model compound.

The work presented is split into three self-contained parts, each with their own defined aims, methods, results and discussion sections.

Part 1 (Chapter 2) aimed to determine the effects of different azoxystrobin concentrations on microbial communities from different trophic levels as well as on broad-scale aspects of the soil community such as biomass and dehydrogenase activity. The specific questions considered were:

i. *Does the concentration of the fungicide azoxystrobin affect the resistance and resilience responses of soil microbial communities from different trophic levels, and are there any indications that impacts on one group of organisms are having knock-on effects on another group or groups?*

ii. *Is there a relationship between azoxystrobin concentration and the diversity of soil microbial communities over time?*

iii. *Do broad-scale analytical methods give similar indications of pesticide impacts as finer-scale molecular methods?*

The main aim of Part 2 (Chapter 3) of this thesis was to build on the work described in Chapter 2 by using T-RFLP analysis in combination with both extracted DNA and RNA to determine the impacts of a single azoxystrobin dose on both the overall and active communities of microorganisms from different trophic levels.The questions considered in Chapter 3 were as follows:

i. *Does the application of azoxystrobin have significant deleterious impacts on active fungal and nematode communities as determined by RNA-derived T-RFLP analyses?*

ii. *Do impacted TRFs in the fungal and nematode communities show high sequence homologies to impacted sequences from the clone libraries produced in Chapter 2?*

Despite its widespread use across the world, there is currently minimal information available about individual microbial genera or species that are capable of degrading azoxystrobin and related strobilurin fungicides. Therefore, Part 3 (Chapter 4) involved the isolation and culturing and identification of azoxystrobin degrading organisms, and the use of T-RFLP analysis to identify possible azoxystrobin degraders amongst the non-culturable bacterial community. More specifically, the work in Chapter 4 aimed to answer the following questions:

i. *Will sequential enrichments with azoxystrobin result in adaptation within the microbial community leading to increased degradation rates?*

ii. *Will any azoxystrobin degrading organisms that are cultured also be active in situ?*

iii. Do the degradation rates of azoxystrobin in culture depend upon the availability of alternative carbon and nitrogen sources?

iv. Will culture-independent methods highlight additional possible azoxystrobin-degrading organisms within the non-culturable bacterial community?

A summary of the techniques and analyses used throughout this project can be seen in Table 1.3

Table 1.3 A table of the microbial groups analysed, and analytical techniques used in this project.

Chapter	Microbial Groups Analysed	Non-Molecular Methods Used	Molecular Methods Used	Culture-Dependent Methods
2	Bacteria Fungi Nematodes General Eukaryotes Archaea Pseudomonads	Biomass Dehydrogenase Activity HPLC (Compound Degradation)	T-RFLP (DNA) Clone libraries (Fungi and Nematodes) qPCR (Bacteria and Fungi)	N/A
3	Bacteria Fungi Nematodes General Eukaryotes	Biomass Dehydrogenase Activity HPLC (Compound Degradation)	T-RFLP (DNA and cDNA)	N/A
4	Bacteria	HPLC (Compound Degradation) ^{14}C-Azoxystrobin degradation	T-RFLP (DNA) Single Colony Sequencing Clone Library	Liquid Enrichment Cultures Growth on solid media

Chapter 2

The Impacts of Azoxystrobin Concentration on Soil Microbial Communities

2.1 Introduction

2.1.1 Stress Impacts on Living Organisms

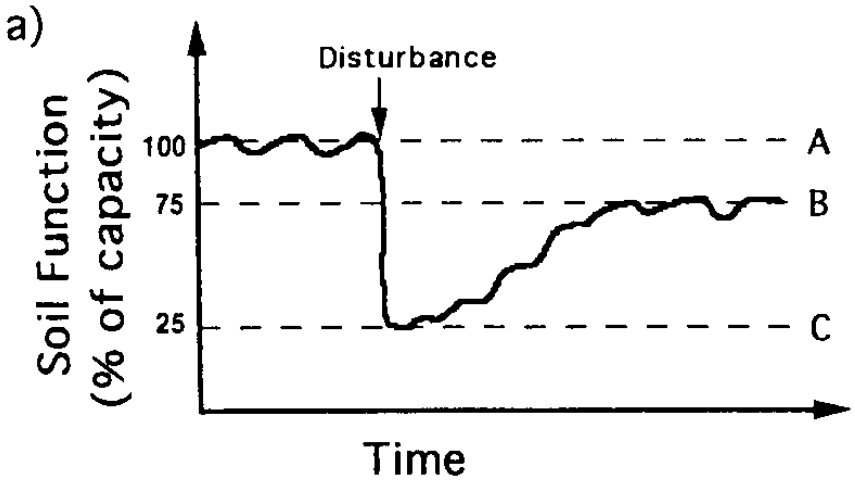
Every community of living organisms is subjected to a range of factors (known as stresses) that have the potential to deleteriously impact some or all of the organisms present, potentially affecting its structure, diversity, and function. These impacts may be classed as direct or indirect. Direct stresses result from the interaction of a particular organism (e.g. a fungus) with a defined stressor (e.g. a fungicide). In this scenario, the impact of this stress could be that the abundance of the stressed organism is markedly reduced within the community, or it could be removed altogether. This, in turn could exert indirect stresses on the community. For example, the fungus may have acted as a food source for higher groups of organisms e.g. nematodes, which would therefore be deleteriously affected by its removal from the community.

Stress effects are usually placed in to three broad categories: biological, physical and chemical. Biological factors can include the introduction of highly-competitive non-native species or the excessive proliferation of, for example, a predator or pathogen. Physical factors include fluctuations in climatic conditions (e.g. extreme temperatures) or in localised conditions (e.g. changes in soil matric potential). Aspects such as changes in pH and salinity, changes in oxygen levels, and contamination with xenobiotic compounds are examples of chemical factors (van Bruggen and Semenov, 2000).

Community responses to stresses can be considered in terms of their resistance and resilience to a particular impact. Resistance refers to the capacity of a community to maintain its size, composition,

and function in the presence of a disturbance, whereas the term resilience describes the ability of an impacted community to recover its initial structure and function following a disturbance (Seybold *et al.*, 1999).

Seybold *et al.* (1999) described resistance/resilience scenarios that may occur in soil communities following a disturbance (Figure 2.1). Figure 2.1a illustrates the general concept of resistance and resilience responses. In this figure following a disturbance the function of the soil decreases noticeably from 100% of the soil function capacity (A) to 25% (C) which is indicative of a low community resistance. Over time, the soil function gradually increases which is representative of a resilience response. In this example, the soil community exhibits a limited resilience to the disturbance as the soil functional capacity at the end of the monitoring period is only 75% that of the un-disturbed soil (B). Figure 2.1b details how soil communities with high natural resistance to stresses, or a low initial resistance but a high resilience, may respond to disturbance.



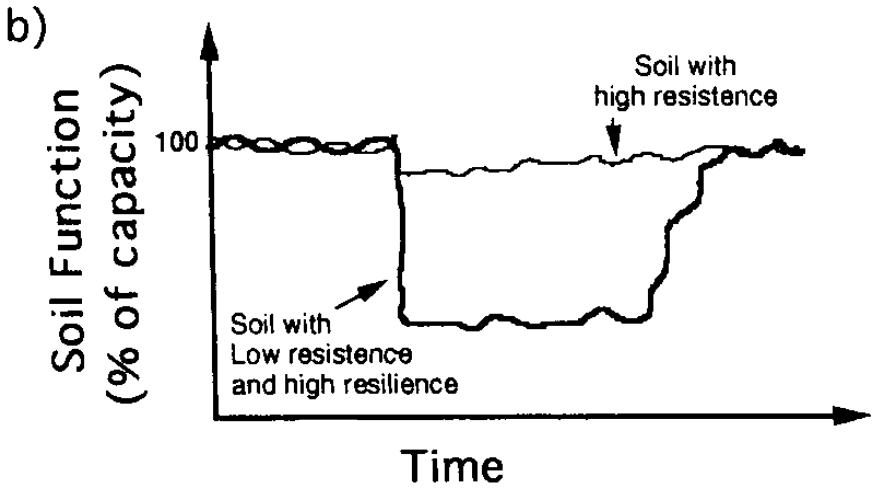


Figure 2.1 Hypothetical resistance and resilience responses of soil communities following disturbances. a) The general concept of resistance and resilience responses. b) Responses in soils with different resistance and resilience capacities. Adapted from Seybold *et al.* (1999).

It has been suggested that the resistance and/or resilience of soil communities to disturbances could be influenced by the initial biodiversity of the system. For example, Girvan *et al.* (2005) studied the impacts of benzene application on the mineralisation of 2,4 dichlorophenol (2,4-DCP) in soils with differing diversities. In both soils the initial resistance to the perturbation was low as mineralisation levels dropped to only approximately 12% of the control in both soils after 1 week. However, in the higher diversity soil mineralisation had recovered to the same levels as the control 9 weeks post-application. In contrast, after 9 weeks the activity in the low diversity soil had not improved relative to the controls. This suggested that the higher diversity soil had a higher natural resilience to the disturbance than the lower diversity one (Girvan *et al.*, 2005).

2.1.1.1 Microbial Community Responses to Stress

The impacts of biotic and abiotic stresses on microbial communities have also been studied, although current knowledge in this area is much lower than for plants. Giller *et al.* (1998) hypothesised two possible relationships between microbial diversity and stress levels (Figure 2.2). Scenario 1 described an extinction response to stress in which the diversity of a microbial community is negatively correlated to an increase in stress levels. The second scenario describes a hump-backed "competitive

exclusion" response. In this situation, a mild stress would enhance the proliferation of resistant organisms, resulting in a higher level of diversity than in a non-stressed system. Alternatively, the removal of dominant organisms from the environment as a result of the stress could promote an increase in diversity as other (normally less-abundant organisms) proliferate to fill the niche. However, once the strength of a stress exceeds a level that even the majority of the resistant organisms can cope with, the community diversity declines rapidly (Giller *et al.*, 1998).

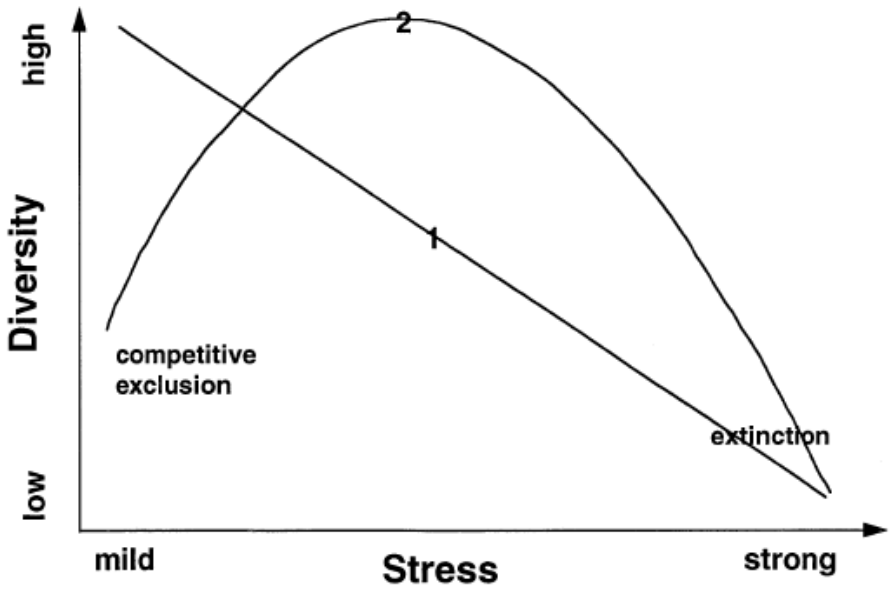


Figure 2.2 Two hypothetical scenarios of the impacts of perturbation on the diversity of a community. From: Giller *et al.* (1998).

Previous research has described how the impacts of a disturbance on a community could be dependent on its scale along with its duration, and that long-term, chronic disturbances could initiate a series of microbial successions which, over an extended period of time, could potentially result in the permanent alteration of the community structure (van Bruggen and Semenov, 2000; Johnsen *et al.*, 2001). Furthermore, Johnsen *et al.* (2001) emphasised the need to determine whether any disturbances are only transient and/or affect a very small area as opposed to severe, widespread impacts that would require methods for mitigation and/or remediation to be developed. It was concluded that large field-scale studies were required in order to determine this.

Permanent changes such as these could also result in alterations in function, such as nutrient cycling. This could, in turn, affect the growth of crops which could be of great economic and ecological significance. Indeed, Sharma *et al.* (2011) reviewed how the structure and diversity of microbial communities could be used as a measure of soil quality and noted how the relationship between community diversity, functional redundancy, and responses to perturbation still needs to be determined.

A range of model stresses have been used to elucidate the responses of microbial communities to stress. These include changes in soil conditions (e.g. pH or water content changes), the addition of either a natural element (such as copper) or a xenobiotic compound (such as a pesticide). Such stresses can be altered with a high level of accuracy and be monitored over an extended period of time to allow both direct, and potential indirect impacts to be analysed.

Degens *et al.* (2001) subjected cropped soils to different stresses (pH decreases, NaCl concentration increases, Cu contamination, multiple wetting and drying cycles, and multiple freezing and thawing cycles). They observed that the "hump-backed" response shown in Figure 2.3 was apparent in soils subjected to the wetting/drying and freezing/thawing cycles (Degens *et al.*, 2001). More recently, research by Zhang *et al.* (2009c) showed that bacterial diversity increased slightly under mild cadmium stress compared to the control. Additionally there was a marked decrease in diversity under higher cadmium stress which seemed to support the "hump-backed" relationship proposed by Giller *et al.* (1998).

Zhang *et al.* (2010) observed an increase in bacterial diversity in both eroded and un-eroded soils 28 days after the application of a single 500 µg of g^{-1} soil dose of copper. In both the eroded and un-eroded soils, the average bacterial diversity in the amended soils was 111% that of the un-amended

controls. This increase in diversity may have represented the first part of the "hump-backed" relationship proposed by Giller *et al.* (1998) although further copper concentrations would have been required to confirm this.

A further potential consideration when analysing the impacts of stress on microbial communities is the functional redundancy of the community. This describes the ability of a structurally altered community to maintain its original functional capabilities due to the presence of multiple different groups of organisms that are able to perform the same processes equally well (Allison and Martiny, 2008). Therefore, it has been suggested that systems with a high functional redundancy would still be able to carry out essential processes (e.g. organic matter decomposition) to the same level even after their diversity has been reduced by perturbation (Nannipieri *et al.*, 2003). This would therefore be indicative of a high level of resistance within the community.

Evidence supporting functional redundancy within microbial communities was provided by Atlas *et al.* (1991). The authors found that even soils that had reduced diversity due to perturbation by either crude oil, leaded petrol, or the herbicide 2,4,5-tricholorophenoxyacetic acid (2,4,5-T) showed resilience to the impacts. The authors found that 21 days post-application the carbohydrate, carboxylic acid, and amino acid utilization capabilities of the amended soils were not significantly different to those of the un-amended controls.

Wertz *et al.* (2007) found that lower community diversity did not deleteriously impact resilience of nitrite-oxidising microbial communities following a 24-hour heat-shock disturbance. There was low initial resistance to the disturbance, as illustrated by a 40% decrease in nitrite-oxidising activity within the first 3 hours post-application. However, after 1 month the nitrite-oxidising activity had returned to levels that were not significantly different from the controls. This appears to show that nitrite-oxidising community had a high level of resilience to this stress. The authors concluded that this may be due to a high level of functional redundancy within the nitrite-oxidising organisms. More recently,

Cravo-Laureau *et al.* (2011) tested the ability of two different microbial communities found in activated sludge sites (one with high and one with low microbial diversity) to degrade 14 different poly-aromatic hydrocarbons (PAHs). They found that degradation was higher in the lower diversity sludge. This raised the point that, although the overall diversity in this treatment was lower, there may have been functional redundancy amongst the narrow niche community involved in PAH degradation.

Whilst the impacts of specific stresses on specific species or groups of organisms have been studied widely, there is little information on how stress impacts on one microbial group can have indirect impacts on another, particularly across different trophic levels. This represents a sizeable knowledge gap as microorganisms play essential roles in all biotic ecosystems.

2.1.2 The Importance of Microbial Biodiversity

Microbial communities perform a number of functions can often be hugely beneficial to mankind and have been termed "ecosystem services" (Costanza *et al.*, 1997). They are involved in the turnover of the microbial biomass, soil nutrients (both in terms of utilisation and excretion), and soil organic matter (Griffiths, 1994). They can also remediate xenobiotic compounds and other pollutants (van Bruggen and Semenov, 2000), are widely involved in soil formation and, particularly in the case of marshland and floodplain soils, atmospheric gas regulation (Costanza *et al.*, 1997). This latter point is becoming ever more important as fears about global climate change grow (Mikola *et al.*, 2004).

Chapin III *et al.* (2000) showed that biodiversity, ecosystem services and human activities are intrinsically linked (Figure 2.3). The authors noted how human activities (1) are increasingly responsible for major ecological changes worldwide (2). These changes have the capacity to significantly alter the natural biodiversity of a system (3), which can in turn render the ecosystem susceptible to succession by invasive, non-native species and the proliferation of sub-dominant species to produce communities with altered structures and functional capabilities. This, therefore leads to altered ecosystem processes (5) and subsequently the provision of ecosystem services (4, 6) and/or further alterations in community biodiversity (7).

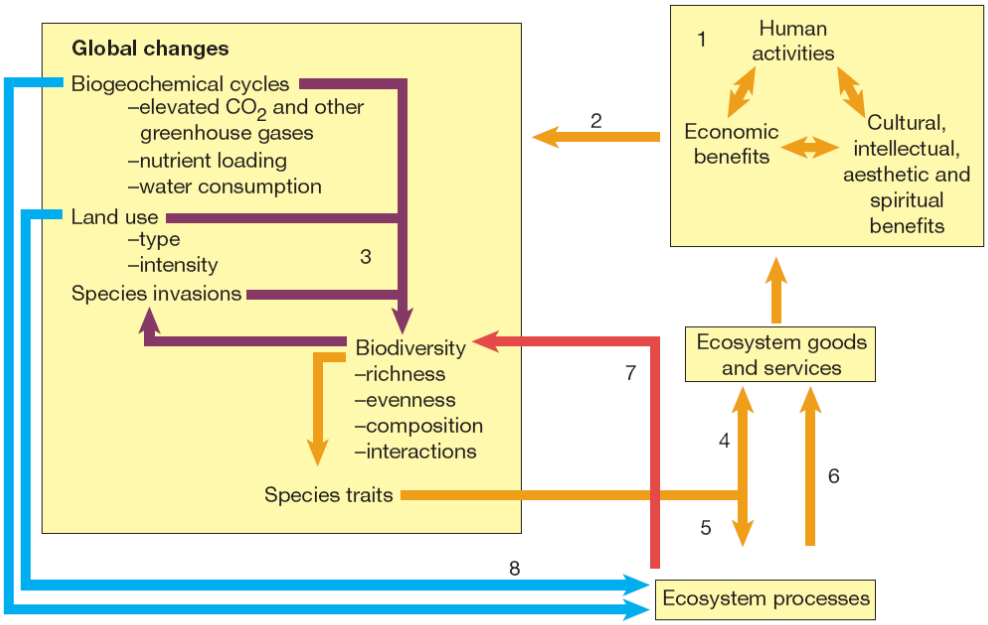


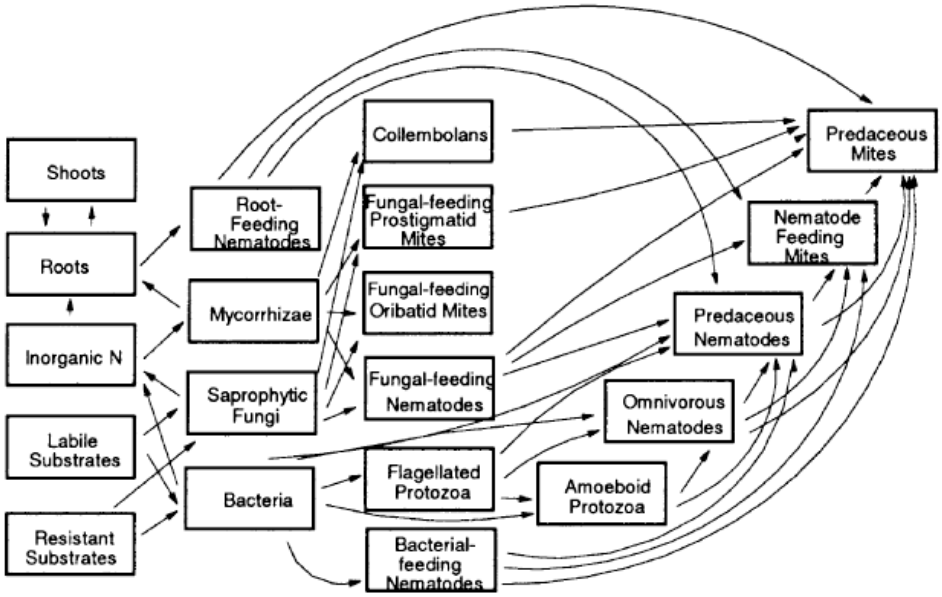
Figure 2.3 The links between human activities, biodiversity and ecosystem services. From: Chapin III *et al.* (2000).

Bailey (2011) built upon these theories by highlighting the role that genetic variation can play in the provision of ecosystem services. The author stated that specific genetic variations within a genus/species etc can influence their interactions between other members of a community or ecosystem and subsequently affect aspects of the community function such as nutrient cycling and energy flow. Therefore, any changes to the community structure and/or diversity in response to stress could have indirect effects on these processes, and eventually on the provision of ecosystem services.

One major role that microorganisms play is as components of food webs (van Bruggen and Semenov, 2000). Using data from Scots pine forest soils, Berg and Bengtsson (2007) described how microbial food webs could be far more complex and variable than models currently predict, and this variability could be significantly affected by factors such as organic matter turnover. Neher (1999) compared the food webs of grassland and cultivated wheat soils (Figure 2.4). Grassland soils (Figure 2.4a) possess generally more complex webs with greater numbers of primary producers and higher trophic level microorganisms (e.g. nematodes and protozoa) than cultivated soils (Figure 2.4b). Soil microorganisms support food webs such as these by acting as saprotrophs of plant organic matter

inputs, and subsequently act as a source of food for organisms from higher trophic levels. For example, saprotrophic fungi can be a food source for collembolans, along with some species of mites and nematodes. These can in turn be a food source for other species of predatory nematodes and mites (Figure 2.4a). Therefore, if a particular stress was applied to the soil system described in Figure 2.4a which resulted in the removal of fungal-feeding nematodes from the community, then this in turn could have indirect effects on the populations of predaceous and nematopathogenic mites, predaceous nematodes as well as the saprotrophic and mycorrhizal fungi.

a)



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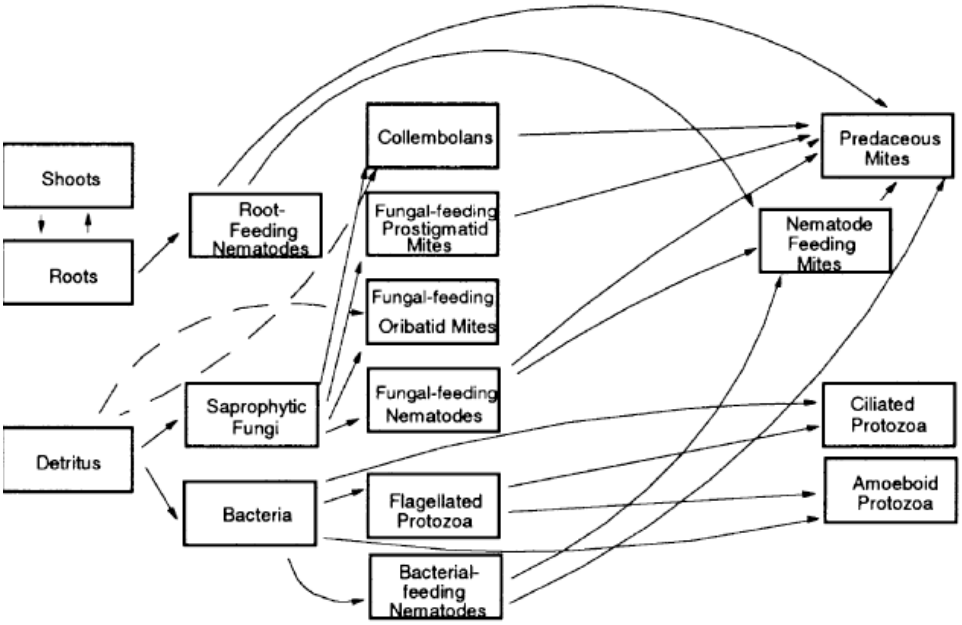


Figure 2.4. Food webs for a) grassland and b) cultivated wheat soils. From: Neher (1999).

Indeed, previous research has shown that grazing habits of nematodes and other protozoa on prokaryotic organisms such as bacteria are widely considered to be beneficial for soil nutrient cycling. These benefits can include increased carbon mineralisation (as a result of the increased turn-over rate of the grazed organisms) and increased nitrogen mineralisation resulting from the excretion of excess N-containing compounds (Mikola *et al.*, 2002). In turn, these releases not only provide nutrients for the soil microfauna, but also for higher plants. For example, increased nitrogen supplies to plant shoots resulting from protozoal grazing have previously been found to increase the shoot biomass (Bonkowski, 2004). Therefore, any impacts on an aspect of these highly complex webs of soil microbial interactions could potentially have far reaching implications. This makes them extremely valuable both environmentally and economically.

2.1.3 Impacts of Organic Pollutants on Soil Microbial Communities

It is thought that microbial communities may be more susceptible to some stresses (e.g. heavy metal concentrations) than plants and other larger organisms (Giller *et al.*, 1998). These stresses could result

from the direct toxic effects of a compound and/or indirect effects caused by impacts to another section of, for example, a plant/soil system. Examples of both direct and indirect impacts of agricultural practices were observed by Ahemad and Khan (2011). The authors noted that the insecticide pyriproxyfen deleteriously affected the phosphate-solubilising activity of *Klebsiella* sp. strain PS19 when applied at a high concentration (3,900 μg L^{-1}). This solubilising process provides plants with a source of phosphorus that is essential for growth. Therefore pyriproxyfen application at high concentrations could have severe indirect effects on plant growth. Examples such as this serve to illustrate why the use of pesticides and their possible wider impacts on the environment are such major issues. As a result, increasing numbers of studies are looking into how to maintain soil community structure, biodiversity and function whilst still using common agricultural practices (Tscharntke *et al.*, 2005). Many of these studies have employed culture-independent molecular methods to give a fuller indication of pesticide impacts on different microbial communities.

2.1.3.1 The Use of Molecular Methods to Determine Impacts on Microbial Communities

The use of "broad-scale" analytical methods such as biomass and dehydrogenase activity have previously been widely used as indicators of pesticide (and other xenobiotic compound) impacts on microbial communities. However, these techniques can only act as broad indicators of community impacts and therefore, they may fail to illustrate more subtle, but still significant impacts. Therefore, recent research has also focussed on the use of a range of molecular techniques to gain a finer-scale appreciation of the impacts of pesticides and other xenobiotic compounds on the structure and function of soil microbial communities, normally bacteria and fungi. Two early examples of this approach come from Engelen *et al.* (1998) and El Fantroussi *et al.* (1999). Using temperature gradient gel electrophoresis (TGGE) Engelen *et al.* (1998) determined that application of the herbicide Herbogil (dinoterb) (250 g L^{-1}) to a soil impacted the structure of the soil bacterial community. There was no return to the original structure observed after the final sampling time (8 weeks post-application) suggesting that the soil bacterial community had a low resistance and resilience to this compound. El Fantroussi and colleagues (1999) found that the bacterial community structures in soils

that had received long-term (over 10 years) applications of phenyl urea herbicides (Diuron, Diuron + Linuron, or Chlorotoluron) differed significantly from that of a nearby non-treated control. This appears to suggest that some soil communities can have low resistance to pesticide application and that resilience responses can be minimal or non-existent even over a period of several years. However, these responses could have also been (at least in part) due to indirect effects such as changes in the plant community.

The work of Wang *et al.* (2008) studied the impacts of 2 different concentrations (2.38 and 23.8 mg kg^{-1} soil) of the broad-spectrum organophosphorus pesticide methamidophos on the structure of bacterial communities in a silt loam soil. Using amplified ribosomal DNA restriction analysis (ARDRA) the authors found that the pesticide had a significant impact on the bacterial community structure, and that this difference was dependent upon the concentration of pesticide applied.

Zhang *et al.* (2009a) used DGGE to determine that a single 6 ml per mass unit of soil emulsifiable concentrate treatment of the insecticide cypermethrin significantly impacted the bacterial community structure in the pepper plant phyllosphere. These changes were illustrated by both the appearance and disappearance of bands in the DGGE gel following pesticide application. Additionally, 2 bands increased in their relative intensities. More recently, Zeng *et al.* (2011) analysed the impacts of a pentachlorophenol (PCP) on the structure of bacterial and fungal communities within a composting pile. The application of a single dose (50 mg kg^{-1} of compost dry weight) had a significant impact on the fungal community structure as determined by DGGE analysis. In contrast, the bacterial community structure was unaffected by PCP application.

Whilst the use of culture-independent methods to determine pesticide impacts on microbial communities has been well-developed for bacteria and fungi few studies have used these methods for determining impacts on higher trophic level organisms. One study that did use these methods was performed by Bending *et al.* (2007). Using DGGE analysis, the authors noted that following the

application of the fungicide chlorothalonil a band showing sequence homologies to two ciliated protozoa (*Arcuospathidium cultriforme* and *Bresslaua vorax*) was absent. Furthermore, a DGGE band showing a sequence homology to the flagellated protozoa *Paraflabellula hoguae* was found to be removed from the soil community following azoxystrobin application.

However, the majority of studies into the impacts of pesticides on higher trophic level microorganisms have involved the use of culture-dependent techniques. A study by Ekelund (1999) analysed the effects of the fungicide fenpropimorph on bacterivorous and fungivorous protozoa in soil microcosms. The author concluded that the application of the compound may have both direct and indirect impacts on the protozoal community. High concentrations of fenpropimorph (6.6-750 mg L^{-1}) were found to have direct deleterious impacts on the soil protozoal community. Indeed, at concentrations of 20 mg L^{-1} or below there was no significant impact on fungal hyphal growth or on the fungivorous protozoa. However, at lower concentrations only the heterotrophic flagellates were significantly impacted, possibly due to the indirect effects of increased predation and/or competition from ciliated protozoa (Ekelund, 1999). Similar direct effects on bacterivorous protozoa were recorded by Ekelund *et al.* (2000). In experiments where the fungicide propiconazole was applied at normal field rates, the growth of bacterivorous flagellates was significantly reduced whereas there were minimal effects on fungivorous protozoa.

Boucard *et al.* (2004) studied the effects of 2 types of sheep dip (organophosphate and synthetic pyrethroid) insecticides on the survival of bacterial and protozoan communities. The protozoa tested included 4 ciliates, 7 flagellates, and 4 amoebae. The authors found that even low concentrations of both formulations inhibited protozoan survival. Minimum inhibitory concentrations (MIC) of 0.003 and 0.005% (v/v) were recorded in the most sensitive species for the organophosphate and synthetic pyrethroid formulations, respectively. Additionally, all of the treatment samples exhibited the presence of amoebic cysts which are produced by amoeba in response to direct abiotic stresses

(Corliss and Esser, 1974). In contrast, bacterial growth was not significantly affected by either formulation (Boucard *et al.*, 2004).

Whilst the studies described give an indication of possible non-target effects of pesticides on higher trophic level microorganisms, the methods used are severely limited by the fact that not all protozoa and nematode species are culturable (Ekelund, 2002). Additionally, the abundance of these non-culturable nematodes and protozoa within the soil community remains unknown (Fredslund *et al.*, 2001). This represents a significant knowledge gap, especially as the evidence from culture-dependent studies detailed above has shown that protozoal communities can be significantly impacted by pesticide application. This emphasises the need to utilise the available culture-independent molecular tools to assess the responses of whole communities following pesticide application, and to tease apart the mechanisms underlying these impacts. The toxicity effects could result from the direct action of the pesticide, or from indirect effects (both within and across different trophic levels) resulting from, for example, the removal of a major food source. Indeed, Bailey (2011) emphasised the importance of integrating observed changes in community and ecosystem genetics with broader concepts such as impacts on ecosystem services.

2.1.4 Aims and Objectives

The work presented here investigated the resistance and resilience responses of a range of microbial communities from different trophic levels (bacteria, fungi, archaea, pseudomonads, nematodes, and general eukaryotes) to perturbation by a range of concentrations of the fungicide azoxystrobin. Bacteria and fungi were chosen for analysis as they have previously been studied in relation to pesticide impacts, they are essential for the functioning of soil ecosystems, and may also potentially be involved in the remediation of the compound. Archaea are also involved in many essential soil processes such as methanogenesis (Kent and Triplett, 2002), denitrification, and ammonia oxidation (Hallin *et al.*, 2009). Pseudomonads are fast-growing organisms and therefore were chosen for analysis as they may have given clearer indications of bacterial community impacts following

azoxystrobin application. Nematodes and general eukaryotes were studied as members of a higher trophic group. This therefore introduced potential predator organisms to the research which could also be susceptible to indirect impacts resulting from impacts on prey organisms. The general eukaryote analysis was also chosen as it encompassed a wide range of organisms including flagellated and ciliated protozoa, amoebae, and algae along with nematodes. Furthermore, members of the flagellated protozoal group have previously been found to be susceptible to direct, non-target impacts by pesticides (Ekelund, 1999; Ekelund *et al.*, 2000).

Azoxystrobin is an important model pesticide as it is widely used worldwide against a broad range of fungal crop pathogens (Bartlett *et al.*, 2002). Additionally, previous research has indicated that azoxystrobin can also deleteriously affect some protozoal species (Bending *et al.*, 2007) although whether any impacts on higher trophic level groups result from direct toxicity, indirect effects (or both) and how these effects alter with application concentration, remains to be elucidated.

The research carried out here aimed to answer the following questions: 1) Does the concentration of the fungicide azoxystrobin affect the resistance and resilience responses of soil microbial communities from different trophic levels, and are there any indications that impacts on one group of organisms are having knock-on effects on another group or groups? 2) Is there a relationship between azoxystrobin concentration and the diversity of soil microbial communities over time? 3) Do broad-scale analytical methods give similar indications of pesticide impacts as finer-scale molecular methods?

2.2 Materials and Methods

2.2.1 Soil collection and preparation

Soil was collected from Hunts Mill field at the Wellesbourne Campus of the University of Warwick School of Life Sciences, UK during January 2008. The soil is a sandy loam of the Wick series with a composition of 73% sand, 12% silt, and 14% clay (Bending *et al.*, 2007). The field has been managed

as set-aside for over a decade (and thus has received no pesticide applications) and is covered with common grass clover ley. Soil was collected from the top 20 cm to comply with OECD guidelines for soil sampling in agricultural soils (OECD, 2011). The soil was then placed on a clear plastic sheet for 24 hours to reduce the moisture content before sieving through a 2 mm sieve. The soils were re-wetted to a matric potential of -33 kPa prior to azoxystrobin application (Bending *et al.*, 2006). This equated to a soil water content of 13.5%.

2.2.2 Soil spiking, storage and sampling

Soils were spiked with a range of azoxystrobin (Greyhound Chromatography, Birkenhead, UK) concentrations (0, 1, 5, 10 and 25 mg kg^{-1} soil). 5 mg kg^{-1} represents the maximum recommended dose of azoxystrobin in the top 1 cm of soil (Bending *et al.*, 2007), so a concentration series was used that encompasses a range above and below this value.

For direct soil spiking, azoxystrobin was first dissolved in acetone and applied to soil using a solvent:soil ratio of 1:20. This value was proposed by Northcott and Jones (2000) as the borderline value between low solvent volume spiking methods, and high solvent volume spiking methods. This borderline ratio was chosen for this experiment to maximise the quality of compound distribution as exhibited by high solvent volume methods, whilst also trying to achieve the minimal residual solvent effects on the microbial community commonly associated with low solvent volume procedures (Northcott and Jones, 2000).

For each pesticide concentration, the dissolved pesticide was initially added to one quarter of the soil, with the remaining three quarters being added gradually during a 15-minute mixing period. Following this, the bags were left in a fume hood for 2 hours to allow evaporation of the acetone.

Following spiking, samples were divided into 120 g portions and placed into sterile 250 ml glass Duran bottles, and the weights recorded. These weights were monitored every 2 weeks as a measure

of the water content of the soil and, if required, sterile distilled water was added to return the sample to its original weight. Each bottle was wrapped in aluminium foil and stored at 15°C in the dark in a fully randomised design. 4 replicates of each treatment were destructively-sampled at time 0, and then on a monthly basis for a period of 4 months.

2.2.3 Soil microbial biomass

For this experiment, soil biomass-N was measured using the method of Joergensen and Brookes (1990). This method is based on the premise that during $CHCl_3$ fumigation ninhydrin-reactive compounds (such as ammonium, amino acids, peptides and proteins) are released from the biomass. The amounts of these compounds that are extractable using the solvents mentioned above is considered to correlate strongly with the initial soil biomass-C, thus making this a rapid method for estimating the SMB (Joergensen and Brookes, 1990).

Briefly, 20 g samples of soil in glass beakers were placed in a dessicator along with a beaker containing 50 ml of alcohol-free $CHCl_3$ (Sigma Aldrich, UK). The dessicator was evacuated causing the $CHCl_3$ to boil vigorously after which the tap was closed, and the samples left to fumigate in the dark for 24 hours. A non-fumigated set of 20 g samples were placed in Duran bottles and stored in the dark for 24 hours.

Following fumigation, fumigated samples were transferred to Duran bottles and 80 ml K_2SO_4 was added to both fumigated and non-fumigated samples. The samples were placed in an inverted mixer for 30 minutes to complete the extraction, and filtered through Whatman No. 1 filter paper until 20 ml of filtrate was collected. The filtrates from the samples were then stored in a freezer at -20°C for 24 hours.

For the ninhydrin assay samples were set up containing the filtrate (or blank) (0.75 ml), a citric acid buffer (0.3 mol, pH 5) (1.75 ml), and 2% ninhydrin solution (1.25 ml) (Sigma Alidrich, UK). 1 litre of

the citric acid buffer contains 42 g citric acid and 16 g sodium hydroxide (NaOH). The pH of the buffer was adjusted to pH 5 using a 10M NaOH solution as required. The samples were placed in a vigorously boiling (100ºC) Grant Sub Aqua 12 water bath (Grant, UK) for 25 minutes. Following cooling of the samples to room temperature, 4.5 ml 95% ethanol:water (1:1 ratio) solution was added to each sample and mixed to stop the reaction. Ninhydrin reactive N absorbance was measured at 570 nm using a Unicam 5625 UV/VIS spectrometer (Unicam, UK). The ninhydrin reactive N content of the experimental samples was determined by comparing the absorbance values with those of a standard series. The standard series was made using different volumes of 0.5 M K_2SO_4 and diluted ammonium sulphate $(NH_4)_2SO_4$. The initial diluted $(NH_4)_2SO_4$ solution was made by first diluting 0.0623 g $(NH_4)_2SO_4$ in 100 ml 0.5 M K_2SO_4. 10 ml of this stock was then taken and diluted into another 100 ml of 0.5 M K_2SO_4. This diluted stock was then used to make up the standard samples to the concentrations detailed in Table 2.1.

Table 2.1 Composition of standard series samples used in the ninhydrin reactive N assay

0.5M K_2SO_4 (µl)	0.005M $(NH_4)_2SO_4$ (µl)	*N* (µg)
0	400	5.28
80	320	4.23
160	240	3.17
240	160	2.11
320	80	1.06
360	40	0.53
400	0	0.00

The moisture content of the soil was used to determine the ninhydrin-N (Nin-N) content of the samples on a per gram dry weight basis. These ninhydrin-N values were then converted to biomass-N using a conversion factor of 3.1 (Amato and Ladd, 1988).

2.2.4 Soil microbial activity

Dehydrogenase activity is one of the major enzymatic processes studied. Dehydrogenases are intracellular enzymes that form an integral part of active microorganisms. Therefore, measuring this

activity within a population provides a good, broad overview of the overall activity of the microbial community (Skujins, 1976). Lenhard (1956) produced the first widely used method for analysing soil dehydrogenase activity. This assay focuses on the production of the water-insoluble, red-coloured compound 2,3,5-triphenyl formazan (TPF), from the reduction of the colourless, water soluble 2,3,5-triphenyltetrazolium chloride (TTC) by the activity of dehydrogenases (Tabatabai, 1994).

In this experiment, dehydrogenase activity was determined using a method based on that of Tabatabai (1994). 6 g of each soil sample was placed in a 50 ml centrifuge tube. 2.5 ml sterile distilled water and 1 ml 3% 2,3,5-triphenyltetrazolium chloride (TTC) solution (Sigma Aldrich, UK) was added to each sample which was then mixed on a vortex and placed in an oven at 37ºC for 24 hours. 5 ml 100% methanol (Fisher Scientific, UK) was then added to each sample. The samples were mixed on a vortex for 10 seconds and centrifuged at 4000 rpm for 2 minutes and the supernatant decanted into 25 ml volumetric flasks. This extraction was carried out a further two times, and methanol was used to make the total amount of each sample to 25 ml.

Experimental samples were analysed by measuring the intensity of the red colour produced using a Unicam 5625 UV/VIS spectrometer (Unicam, UK) at a wavelength of 485 nm. The absorbance values of the experimental samples were compared with those of reagent standards containing known amounts of TPF. Reagent standards were produced by dissolving TPF (Sigma Aldrich, UK) in HPLC-grade methanol (Fisher Scientific, UK) to produce concentrations of 0, 1, 2, 5, 10 and 15 mg TPF L^{-1}.

2.2.5 Monitoring azoxystrobin concentration using high-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a widely-used technique for the quantitative detection and separation of different compounds within a solvent. Differentiation between compounds is based on the retention times of the compounds within an HPLC column. This retention time varies based on factors such as the column width used, the flow rate of the mobile phase, and the nature of

the compound itself.

Di Corcia and Marchetti (1992) demonstrated the potential usefulness of this technique for monitoring pesticides in the environment. The authors were able to successfully detect a total of 89 pesticides from groundwater and river water following liquid-solid extraction. For the majority of the compounds tested the limits of quantification were found to be lower than 0.1 μg L^{-1} (Di Corcia and Marchetti 1992).

For this experiment, pesticides were extracted from the soil samples by adding 10 g of soil to 50 ml centrifuge tubes and mixing it with 20 ml of HPLC-grade acetonitrile (Fisher Scientific, UK). The tubes were shaken by hand and placed on a shaker for 1 hour. Following shaking, the samples were left for 30 minutes to settle and then centrifuged at 4000 rpm for 2 minutes. Two millilitres of the supernatant was decanted into a 2 ml screw-top glass HPLC vial (Chromacol Ltd, UK) using a plastic 3 ml Pasteur pipette, before a screw-top lid with a seal was added. The samples were placed in a freezer at -20°C until required for analysis.

Samples were analysed using an Agilent 1100 series unit with a diode array detector (DAD) and LiChrospher® 100 RP-18e (5 μm) HPLC column (Agilent, UK). A liquid phase composed of 75% HPLC-grade acetonitrile (Fisher Scientific, UK) and 25% distilled water was used at a flow rate of 1.30 ml min^{-1}. 25 μl of each sample was injected and the concentration of azoxystrobin determined by monitoring the absorbance at 230 nm. The run time per sample was 4 minutes with the azoxystrobin retention time recorded as approximately 3 minutes. The azoxystrobin recovery efficiency of the equipment was monitored using control samples containing the equivalent of 10 mg kg^{-1} azoxystrobin dissolved in acetonitrile. 1 control sample measurement was taken after every 10 experimental sample measurements. Recovery of control samples varied from 94.2% to 88.6% of the applied concentration.

2.2.6 Extraction of soil DNA, and Terminal Restriction Fragment Length Polymorphism analysis

DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis was used to determine the effects of azoxystrobin concentration on the community structure of microorganisms from different trophic levels. The organism groups studied were bacteria, fungi, archaea, pseudomonads, nematodes, and general eukaryotes.

DNA was extracted using a FastDNA® Spin Kit (Qbiogene, UK) and its quantity and quality determined using a NanoDrop® ND-1000 spectrophotometer (Labtech International Ltd., UK). Sample quality was determined based on the absorbance ratios recorded for 260 nm: 280 nm and 260 nm: 230 nm. A 260 nm: 280 nm ratio value of ~1.8 was indicative of a pure DNA sample. Values noticeably below this would have been considered indicators of possible contamination by compounds such as phenolics or proteins. A value of ~2 for the 260 nm: 230 nm ratio indicated that the DNA obtained was pure, with values below this suggesting the presence of contaminants (Thermo Scientific, 2008).

The DNA extracts were diluted with distilled water to a concentration of 10 ng μl^{-1} and a PCR reaction was set up containing these dilutions along with the fluorescently-labelled primer(s) required for T-RFLP.

The fluorescent labels used were 6-FAM (6'- carboxyfluorescein ($C_{21}H_{12}O_7$), emission at 520 nm), NED (emission at 580 nm), VIC (emission at 550 nm) and PET (emission at 595 nm). All labelled primers were obtained from Applied Biosystems, Warrington, UK and all un-labelled primers from Invitrogen, Paisley, UK.

Table 2.2 describes the primers used, restriction enzymes, and annealing temperatures required for each of the primer pairs.

Table 2.2: The primer pairs used for the analysis of different groups of organisms

Organism Group	Forward Primer	Reverse Primer	Annealing Temperature	Approximate Fragment Size (bp)	Restriction Enzyme(s) for T-RFLP
Fungi (1)	**ITS1f-PET:** 5' CTT GGT CAT TTA GAG GAA GTA A 3' (Gardes and Bruns, 1993)	**ITS4r-FAM:** 5' CAG ACT T(G/A)T A(C/T)A TGG TCC AG 3' (White *et al.,* 1990)	55°C	750	*Hha*I, *Msp*I
Fungi (2)	**EF4f-FAM:** 5' GGA AGG G(G/A)T GTA TTT ATT AG 3 (Smit *et al.*, 1999)	**EF3r:** 5' GTT TGA ACC AGT AAA TCT CCT 3' (Smit *et al.*, 1999)	48°C	1,400	*Hha*I, *Msp*I
Bacteria	**63f-NED:** 5' CAG GCC TAA CAC ATG CAA GTC 3' (Marchesi *et al.*, 1998)	**1087r-VIC:** 5' CTC GTT GCG GGA CTT ACC CC 3' (Hauben *et al.*, 1997)	55°C	1,000	*Hha*I, *Msp*I
Archaea	**AR3f:** 5' TTC CGG TTG ATC CTG CCG GA 3' (Giovannoni, 1988)	**AR927r-NED:** 5' CCC GCC AAT TCC TTT AAG TTT C 3' (Jurgens *et al.*, 1997)	55°C	600	*Hha*I, *Alu*I
Pseudomonads	**PS16Sf-FAM:** 5' ACT GAC ACT GAG GTG CGA AAG GC 3' (Locatelli *et al.*, 2002)	**PS23Sr:** 5' ACC GTA TGC GCT TCT TCA CTT GAC C 3' (Locatelli *et al.*, 2002)	55°C	750	*Hha*I, *Alu*I
Nematodes	**Nem18Sf-VIC:** 5' CGC GAA T(G/A) G CTC ATT ACA ACA GC 3' (Floyd *et al.*, 2005)	**Nem18Sr:** 5' GGG CGG TAT CTG ATC GCC 3' (Floyd *et al.*, 2005)	56°C	900	*Aci*I, *Hae*III
General Eukaryotes	**Euk20f-FAM:** 5' TGC CAG TAG TCA TAT GCT TGT 3' (Kowalchuk *et al.*, 1997)	**Euk516r:** 5' ACC AGA CTT G(C/T)C CTC C 3' (Amann *et al.*, 1990)	55°C	500	*BstU*I

All PCR reactions were carried out using the MegaMix ready PCR Mix (Microzone Ltd, UK). This mix contains *Taq* polymerase, dNTPs, reaction buffer and stabiliser. PCR reactions were set up based on the cycling profile provided by Microzone Ltd. Each PCR reaction contained 47 µl MegaMix, 1 µl forward primer, 1 µl reverse primer, and 1 µl 10 ng μl^{-1} sample DNA. Cycling conditions and primer concentrations used were altered based on the primer pair used.

The PCR conditions for the ITS1f/ITS4r and 63f/1087r primer pairs were: 95°C 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute. This was followed by a final extension step of 10 minutes at 72°C. The primer concentrations used were 5 µM for 63f and 1087r, and 25 µM for ITS1f and ITS4r.

Ar3f/Ar927r and PS16Sf/PS23Sr PCR conditions were the same as those mentioned above but 40 cycles were performed instead of 30. The primer concentration used for the Ar3f, Ar927r, and PS16Sf was 15 µM. A concentration of 25 µM was used for the PS23Sr primer.

For the EF4f/EF3r primer pair the conditions used were as follows: 94°C for 3 minutes, followed by 40 cycles of 94°C for 1 minute, 48°C for 1 minute, 72°C for 3 minutes. The final extension phase used was 15 minutes at 72°C. A primer concentration of 20 µM was used for both EF4f and EF3r.

The Nem18Sf and Nem18Sr primers were used at a concentration of 20 µM each. The PCR conditions were: 94°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute. The final extension for this reaction was 72°C for 10 minutes.

The primers Euk20f and Euk516r were both used at a concentration of 20 µM. The cycling conditions were: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 70°C for 1 minute. The final extension phase was 70°C for 10 minutes.

All PCR reactions were carried out using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Warrington, UK). Following amplification, PCR products were analysed using a 1% agarose gel to confirm that fragments of the correct size had been amplified. The remaining PCR product was then stored at -20°C until required for PCR purification. PCR products were purified using a QIAquick® PCR Purification Kit using the manufacturer's instructions and quantified using the NanoDrop® machine as previously.

A restriction digest reaction was set up containing the sample and the required restriction enzymes. Each restriction digest sample contained: 500 ng PCR product, 2 µl 10x restriction digest buffer (New England Biolabs, Hitchin, UK), 0.2 µl bovine serum albumin (BSA) (*Hha*I only), and 5 units (U) restriction enzyme (New England Biolabs, Hitchin, UK). This mixture was then made up to 20 µl using sterile distilled water.

The restriction digest conditions used were the same for all enzymes. The conditions used were 37°C for 4 hours followed by 15 minutes at 95°C. Samples were then returned to 12°C before being sent for T-RFLP analysis or storage at -20°C as required.

T-RFLP analysis was carried out by the Genome Centre at the University Of Warwick, School Of Life Sciences, Wellesbourne Campus. All samples were analysed using an Applied Biosystems 3130XL Genetic Analyzer (Applied Biosystems, Warrington, UK) and results were analysed using the GeneMarker® software (Softgenetics®, USA).

All GeneMarker® samples were analysed using LIZ-1200 standards and a chromatogram for each sample produced. Analysis was carried out with the automatic baseline subtraction, pull-up correction, spike removal, and stutter peak filter enabled at the default software settings. Only peaks with an intensity value of 50 or over were selected and used for further analysis (Hackl *et al.*, 2004). A bin table of results was produced and transferred to Microsoft Excel for further analysis.

Each peak on the chromatograms was representative of a different terminal restriction fragment (TRF). In other words, they represent the labelled DNA fragments of different sizes produced by the restriction digest reaction. However, as some microorganisms can have the same restriction sites even between different species, a single TRF may be representative of multiple different organisms.

2.2.7 Clone library production and sequencing of extracted DNA

Clone libraries were produced for nematodes and fungi (using the EF4f/EF3r primer pair) from 0 mg kg^{-1} and 25 mg kg^{-1} (1 month) samples. The main aim of these libraries was to try and identify sequence homologies to the taxa responding to azoxystrobin application as identified by T-RFLP analysis. Additionally, analyses were carried out to see whether the clone libraries gave a similar indication of azoxystrobin impacts on community structure and diversity to the T-RFLP and Shannon diversity analyses.

For clone library production, DNA extracts from the samples described above were first diluted to a concentration of 10 ng μl^{-1} and quantified using Nanodrop analysis.These diluted DNA samples were used in a PCR with unlabelled EF4f/EF3r and Nem18Sf/Nem18Sr primer pairs with 4 experimental replicates being used for each PCR reaction. The PCR conditions for both of these primer pairs were the same as detailed previously. Following PCR, the 4 replicates were pooled together and the samples were run on a 1% agarose gel. DNA bands were then excised from the gel under a UV lamp using a clean, sharp scalpel. Gel extraction was carried out using the QIAquick Gel Extraction kit following the manufacturer's instructions, with the DNA being eluted into 30 µl buffer EB. A ligation reaction mix was then set up using the Qiagen PCR cloning kit. This mix contained 1 µl pDrive Cloning Vector (50ng/µl), 2 µl PCR product, 2 µl distilled water, and 5 µl of 2x Ligation Master Mix. The ligation reaction was carried out at 10ºC for 2 hours in a thermal cycling block and samples were subsequently stored at -20ºC until use. The transformation reaction was carried out using Qiagen EZ Competent Cells and liquid SOC medium following the manufacturer's instructions. 2 µl ligation-reaction mixture was used per sample, and volumes of 20, 40, 60, 80 and 100 µl of each sample were

plated onto individual LB plates containing 1.5 ml L^{-1} ampicillin and 2 ml L^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma Aldrich, UK). Samples were stored at 37ºC overnight before being transferred to 4ºC for a further overnight incubation to enhance the colour of any blue colonies and therefore facilitate differentiation between colony types.

Sequencing reactions were prepared using the TempliPhi DNA Sequencing Template Amplification Kit (GE Healthcare Life Sciences, UK) following the manufacturer's instruction. Replicate samples of the white bacterial colonies containing the required insert used were inoculated into 96-well plates containing 100 µl LB + ampicillin before being shaken at 150 rpm overnight at 37ºC. Following shaking, 50 µl of glycerol:water solution (50:50) was added and the samples were placed at -80ºC for long-term storage. The sequencing reaction consisted of (per sample): 3 µl template DNA, 2 µl BigDye, 2 µl 5x buffer, 3.2 µM primer (either M13R or M13F). This was made up to 7 µl using sterile distilled water. The cycling conditions involved an initial step of 96ºC for 1 minute. This was followed by 25 cycles of 96ºC for 10 seconds, 50ºC for 5 seconds, and 60ºC for 4 minutes. Samples were analysed at the Genome Centre at the University Of Warwick, School Of Life Sciences, Wellesbourne Campus using an Applied Biosystems 3130XL Genetic Analyzer (Applied Biosystems, Warrington, UK).

Sample sequences were analysed using the SeqMan programme (DNASTAR Inc., USA). For each sample, the sequences from the forward and reverse primer reactions were aligned with each other. Next, the insert sequence was determined by searching for the forward and reverse primer sequences and a consensus sequence was produced.

The consensus sequences were used to search for restriction sites for the relevant restriction enzymes used for the fungal and nematode T-RFLP analysis (*Hha*I and *Msp*I for fungi, *Aci*I and *Hae*III for nematodes). This analysis was carried out using the EditSeq programme (DNASTAR Inc., USA). Using the search option, the first restriction site for each enzyme of interest was identified. This

enabled estimated TRF sizes to be determined for each sequence which could then be compared with actual TRFs obtained by T-RFLP analysis.

Sequences were identified using nucleotide BLAST (NCBI, url: http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) and Ribosome Database Project (RDP) (Michigan State Univeristy, url: http://rdp.cme.msu.edu/) searches.

Collector's curves were applied to the obtained data. These were produced by analysing the identities ascribed to each sequence in the order in which they were analysed. From this list, a Collector's curve was plotted for each clone library with the total number of clones recorded on the *x* axis and the number of unique clones recorded on the *y* axis.

2.2.8 Quantitative PCR analysis of bacterial and fungal samples

Quantitative PCR (qPCR) analysis was carried out to compare the bacterial (16S rRNA) and fungal (18S rRNA) copy numbers present in 0 mg kg^{-1} and 25 mg kg^{-1} samples 1 month post application.

Each 20 µl qPCR reaction contained the following: 10 µl 2 x SYBR Green mix (Applied Biosystems, UK), 1 µl forward primer, 1 µl reverse primer, 0.2 µl bovine serum albumin (40 mg ml^{-1}), 6.8 µl double distilled molecular biology grade water (Microzone Ltd., UK), and 1 µl DNA standard or environmental sample. Each qPCR reaction was carried out in triplicate. The primers Eub338 (5' ACT CCT ACG GGA GGC ACG AG 3') and Eub518 (5' ATT ACC GCG GCT GCT GG 3') were used for bacterial community analysis, and 5.8s (5' CGC TGC GTT CTT CAT CG 3') (Fierer et al., 2005) and ITS1F (5' CTT GGT CAT TTA GAG GAA GTA A 3') for fungi (Gardes and Bruns, 1993).All primers were obtained from Invitrogen™, UK and were used at concentrations of 20 µM for the bacterial pair, and 5 µM for the fungal pair. All environmental samples were used at a concentration of 1 ng $µl^{-1}$.

Standard samples were produced using plasmid DNA from the bacterial and fungal clone libraries produced during this experiment. Purified products from these samples were obtained using the QIAfilter Plasmid Midi Kit (Qiagen, UK) using the manufacturer's instructions and elution into 50 µl TE Buffer (pH 8.0). Purified extracts were quantified and their quality determined using the NanoDrop® machine, as previously. Standard samples were sequenced by the Genomics Centre at the University Of Warwick, School Of Life Sciences, Wellesbourne Campus to make sure that the DNA sequence extracted was the same as the one recorded in the clone library sample it was taken from. The bacterial standard used had a 97% sequence homology to the uncultured α-Proteobacterium clone AKYH1384 (AY921937.1), and the fungal standard had a 98% sequence homology to the species *Phoma exigua* var. exigua (AB454232.1).

The purified samples were then diluted to produce the required qPCR standard series. For both bacterial and fungal samples the standard series concentrations were (in pg μl^{-1}) 800, 160, 32, 6.4, 1.28, 0.26, 0.05, and 0.01 along with a non-template control containing no DNA. The 20 µl samples were pipetted into individual wells of a 384-well PCR plate and covered with an optical adhesive cover (Applied Biosystems, UK). They were then centrifuged at 1000 rpm for 1 minute before being used for qPCR analysis.

qPCR was carried out using a ABI Prism 7900 HT sequence detection system (Applied Biosystems, UK). The qPCR procedure for both primer sets was: 50°C (2 minutes), 95°C (10 minutes), followed by 40 cycles of 95°C (15 seconds), 53°C (30 seconds), and 72°C (30 seconds). Following this, a dissociation stage was added to enable melting curve analysis to be carried out. The conditions for this were: 95°C (15 seconds), 60°C (15 seconds) and 95°C (15 seconds).

All samples were analysed using the SDS 2.1 software (Applied Biosystems). The sample type (non-template control, standard, or unknown (environmental)) was inputted manually, and the detector set

for pBSK + SYBR for each sample. Samples were run by highlighting the relevant wells on the plate display and using the analyse function.

Analysis was carried out using on the amplification curves and standard plots. These aspects of the qPCR were considered as being successful if the R^2 value was greater than 0.99 and the slope value was between -3.0 and -3.4. Amplification plots were analysed to ensure that there was an even spacing between the curves of the standard samples. If this was case, it was considered that the samples contained the intended concentrations of DNA. For the first set of fungal qPCR data produced this was not the case. Therefore, the fungal qPCR procedure was repeated and the correct curve spacing was observed in this case.

Melting curve analysis was carried out to ensure that there was only 1 curve present, indicating the amplification of the intended product. Experimental samples and standards were compared with the non-template control to ensure that no primer dimers were present. qPCR products were run on a 1% agarose gel to ensure that only the intended fragment was amplified.

The data obtained was analysed using Microsoft Excel. First, the mean quantities (in pg μl^{-1}), standard errors, and standard deviations were calculated for each triplicate set. Following this, the copy number of DNA present in 1 pg was calculated as detailed by Whelan *et al.* (2003). Briefly, these calculations described how the copy number of a gene can be ascertained provided the length (in base pairs) and the molecular weight of the plasmid (including the insert) are known (Whelan *et al.*, 2003). This concept is summarised in Figure 2.5:

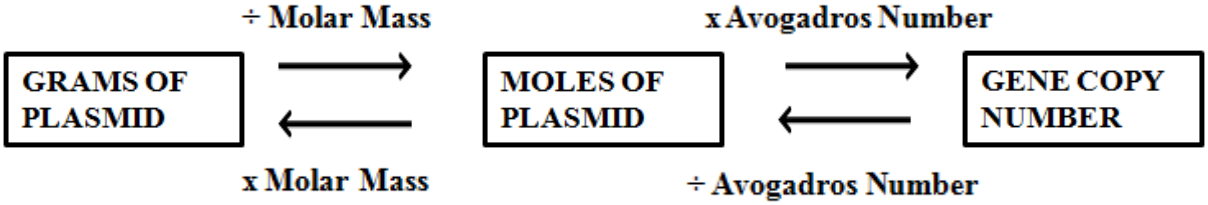


Figure 2.5. The calculations used for determining bacterial and fungal copy numbers from plasmid weights.

An example of the calculations performed is shown using the bacterial primer pair:

Avogadros number = no. molecules in a mole = 6.022×10^{23}

One double-stranded DNA bp has an average MW (molecular mass) of 660 daltons.

Plasmid pDrive cloning vector = 3851 bp

Plasmid + 180 bp insert (PCR product) = 4031 bp \has a MW of $4031 \times 660 = 2.7 \times 10^6$ daltons.

Using the PCR product size, the moles for different masses of plasmid can be calculated. For example, for a plasmid with a mass of 1g the following calculations can be used:

$$\textbf{moles} = \frac{\textbf{mass (g)}}{\textbf{Product size (Daltons)}}$$

$$= \frac{1}{2.7 \times 10^6}$$

$$= 3.704 \times 10^{-7}$$

From this value the DNA copy number can then be calculated:

copy number = moles of plasmid x Avogadros Number

$$= (3.704 \times 10^{-7}) \times (6.022 \times 10^{23})$$
$$= 2.23 \times 10^{17}$$

2.2.9 Statistical Analyses

2.2.9.1 Biomass and Dehydrogenase Analysis

Significant differences between different azoxystrobin concentrations were determined using 2-way ANOVA analysis with a *p* value of ≤ 0.05 considered indicative of a significant difference. If an overall significant difference was observed, Fisher's least significant difference (LSD) analysis was

then carried out using the information provided in the ANOVA table. This technique offers a further level of detail of data analysis compared with the ANOVA. For example, whilst a 2-way ANOVA could show that azoxystrobin concentration had a significant effect on soil dehydrogenase activity over the course of the experiment, LSD analysis can take this one step further and give precise details of where the significant differences occur e.g. between 0 mg kg^{-1} and 25 mg kg^{-1} samples after 1 month. The first stage of LSD analysis requires the calculation of the critical t value using the formula:

Critical t = TINV(significance level, error degrees of freedom (df))

where TINV is a function analysing the inverse probability of a data set and the significance level refers to the *p* value below which a difference is considered significant (e.g. 0.05). The error degrees of freedom value can be found in the Excel ANOVA table.

After this, the LSD value can be calculated using the following formula:

LSD = Critical t x √(2 x MSE/Number of columns)

where MSE is the mean squared error from the ANOVA table and the number of columns refers to the number of columns in the data set analysed. Once the LSD value has been obtained it can be used to compare individual data points within a data set e.g. 0 mg kg^{-1} and 25 mg kg^{-1} samples after 1 month. If the difference between these two values is equal to, or higher than the calculated LSD value, then the two data points are significantly different to each other.

2.2.9.2 HPLC Analysis of Azoxystrobin Degradation

For the degradation assays DT_{50} values were calculated using the vinterpolate function in the GenStat Version 12 statistics programme (VSN International, UK). An example of the code used to determine

the DT_{50} values for a sample data set is shown in Appendix I.

2.2.9.3 T-RFLP Data Analysis

Once the data had been transferred to Microsoft Excel, traces were analysed individually and the total number of TRFs calculated along with the total trace intensity. Following this, any TRFs that were found to represent less than 1% of the total intensity of a trace were discarded and recorded as "0" (MacDonald *et al.*, 2007). Subsequently, the 4 replicates of each sample were pooled to produce an average T-RFLP trace for each sampling point and pesticide concentration (Lukow *et al.*, 2000). 2-way ANOVA was performed to investigate the impact of pesticide application on the number of terminal restriction fragments (TRFs) recorded in each sample. If ANOVA analysis produced a significant result (p = <0.05) for the effect of pesticide concentration on TRF numbers, then LSD analyses were performed as described for the biomass and dehydrogenase assays.

Shannon diversity analysis and (if required) LSD analysis were used to determine the impacts of azoxystrobin application on soil microbial diversity. Additionally, these analyses were used to ascertain whether the diversity responses of the communities in this experiment supported either of the theories proposed by Giller *et al.* (1998). The Shannon diversity index can be used to determine the diversity of a particular community based on the relative abundances of its members. In the case of this experiment the members of the community were the individual TRFs present in any single T-RFLP trace. The index value can be calculated using the following formula:

$$H' = -\Sigma\, p_i \ln (p_i)$$

where *H'* is the Shannon diversity index value, -Σ represents the minus sum value of the formula, p_i is the relative abundance of a member in the population and ln p_i is the natural logarithm of the relative abundance. A high Shannon Index value (e.g. 3.5) is indicative of a highly diverse population. ANOVAs of the H' vales were performed using Genstat version 12 (VSN International, UK) and the

p value recorded. An additional "contrast" function was added to the ANOVA which enabled broad comparisons to be made (e.g. are the results for the 25 mg kg^{-1} treatment significantly different to all of the other treatments?). An example of the Genstat code used for these modified ANOVAs is shown in Appendix II.

T-RFLP data was analysed using non-metric multi-dimensional scaling (NMDS) using the Primer6 software (Primer-E Ltd., UK) in order to determine the impacts of pesticide concentration on microbial community structure. This method compares the dissimilarities between each sample set (e.g. each T-RFLP trace) and plots the distances between each sample on a simplified 2D ordination plot. Multiple iterations of the data are then calculated by the programme to produce the orientation of the sample points that produces the lowest possible 2D stress value (Ramette, 2007). These values illustrate the discrepancy between the multidimensional data from the NMDS and its eventual 2D configuration (Cai and Franco, 2009). Once the data has been plotted, differences can be determined by the visual analysis of how the data points group in the 2D configuration and this can be supplemented with statistical analyses to determine significant differences.

The first stage of analysis was to input the data into the programme as abundance data, and to then carry out a resemblance analysis on it using the Bray-Curtis dissimilarity formula. This formula is used to compare the compositional differences between two different data sets (e.g. two individual T-RFLP traces). The analysis uses the formula detailed below:

$$BC_{ij} = \frac{S_i + S_j - 2C_{ij}}{S_i + S_j}$$

where C_{ij} is the sum of the minimum abundances of each data point (e.g. individual TRFs) across two different data sets; and S_j and S_i are the total number of data points (e.g. total TRF numbers) across the two different data sets (Bray and Curtis, 1957). Primer 6 calculated the relative stress values between the different points, and configured the points in the orientation that produced the lowest value. From

this the NMDS analysis was carried out using the default programme settings and a scatter plot of results produced to compare the impacts of different factors e.g. sampling time and/or pesticide concentration on microbial community structure. Further analyses were then carried out with cluster analysis being used to group similar samples and bubble plots being used to determine the relative abundances of particular TRFs of interest in different samples.

To test for significant differences between individual sample points an analysis of similarity (ANOSIM) was performed. This analysis provides a significance level as a percentage for each comparison. In order to obtain a *p* value for each comparison this percentage was divided by 100. Any comparisons that produced a *p* value of less than 0.05 were considered to be significantly different.

Similarity percentage (SIMPER) analysis was used to determine to what extent different TRFs contributed to the overall community, and to work out how much different TRFs contributed to the community variation between two defined populations.

2.2.9.4 Clone Library Analysis

For comparing clone libraries of non-perturbed and perturbed soils, the impacts on diversity were analysed using a Chao1 estimate of population size, including a calculation of the Chao1 variance to determine whether any changes are significant (Chao, 1987).

The Chao1 estimate of population size is calculated as follows:

$$S = D + \frac{f1(f1-1)}{2(f2 + 1)}$$

Where S is the Chao1 estimate of the actual population size, D is the observed population size (i.e. the number of categories) and f1 and f2 are the number of categories which are represented by 1 and 2 individuals respectively.

Chao1 variance can then be calculated using the formula:

Chao1 variance = (f2+1)(0.25R^4 + R^3 + 0.5R^2), where R = (f1-1)/(f2+1)

A Mann-Whitney U-test was used to determine whether there was a significant difference in the community structure of clone libraries from perturbed and un-perturbed soils. For this analysis the samples in each library were first ranked from highest to lowest according to the proportion of the population they represent.

The U value was then calculated as follows:

U = (n1xn2) – (n1(n1+1))/2 – R1

Where n1 and n2 are the numbers of samples in populations 1 & 2 and R1 is the sum of the ranks for the samples in population 1. The data values for U, n1 and n2 were then entered into the Mann-Whitney U-Test page of the *Caenorhabditis elegans* WWW Server (url: http://elegans.swmed.edu/~leon/stats/utest.html) where the probability value for U was calculated and the 2 populations compared.

2.2.9.5 Quantitative PCR Analysis

Average copy numbers were calculated and plotted in 2-D bar charts with error bars denoting the average values plus and minus the standard error of the mean. 2-way ANOVAs were used to identify whether azoxystrobin concentration had a significant impact on copy number, and least significant

difference analysis was used to show which comparisons within the data set (e.g. 0 mg kg^{-1} vs. 25 mg kg^{-1}) were significantly different.

2.3 Results

2.3.1 Azoxystrobin recovery using HPLC

The results of the HPLC analysis showed a marked decrease in azoxystrobin concentration in all samples after 1 month (Figure 2.6). After this, there was little further degradation over the final 3 months of the experiment. The highest degradation within the first month was in the 5 mg kg^{-1} treatment with an average of 21% of the initial azoxystrobin applied remaining. The lowest degradation rate was in the 25 mg kg^{-1} samples with 54% of the added azoxystrobin still remaining after 1 month.

After 4 months the 5 mg kg^{-1} treatments showed the highest percentage degradation with 10% of the added pesticide still remaining, in contrast to the 25 mg kg^{-1} treatments where 37% of the initial applied azoxystrobin was still present.

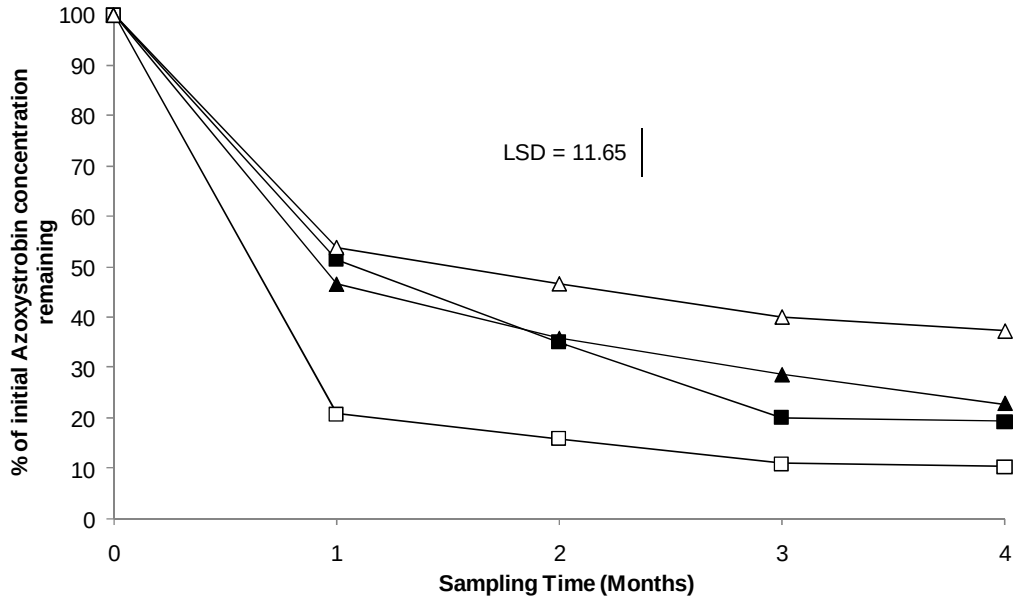


Figure 2.6. Percentages of initially applied azoxystrobin extracted using HPLC analysis. ■ 1 mg kg^{-1} □ 5 mg kg^{-1} ▲ 10 mg kg^{-1} △ 25 mg kg^{-1}. Each data point represents the mean of 4 experimental replicates.

DT_{50} values as determined by vinterpolate analysis showed the quickest degradation was in the 5 mg kg^{-1} treatment with an average predicted value of 19 days. This contrasts with the 33, 32, and 47 days recorded for 1, 10 and 25 mg kg^{-1} treatments respectively. LSD analysis of the DT_{50} data produced a value of 11.65. Using this value it was determined that there was no significant difference in the DT_{50} of the 1 and 10 mg kg^{-1} treatments. However, the DT_{50} of the 5 mg kg^{-1} treatment was significantly lower than in the other treatments, and the value for the 25 mg kg^{-1} treatment was significantly higher.

2.3.2 Dehydrogenase Activity

The results of a 2-way ANOVA showed that the concentration of azoxystrobin applied had a highly significant (p = <0.01) effect on soil dehydrogenase activity.

There was a marked decrease in dehydrogenase activity in all treatments after 1 month, relative to the 0 mg kg^{-1} control (Figure 2.7). This decrease was dependent upon the concentration of azoxystrobin applied with the 25 mg kg^{-1} treatment showing an activity that was only 45.4% that of the control. This compares to values of 54.6, 65.0, and 75.0% recorded for 10, 5, and 1 mg kg^{-1} treatments respectively. The dehydrogenase activity levels remained at these levels at the 2-month sampling point. By 3 months, there had been a marked increase in dehydrogenase activity in all treatments. Indeed, the 1 and 5 mg kg^{-1} treatments recorded activity levels that were 102.9 and 103.6% those of the control. Values of 88.4 and 98.8% were recorded for 10 and 25 mg kg^{-1} treatments respectively. After 4 months the activities in the 10 and 25 mg kg^{-1} treatments were higher than those of the controls (137.4 and 133.2%, respectively). The 1 and 5 mg kg^{-1} treatments recorded values of 87.1 and 97.8% compared to the control.

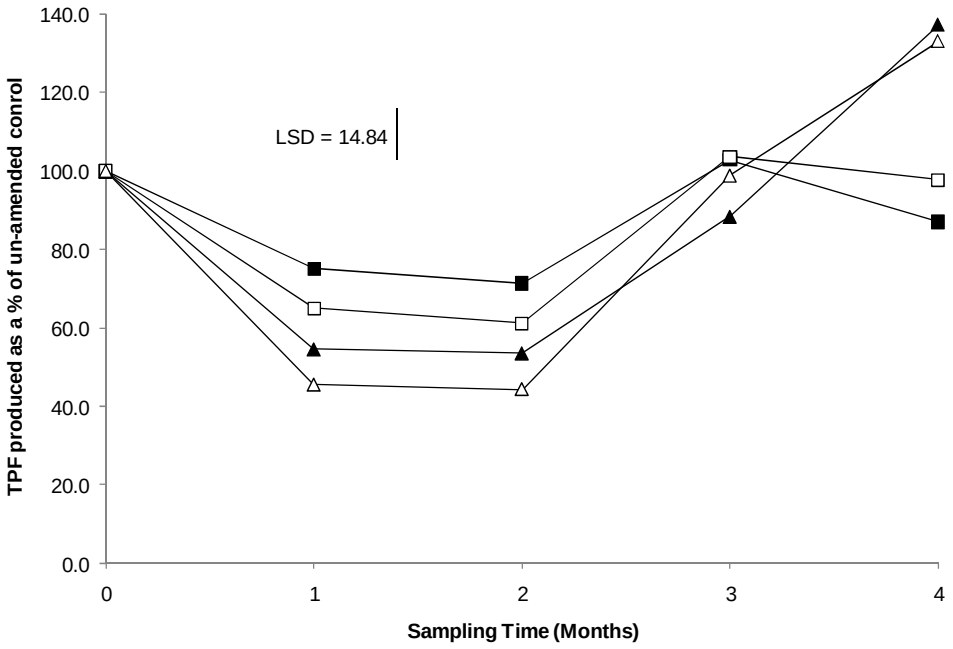


Figure 2.7. Percentage dehydrogenase activity in azoxystrobin-amended soils compared to 0 mg kg^{-1}controls. TPF = Triphenyl formazan. ■ 1 mg kg^{-1} □ 5 mg kg^{-1} ▲ 10 mg kg^{-1} △ 25 mg kg^{-1}. Each data point represents the mean of 4 experimental replicates.

2.3.3 Soil Microbial Biomass

2-way ANOVA analysis showed that overall, pesticide concentration had no significant effect on biomass over the course of the experiment (p = 0.149). However, sampling time (p = 0.05) did significantly affect the biomass.

The average biomass values during the experiment ranged from 228.8 µg to 112.8 µg g^{-1} of soil.The notable result from the data set was that after 1 month (Figure 2.8) the 25 mg kg^{-1} samples had a significantly higher biomass than the control. A value of 228.8 µg biomass-N per gram of soil was recorded in these samples compared to 129.6 µg g^{-1} in the control. This could represent an artefact of the biomass quantification method. For example, Joergensen and Brookes (1990) noted how biomass extractions using K_2SO_4 could occasionally cause erratic calorimetric measurements. There were no significant differences between the control and any of the other concentrations used. From 2 to 4 months, there were no significant differences in biomass between the control and amended soils. At these time points, all biomass values were between 137.6 and 101.0% those of the 0 mg kg^{-1} controls

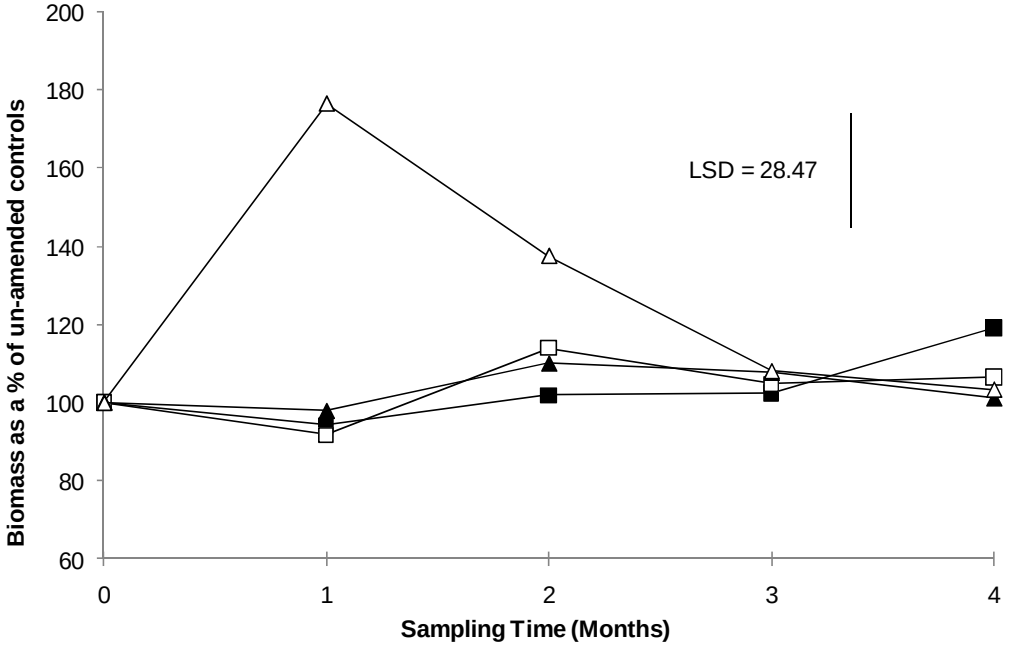


Figure 2.8. Microbial biomass in soils amended with different concentrations of azoxystrobin, recorded as a percentage of 0 mg kg^{-1} controls at each time point. ■ 1 mg kg^{-1} □ 5 mg kg^{-1} ▲ 10 mg kg^{-1} △ 25 mg kg^{-1}. Each data point represents the mean of 4 experimental replicates.

2.3.4 T-RFLP Analysis of Microbial Community Structure

2.3.4.1 Eukaryotes

2.3.4.1.1 Fungi

2.3.4.1.1.1 ITS1F and ITS4r Primer Pair

There were a total of 123 unique TRFs with between 3 and 47 occurring in individual traces. A 2-way ANOVA showed that azoxystrobin concentration did not have a significant impact (p = 0.151) on the number of TRFs, whereas sampling time did (p = <0.01).

Shannon diversity analysis demonstrated that there was no significant impact of azoxystrobin concentration on fungal community diversity using the ITS1f/ITS4r primer pair (p = 0.154) (Figure 2.9). Contrast analysis also showed no significant differences in diversity between individual pesticide concentrations over the 4-month experimental period.

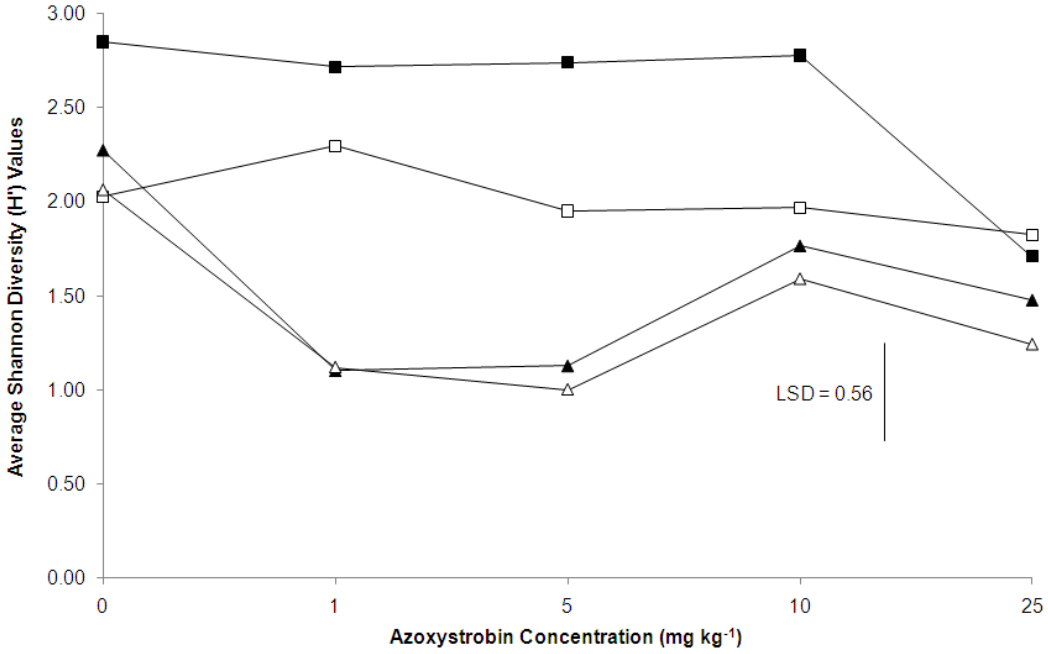
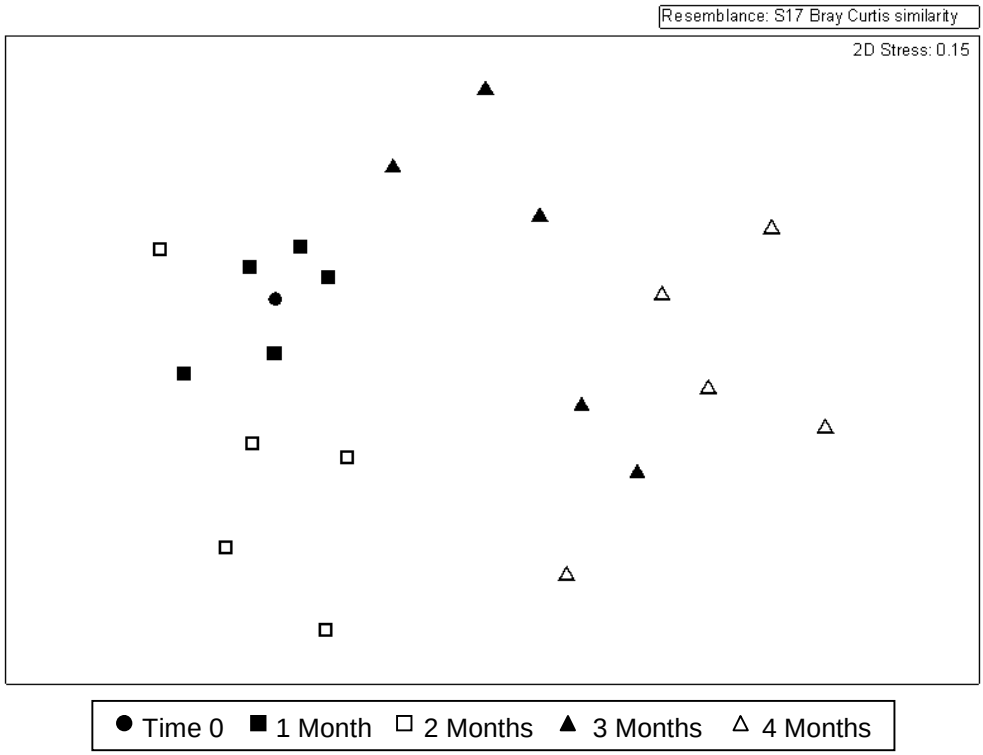


Figure 2.9. Average fungal Shannon diversity (H') values recorded using the ITS1f and ITS4r primer pair over 4 months following azoxystrobin application. ■ 1 month □ 2 months ▲ 3 months △ 4 months. Each data point represents the mean of 4 experimental replicates.

Analysis of the fungal community structure using the ITS1f and ITS4r primer pair showed no significant impact of azoxystrobin on fungal community structure. Indeed, ANOSIM analyses using Primer 6 showed that sampling time had a greater impact on fungal community structure (p = 0.056) than azoxystrobin concentration (p = 0.229). Pair-wise comparisons of different azoxystrobin concentrations showed no significant differences for any pesticide combination at any time point. This was backed up by non-metric multidimensional scaling (NMDS) analysis which showed a clear grouping of points when sampling times were compared (Figure 2.10a) as opposed to azoxystrobin concentration (Figure 2.10b).

a)



b)

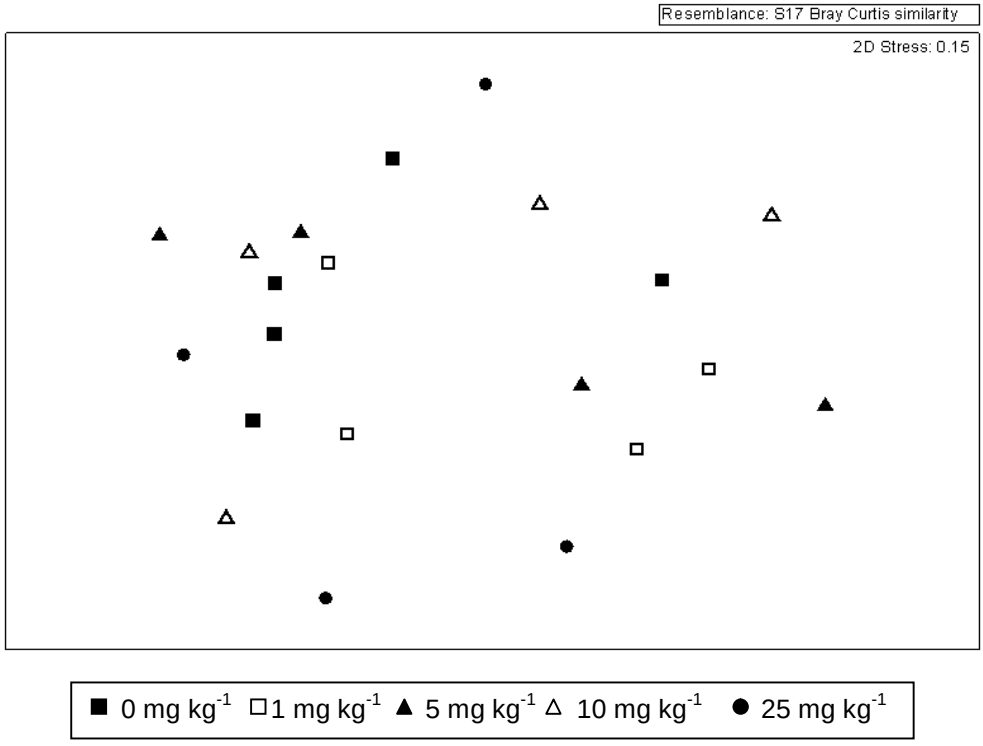


Figure 2.10. NMDS analysis of fungal community structure produced using the primer pair ITS1f and ITS4r and grouped by a) sampling time and b) azoxystrobin concentration. Each data point represents the mean of 4 experimental replicates. In figure b) the time 0 point is included within the 0 mg kg^{-1} samples.

2.3.4.1.1.2 EF4f and EF3r Primer Pair

In total 64 unique TRFs were observed with between 12 and 24 recorded in an individual trace. There was no significant difference in the overall number of TRFs recorded in the different treatments (p = 0.43). Sampling time also didn't have a significant impact on TRF numbers (p = 0.08).

Using the EF4f and EF3r primer set there was a significant impact of azoxystrobin concentration on fungal diversity (p = < 0.01). Contrast analysis showed that the diversity in the 25 mg kg^{-1} treatment was significantly different to all of the other treatments (p = < 0.01).

The diversity of the 0 mg kg^{-1} control remained relatively stable throughout the course of the experiment (Figure 2.11). After 1 month, there was no significant difference in the diversity in the 1 to 10 mg kg^{-1} treatments, compared to the controls. Conversely, in the 25 mg kg^{-1} treatment, the diversity was only 79% that of the un-amended controls. Indeed, the diversity in the 25 mg kg^{-1} treatment was significantly lower than in the un-amended control throughout the experimental period. In this treatment, the diversity was between 78 and 80% that of the controls. After 3 months, the diversity increased in the 1 to 10 mg kg^{-1} treatments and these levels were also observed after 4 months. Indeed, after 4 months the diversity in the 1 mg kg^{-1} treatment was significantly higher than in the control, as determined by LSD analysis.

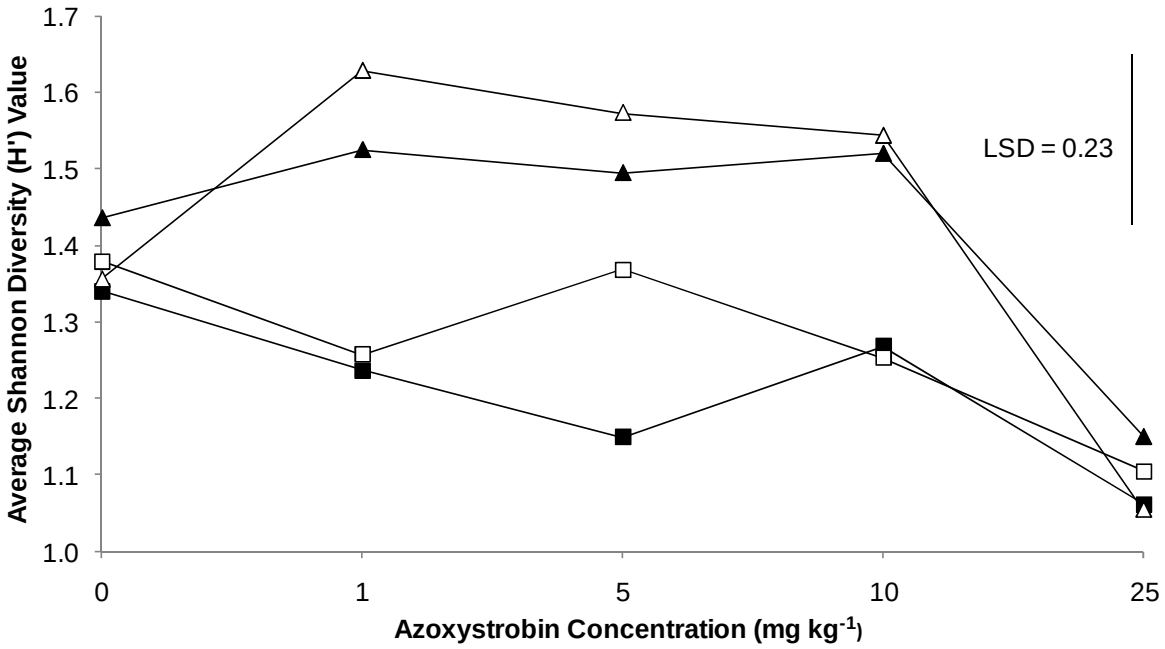
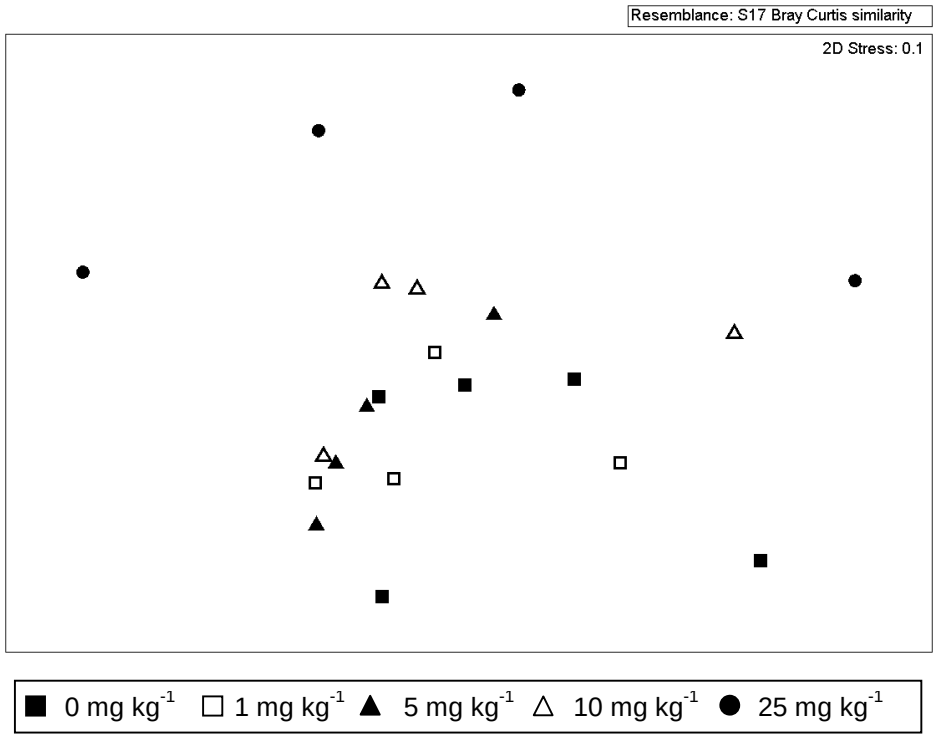


Figure 2.11. The average Shannon diversity (H') of fungal communities recorded using the EF4f/EF3r primer pair over 4 months following azoxystrobin application. ■ 1 month □ 2 months ▲ 3 months △ 4 months. Each data point represents the mean of 4 experimental samples.

When the EF4f and EF3r primer set was used to determine the impacts of azoxystrobin concentration on fungal community structure, the results were markedly different to those observed using the ITS1f and ITS4r primer pair. Although ANOSIM analysis showed no overall significant impact of azoxystrobin concentration on fungal community structure (p = 0.168), pair-wise analysis did show some significant impacts. These impacts were found in the 25 mg kg^{-1} treatment relative to the 0 (p = 0.032), 1 (p = 0.029), and 5 mg kg^{-1} (p = 0.029) treatments.

The results of the ANOSIM were backed up by NMDS analysis which showed no noticeable grouping of samples based on azoxystrobin concentration (Figure 2.12a). However, the points representing the 25 mg kg^{-1} treatment showed a significant difference in fungal community structure compared to the 0 mg kg^{-1} control (p = 0.032). Unlike with the ITS1f and ITS4r primer pair, no grouping was observed based on sampling time (Figure 2.12b).

a)



b)

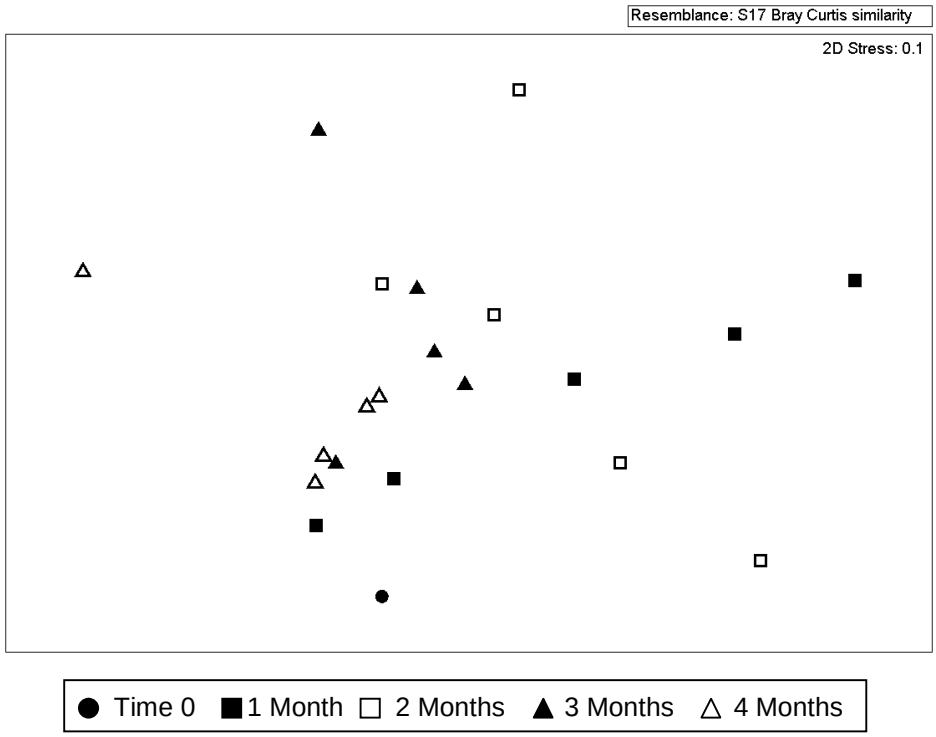


Figure 2.12. NMDS analysis of fungal community structure determined using the primer pair EF4f and EF3r and grouped by a) azoxystrobin concentration and b) sampling time. Each data point represents the mean of 4 experimental replicates. In figure a) the time 0 point is included within the 0 mg kg^{-1} samples.

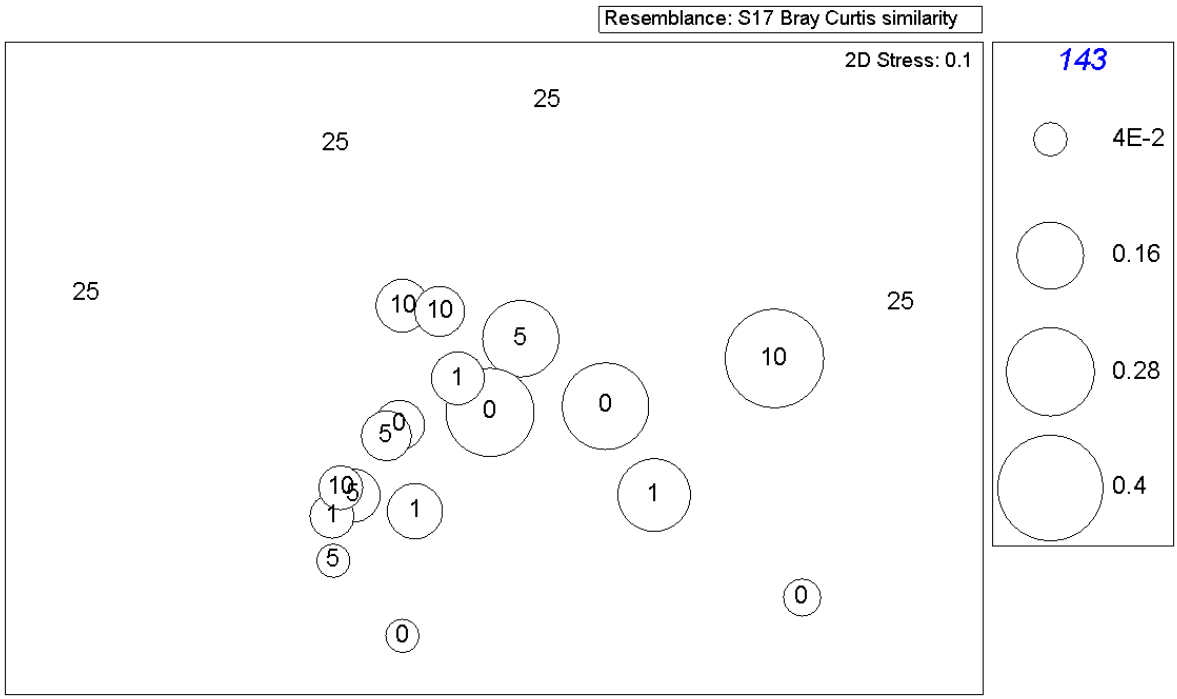
SIMPER analysis identified TRFs at 143, 146, and 148 bp that were dominant in the at the 0-10 mg kg^{-1} concentrations, but were absent in the 25 mg kg^{-1} treatment suggesting that the fungi represented by these TRFs were particularly susceptible to high concentrations of azoxystrobin.

TRF 143 bp accounted for an average of 20.6% of the intensity in the 0-10 mg kg^{-1} treatments. The absence of this TRF from the 25 mg kg^{-1} accounted for an average of 11.8% of the total variation in community structure when comparing these samples to the other treatments. The TRF at 143 bp was present to varying degrees in the 0-10 mg kg^{-1} treatments (i.e. the abundance was not dependent on the concentration of azoxystrobin applied) and was only absent in the 25 mg kg^{-1} treatment (Figure 2.13a).

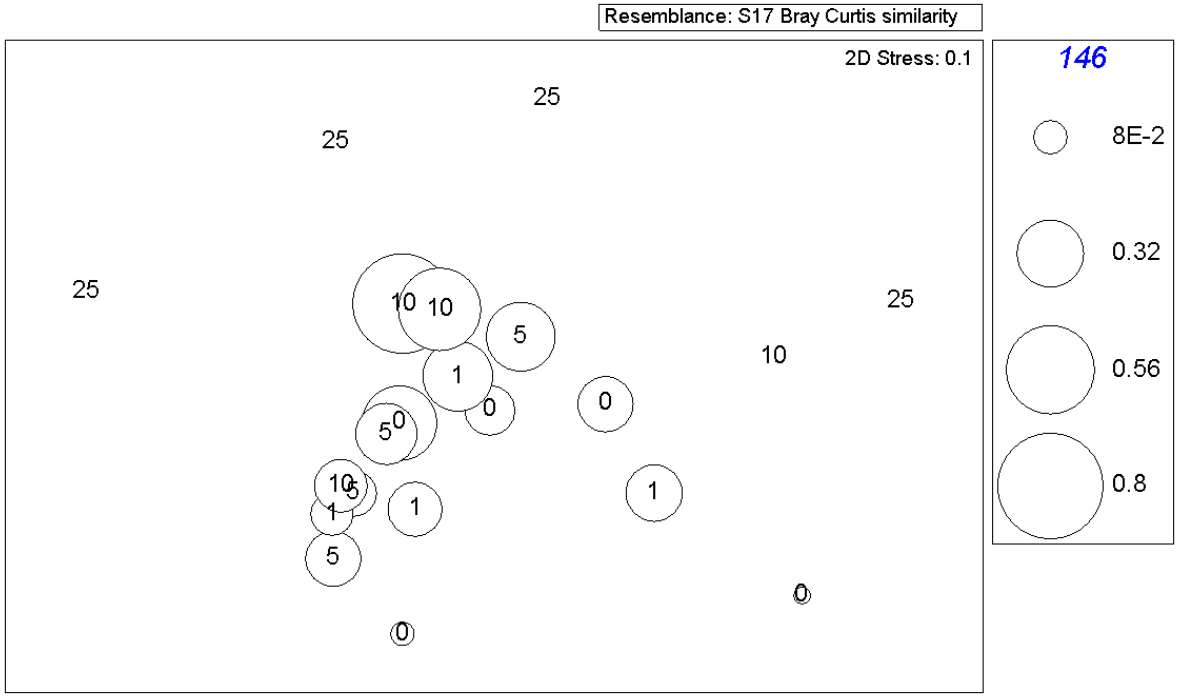
Changes in the intensity of TRF 146 were found to contribute even more to the variation between the 25 mg kg^{-1} treatment and the other treatments (22.9% of the average variation). TRF 146 bp represented an average of 33.4% of the total intensity in the 0-10 mg kg^{-1} treatments. This TRF was present in all of the treatments apart from the 25 mg kg^{-1} treatment and one of the 10 mg kg^{-1} treatments (Figure 2.13b). Once again, with the exception of the 25 mg kg^{-1} treatment the recorded abundances did not appear dependent on the concentration of azoxystrobin applied.

The values for TRF 148 bp were in-between those of the other two TRFs. This TRF represented an average of 22.2% of the total intensity in the 0-10 mg kg^{-1} samples and accounted for an average of 16.7% of the variation between the 25 mg kg^{-1} samples and the other treatments. This TRF was also present in fewer samples than the other two TRFs, although the intensities in the samples where it was present were similar to those of 143 and 146 bp (Figure 2.13c). It was not present at all in the 25 mg kg^{-1} treatment samples and in only three of the 10 mg kg^{-1} treatment samples. Additionally, it was not present in one of the 0 and one of the 1 mg kg^{-1} treatment samples.

a)


Resemblance: S17 Bray Curtis similarity
2D Stress: 0.1
143
4E-2
0.16
0.28
0.4

b)


Resemblance: S17 Bray Curtis similarity
2D Stress: 0.1
146
8E-2
0.32
0.56
0.8

c)

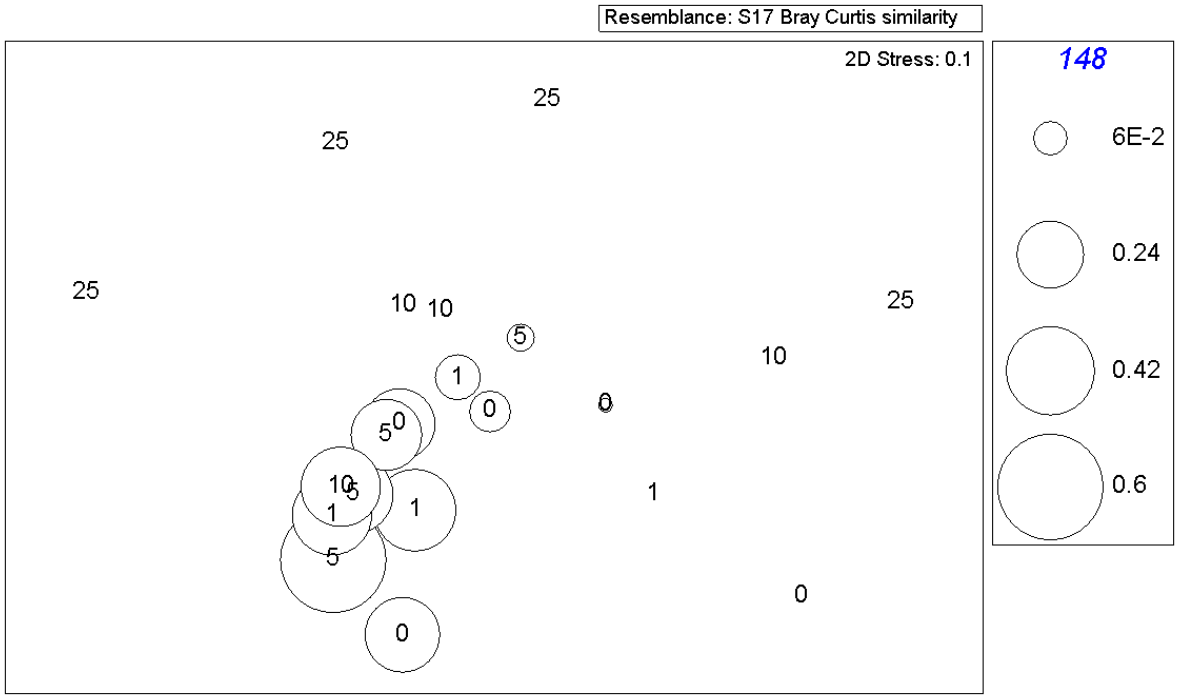


Figure 2.13. Bubble plots of TRF abundances in EF4f/EF3r fungal samples a) 143 bp b) 146 bp c) 148 bp. The numbers in the bubbles denote the pesticide concentration (mg kg^{-1}). The numbers in the keys denote the relative abundance of the TRF within a single trace. Each sample point represents the average of 4 experimental replicates. The time 0 point is included within the 0 mg kg^{-1} samples

2.3.4.1.2 Nematodes

A total of 67 different nematode TRFs were obtained with between 8 and 29 being recorded in a single trace. There was no significant impact of azoxystrobin concentration on the number of nematode TRFs (p = 0.538). Time also did not significantly impact TRF numbers (p = 0.537).

Azoxystrobin concentration had a significant impact on nematode community diversity (p = 0.049) (Figure 2.14). Contrast analysis showed that the diversity in the 25 mg kg^{-1} treatment was significantly different to the other treatments (p = < 0.01).

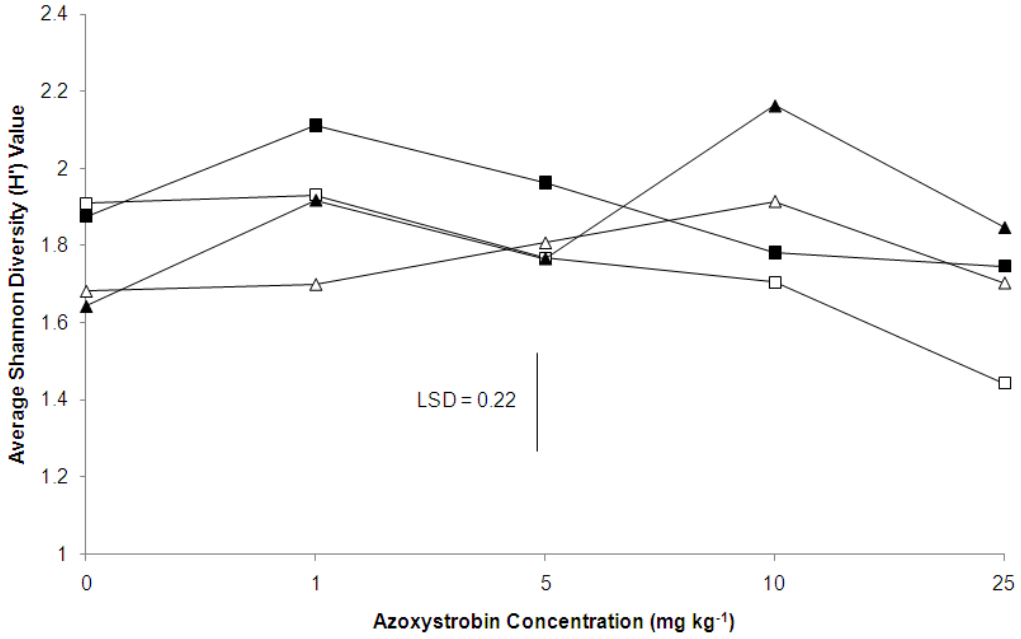
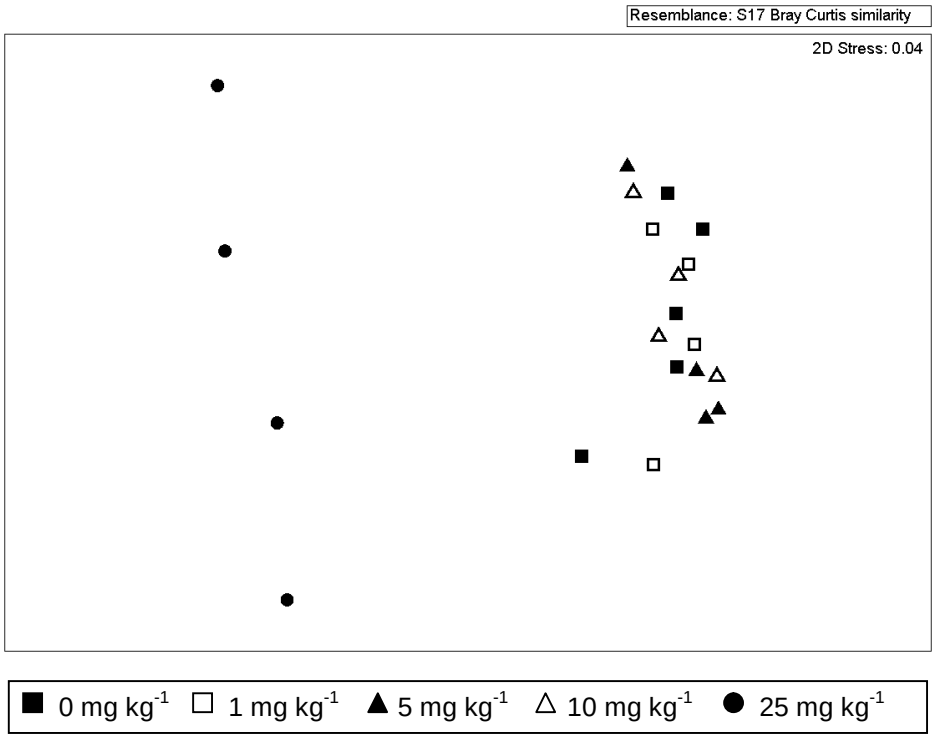


Figure 2.14. The average Shannon diversity (H') of nematode communities recorded using the Nem18Sf/Nem18Sr primer pair over 4 months following azoxystrobin application. ■ 1 month □ 2 months ▲ 3 months △ 4 months. Each sample point represents the average of 4 experimental replicates.

There was a significant impact of azoxystrobin application on the structure of the nematode community (p = 0.019). ANOSIM analysis was able to pinpoint where these significant differences occurred. These differences were found to be in every comparison between the 25 mg kg^{-1} treatment and another azoxystrobin concentration. A p value of 0.029 was recorded for comparisons with the 1, 5 and 10 mg kg^{-1} treatments whereas the value was 0.008 compared with the 0 mg kg^{-1} control.

The ANOSIM results were backed up those of the NMDS analysis that showed a noticeable grouping of the 25 mg kg^{-1} treatment samples which was distinct from the other pesticide concentrations (Figure 2.15a). There was no discernable grouping of data points based on sampling time (Figure 2.15b).

a)



b)

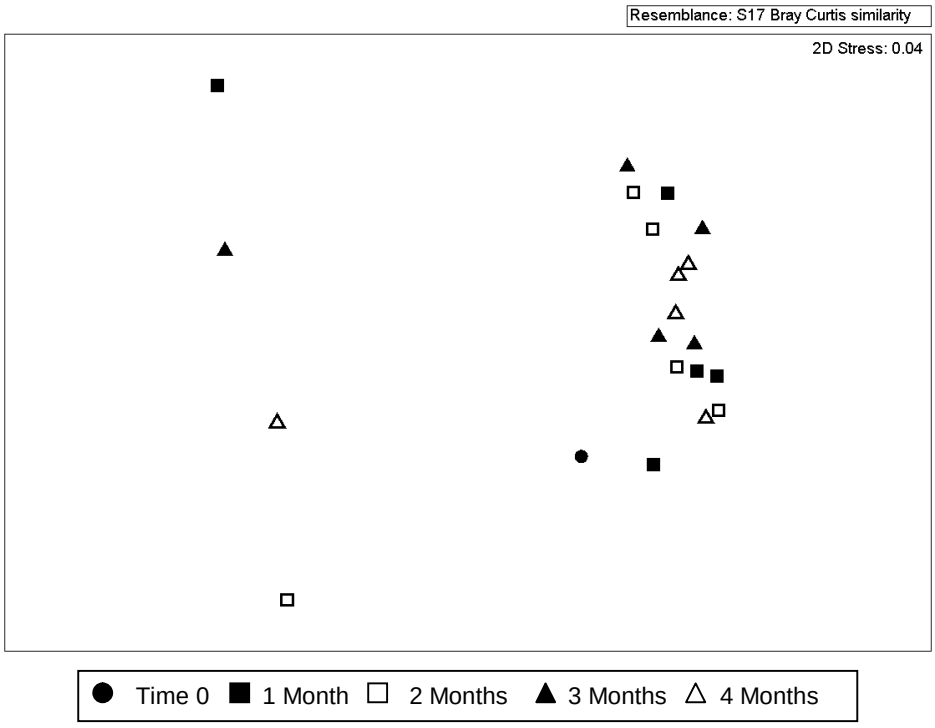


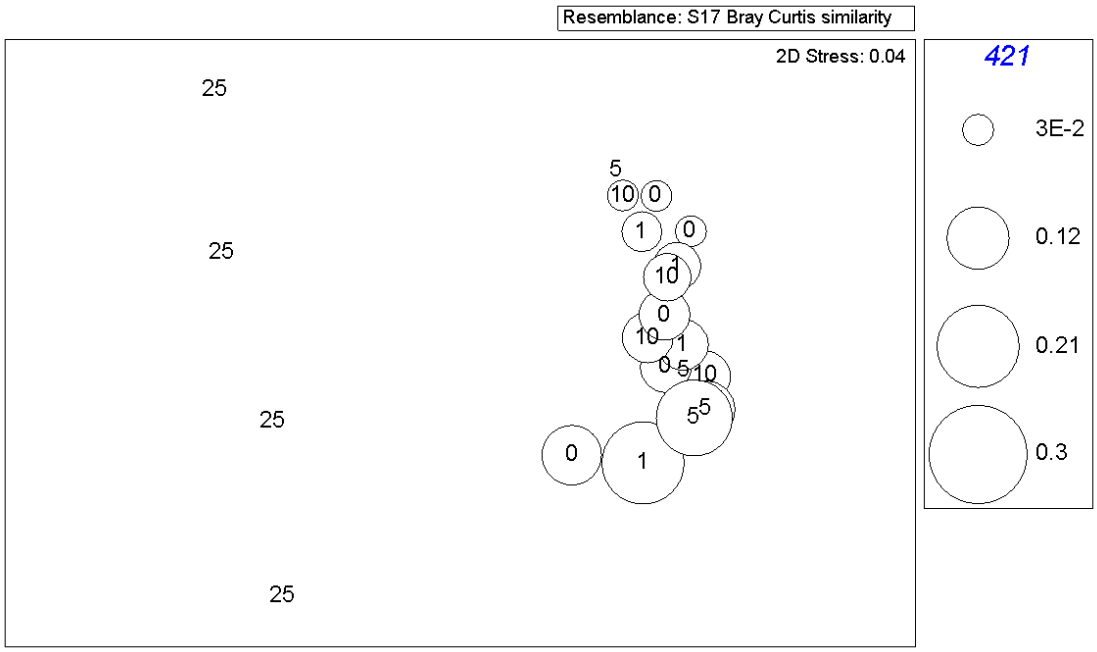
Figure 2.15. NMDS analysis of nematode community structure produced using the primer pair Nem18Sf/Nem18Sr grouped by a) azoxystrobin concentration and b) sampling time. Each sample point represents the average of 4 experimental replicates. In figure a) the time 0 point is included within the 0 mg kg^{-1} samples.

SIMPER analysis showed that the TRF of 421 and 458 bp were impacted by high azoxystrobin concentrations. Bubble plot analysis was used to compare the changes in the abundances of these TRFs in response to azoxystrobin concentration (Figure 2.16).

TRF 421 bp represented an average of 7.5% of the total sample intensities. However, this TRF was responsible for an average of 12.5% of the variation between the 25 mg kg^{-1} samples and the other treatments. The abundance of TRF 421 bp (Figure 2.16a) didn't appear to be dependent upon the concentration of azoxystrobin at concentrations ranging from 0 to 10 mg kg^{-1}. However, it was not present in any of the 25 mg kg^{-1} treatment samples.

TRF 458 bp was slightly less abundant in the communities, representing an average of 6.9% of the total sample intensities. This TRF was responsible for an average of 12.2% of the community variation when the 25 mg kg^{-1} treatment was compared to the other samples. It was more variable in abundance between different azoxystrobin concentrations than TRF 421 bp (Figure 2.16b). However, at the highest azoxystrobin concentration it was not present in the nematode community apart from after 1 month.

a)



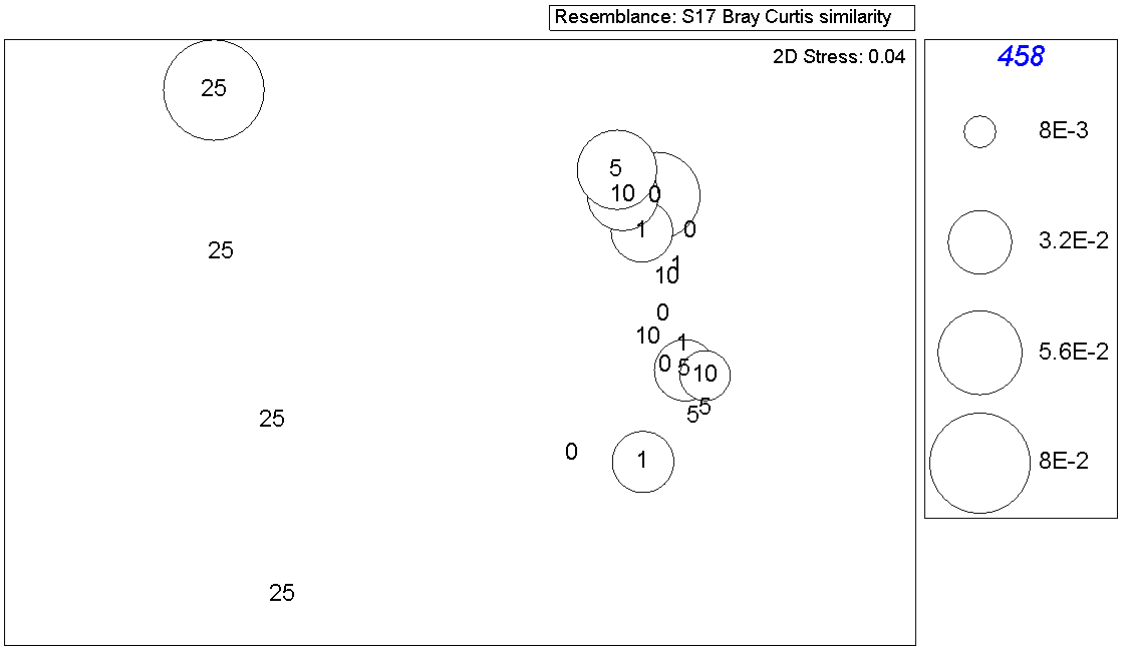


Figure 2.16. Bubble plots of TRF abundances in nematode samples a) 421 bp b) 458 bp. The numbers in the bubbles denote the pesticide concentration (mg kg^{-1}). The numbers in the keys denote the relative abundance of the TRF. Each sample point represents the average of 4 experimental replicates. The time 0 point is included within the 0 mg kg^{-1} samples.

2.3.4.1.3 General Eukaryotes

50 unique general eukaryote TRFs were obtained in total. The numbers recorded in a single trace ranged from 8 to 18. 2-way ANOVA indicated that azoxystrobin concentration significantly affected TRF numbers (p = <0.01), but sampling time did not (p = 0.051). Azoxystrobin application did not significantly impact the eukaryotic diversity (p = 0.066) (Figure 2.17). However, contrast analyses showed that the diversity in the 10 and 25 mg kg^{-1} treatments was significantly different to the diversity in the 1 and 5 mg kg^{-1} treatments.

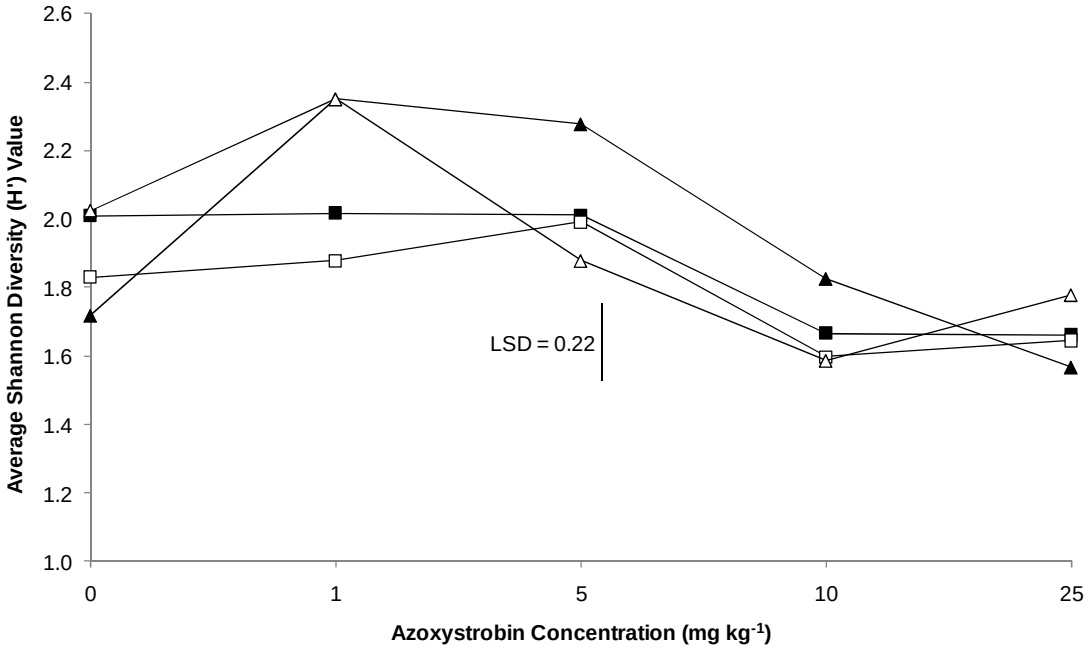
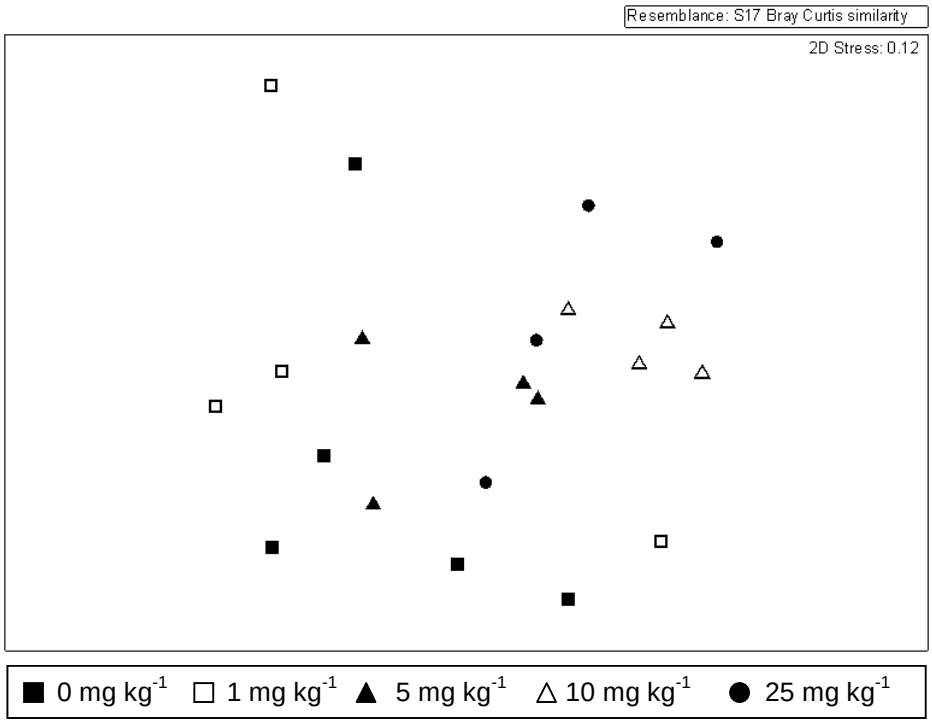


Figure 2.17. The average Shannon diversity (H') of general eukaryote communities comparing sampling time. ■ 1 month □ 2 months ▲3 months △ 4 months. Each sample point represents the average of 4 experimental replicates.

ANOSIM analysis of general eukaryote community structure showed no significant impacts of either azoxystrobin concentration or sampling time (p = 0.100 and 0.360, respectively). This observation was backed up by the pair-wise comparisons which showed no significant differences between any of the combinations, at any time point. Additionally, NMDS analyses (Figure 2.18) demonstrated little or no grouping of points based on either azoxystrobin concentration (Figure 2.18a) or sampling time (Figure 2.18b).

a)



b)

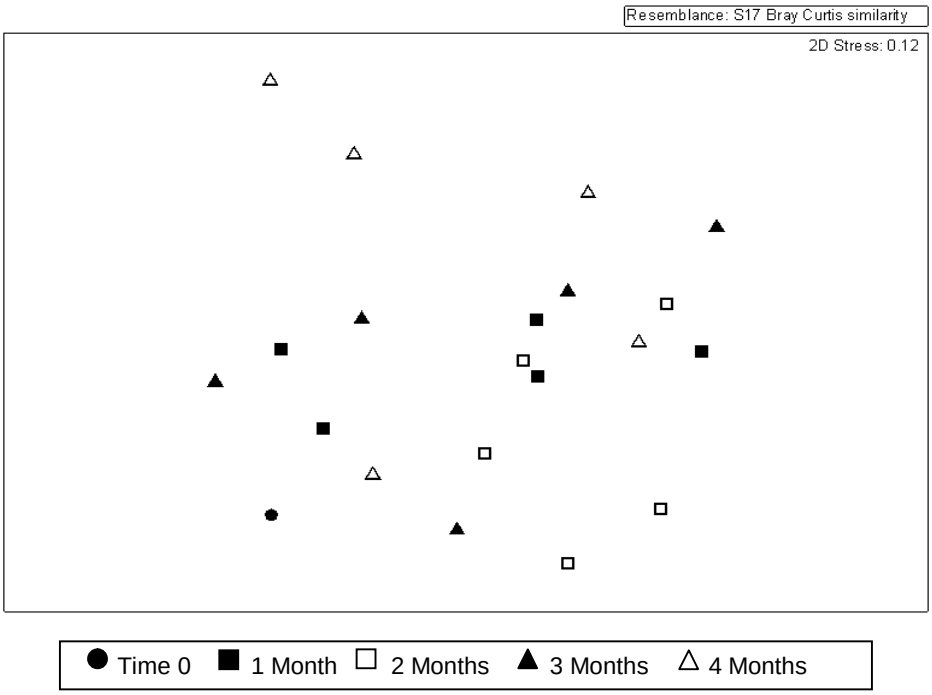


Figure 2.18. NMDS analysis of general eukaryote community structure grouped by a) azoxystrobin concentration and b) sampling time. Each sample point represents the average of 4 experimental replicates. In figure a) the time 0 point is included within the 0 mg kg^{-1} samples.

2.3.4.2 Prokaryotes

2.3.4.2.1 Bacteria

A total of 159 individual TRFs were recorded for the bacterial samples. In a single trace, the number of TRFs varied markedly from 33 to 109. Neither azoxystrobin concentration (p = 0.994) nor sampling time (p = 0.123) had a significant impact on bacterial TRF.

There was no significant impact of azoxystrobin concentration on the diversity of the bacterial community as determined using Shannon analysis (Figure 2.19). Contrast analysis also showed no significant differences between individual pesticide concentrations. At time 0 the diversity was 2.848. Throughout the 4 month experimental period the diversity in all samples at all sampling times fluctuated within a very narrow range around this value. The highest diversity recorded was 2.916 in the 25 mg kg^{-1} samples after one month whereas the lowest was 2.834 recorded in the 1 mg kg^{-1} samples after 3 months.

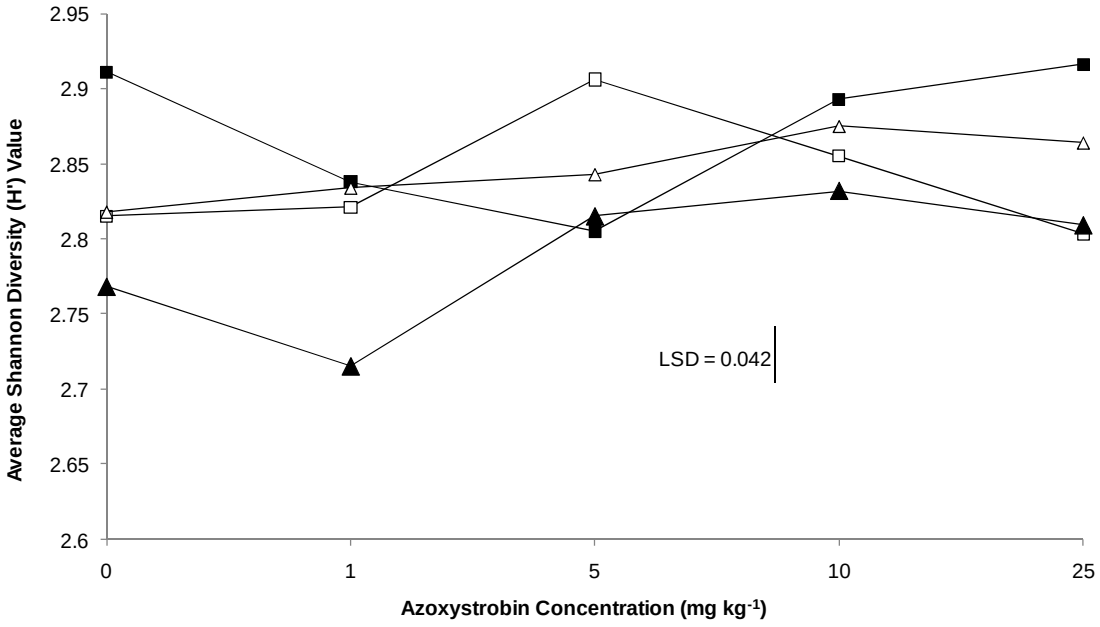
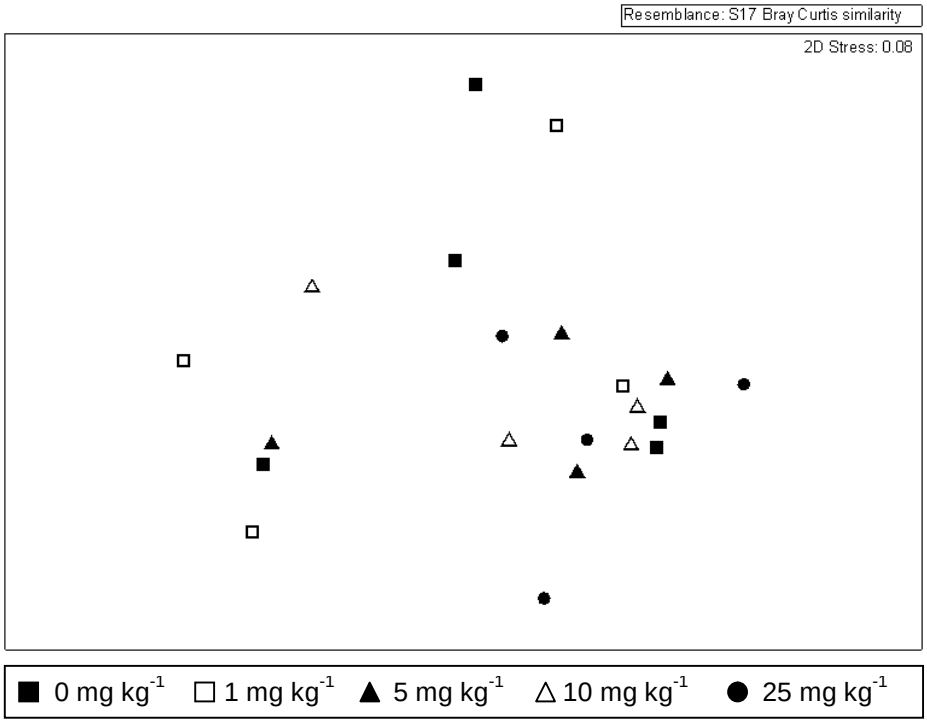


Figure 2.19. The average Shannon diversity (H') of bacterial communities comparing sampling time. ■1 month □2 months ▲3 months △4 months. Each data point represents the average of 4 experimental replicates.

Azoxystrobin application did not have a significant impact on the structure and diversity of the bacterial community. ANOSIM analysis showed that the bacterial community was not significantly impacted by either the concentration of azoxystrobin applied (p = 0.636) or the sampling time (p = 0.806). These observations were backed up by NMDS analyses which showed no discernable grouping of sampling points based on either azoxystrobin concentration or sampling time (Figure 2.20). However, three individual TRFs (470, 472, and 814 bp) increased in relative intensity with increasing azoxystrobin concentrations.

a)



b)

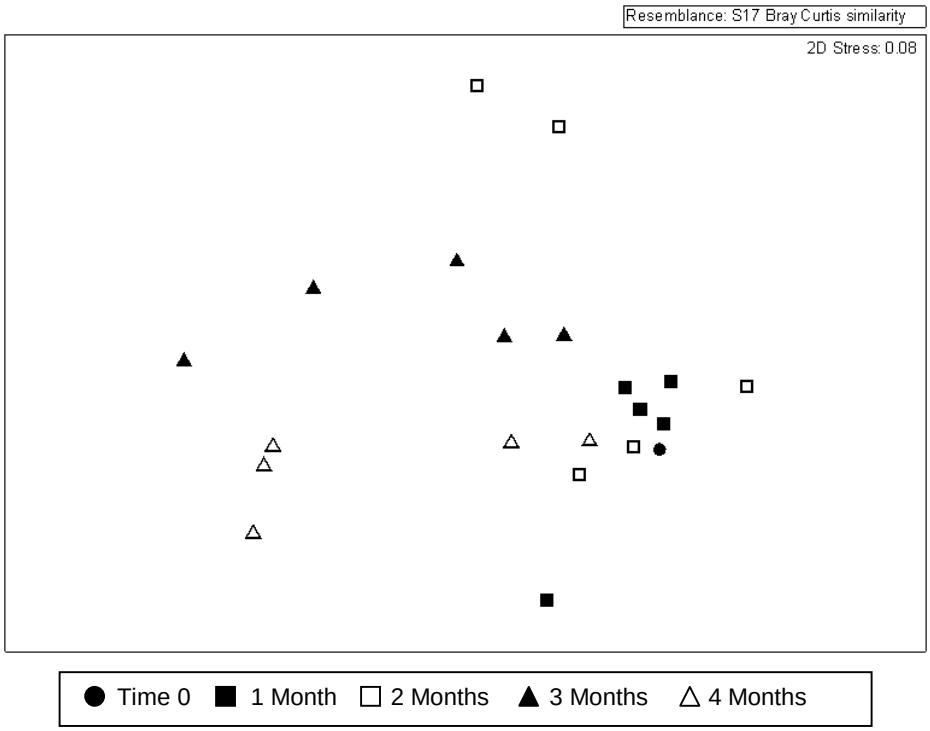


Figure 2.20. NMDS analysis of bacterial community structure grouped by a) azoxystrobin concentration and b) sampling time. Each sample point represents the average of 4 experimental replicates. In figure a) the time 0 point is included within the 0 mg kg^{-1} samples.

2.3.4.2.2 Archaea

T-RFLP analysis produced a total of 98 individual archaeal TRFs with between 8 and 33 being present in an individual trace. A 2-way ANOVA showed that neither azoxystrobin concentration (p = 0.352) nor sampling time (p = 0.131) had a significant impact on TRF numbers. There was no significant impact of azoxystrobin on archaeal diversity as demonstrated by Shannon analysis (Figure 2.21) (p = 0.754). Contrast analysis also indicated no significant differences in diversity between individual treatments.

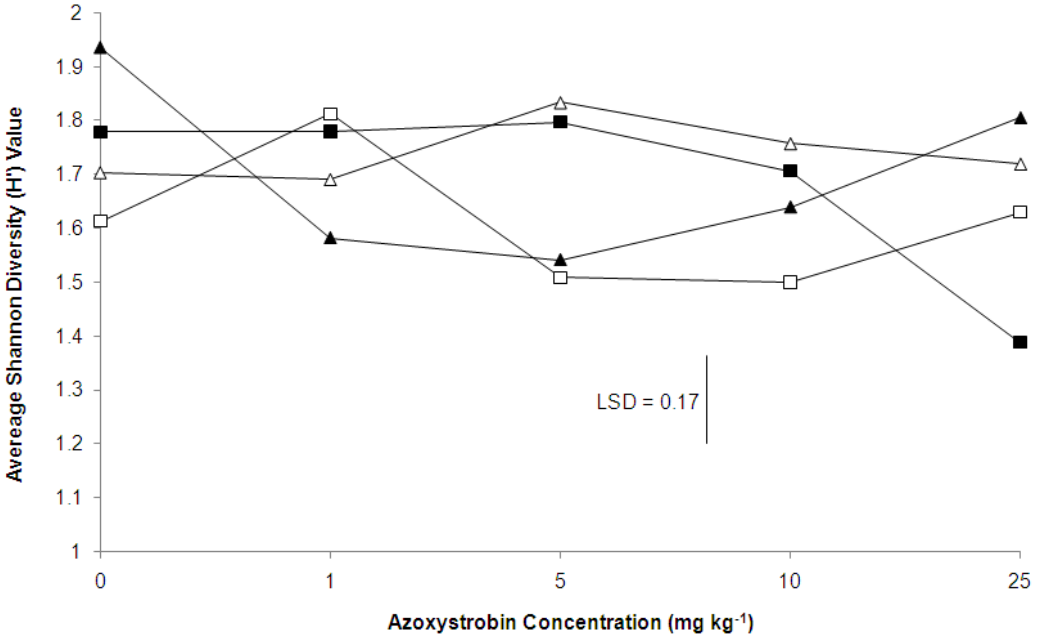
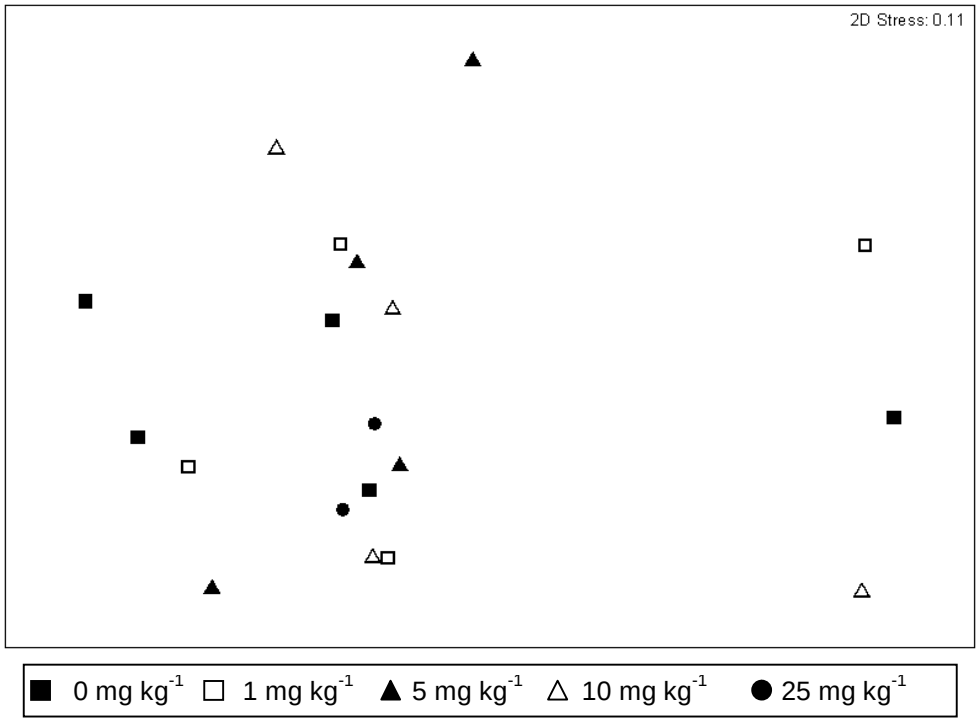


Figure 2.21. The average Shannon diversity (H') of archaeal communities recorded using the Ar3f/Ar927r primer pair over 4 months following azoxystrobin application. ■ 1 month □ 2 months ▲ 3 months △ 4 months. Each sample point represents the average of 4 experimental replicates.

Azoxystrobin concentration did not have a significant impact on archaeal community structure. ANOSIM analyses produced *p* values of 0.890 and 0.001 for pesticide concentration and sampling time, respectively indicating that sampling time had a highly significant impact on archaeal community structure. Pair-wise comparisons showed no significant differences between any of the treatments, at any time point. NMDS analyses backed this conclusion as there was no clear grouping of data points when the samples were grouped by azoxystrobin concentration (Figure 2.22a). In contrast, when samples were grouped by sampling time (Figure 2.22b) grouping of points was observed particularly in the samples taken after 2 and 4 months.

a)



b)

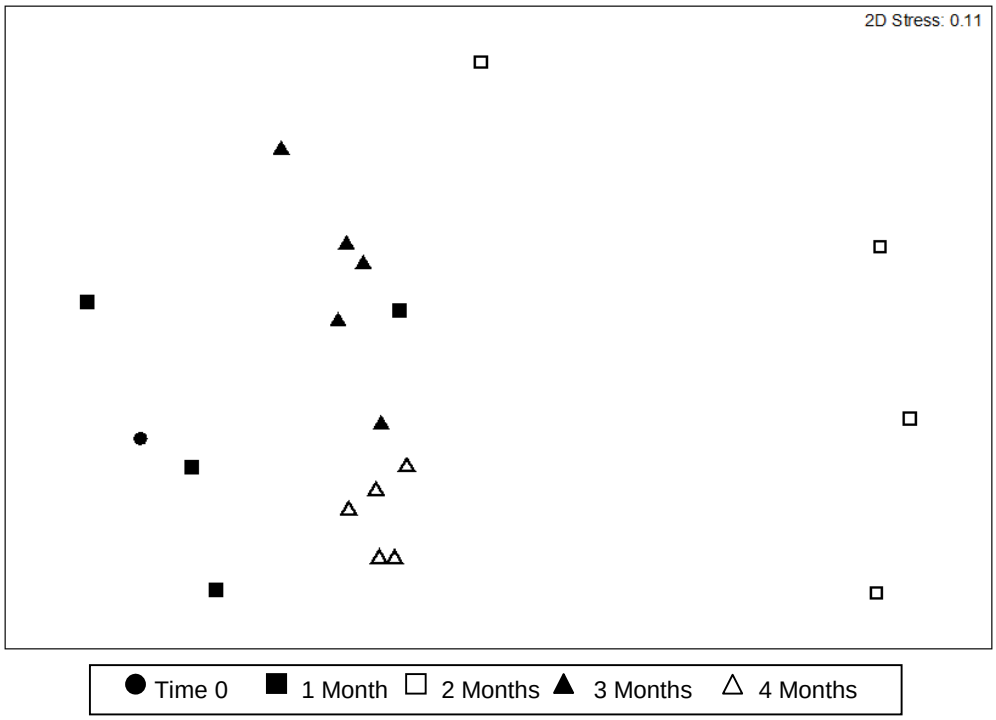


Figure 2.22 NMDS analysis of archaeal community structure grouped by a) azoxystrobin concentration and b) sampling time. Each sample point represents the average of 4 experimental replicates. In figure a) the time 0 point is included within the 0 mg kg^{-1} samples.

2.3.4.2.3 Pseudomonads

A total of 73 individual pseudomonad TRFs were recorded with between 9 and 19 being present in an individual trace. A 2-way ANOVA of the TRF data confirmed that azoxystrobin concentration did not have a significant impact on TRF numbers (p = 0.905). Sampling time, however, did have a significant impact on the number of TRFs recorded (p = <0.01).

Azoxystrobin concentration had no significant impact on pseudomonad diversity as determined by ANOVA (p = 0.147) and contrast analysis, but sampling time did (p = < 0.01) (Figure 2.23).

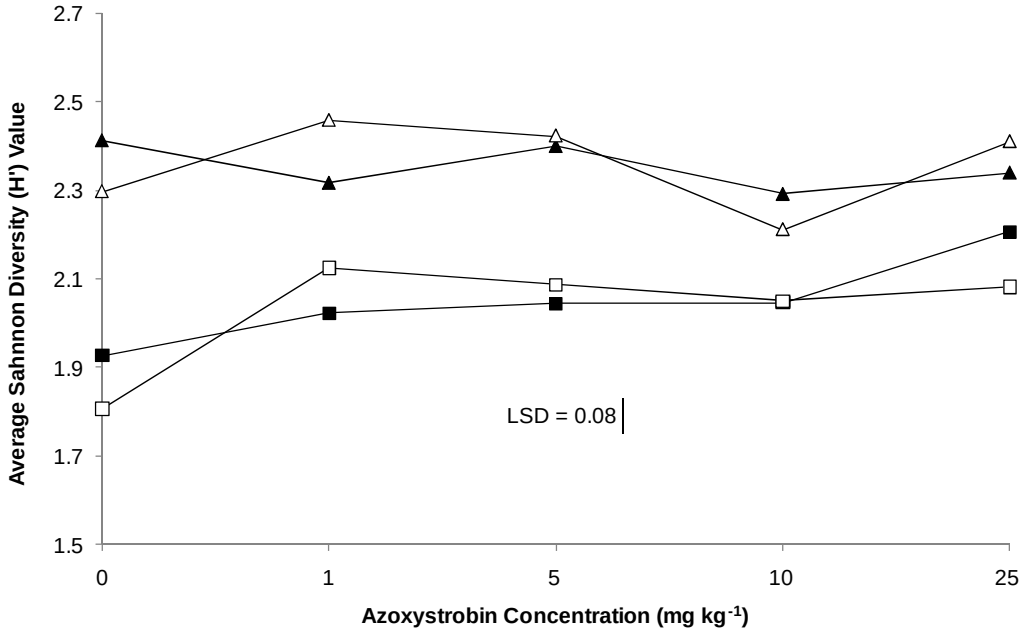
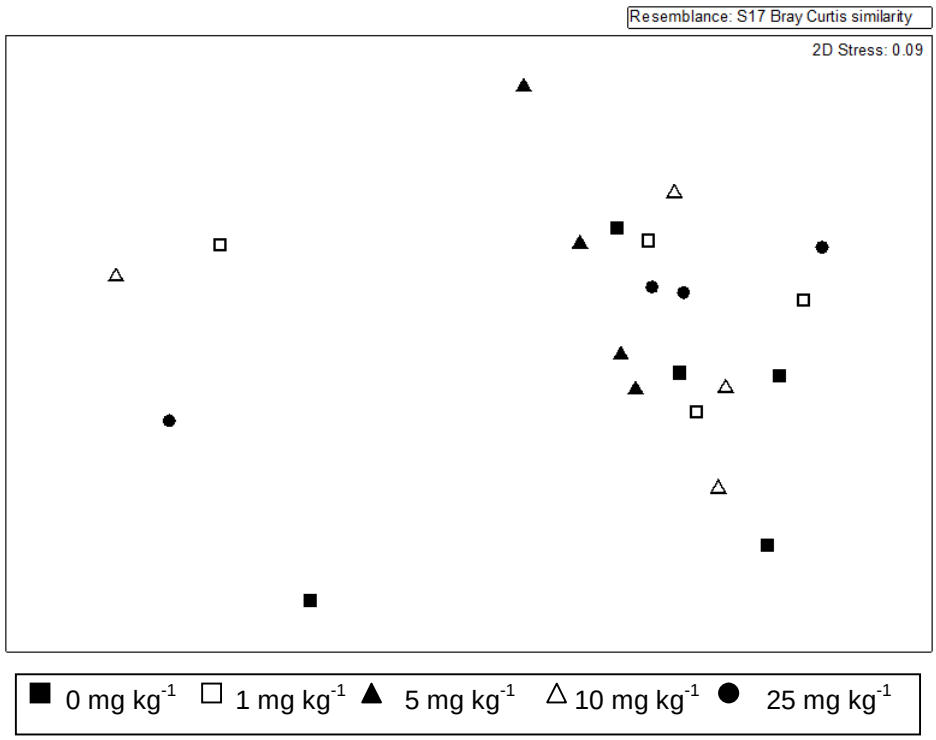


Figure 2.23. The average Shannon diversity (H') of pseudomonad communities recorded using the PS16Sf/PS23Sr primer pair over 4 months following azoxystrobin application. ■ 1 month □ 2 months ▲ 3 months △ 4 months. Each data point represents the average of 4 experimental replicates.

As with the archaeal samples, sampling time had a greater bearing on the structure of the pseudomonad community than azoxystrobin concentration. ANOSIM analysis produced p values of 0.941 and 0.001 for comparisons between azoxystrobin concentration and sampling time respectively. Pair-wise comparisons failed to show any significant differences between any of the pesticide combinations. NMDS analysis backed up these findings. There was no discernable grouping of points based on azoxystrobin concentration (Figure 2.24a), whereas figure 2.24b shows a clear grouping of

points based on sampling time, particularly at the 1 and 2 month sampling points.

a)



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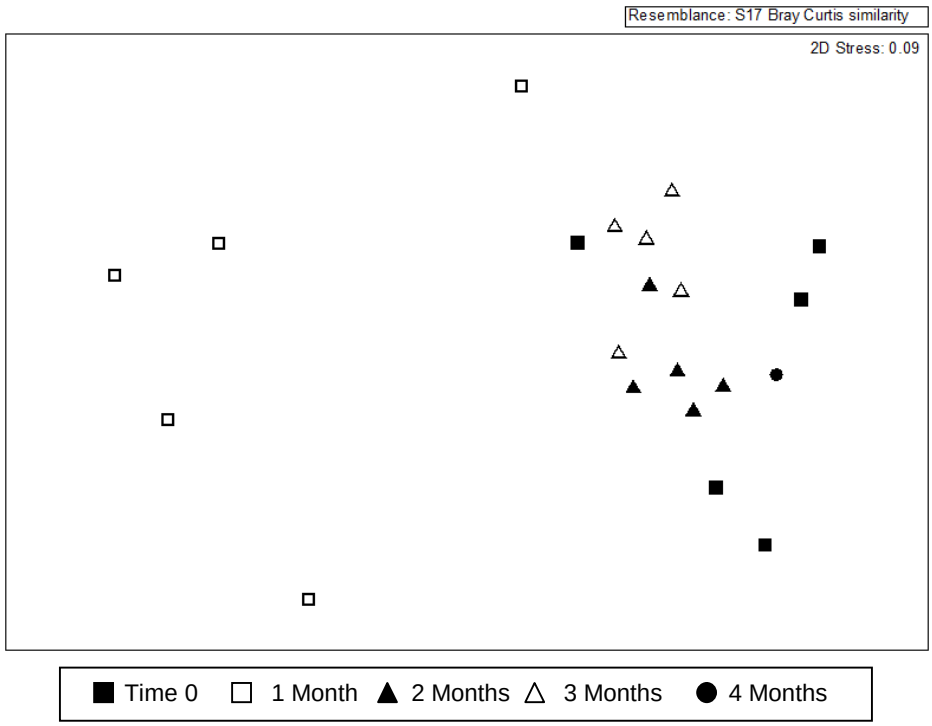


Figure 2.24 NMDS analysis of pseudomonad community structure grouped by a) azoxystrobin concentration and b) sampling time. Each sample point represents the average of 4 experimental replicates. In figure a) the time 0 point is included within the 0 mg kg^{-1} samples.

2.3.5 Fungal and Nematode clone library analysis

Clone libraries were produced for fungi and nematodes using the 18S small subunit rRNA gene regions, as used for T-RFLP analysis. The libraries enabled the structure and diversity of un-amended and amended (25 mg kg^{-1}) treatments to be determined and compared. The libraries also allowed TRF sizes for each sequence to be predicted and compared with the results obtained previously using T-RFLP analysis. Tentative identifications were then able to be made for individual TRFs, with those of the dominant community organisms and those that appear to have been impacted by the application of azoxystrobin being of particular interest.

2.3.5.1 Fungi

Two clone libraries were produced from the PCR products of 0 mg kg^{-1} and 25 mg kg^{-1} samples taken 1 month post-application using the EF4f and EF3r primer pair. The clone libraries produced included 80 individual sequences for the 0 mg kg^{-1} samples and 83 individual sequences for the 25 mg kg^{-1} samples.

Figure 2.25 shows the collector's curves produced from the 0 mg kg^{-1} and 25 mg kg^{-1} clone libraries. The curves demonstrate the higher number of unique clones in the 0 mg kg^{-1} samples (32) compared with those obtained in the 25 mg kg^{-1} library (24). Unique clones were identified at regular intervals throughout the library, suggesting that the full extent of fungal diversity wasn't accounted for.

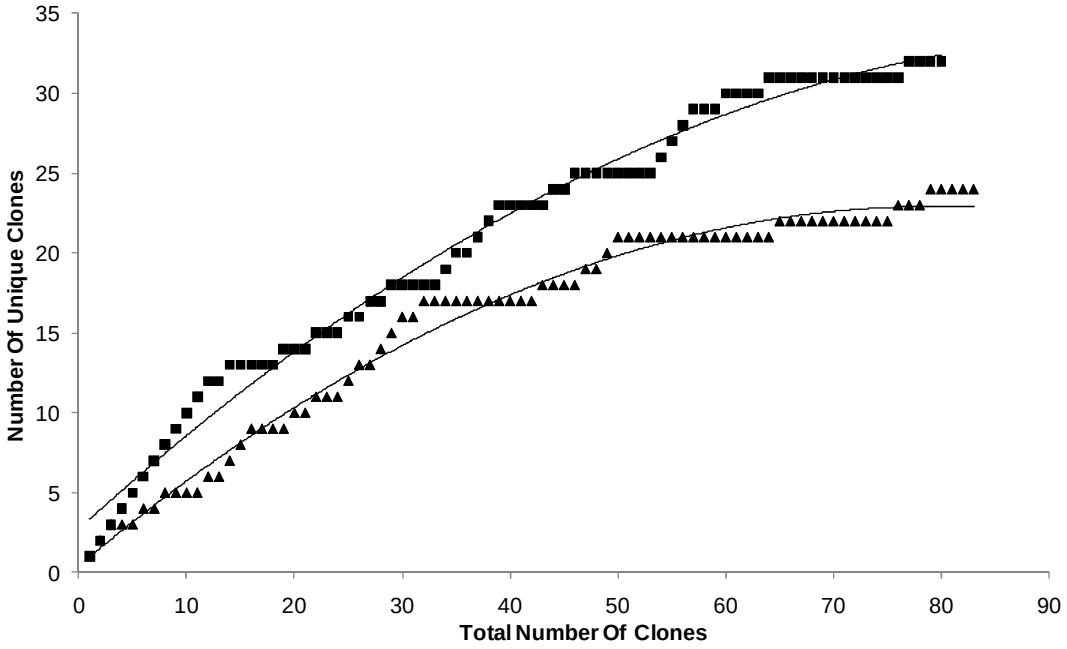


Figure 2.25. Collector's curves for the 0 and 25 mg kg^{-1} clone libraries produced using the EF4f/EF3r fungal primer pair. ■ 0 mg kg^{-1} ▲ 25 mg kg^{-1}

The percentage distribution of different fungal groups within the clone libraries is shown in Figure 2.26. The 0 mg kg^{-1} library consisted primarily of ascomycete fungi (66.5%), and the 25 mg kg^{-1} library consited mostly of zygomycete fungi (54.0%). This was mostly due to the increase in the prevalence of sequences showing a homology to Zygomycete sp. AM-2008a from 9.0% in the un-amended treatment library to 49.5% in the 25 mg kg^{-1} library.

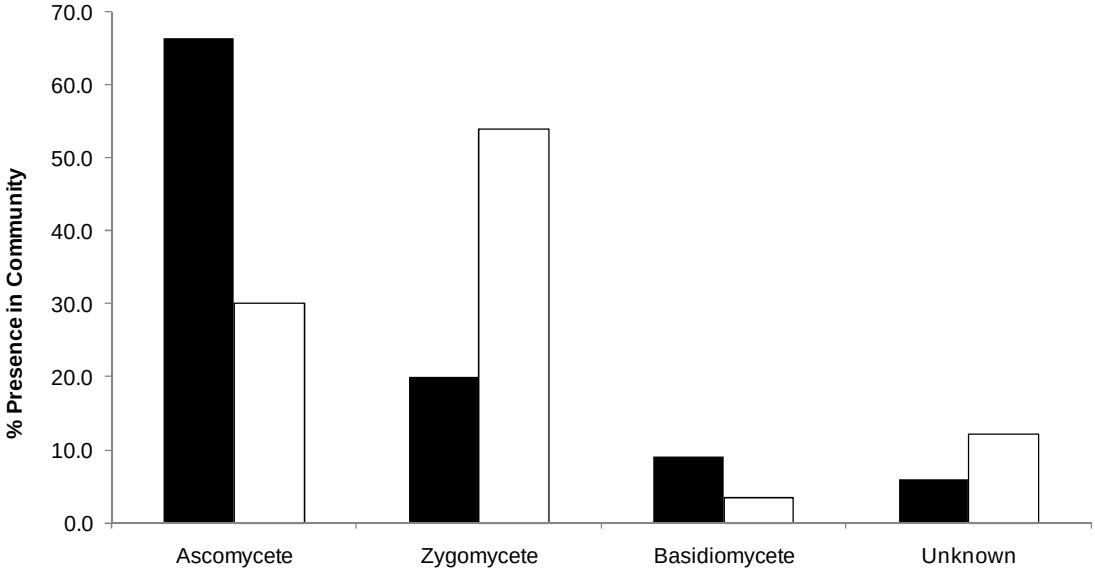


Figure 2.26. The prevalence of different fungal groups within clone libraries produced from 0 and 25 mg kg^{-1} (1 Month) samples using the primer pair EF4f and EF3r. ■ 0 mg kg^{-1} □ 25 mg kg^{-1}

Table 2.3 details the main sequences obtained in the two clone libraries and the reference for the top hit in the BLAST searches (at a sequence similarity of 90% or greater) for the un-inoculated control and the 25 mg kg^{-1} samples, respectively.

Table 2.3. Sequence homologies from 0 and 25 mg kg^{-1} fungal clone libraries produced using the EF4f and EF3r primer pair. All fungal classifications had a grouping similarity of ≥ 97%

Fungal Classification from BLAST Search	Accession Number of Nearest Match	% Sequence Homology to Nearest Match	% Presence in 0 mg kg^{-1} Library	% Presence in 25 mg kg^{-1} Library
Uncultured Zygomycete	AJ 506030.1	98	9.0	4.0
Exophiala pisciphila	NG 013192.1	98	9.0	1.0
Zygomycete sp. AM-2008a	EU 428773.1	100	9.0	49.5
Penicillium sacculum (Eladia saccula)	AB 031391.1	99	7.5	-
Fusarium oxysporum	DQ 916150.1	100	6.5	-
Uncultured Fungal Species	HQ 404549.1	98	6.5	3.0
Cryptococcus sp.	AB 032614.1	99	6.5	1.0
Nectria lugdunensis	AY 357278.1	98	4.0	3.5
Penicillium sp.	DQ 810194.1	97	4.0	5.0
Pochonia suchlasporia	AB 214658.1	99	4.0	-
Uncultured Eimeriidae	EF 023204.1	95	2.5	4.0
Byssoascus striatosporus	AB 015776.1	97	2.5	-
Collophora rubra	GQ 154629.1	95	2.5	-
Cryomyces minteri	DQ 066714.1	94	2.5	-
Geosmithia putterillii	AY 231439.1	96	2.5	-
Metatrichia vesparium	AF 542044.1	90	2.5	-
Cladophialophora boppii	FJ 358301.1	96	2.5	-
Nomuraea rileyi	AB 268359.1	98	1.0	-
Pleosporales sp.	DQ 085396.1	99	1.0	3.5
Phacidiopyanis washingtonensis	FJ 237044.1	97	1.0	-
Tetracladium maxilliforme	EU 883430.1	95	1.0	-
Cladophialophora Sp.	AJ 232953.1	95	1.0	-
Giberella fujikuroi	AB 237662.1	100	1.0	-
Tremella foliacea	L 22262.1	91	1.0	-
Nectria cinnabarina	AB 237663.1	96	1.0	2.5
Toxicocladospoium irritans	GU 214619.1	92	1.0	-
Tetracladium sp.	EU 883433.1	98	1.0	-
Uncultured Dothideomycete	AY 275187.1	90	1.0	-
Curcurbitaria elongata	DQ 678009.1	98	1.0	-
Pseudophacidium ledi	AF 315623.1	95	1.0	-
Uncultured Sarcosomataceae	EF 023647.1	99	1.0	-
Fusarium merismoides	AF 141950.1	96	1.0	-
Phoma sp.	FJ 430776.1	98	-	2.5
Paecilomyces carneus	AB 258369.1	99	-	2.5
Thaumalomonadida sp.	EF 023506.1	99	-	1.0
Arthrobotrys oligospora	DQ 157745.1	90	-	1.0
Myrothecium verrucaria	EF 211127.1	96	-	1.0
Cyathicula microspora	EU 940015.1	99	-	1.0
Pyrenochaeta sp. GMG_PPb7	FJ 439593.2	99	-	1.0
Trichosporon loubieri	AB 001759.2	97	-	1.0
Sordariomycete sp. pgp-hsf	AF 292054.1	98	-	1.0
Psilolechia lucida	AF 455132.1	91	-	1.0
Leohumicola sp.	AY 706331.1	97	-	1.0
Bionectria ochroleuca	GU 112755.1	99	-	1.0

The predominant sequences in the 0 mg kg^{-1} samples (Table 2.3) showed a high sequence homology to *Exophiala pisciphila* (a black yeast found widely in the environment) and Zygomycete sp. AM-2008a (a cold-tolerent zygomycete) and uncultured zygomycetes, which represented 9% of the total number of sequences each. *Cryptococcus* sp. (a soil yeast) and the plant pathogen *Fusarium oxysporum* each represented 6.5% of the total sequences. Additionally, 6.5% of the sequences were classified as "uncultured fungal species".

In contrast the clone library of the 25 mg kg^{-1} samples was dominated by Zygomycete sp. AM-2008a which accounted for 49.5% of the total sequences. Sequences with high homologies to *Nectria lugdunensis* (a wood-decaying fungus) which represented 3.5% of the total sequences, and the plant pathogen *Pleosporales* sp. (which actually increased slightly in prevalence from 1% of the total sequences in the 0 mg kg^{-1} samples to 3% in the 25 mg kg^{-1} samples) were also present within the library. Two of the prevalent sequence groups in the 0 mg kg^{-1} samples (which showed homologies to *Penicillium sacculum* and *Fusarium oxysporum*) were not found at all in the 25 mg kg^{-1} samples. The abundance of sequences with homologies to *Exophiala pisciphila* and *Cryptococcus aerius* were reduced from 9% and 6.5% respectively in the 0 mg kg^{-1} clone library to 1% each in the 25 mg kg^{-1} library.

In silico analysis of the sequences with homologies to *Fusarium oxysporum* and *Exophiala pisciphila* showed that they would have both produced a TRF at 145 bp using the restriction enzyme *Hha*I and this matches quite well with the peak changes in the T-RFLP traces where TRFs at 143, 146 and 148 bp are absent in the 25 mg kg^{-1} samples, but were present in the un-amended controls.

The sequences with a homology to Zygomycete sp. AM-2008a in the 0 mg kg^{-1} library would have all produced a TRF of 271 bp using the *Hha*I restriction enzyme. In contrast, of the 41 sequences that showed a homology to this fungus in the 25 mg kg^{-1} library, 24 would have produced a TRF at 241 bp, 16 at 240 bp and 1 at 89 bp. However, no TRF of 271 bp was recorded in the fungal T-RFLP traces.

TRFs at 88, 90 and 244 bp were recorded in the T-RFLP traces but there was no evidence that they became more predominant following azoxystrobin application.

Chao1 analysis was carried out to determine whether azoxystrobin application had a significant impact on fungal **diversity**. The result produced for this analysis was non-significant. However, the Chao1 estimate of population size indicated that only a low percentage of the predicted total diversity had been represented within these clone libraries (44% for the 0 mg kg^{-1} library and 37% for the 25 mg kg^{-1} library). This means that a much larger clone library would have to be produced to enable fungal diversity to be accurately analysed in this way.

However, analysis of the fungal **community structure** using the Mann Witney U-Test indicated that azoxystrobin application did have a significant impact, producing a *p* value of 0.017.

2.3.5.2 Nematodes

Clone libraries produced using the primer pair Nem18Sf and Nem18Sr included a total of 81 sequences for the 0 mg kg^{-1} samples and 79 for 25 mg kg^{-1} samples.

Figure 2.27 shows the collector's curves for the 0 and 25 mg kg^{-1} clone libraries. In the 0 mg kg^{-1} library a large number of unique clones were found in the early sequences. However, none of the final 37 clones observed was from a unique group. This suggested that the majority of the diversity had been accounted for in this library. In contrast, the 25 mg kg^{-1} library showed new clones being identified throughout the library.

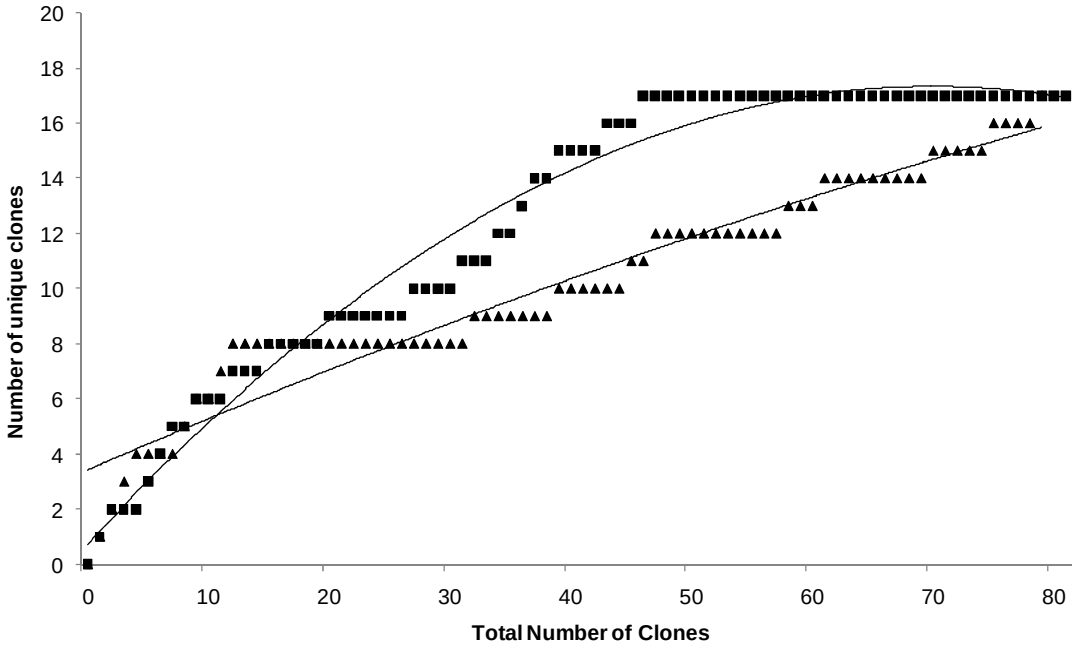


Figure 2.27. Collector's curves for the 0 and 25 mg kg^{-1} clone libraries produced using the Nem18Sf/Nem18Sr nematode primer pair. ■ 0 mg kg^{-1} ▲ 25 mg kg^{-1}

Taxonomic analysis of the 0 mg kg^{-1} clone library showed that the majority of the clones came from the orders Enoplida (45%) and Tylenchida (27%) (Figure 2.28). The remainder were from the orders Araeolaimida (3.5%), Aphelenchida (2.5%), and Rhabditida (1%). 5% of the clones could not be assigned to an order due to them being recorded as "uncultured nematodes".

The 0 mg kg^{-1} library was represented by 12 different named genera plus the 5 of the sequences which were categorised as "unclassified nematode species" in Blast searches. The most common sequence recorded (Table 2.4) showed homology to the plant-pathogenic nematode *Pratylenchus neglectus* which accounted for 26% of the clone library sequences. Sequences that showed high homologies to *Xiphinema rivesi* (a plant pathogen), *Achromadora* sp and *Trichistoma* sp. (genera of predatory nematodes) constituted 17%, 13.5%, and 10% of the clone library sequences, respectively.

Following azoxystrobin application the major change that occurred was the increase in prevalence of nematodes from the order Araeolaimida to 26.5% of the total clones (Figure 2.28). This was due to a

large increase in sequences identified having a sequence homology to the genus *Plectus* sp. within the clone library. There was also an increase of 6.5% in the number of clones that showed sequence homologies to the order Tylenchida. Conversely, those with homologies to the order Enoplia decreased by 14.5%. There were no clones present that showed sequence homologies to the orders Aphelenchida or Rhabditida and 6% of the clones could not be ascribed to an order.

The 25 mg kg^{-1} clone library also consisted of 12 named genera. 6% of the sequences originated from "unclassified nematode species" (Figure 2.28). The dominant organisms in this library were *Pratylenchus neglectus* (32.5% of the sequences), *Xiphinema* sp. (21% of the sequences), and *Plectus rhizophilus* (12.5% of the sequences). Sequences for the genera *Achromadora* were not found at all in the 25 mg kg^{-1} library and *Trichistoma* sp. sequences only represent 2.5% of the sequences (as opposed to 10% in the 0 mg kg^{-1} library). Conversely, the prevalence of *Plectus rhizophilus* increased by 9% in the 25 mg kg^{-1} library samples.

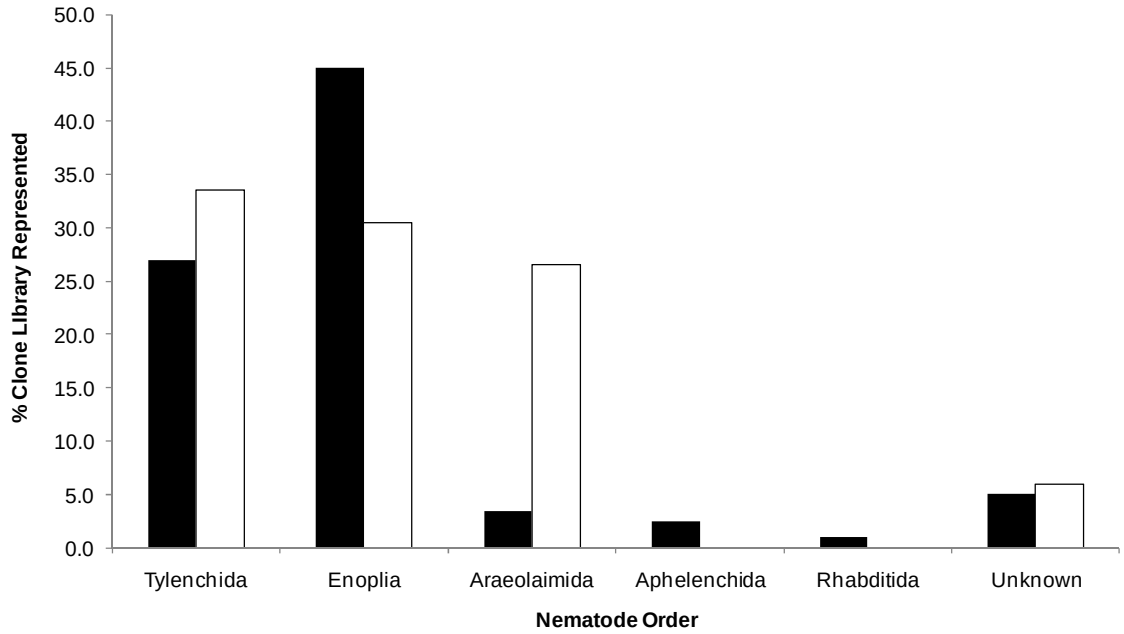


Figure 2.28. Structure of nematode clone libraries produced from 0 mg kg^{-1} and 25 mg kg^{-1} samples using the primer pair Nem18Sf and Nem18Sr. ■ 0 mg kg^{-1} □ 25 mg kg^{-1}

Table 2.4 details the sequence matches and percentage sequence homologies for the un-inoculated control and the 25 mg kg^{-1} samples, respectively.

Table 2.4. Sequence identifications from 0 and 25 mg kg^{-1} nematode clone libraries produced using the Nem18Sf and Nem18Sr primer pair. All nematode classifications had a grouping similarity of $\geq$ 97%

Nematode classification from BLAST search	Accession Number of Nearest Match	% Sequence Homology to Nearest Match	% Presence in 0 mg kg^{-1} samples	% Presence in 25 mg kg^{-1} samples
Pratylenchus neglectus	EU 669924.1	100	26.0	32.5
Xiphinema rivesi	HM 921344.1	100	17.0	5.0
Achromadora sp.	AY 593940.1	98	13.5	0.0
Trichistoma sp.	GQ 503079.1	99	10.0	2.5
Xiphinema chambersi	HM 191718.1	98	8.5	0.0
Pseudocella sp.	FN 43900.1	100	7.5	0.0
Plectus rhicophilus	AY 593929.1	99	3.5	12.5
Aphelenchoides sp.	EU 287591.1	100	2.5	0.0
Xiphinema sp.	HM 921342.1	98	1.0	21.0
Deontostoma sp.	FN 433899.1	98	1.0	1.0
Acrobeloides maximus	EU 306344.1	97	1.0	0.0
Bitylenchus dubius	AY 284601.1	98	1.0	0.0
Plectus sp.	U 61761.1	99	0.0	9.0
Anaplectus porosus	FJ 040453.1	100	0.0	2.5
Wilsonema otophorum	AY 593927.1	98	0.0	2.5
Thoracostoma microlabatum	FN 433903.1	99	0.0	1.0
Laimaphelenchus preissii	EU 287590.1	98	0.0	1.0
Coslenchus costatus	AY 284581.1	96	0.0	1.0

Chao1 analysis indicated that azoxystrobin application had no significant impact on the **diversity** of the nematode community. However, the Chao1 estimate of population size indicates that these clone libraries only account for 49% of the total nematode diversity in the 0 mg kg^{-1} library and 65% in the 25 mg kg^{-1} library so the accuracy of this result is not sufficient to form a firm conclusion. These results were also in complete contrast to the indications provided by the collector's curve analysis.

Mann-Witney U-test analysis showed that azoxystrobin application did have a significant impact on nematode **community structure** ($p = 0.026$).

2.3.6 Quantitative Polymerase Chain Reaction Analysis

2.3.6.1 Bacteria

Figure 2.29 illustrates the average bacterial copy numbers present in DNA samples taken from soils exposed to different azoxystrobin concentrations, one month post-application. 2-way ANOVA showed that azoxystrobin application did not have a significant effect on bacterial copy number ($p = 0.622$)

An average bacterial copy number of 3.15×10^6 was recorded for the 0 mg kg^{-1} control. Copy numbers in the amended samples ranged from 2.275 to 3.775×10^6.

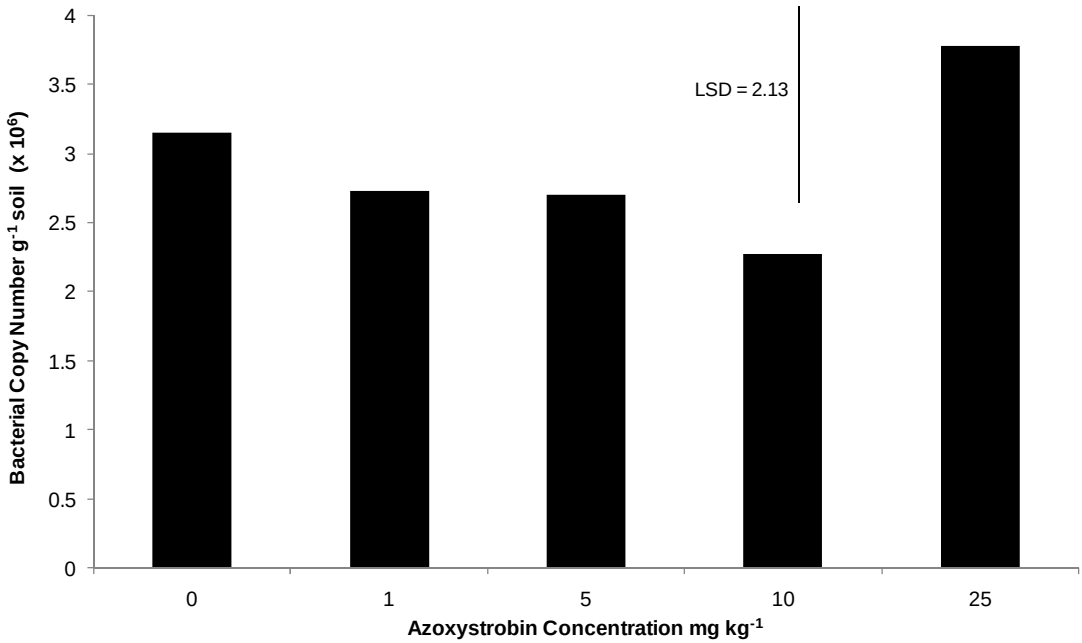


Figure 2.29. Average bacterial copy numbers recorded for soils exposed to different azoxystrobin concentrations 1 month prior to sampling. Each copy number value represents the mean of 4 experimental replicates.

Melting curve analysis of these samples showed a single curve at approximately 85°C (Figure 2.30). The slope of the standard curve was -3.102 and the R^2 value was 0.991. Analysis of the amplification plot showed that the majority of the amplification occurred between the 12th and 28th cycles of the quantitative PCR procedure.

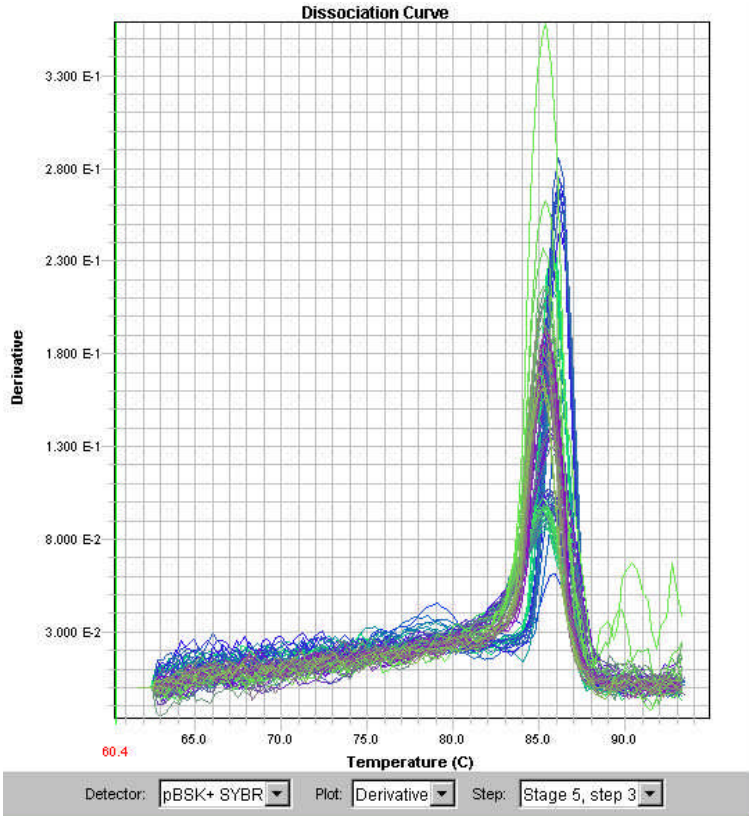


Fig. 2.30 Melting curve for bacterial qPCR analysis using the Eub338f/Eub518r primer pair.

2.3.6.2 Fungi

A 2-way ANOVA produced a *p* value of 0.043 which suggested that azoxystrobin concentration did have a significant impact on fungal copy number. The changes in average fungal copy number in response to azoxystrobin application can be seen in Figure 2.31. Fungal copy numbers were highest in the 0 mg kg^{-1} samples with an average value of 0.83 x 10^4 being recorded. Fungal copy numbers for the 1, 5, and 10 mg kg^{-1} samples were similar to each other with values of 0.58, 0.57, and 0.55 x 10^4 respectively being obtained. The 25 mg kg^{-1} samples showed a lower average fungal copy number than the other treatments, recording a value of 0.43 x 10^4.

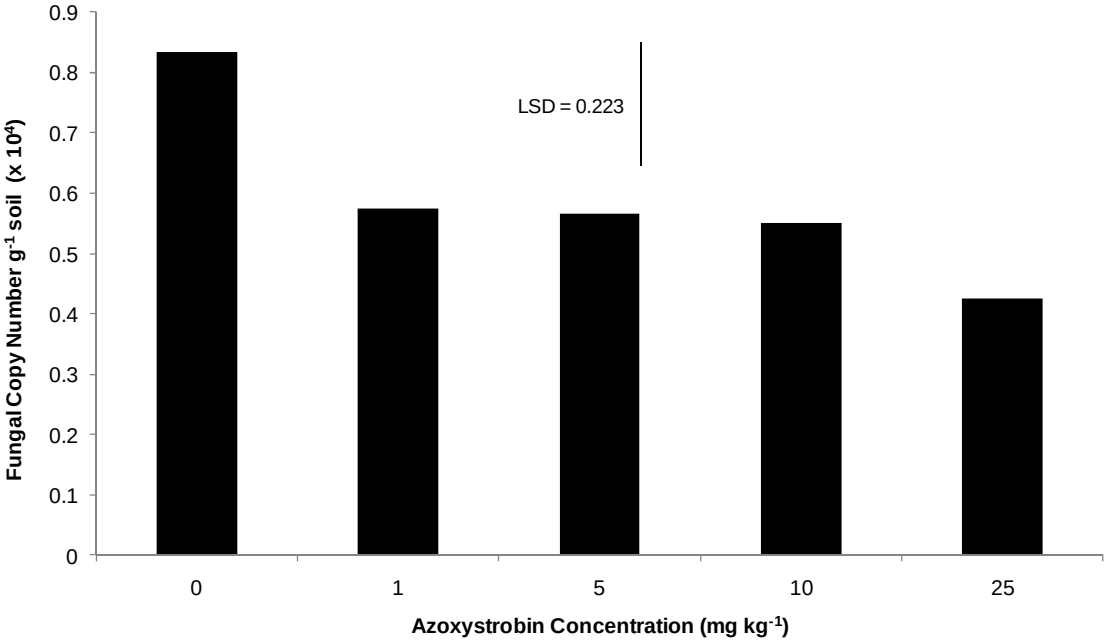


Figure 2.31. Average fungal copy numbers recorded for soils exposed to different azoxystrobin concentrations 1 month prior to sampling. Each copy number value represents the mean of 4 experimental replicates.

Melting curve analysis produced a main peak at approximately 84°C with a smaller peak observed at approximately 81°C (Figure 2.32). This was considered to be due to natural sequence variations in the ITS region amplified after analysis of the PCR products on a 1% agarose gel produced a single clear band of the correct size. Additionally, further analysis using the non-template control showed that primer dimerisation would have produced a peak at 75°C so this was ruled out as a cause of the secondary peak.

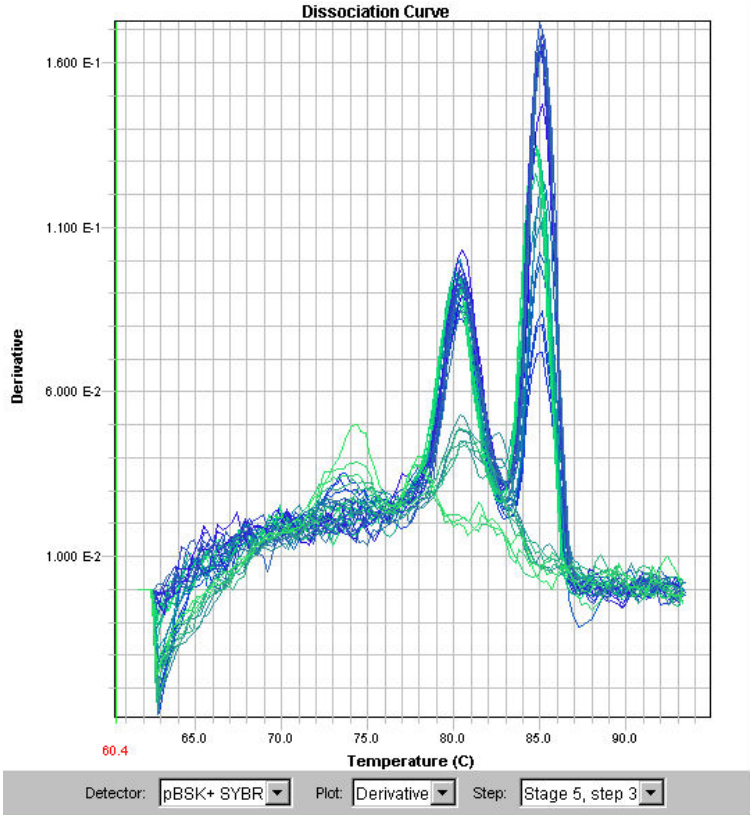


Fig. 2.32 Melting curve for fungal qPCR analysis using the 5.8s/ITS1f primer pair.

The slope of the standard curve was -3.37 and the R^2 value was 0.991. Analysis of the amplification plot showed that the majority of the amplification occurred between the 13th and 35th cycles.

2.4 Discussion

The resistance and resilience responses of soil micro-organisms to perturbation have been documented in a number of previous publications (Atlas *et al.*, 1991; Girvan *et al.*, 2005; Wertz *et al.*, 2007). However, despite this, there is still relatively little known about the impacts of pesticides and other similar xenobiotic compounds on these responses in soil microbial communities.

The main aim of the work undertaken was to ascertain the impacts of pesticides on soil microbial resistance and resilience responses at different trophic levels, using the widely-used, broad-spectrum

fungicide azoxystrobin as a model compound. Analysis was carried out for a range of pesticide concentrations (0, 1, 5, 10, 25 mg kg^{-1}) over a 4-month sampling period.

2.4.1 Analysis of azoxystrobin recovery using HPLC

The DT_{50} values recorded in this experiment were 33, 19, 32, and 47 days for the 1, 5, 10 and 25 mg kg^{-1} treatments, respectively. These values differ markedly from the observations of Bending *et al.* (2006) who recorded a DT_{25} value of 75 days for a 5 mg kg^{-1} dose of azoxystrobin in a sandy loam soil.

Ghosh and Singh (2009b) analysed the degradation of azoxystrobin in aerobic and anaerobic soils in the presence and absence of an amendment with 5% compost. The results showed that in the compost-free treatments the azoxystrobin DT_{50} values were 107 and 63 days for the aerobic and anaerobic soils, respectively. Compost amendment reduced the DT_{50} values to 73 and 39 days for the aerobic and anaerobic soils respectively. In contrast, Adetutu *et al.* (2008) analysed the degradation of a low concentration of azoxystrobin (0.33 mg kg^{-1}) in soils under light and dark conditions. Under both light regimes the azoxystrobin DT_{50} values were between 3 and 7 days. More recently, the work of Karanasios *et al.* (2010) showed a wide range of DT_{50} values for 2 azoxystrobin concentrations (3.1 and 31 mg kg^{-1} of soil) when its degradation was analysed in one soil type along with 5 different biobed mixtures and 2 biobed mixtures containing additional peat. In the soil treatment DT_{50} values of 69.3 and 38.5 days were recorded for the high and low dose rates, respectively. The DT_{50} values in the 5 biobed mixtures ranged from 2.7 to 53.3 days in the high concentration treatments, and between 7.1 and 17.3 days in the low concentration treatments. DT_{50} values of 13.1 and 33.0 days were recorded for the high azoxystrobin treatments in the 2 biobed mixtures containing additional peat. For the low azoxystrobin treatments these two values were 1.6 and 6.3 days, respectively.

Overall, the research detailed above, along with the results of the HPLC analyses in this chapter shows a high level of variation in the degradation of azoxystrobin in terrestrial systems. This

variability could be due to the physical characteristics of the amended substrate which could affect the sorption of the compound and as a result its bioavailability. Additionally, azoxystrobin degradation may also be mediated by the structural and functional resistance, resilience (and therefore degradative capabilities) of the indigenous microorganisms.

Another point to note is that azoxystrobin degradation within the first month was significantly higher in the 5 mg kg^{-1} treatment than in the other treatments. This may have been indicative of the presence of a preferred concentration threshold for azoxystrobin degradation. Over thirty years ago, Boethling and Alexander (1979) stated that a minimum pollutant concentration threshold had to be reached before microbial degradation can occur. However, over ten years later Fogarty and Tuovinen (1991) remarked that the pollution concentration thresholds required for microbial degradation pathways to be activated remained unknown.

Toräng *et al.* (2003) monitored the degradation of the ^{14}C-labelled phenoxy acid herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) applied at 5 different concentrations, to two different liquid culture types. The first culture contained 200 g of aquifer sediment and 250 ml of groundwater, and the second culture contained 400 ml of groundwater spiked with sediment fines at a concentration of 4.4 g L^{-1}. The type of liquid culture did not significantly impact the degradation of the compounds. In contrast, the initial pesticide concentration had a significant impact on degradation. DT_{50} values of 12 days were recorded for the 100 μg L^{-1} treatments of both cultures, whereas for treatments containing 5 μg L^{-1} of 2,4-D the DT_{50} values were approximately 15 and 20 days for the 250 and 400 ml groundwater treatments, respectively. 50% degradation was not achieved in any cultures containing a 2,4-D starting concentration below 5 μg L^{-1} in the 30-day experimental period.

Similarly, Janniche *et al.* (2010) observed that mecoprop (and to an extent isoproturon) mineralisation increased when the pesticides were applied at higher doses in a sandy aquifer. In contrast, acetochlor concentration did no significantly impact mineralisation when applied to this substrate. The authors

also analysed the degradation of these compounds in a deep, un-saturated limestone aquifer. The application concentration did not significantly impact the mineralisation of the three pesticides in this substrate.

Zhang *et al.* (2006) analysed the biodegradation of benzonitrile at three concentrations (0.1, 1.06 and 10.2 mg L^{-1} of culture) in an un-amended soil slurry and a slurry containing 1% wheat char. The assay results showed that in the un-amended slurry, benzonitrile degradation was negatively correlated with an increase in pesticide concentration. Over the 4-hour experimental period 85, 62, and 35% of the applied benzonitrile was degraded in the 0.1, 1.06, and 10.2 mg L^{-1}amendments, respectively. Conversely, in the char-amended slurry benzonitrile degradation increased with increased compound concentration. In this slurry no degradation was recorded in the 0.1 mg L^{-1} treatment after 4 hours, with 12 and 30% being degraded in the 1.06 and 10.2 mg L^{-1} treatments, respectively.

Furthermore, the presence of azoxystrobin in all treatments even after 4 months was also a point of consideration. This may have been due to a reduction in compound bioavailability over time (pesticide ageing), resulting from increased binding to the soil matrix. Additionally, a reduction in degradation may have been (at least in part) due to the removal of a required co-factor from the environment, or an increase in the concentration of a metabolite or metabolites. This point was considered by Aislabie and Lloyd-Jones (1995) and experimental observations with some pesticide compounds have supported it. For example, Wang *et al*. (2011a) noted that the chlorothlonil metabolite 4-hydroxy-2,5,6- trichloroisophthalonitrile (HTI) significantly inhibited degradation of the parent compound at concentrations > 0.1 μg g^{-1}. In contrast, Racke *et al.* (1990) did not observe any effects of the chlorpyrifos metabolite, 3,5,6-trichloro-2-pyridinol on the degradation of the parent compound. It is possible that a degradation product (or products) of azoxystrobin could inhibit degradation of the parent compound. However, a current lack of information regarding the possible toxicity of these metabolites to degrader communities means that this cannot be definitively determined at present.

The research described in this chapter illustrates how difficult it is to try and establish degradation thresholds for different compounds, especially with respect to the possible effects chemical and physical stresses may have on soil microbial communities. There was no apparent link between degradation rates and the soil microbial biomass and dehydrogenase activity. Firstly, there was no apparent change in microbial biomass despite the fact that there had been changes in dehydrogenase activity, and degradation of azoxystrobin was occurring. Furthermore, there was resilience response recorded in dehydrogenase activity in the 1 and 5 mg kg^{-1} treatments (as illustrated by a recovery in activity) despite the HPLC analysis showing that there was still azoxystrobin remaining in the system. This suggests that different factors affect the resistance and resilience of the microbial community in terms of its degradative capabilities compared with dehydrogenase activity. However, in the 10 and 25 mg kg^{-1} treatments, dehydrogenase activity was significantly higher than in the control samples. This may have resulted from increased stress levels within the community, and as a result, this response could not be considered an indication of community resilience.

2.4.2 Effects of azoxystrobin concentration on soil microbial biomass

Soil microbial biomass was not significantly affected by any of the azoxystrobin concentrations, at any time point. This observation matches that of Bending *et al.* (2007). However, the broad-scale nature of this assay means that the possibility of finer-scale (yet still ecologically significant) impacts taking place which could have resulted in a changed community structure and/or function, but a relatively unaltered microbial biomass cannot be ruled-out. Bending *et al.* (2007) also observed no significant impact of a single dose of tebuconazole (5 mg kg^{-1} soil) or chlorothalonil (10 mg kg^{-1} soil) on microbial biomass over a 3-month experimental period. This supports earlier work by Chen *et al.* (2001) who studied the impacts of benomyl, captan, and chlorothalonil on microbial biomass N when applied at approximate field rates (51 mg kg^{-1} of soil for benomyl, 125 mg kg^{-1} of soil for captan, and 37 mg kg^{-1} of soil for chlorothalonil). There was no significant impact of any of the three fungicides on microbial biomass N over the 8-week experimental period. Okur *et al.* (2010) studied how the application of two organic insecticides derived from Sea Squill (*Urginea maritima*) and Myrtle

Spurge (*Euphorbia myrsinites*) plants affected soil microbial biomass when applied at 3 different concentrations. The insecticides were applied on 4 occasions, with a gap of 1 month between each application. At each sampling point, the treatments amended with the insecticide derived from *E. myrsinites* all of the samples had a reduced biomass compared with the un-inoculated controls. In contrast, the insecticide derived from *U. maritima* only reduced microbial biomass at the highest concentration applied. Furthermore, research by Widenfalk *et al.* (2008) showed that in a river sediment, microbial biomass was unaffected by applications of either glyphosate, isoproturon (both herbicides), captan (a fungicide), and pirimicarb (an insecticide) even when the compounds were applied at concentrations 1000 x higher than the Maximum Permissible Concentrations.

In contrast, Perucci *et al.* (2000) analysed the effects of sewage sludge plus either rimsulfuron (a sulfonylurea herbicide) or imazethapyr (an imidazolinone herbicide) on soil microbial biomass when applied at either field rates, or 10 x field rates. Although there was no significant impact on biomass when the compounds were applied at field rates, both rimsulfuron and imazethapyr caused a significant decrease in biomass when applied at 10 x field rates. This suggests that some currently used pesticides may deleteriously affect the soil microbial biomass in situations of relatively mild contamination.

2.4.3 Effects of azoxystrobin concentration on soil dehydrogenase activity

The application of azoxystrobin had a significant, concentration-dependent, deleterious impact on soil dehydrogenase activity. This impact was observed 1 month post-application with a recovery to control levels being observed after 3 months. This suggests that initially the microbial community has a low resistance to the pesticide and that pesticide concentration is important in determining the level of resistance. The return of dehydrogenase activity levels to those of the control samples suggests that it takes between 2 and 3 months for a full resilience response from the microbial community to be observed in the 1 and 5 mg kg^{-1} treatments. In contrast, the activity in the 10 and 25 mg kg^{-1} treatments had not returned to the levels observed in the un-amended controls by the end of the experimental

period. This indicated that the soil microbial community had a lower level of resilience to these amendment concentrations. Over a longer period of time the activity may have dropped back down to levels comparable to those of the controls. Sebiomo *et al.* (2011) studied the impacts of 4 herbicides (atrazine, primeextra, paraquat and glyphosate) on soil dehydrogenase activity over a 6-week sampling period. The results showed a significant decrease in dehydrogenase activity in all treatments after 4 days, relative to control soils. A subsequent recovery of activity within the treatments was observed between 2 and 6 weeks which could have been indicative of resilience responses within the microbial communities. Monkiedje *et al.* (2002) also recorded significant impacts of pesticides (mefenoxam and metalaxyl) on soil dehydrogenase activity.

A contrast was observed by Cycoń *et al.* (2010) who analysed the impacts of 2 fungicides (mancozeb and dimethomorph) on soil dehydrogenase activity over the course of 28 days when applied at 3 different concentrations (15, 75 and 1500 mg kg^{-1} of soil). In the mancozeb treatments there was a significant, concentration-dependent decrease in activity after 1 day with no noticeable recovery even after 28 days. This suggests that this community had both a low resistance and resilience to this compound. On the other hand, the 15 and 75 mg kg^{-1} doses of dimethomorph did not significantly impact dehydrogenase activity at any point during the experiment. However, in the 1500 mg kg^{-1} treatment, activity levels after 1 day were only 67% of the un-amended controls. This was followed by a further decrease to only 19% of the controls after 7 days. There was no subsequent recovery in activity throughout the rest of the experiment.

Bending *et al.* (2007) found that the fungicides chlorothalonil (10 mg kg^{-1}) and tebuconazole (5 mg kg^{-1}) both caused a significant reduction in dehydrogenase activity. In the case of tebuconazole this was a short term effect with a significant decrease of 10% recorded after 1 month only. In contrast, chlorothalonil reduced activity by between 23 and 39% with no recovery of activity observed over the 4-month experimental period. A 5 mg kg^{-1} dose of azoxystrobin resulted in a significant decrease in activity of between 10.7 and 13.7% after 1 month, with recovery observed after 2 months. This is in

marked contrast to the 35% decrease recorded at the same time point in the work carried out here. Additionally, the work here indicates that up to an extra month was required for activity levels to return to those of the control samples, indicating a lower resilience within the soil community in these experiments.

It may be important to consider the fact that both azoxystrobin and chlorothalonil had inhibitory effects on dehydrogenase activity. These two compounds are also licensed within the UK for use as a combination application (British Crop Protection Council, 2008). This therefore, raises the question of whether the application of these compounds in combination would exacerbate their impacts on soil dehydrogenase activity and/or other aspects of soil microbial community structure and function.

Schuster and Schröder (1990b) noted that when the fungicides Sportak Alpha, Bayleton DF, and Corbel were applied sequentially, soil microbial dehydrogenase activity decreased significantly with the addition of each additional compound. After 144 days the activity levels were still significantly lower than those of the un-amended controls. Moreover research by Gundi *et al.* (2005) noted how, when applied separately, the insecticides monocrotophos, quinalphos, and cypermethrin did not significantly affect soil microbial dehydrogenase activity. However, when monocrotophos or quinalphos were applied in combination with cypermethrin, there was a significant negative impact on dehydrogenase activity and the extent of these impacts was dependent on the concentration of the compounds applied.

Pampulha and Oliveira (2006) showed that the application of a herbicide combination (60% Bromoxynil, 3% Prosulfuron) had a significant deleterious impact on soil dehydrogenase activity and there was no recovery in activity 40 days post-application. Herbicide concentration had a significant impact on activity. After 40 days, activity in a 1 ppm treatment was approximately 12.5% that of the un-amended controls. This compared with values of 2.5% and 1% for treatment concentrations of 10 and 100 ppm, respectively.

The results of the studies mentioned above, combined with the results of the dehydrogenase assays presented in this chapter indicate that soil dehydrogenase activities can be highly sensitive to perturbation by pesticides (i.e. there is a low community resistance). However, the recovery of activity in some of the experiments described suggests that soil communities can have a high level of resilience to any impacts, although the time differences recorded for dehydrogenase activity recovery to occur may suggest that resilience levels vary between different soil ecosystems and may also be dependent upon the pesticide applied.

These results also agreed with previous research into the impacts of pesticides and perturbations on different aspects of soil microbial activity. Girvan *et al.* (2005) noted how the amendment of soils with benzene reduced soil microbial activity as illustrated by a reduction in the ability of the community to mineralise a 100 mg kg^{-1} dose of 2,4 dichlorophenol (2,4-D) in both high and low diversity soils. There was no apparent resilience response from the low diversity soil, with no increase in mineralisation recorded 9 weeks post-application. In contrast, the activity in the high diversity soils had returned to levels comparable to the un-amended controls after 9 weeks indicating a higher level of community resilience.

In a study monitoring the impacts of heat-shock treatment on soil nitrite oxidising ability Wertz *et al.* (2007) observed a similar resistance and resilience response to perturbation as was observed for dehydrogenase activity in this chapter. The heat shock treatment reduced soil nitrite oxidising activity to 60% of the control within the first 3 hours of the experiment. After 1 month the recorded activity in these treatments was not significantly different to the controls.

However, some previous research has shown that some soil measures of microbial activity can be resistant to the application of pesticides and other xenobiotic compounds. For example, Atlas *et al.* (1991) observed that the application of a 100 ppm dose of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)

did not significantly affect microbial activities in a silt loam soil, as determined by the capacity of the microbial community to utilise carboxylic acids, carbohydrates and amino acids.

The results obtained here serve to highlight the inherent difficulties associated with determining the impacts of stresses on soil microbial communities. This point is further illustrated by the fact that the biomass analysis gave contrasting indications of the impacts of azoxystrobin on soil communities. This, in turn emphasises the need to use a variety of analytical techniques (both broad-scale and molecular) to give a more accurate picture of impacts on microbial structure, function and diversity.

2.4.4 Effects of azoxystrobin concentration on soil community structure and diversity

The resistance and resilience of different microbial groups to the application to a range of azoxystrobin concentrations was assessed.

2.4.4.1 Fungi (EF4f/EF3r) Primer Pair

There were no significant impacts of azoxystrobin application on fungal community structure or diversity when the ITS1F/ITS4r was used. However, using the EF4f and EF3r primer a significant decrease in fungal diversity was observed and this persisted for the duration of the experiment. Furthermore, analysis of the fungal clone library involved using the Mann-Witney U Test to determine possible impacts on community **structure** and the Chao1 estimate to analyse possible impacts on community **diversity**. The Mann-Witney U Test showed that there was a significant impact on fungal community **structure** (p = 0.017). However, there was insufficient coverage of the community to enable the use of the Chao1 estimate to determine impacts on **diversity**. Average coverage percentages were 44 and 37% for unperturbed and perturbed soils, respectively. The different observations recorded when using the EF4f/EF3r and ITS1F/ITS4r primers may have resulted (at least in part) from the relative bias of the different primer pairs. More specifically, the ITS primers may have given the impression of fungal community resistance by selectively amplifying species that with a lower sensitivity to azoxystrobin. Smit *et al.* (1999) described a bias towards the

amplification of basidiomycetes and zygomycetes using the EF4f/EF3r primer pair compared with other fungal groups. In contrast, Anderson *et al.* (2003b) studied the specificities of four 18S rDNA fungal primer pairs (nu-SSU-0817f/nu-SSU-1196r, nu-SSU-0817f/nu-SSU-1536r, EF4f/EF3r, and EF4f/fung5r) and one internal transcribed spacer (ITS) primer pair (ITS1F/ITS4r), in order to determine any possible issues with primer bias. The authors concluded that primer bias amongst these primer pairs may be less of an issue than previously thought, although it must still be considered. Anderson and Cairney (2004) suggested that targeted analyses using different primer pairs to determine the structures of synthetic fungal communities was the way forward in resolving the issue of primer bias.

In order to determine whether primer bias was a possible cause of the differences observed, further clone libraries would also have to be produced using the ITS1f and ITS4r primer pair to enable a full comparison. These observations emphasise the fact that, although they are an essential part of contemporary microbial community studies, PCR-based analytical methods are far from infallible. Therefore, issues such as primer bias should always be considered when performing these techniques.

The impacts of azoxystrobin on fungal community diversity appeared to be dependent upon the concentration of the pesticide. In the 25 mg kg^{-1} treatment, there was a significant decrease in diversity after 1 month relative to the controls, with no recovery seen over the experimental period. This differs from the results observed with the dehydrogenase analysis where a recovery in activity was observed by three months. This suggested that dehydrogenase activity was more resilient to azoxystrobin application than the overall fungal diversity. This could mean that the remaining soil biota, after a 3-month adaptation phase, were able to compensate for any deleterious impacts on dehydrogenase activity resulting from the reduction in diversity. Alternatively, this compensatory effect could have been carried out (at least in part) by other organism groups (e.g. bacteria) which may have had a higher level of functional redundancy resulting from a higher natural diversity.

Decreases in fungal diversity following the application of azoxystrobin have been previously documented by Adetutu *et al.* (2008). In their analyses samples spiked with the equivalent of only 0.33 mg azoxystrobin per kg soil displayed a significant decrease in fungal diversity (as determined by Shannon-Weaver diversity analysis) when the soils were incubated in the dark. After 28 days the diversity in these samples was only 77% that of the un-amended controls. This remained the case after 56 days as well. However, after 84 days the diversity had recovered to levels comparable with the un-amended controls indicating a resilience response from the community. Conversely, Girvan *et al.* (2004) had previously shown no significant impact of azoxystrobin application on fungal diversity over a 1-year experimental period. Crouzet *et al.* (2010) studied the impacts of the herbicide mesotrione on bacterial and fungal communities when applied either as a pure compound, or as a 100 g L^{-1} commercial formulation over a 95-day experimental period. The authors only recorded slight changes in fungal diversity for both treatments over the experimental period. This suggests that the fungal community had a high resistance to this compound.

Analysis of the Shannon diversity results in relation to the hypotheses proposed by Giller *et al.* (1998) failed to show any distinct "extinction" or "hump-backed" relationships between azoxystrobin concentration and fungal diversity using either primer pair. With the ITS1F/ITS4r primer pair, no noticeable azoxystrobin/fungal diversity relationships were observed. However, using the EF4f/EF3r primer pair, there appeared to be a "threshold" effect of azoxystrobin concentration on fungal diversity. More specifically, there appeared no significant impact of azoxystrobin on fungal diversity at concentrations from 1 to 10 mg kg^{-1} but in the 25 mg kg^{-1} treatments the diversity was lower at all sampling times, but most notably after 3 and 4 months. This may indicate that the fungal community had a sufficient natural resistance to azoxystrobin to enable it to maintain its diversity at concentrations up to 10 mg kg^{-1}, and that the azoxystrobin resistance threshold was somewhere between the 10 and 25 mg kg^{-1} concentrations. These results were contrary to previous observations of microbial communities exhibiting a "hump-backed" relationship between different amendments and community diversity. Research by Griffiths *et al.* (2000) analysed how chloroform fumigation

impacted bacterial diversity in a clay loam soil sampled in two different years. For the 1st year soil samples, bacterial diversity was highest when the soil was fumigated for 0.5 hours (104% of the un-fumigated control). However, there was then a significant decrease in diversity following 2 or 24 hours of fumigation. The diversity in the 24-hour fumigation treatment was only 85% that of the 0.5 hour treatment. These impacts were more pronounced in the second year. In the 0.5-hour fumigation treatment the diversity was 114% that recorded in the un-fumigated control. The diversity in the 24-hour fumigation treatment was only 73% of the value recorded for the 0.5-hour treatment. Degens *et al.* (2001) found a "hump-backed" relationship when soil microbial communities were exposed to 1, 2, 4 or 8 freeze/thaw or wet/dry disturbance cycles. The freeze/thaw treatment involved freezing the samples at -30ºC for 24 hours, followed by thawing and incubation at 25ºC for 48 hours. Wet/dry disturbances involved air drying for 24 hours at 20-25ºC followed by re-wetting to 60% of the maximum water holding capacity. This was followed by a 48-hour incubation period in the dark at 25ºC. In a low diversity soil, the diversity recorded gradually increased between 1 and 4 disturbance cycles for both treatments. However, the diversity decreased markedly after 8 disturbance cycles. This was particularly evident for the wet/dry treatment where the diversity after 8 cycles was only 77% that recorded in the 4-cycle treatment. In a high diversity soil the same pattern was observed for the wet/dry treatment but not for the freeze/thaw one. Zhang *et al.* (2009c) observed a "hump-backed" relationship when analysing the impacts of cadmium amendment on soil bacterial diversity. In two of the three sample sites, a significant concentration-dependent increase in diversity was recorded in 0, 1, and 2 mM cadmium amendment treatments. However, when cadmium was applied at a concentration of 4 mM, the diversity was, on average, only 87% that of the 2 mM treatment. There was no significant impact of cadmium amendment on diversity at the third sample site (Zhang *et al.*, 2009c).

Previous research has also shown examples of "extinction" relationships between amendment concentration and microbial diversity. Li *et al.* (2006) analysed the changes in bacterial community diversity in rice paddy fields contaminated with copper, zinc, or cadmium from nearby smelting plants. The results showed that bacterial diversity decreased steadily with increasing concentrations of

each amendment. More recently, Bamborough and Cummings (2009) showed that enrichments of a grassland soil with zinc or lead resulted in significant decreases in bacterial diversity. The extent to which the bacterial diversity decreased was dependent on the amount of each compound applied. At the highest amendment concentration, bacterial diversity was only 74% that of the un-amended controls for both treatments.

SIMPER analysis of fungal T-RFLP traces produced using the EF4f and EF3r primer pair showed that the TRFs at 143, 146 and 148bp predominated in the 0-10 mg kg^{-1} and were absent in the 25 mg kg^{-1} samples. Predicted TRF sizes within this range were also absent from the clone library produced from 25 mg kg^{-1} samples. However, in the 0 mg kg^{-1} clone library several sequences produced TRFs within this size range. These showed homologies to a variety of different species including *Byssoascus striatosporus* (97%) along with the known plant pathogens *Gibberella fujikuroi* (100%) and *Fusarium oxysporum* (99-100%). Incidentally, all three of the abovementioned species are ascomycete fungi. This raises the possibility that ascomycete fungi may be more susceptible to azoxystrobin application. Indeed, of the 24 named species from the clone library that decreased following the application of azoxystrobin, 22 of these were ascomycetes with the other two belonging to the basidiomycetes. This appears to contradict the widely-accepted view of the broad-spectrum nature of this compound.

2.4.4.2 Nematodes

The nematode samples showed the hump-backed diversity proposed by Giller *et al.* (1998) after 1 month. The peak in nematode diversity after 1 month was observed for the 1 mg kg^{-1} amendment (113% that of the un-amended control). Nematodes and other soil eukaryotes have been previously used as indicators of community stress. Griffiths *et al.* (2000) observed an “extinction” relationship between the length of time a soil was fumigated using chloroform, and protozoal community diversity. Overall protozoal diversity was 65, 54, and 26% that of the un-fumigated controls following fumigation for 0.5, 2 and 24 hours, respectively. DGGE analysis showed that there were no fungivorous protozoa present in soils subjected to fumigation for 24 hours, and the numbers of

bacteriovorous protozoa were also reduced. Subsequent work by Griffiths *et al.* (2001) suggested that protozoal communities could potentially be used as indictors of soil perturbation, with protozoan biomass being lower in polluted, industrial soils than in grassland or agricultural soils. The highest biomass was observed in the agricultural soils. In particular, there was a significant decrease in flagellated protozoal biomass in the polluted soils. Additionally, culture-dependent analyses by Ekelund *et al.* (2003) showed that copper amendments of 64 $\mu g\ g^{-1}$ or above caused a concentration-dependent decrease in protozoan diversity over a 70-day sampling period.

Soil nematode communities were also found to be impacted by azoxystrobin application with both community structure and diversity found to be significantly impacted. This is a potentially important finding as it would represent non-target impacts of azoxystrobin on higher trophic level organisms. When the community structure and diversity results obtained from the T-RFLP analyses were compared along with the clone library data, three possible broad scenarios emerged regarding the possible relationship between nematode and fungal communities following azoxystrobin amendment.

1. **The impacts on fungal and nematode diversity were solely due to direct azoxystrobin effects.**

The diversity in the nematode samples decreased to its lowest levels after 2 months with a recovery observed after 3 months. This suggests that initially the nematode community was more resistant to azoxystrobin application than the fungal community because the reduction in diversity took longer to occur. Additionally, this would also suggest that the nematode community was more resilient than the fungal community, with recovery to a diversity comparable to that of the control being observed only 1 month after the decrease.

One possible cause of these impacts could have been due to the direct toxicity of azoxystrobin to the nematode community. Azoxystrobin acts by preventing ATP production within the mitochondrial

cells. More specifically, the compound binds to the Q_o site of the cytochrome b part of the cytochrome bc_1 complex which is located on the inner membrane of the fungal mitochondria. This binding disrupts the transfer of electrons from cytochrome b to cytochrome c_1, which in turn prevents the production of ATP. However, other eukaryotes (including nematodes) also possess cytochrome bc_1 complexes within their mitochondria (Bartlett *et al.*, 2002). This, therefore leads to the possibility that azoxystrobin could also be directly toxic to nematodes.

However, despite this, the current understanding is that soil ecosystems are highly complex and involve a multitude of interactions both within and across organism groups e.g. food webs and nutrient cycles. Therefore, it seems unlikely that any impacts of azoxystrobin would affect individual groups in isolation.

2. A decrease in fungal community abundance caused the subsequent decrease in nematode diversity

Another possibility is that the reduction in nematode diversity occurred as a knock-on effect of the impacts on the fungal community. This theory works on the premise that the fungal communities represented an essential nutrient source for the nematodes. The results obtained in the Shannon diversity analyses could support this as the nematode (predator) diversity decreased after the decrease in fungal (prey) diversity.

More recently, Sánchez-Moreno *et al.* (2010) used nematodes as indicators of non-target impacts of the soil fumigants 1,3-dichloropropene (1,3-D), chloropicrin (Pic), and a combination of the two, at two different sites, over a 35-week sampling period. The results showed that the abundance of fungivorous nematodes decreased following amendment. The authors concluded that this may have been due to direct effects of the fumigant and/or as a knock-on effect following impacts on saprotrophic fungi.

Unfortunately, the grazing habits of several of the nematode species that had high sequence homologies with the sequences obtained in the clone libraries remain unknown. Some of the sequences however did show homologies to nematodes with known grazing habits. These included:

1. **Plant Pathogens:** *Pratylenchus neglectus*, *Xiphinema rivesi*, *Xiphinema chambersi*, Uncultured *Xiphinema* sp., *Bitylenchus dubius*, and *Coslenchus costatus*.
2. **Bacterivorous Nematodes:** *Acrobeloides maximus* and *Wilsonema otophorum*.
3. **Fungivorous Nematodes:** *Aphelenchoides* sp.

Sequences with homologies to *Xiphinema rivesi* (17% to 5% of sequences) and *Xiphinema chambersi* (8.5% to 0% of sequences) and *Bitylenchus dubius* (1% to 0% of sequences) decreased in abundance following the application of azoxystrobin, whereas sequences with homologies to *Pratylenchus neglectus* (26% to 32.5% of the sequences) and an uncultured *Xiphinema* sp. (1% to 21% of the sequences) markedly increased in abundance. Amongst the sequences that showed homologies to bacterivorous nematodes *Acrobeloides maximus* decreased in abundance from 1% of the sequences to 0% following azoxystrobin application. Conversely, sequences with homologies to *Wilsonema otophorum* increased from 0% of the sequences to 2.5% following pesticide amendment. Sequences with homologies to the fungivorous nematode *Aphelenchoides* sp decreased from 2.5% of the sequences to 1% after azoxystrobin application. This relative lack of information on the grazing habits of the nematode species showing high sequence homologies to the clone library samples means it was not possible to determine whether the impacts recorded were indirect ones resulting from changes in the fungal community, although the evidence gathered suggests that this was not the case. If clear indirect impacts had occurred the expected result would have been a decline in the presence of sequences with homologies to fungivorous nematodes as a result of impacts on the fungal community. However, only a small number of sequences from the libraries showed homologies to a fungivorous nematode genus, and the change in the abundance of these sequences following azoxystrobin application was minimal.

3. The altered structure of the fungal community was responsible for the decrease in nematode diversity.

The third possibility emerged when nematode Shannon diversity analysis was compared with the data from the fungal and nematode clone libraries. Instead of the expected decrease in fungivorous nematodes in amended samples, the fungal clone libraries showed the presence of the nematopathogenic fungi *Paecilomyces carneus* and *Arthrobotrys oligospora* in the 25 mg kg^{-1} clone library. No nematopathogenic fungal sequences were observed in the clone library from the un-amended soil, whereas in the amended community library they represented 3.5% of the total sequences. A possible reason for this could have been that the nematopathogenic fungi were sub-dominant species in the un-amended treatment. However, the removal of dominant fungal species (e.g. *Exophiala pisciphila*, *Penicillium sacculum*, or *Fusarium oxysporum*) from the amended treatment community, could have allowed *P. carneus* and *A. oligospora* to become more prevalent. This scenario could also back up research by de Ruiter *et al.* (1995) where, using material flow diagrams and Lokta-Volterra equations, they predicted the major predator-prey interactions within soils and the level that these interactions impacted the communities present. From this work it was observed that nematodes feature prominently within the community as both a food source, and as a consumer. Further clone library analysis from a range of time periods would be required in order to determine whether nematodes the nematode community changes observed in this experiment were due to them being used as a food source in increased amounts.

The findings of the work carried out in this chapter serve to emphasise the fact that current knowledge of the fine-scale aspects of soil nematode community dynamics (especially in relation to responses to perturbation) is very limited, particularly in comparison with other microbial groups such as bacteria and fungi. Edel-Hermann and colleagues (2008) raised this point, and allied it to the fact that different microbial groups do not exist in isolation within the soil, but interact extensively in areas such as nutrient cycling, competition and predation. Therefore, the perturbation of a soil with a xenobiotic

compound such as a pesticide has the potential to not only directly impact organisms, but also have a range of knock-on effects on microbial groups that interact with it. The authors used T-RFLP to analyse the structure of bacteria, fungi, protozoa, and nematodes in two soils amended with either 5% compost or 5% manure, compared with un-amended controls. No significant differences in nematode community structure were observed as a result of amendment although it was suggested that the 30-day experimental period may not have been sufficiently long to enable indirect impacts to be observed (Edel-Hermann *et al.* 2008). However, the other microbial groups tested were found to be impacted in different ways. For example, in one of the soils the amendment treatments had significantly different bacterial and protozoal community structures compared with the controls. Additionally, the amendment with 5% compost significantly reduced the abundance of bacteria (as determined by culture-dependent methods) compared with the controls in one of the soil types. Therefore, it was concluded that soil ecological studies should be carried out using analysis of a range of organisms covering different trophic levels, and that these should be carried out using molecular methods such as T-RFLP (Edel-Hermann *et al.* 2008).

This conclusion is backed up by the results obtained from the work carried out here. The work showed a significant impact of azoxystrobin on soil nematode communities. However, a lack of current knowledge on individual nematode genera and their functions within soil communities makes the task of determining the possible impacts of its removal from the soil community practically impossible. This represents an extensive knowledge gap considering the role that nematodes are considered to play in the structure and function of the soil microbial community as a whole.The lack of any additional differences may have been due to the fact that DNA was used for the T-RFLP analysis. The use of DNA could highlight the full range of organisms within a community i.e. active, dormant, and dead, which could have resulted in significant changes in the active community being masked. Therefore further work in this thesis will cover the impacts of azoxystrobin on active soil communities based on T-RFLP carried out using RNA extraction procedures and reverse transcription PCR (RT-PCR).

2.4.4.3 General Eukaryotes

There were no significant differences in general eukaryote community structure following azoxystrobin application at any concentration. These results suggest that the community structure had a high natural resistance to both direct azoxystrobin impacts at all concentrations, and to any potential indirect impacts that may have been caused by the effects that the compound had on either the fungal or nematode communities. This could mean that organisms such as bacterivorous protozoa are more resistant to direct non-target effects by azoxystrobin, than previous research has shown them to be to other fungicidal compounds such as fenpropimorph or propiconazole (Ekelund, 1999; Ekelund *et al.*, 2000). However, contrast analysis did indicate that eukaryote diversity was significantly lower in the 10 and 25 mg kg^{-1} treatment samples compared with the other treatments which would support the observations of the nematode analysis that azoxystrobin could have negative, non-target impacts on higher trophic level organisms. Analysis of the T-RFLP did not show any specific TRFs that appeared consistently responsible for these changes in diversity at the higher pesticide concentration treatments which suggests that the changes in diversity were not due to specific, direct impacts on particular organisms. Instead, the impacts may have been due broader, non-specific indirect impacts that could not be elucidated using the data obtained.

2.4.4.4 Bacteria, Pseudomonads, and Archaea

There was no significant impact of azoxystrobin on bacterial community structure and diversity. This was backed up by quantitative PCR analysis which showed that there was no significant difference in bacterial copy number in the 25 mg kg^{-1} treatment after 1 month, compared with the un-amended control. These results back up the results of the biomass assay which could possibly mean that the chloroform fumigation method preferentially quantified the bacterial biomass. Therefore, future research in this area could possibly utilise techniques such as ergosterol analysis to determine impacts on fungal biomass. Alternatively, phospholipid fatty acid (PLFA) analyses could be used for both fungal and bacterial biomass determination (Bardgett *et al.*, 1996). Furthermore, it could be expected that the recorded impacts on fungal communities (that compete with the bacteria for nutrients), and

nematodes (that represent potential predators) would affect the bacterial community. This, however did not appear to be the case. This may have been because the interactions between bacteria and the impacted fungi and nematodes represented only a small part of the total interactions of bacteria within the natural environment. Additionally, the techniques used could have lacked the required sensitivity to detect impacts on the bacterial community. However, three individual TRFs (470, 472 and 814 bp) increased in relative intensity with increasing azoxystrobin concentrations. This could potentially have resulted from the increased proliferation of bacteria involved with the degradation of the compound.The results of the Pseudomonad analysis also illustrated that the apparent high levels of resistance and resilience observed for the overall bacterial community may also extend more specifically to these organisms, despite their fast-growing nature and relatively lower diversity suggesting that they may be more susceptible. Similar results were also observed for the archaea which suggests that this community also had a high natural resistance to azoxystrobin at all of the concentrations tested.

Chapter 3

Characterising the Effects of Azoxystrobin on Active Soil Microbial Communities

3.1 Introduction

3.1.1 DNA and RNA community analyses: A comparison

Over the last 20 years there has been a dramatic rise in the use of culture-independent techniques for monitoring the structure of microbial communities in a range of ecosystems and environments. Generally, these techniques involve the extraction of DNA from a particular sample (soil, water etc) followed by PCR amplification of a group-specific (e.g. 16/18S rDNA), or function-specific (e.g. Amo*A*) gene. This is then followed by purification and analysis using techniques such as DGGE or T-RFLP.

Whilst this approach gives a good broad indication of microbial community structure, DNA analysis cannot identify whether observed organisms are metabolically active, dormant, or dead (Sheridan *et al.*, 1998). This is because extracellular DNA can still persist in the environment even after the death of a cell so the viability of particular species cannot be determined (Mengoni *et al.*, 2005).

This poses a major problem for many fields of research as microorganisms are responsible for a wide variety of ecosystem functions such as nutrient cycling and the degradation of xenobiotic compounds. In these situations there is a need to isolate and identify specific metabolically-active organisms (or groups of organisms) that are, for example, capable of degrading a particular xenobiotic compound (Nogales *et al.*, 2001).

One method that has been used to try and measure impacts on metabolically active organisms is stable isotope probing (SIP). The technique involves the application of a stable isotope-labelled (e.g. ^{13}C or ^{15}N) substrate to an experimental sample, which is then monitored for the incorporation of the labelled substrates into nucleic acids (normally DNA and/or rRNA) as a measure of metabolic activity (Lueders *et al.*, 2004). These nucleic acids are then extracted and the radio-labelled DNA or rRNA separated from the un-labelled genetic material by density-gradient centrifugation. The separated stable isotope-labelled nucleic acids can then be used for e.g. specific gene amplification and sequence analysis (Dumont and Murrell, 2005).

Radajewski *et al.* (2000) studied the formation of ^{13}C-DNA by methanol-utilising microorganisms grown on ^{13}C-labelled substrates (^{13}C-Methanol or ^{13}C-Methane). The results of the SIP and sequencing analyses showed that two phylogenetically distinct groups of bacteria (*Acidobacterium* sp. and members of the α-Proteobacterium) utilised methanol as a carbon source. More recently, Pumphrey *et al.* (2011) used similar ^{13}C-DNA SIP techniques alongside PCR amplification of the 16S rRNA and *hydB* and sequence analysis to identify autotrophic hydrogen-oxidising bacteria present in vetch and clover rhizosphere soils. The results identified 5 β-Proteobacterial species that had high ($\geq$ 97%) sequence homologies to the species *Aquincola tertiaricarbonis*, *Pelomonas saccharophila*, *Methylibium petroleiphilum*, *Ideonella dechloratans*, and *Aquaspirillium articum*. Bell *et al.* (2011) took a slightly different approach and monitored the uptake of ^{15}N into the DNA of bacteria in petroleum-contaminated Arctic soils followed by pyrosequencing in order to identify potential petroleum-degrading organisms. The authors observed that in contaminated soils, members of the family *Sphingomonadaceae* were dominant within the community (20% of the sequences), followed by the *Pseudomonadaceae* and *Comamonadaceae* (18 and 13% of the sequences, respectively).

Alternatively, RNA-SIP is also becoming widely used in research. RNA-SIP can be preferable to DNA-SIP because RNA synthesis within active cells is faster than DNA synthesis. Therefore, RNA-SIP allows radio-labelled samples to be obtained much quicker. However, one drawback is that, for

example, ^{13}C-labelled RNA can form multiple bands following density-gradient centrifugation which then each need to be analysed (Dumont and Murrell, 2005). Manefield *et al.* (2002) used ^{13}C-labelled phenol and RNA-SIP to monitor phenol degradation in an adapted community sampled from an aerobic industrial bioreactor. The authors found that the phenol-degrading community was dominated by members of the β-Proteobacterial genus *Thauera* sp. Work by Lu and Conrad (2005) used pulses of $^{13}CO_2$ and subsequent uptake of ^{13}C into the microbial rRNA to determine possible organisms involved with the production of methane in rice rhizosphere communities. Following $^{13}CO_2$ pulsing, there was a significant increase in the prevalence of sequences with high sequence homologies to the rice cluster I (RC-I) group of archaea suggesting that these may have been involved in methane production in this system. More recently, Hunger *et al.* (2011) identified CO_2 and formate-utilising organisms present within and anaerobic methane-emitting fen soil. The authors combined SIP using pulses of either $^{13}CO_2$ or ^{13}C-formate with 16S rRNA, *mcrA*, and *fhs* and sequence analysis to achieve their objectives. The results showed that the ^{13}C-formate treatment selected for bacteria with high sequence homologies to the *Methanocellaceae*, *Methanobacteriaceae*, *Acetobacteriaceae*, and *Rhodospirillaceae* families. In contrast, $^{13}CO_2$ pulsing resulted in the predominance of sequences with high homologies to the families *Methanosarcinaceae*, *Conexibacteraceae* and *Solirubrobacteraceae*.

3.1.2 RNA extraction and community analysis in current research

Another possibility for carrying out the culture-independent analysis of active microbial communities is the use of extracted RNA alongside methods such as DGGE or T-RFLP. Whilst extracted DNA can be representative of organisms in the community that are either active, dormant or dead, RNA in contrast, is labile and does not persist outside dead cells due to it being rapidly degraded (Hirsch *et al.*, 2010). This, therefore, highlights the possibility of using extracted RNA as a measure of metabolically active organisms within a community. These RNA-based methods can be carried out using either extracted ribosomal RNA (rRNA) or messenger RNA (mRNA). rRNAs are thought to be a good indicator of active organisms as ribosome numbers have previously been correlated with cellular metabolic activity. They are also present in high numbers within metabolically active cells,

thus making them an attractive target for analysis. For example, Matsuda *et al.* (2007) stated that there are approximately 10^3 copies of each rRNA type (5S, 16S, and 23S) within a metabolically-active *E. coli* cell. This, therefore, helps to increase the level of detection achieved by reverse-transcriptase PCR (RT-PCR) processes (Matsuda *et al.*, 2007). Following this, the complementary DNA (cDNA) produced can be used to determine active community structures using techniques such as TGGE, DGGE or T-RFLP (Curlevski *et al.*, 2011). mRNA has previously been analysed as a possible alternative to rRNA as indicator for determining the structures of active microbial communities. mRNA is an attractive proposition for use in community analysis as it can be used to analyse specific functions within a community, as opposed to simply overall activity. However the use of mRNA has also (until relatively recently) been seen as very challenging particularly because mRNA is rapidly degraded within the environment, and comprises only a small proportion of the total RNA within a cell (Frias-Lopez *et al.*, 2008). Indeed, McGrath *et al.* (2008) noted that only approximately 1 to 5% of the RNA within a community is mRNA, with the majority being rRNA.

RNA extraction from soils and other environmental samples initially involved multiple step-wise solvent purification methods, which although successful were very labour-intensive and therefore considered unsuitable for large sample sets (Griffiths *et al.*, 2000). Following this, Griffiths *et al.* (2000) devised a method of co-extracting pure DNA and rRNA from soils. This method involved a single initial extraction using 0.5 ml of hexadecyltrimethylammonium bromide (CTAB) buffer and 0.5 ml of phenyl-chloroform-isoamyl alcohol (25:24:1). Following extraction and centrifugation the supernatant was extracted and the excess phenol removed using chloroform-isoamyl alcohol (24:1). The nucleic acids were precipitated using polyethylene glycol and 1.6 M sodium chloride (NaCl) the samples were then centrifuged, the supernatant removed, and the pellet washed in 70% (vol/vol) ethanol before being air-dried. The pellet was then re-suspended in RNase-free Tris EDTA buffer and the DNA and RNA yields determined by gel electrophoresis. Pure DNA was obtained by the addition of RNase A at a concentration of 100 µg ml^{-1} and storage at 37°C for 10 minutes. Additionally, pure RNA was obtained using 3 units of RNase-free DNase (Griffiths *et al.*, 2000).

A co-extraction method was also devised by Hurt *et al.* (2001), this time for the extraction of DNA and mRNA. Frozen soil samples were thawed with 1 ml of denaturing solution and re-frozen with liquid nitrogen. These samples were then ground with a mortar and pestle and re-frozen and thawed 3 times in liquid nitrogen. Following the third thawing cycle, the samples were incubated in extraction buffer (pH 7) at 65°C for 30 minutes before being centrifuged and the supernatant collected. Nucleic acids were extracted from the supernatant using chloroform and precipitated using isopropyl alcohol. The pellet was then suspended in 1 ml of water before the RNA and DNA was separated in a Qiagen resin column. Purified RNA was obtained using the Qiagen followed by treatment with RNase-free DNase I. Purified DNA was produced using column purification.

3.1.2.1 The Use of Ribosomal RNA (rRNA) in Active Microbial Community Analysis

3.1.2.1.1. Comparisons Between Community Analyses Performed using Extracted DNA and rRNA

Significant differences in marine bacterioplankton community structure using amplified 16S rDNA and rRNA genes and T-RFLP analysis were observed by Moeseneder *et al.* (1999). The authors noted that a greater number of TRFs were observed in the RNA-derived communities than in the DNA-derived ones. This suggested that some organisms, despite only being present at low levels within the community, may have been very active within it. Further research in this area showed that significant differences were also observed following clone library production and sequencing. In bacterioplankton clone libraries Moeseneder *et al.* (2005) observed that 25.5% of the sequences obtained in a 16S rDNA library did not have close homologies to any sequences in a parallel 16S rRNA library. Additionally, 21.5% of the sequences in the rRNA library did not show high homologies to sequences in the rDNA library.

Griffiths *et al.* (2000) used PCR amplification of the 16S rDNA or rRNA region along with DGGE to compare overall and active soil microbial community structures following DNA and RNA co-

extraction. Cluster analysis showed that the communities produced using extracted DNA grouped separately from those produced using RNA. This indicated that the extracted DNA could have been representative of the overall community, whilst the RNA could have shown the active community.

Mengoni *et al.* (2005) also observed differences between DNA and RNA-derived community structures whilst studying the impacts of chromate amendment on bacterial community structure in previously polluted and un-polluted soils. Using PCR and RT-PCR of extracted DNA and RNA, followed by T-RFLP analysis the authors concluded that amendment with chromate (Cr(VI)) at a concentration of 250 mg kg^{-1} soil dry weight had a significant impact on the bacterial communities in both soil types. Between the two soil types an average of 57% of the TRFs were shared between the DNA- and RNA- derived profiles. An average of 28% of the TRFs were found in the DNA-derived profiles with 15% of the TRFs being unique to the RNA-derived samples (Mengoni *et al.*, 2005).

More recently, Lillis *et al.* (2009) used DNA/RNA co-extraction followed by PCR or RT-PCR to amplify 16S bacterial gene products from soils exposed to a 100 mg kg^{-1} dose of the chlorinated phenol 2,4-dichlorophenol (2,4-DCP). T-RFLP and clone library analyses were then used to determine the impacts of perturbation on the soil bacterial communities. The authors observed significant differences in the bacterial community structure in both the DNA- and RNA-derived communities in response to 2,4-DCP application. Both clone libraries were dominated by Proteobacteria. One noticeable difference was in the class Bacilli which represented 10% of the RNA-derived library compared with 3.5% for the DNA-derived samples. Some of the organisms present in the RNA-derived library such as members of the genus *Bradyrhizobium* have previously been identified as being involved in 2,4-DCP degradation (Lillis *et al.*, 2009).

However, such differences between DNA and RNA-derived community profiles have not always been observed. Research by Nogales *et al.* (2001) used extracted DNA and RNA followed by RT-PCR and clone library production to determine the differences in the overall and active bacterial community

structures in the same PCB contaminated soil. The results showed that the major bacterial groups present in the DNA-derived clone library were also present in the RNA-derived samples, with differences between the libraries being restricted to species represented by only one or two sequences such as the members of the actinobateria which were only present in the RNA libraries (Nogales *et al.*, 2001).

3.1.2.1.2 The use of Extracted rRNA to Determine the Effects of Perturbations on Active Microbial Communities

Following this, researchers began to use these techniques to determine the impacts of perturbation on microbial communities. One early target for this research was the impact of polychlorinated biphenyls (PCBs) on active bacterial communities in soils. Nogales *et al.* (1999) used RNA extraction and RT-PCR with bacterial primers followed by clone library production and sequencing to determine the active community structure of a moorland soil contaminated with PCBs. The major finding of this work was that the soil contained a high proportion of beta proteobacteria from the genera *Burkholderia* and *Variovorax*. Additionally, some of the sequences obtained were identified as organisms that had previously been associated with the degradation of organic pollutants. The authors concluded that these organisms may have been using the PCBs as carbon and energy sources (Nogales *et al.*, 1999).

Ravenschlag *et al.* (2000) successfully combined 16S rRNA gene amplification and rRNA slot blot hybridisation to determine the structure of the active sulphur-reducing community within marine arctic sediments. The observations showed that the sulphur reducing community in these sediments was dominated by organisms showing high sequence homologies to the genera *Desulfosarcina* and *Desulfococcus*.

Further work by Edlund *et al.* (2008) used 16S rRNA gene cloning and sequencing to identify the wide array of bacteria involved in nitrogen and sulphur cycling across vertical redox gradients in

Baltic Sea sediments. More recently, Wrighton *et al.* (2010) used a combination of 16S rRNA and fluorescent *in situ* hybridisation (FISH) to analyse how bacterial community structure affected nitrate reduction within cathodic systems. The results of the analyses showed that higher bacterial richness and evenness corresponded to a higher level of cathode performance, with the phyla Proteobacteria, Firmicutes and Chloroflexi dominating the community. Furthermore, Cagnon *et al.* (2011) analysed how marine bacterial community structure was impacted by different levels of contamination with hydrocarbons. The results of 16S rRNA gene and T-RFLP analyses showed significant differences in community structure, which may have been indicative of adaptation to the presence of the hydrocarbons.

However, despite the widespread use of rRNA to determine the structures of active microbial communities, some potential issues have arisen with these techniques. One of the main issues is that rRNA could potentially still be obtained from metabolically inactive organisms. This is due to the fact that rRNA possesses a secondary structure and therefore could potentially persist for months after the death of a cell (Hirsch *et al.*, 2010). During their study of the benthic eukaryote community structure in a hydrothermal vent ecosystem, Edgcomb *et al.* (2002) noted how many of the rRNA sequences obtained could have been from inactive members of the population. Additionally, whilst rRNA can be used to determine the structure of active communities within soils, its use in ascertaining specific functions within the community is limited. Therefore, research has also focussed on the use of mRNA to characterise the active members of microbial communities.

3.1.2.2 The Use of Messenger RNA (mRNA) in Active Microbial Community Analysis

McGrath *et al.* (2008) used total RNA extraction to obtain RNA from a range of soil, water and commensal samples. These extracts were then run on 1.5% agarose gels and the 23S, 16S and 5S rRNA bands identified. In order to isolate pure mRNA the regions between these bands were excised and used for cDNA synthesis using random hexamers. The authors concluded that this method produced clean mRNA products which they were able to use to produce clone libraries.

Research by Kotiaho *et al.* (2010) used mRNA extraction, RT-PCR and T-RFLP analysis to determine the expression of the methanogen-specific gene *mcrA* (for the α-subunit of methyl coenzyme M reductase) in two boreal fens with different water table heights. The T-RFLP profiles indicated that a single TRF from the mRNA-derived profiles (462 bp) predominated in both communities.

Im *et al.* (2011) also used mRNA extraction combined with RT-PCR to determine the effects of nitrogen and phenylacetylene application on the expression of the genes *amoA*, *mmoX*, *pmoA*, *nirK*, and *norB* in landfill cover soils. The results showed that *pmoA* was detected in all treatments, but the expression levels increased following amendment with nitrogen and decreased with phenylacetylene amendment. Archaeal *amoA* was present in the nitrogen-amended treatment, but was absent when phenylacetylene was applied.

PCR-independent methods have also been developed for use with mRNA to analyse active microbial communities. Urich *et al.* (2008) developed a simultaneous rRNA and mRNA-based method for analysing the structure and function of soil microbial communities. This approach involved cDNA production using random hexamer-primed reverse transcription, followed by direct pyrosequencing and taxonomic analysis. This method enabled the analysis of community structure and function across different trophic levels in a single experiment. The lack of a PCR step within the procedure meant that any of the commonly-associated issues such as primer bias were avoided.

PCR-independent mRNA extraction, reverse transcription and pyrosequencing were also used by Gilbert *et al.* (2008) to analyse the structure of complex marine microbial communities. The authors observed that during phytoplankton blooms about 50% of the mRNA transcripts were shared across samples whereas under post-bloom conditions this value was approximately 90%.

More recently, Gifford *et al.* (2011) used bacterioplankton mRNA extracted from coastal seawaters along with 454 FLX pyrosequencing to produce a library of approximately 500,000 potential protein-encoding sequences. These sequences were analysed for the expression levels of 82 genes involved in the marine nitrogen, phosphorus, and sulphur cycles. Of these 82 genes, 36 were found to be present at levels below the detection limit of the procedure ($< 1 \times 10^6$ transcripts L^{-1}). Other genes such as those for an ammonia transporter (*amt*) and for the ammonia monooxygenase subunit *amok* were present in much higher amounts ($> 2.7 \times 10^9$ transcripts L^{-1}).

These examples are just a small proportion of a rapidly-increasing body of research that is using extracted RNA (both rRNA and mRNA) and high throughput techniques to shed light on the activity of a wide variety of microbial communities within an extensive range of ecosystems.

3.1.3 Applying RNA-based analyses to this project: experiment aims and hypotheses

The work presented in Chapter 2 showed that azoxystrobin application had a significant deleterious impact on soil dehydrogenase activity. However, this impact was not illustrated in the DNA-derived bacterial, pseudomonad, archaeal, and general eukaryote T-RFLP analyses. This may have been due to the presence of DNA from dormant or dead organisms masking any impacts.

Similar results were observed by Bending *et al.* (2007) who observed that changes in eukaryote DGGE band patterns showed no correlation with impacts on dehydrogenase activity in soils amended with the fungicides azoxystrobin, chlorothalonil, or tebuconazole.

Furthermore, fungal quantitative PCR analysis (qPCR) in Chapter 2 showed a significant reduction in 18S rRNA gene copy number following azoxystrobin application, particularly at the highest amendment concentration. This indicated a possible impact specifically on the fungal community but this was not associated with a shift in community composition in fungal DNA samples.

Thirdly, Mann Witney U test analyses of the fungal and nematode community structure clone libraries produced in showed significant differences between amended and un-amended treatments for both organism groups. Additionally, Shannon diversity analyses also showed significant impacts of azoxystrobin on fungal and nematode communities.

Therefore, the work presented in this chapter attempted to determine the impacts of perturbations on soil microbial communities by analysing the impacts of a single 25 mg kg^{-1} dose of azoxystrobin on the overall and active microbial community structures for organisms from different trophic levels using RNA-derived T-RFLP analysis. In addition, a 2-week sampling point was added to determine impacts on the community in the early stages post-application. Broad-scale analyses (biomass and dehydrogenase activity) were performed along with HPLC analysis of compound degradation as in the previous chapter.

The hypotheses tested in this chapter were: 1) The application of azoxystrobin will have significant deleterious impacts on active fungal and nematode communities as determined by RNA-derived T-RFLP analyses; 2) Impacted TRFs in the fungal and nematode communities will show high sequence homologies to impacted sequences from the clone libraries produced in Chapter 2.

3.2 Materials and Methods

3.2.1 Soil Collection and Preparation

Soil was collected from Hunts Mill field at the Wellesbourne site of the University of Warwick School of Life Sciences, UK during January 2009. A description of the soil characteristics can be found in the methods section of the previous chapter. Soils were collected and prepared as described in Chapter 2.

3.2.2 Soil Spiking, Storage and Sampling

Soils were spiked with azoxystrobin (Greyhound Chromatography, Birkenhead, UK) to a concentration of 25 mg kg^{-1} soil using the methods described in Chapter 2. Additionally, a non-spiked control soil was also prepared.

After spiking, the soils were divided up into 250 ml glass Duran bottles, and the weights recorded and monitored as in Chapter 2. The bottles were wrapped in aluminium foil and stored in the dark at 15°C in an augmented Trojan design. This design is generally used when the number of treatments (e.g. pesticide concentrations) is low compared with, for example, the number of replicates. This design uses the formula shown below:

$$((n\text{-}1)\neg n)\}k$$

Where n is the number of treatments (e.g. the number of pesticide concentrations), and k is the number of plots per block (e.g. the number of replicates) (Edmondson, 1998).

5 replicate samples of each treatment were destructively sampled at time 0, and then 2, 4, 8, and 16 weeks post-amendment.

3.2.3 Soil Microbial Biomass

Soil biomass-N was measured using the chloroform fumigation method of Joergensen and Brookes (1990), and the conversion factor of 3.1 proposed by Amato and Ladd (1988) for the conversion of ninhydrin-N values to biomass-N. All analyses were set up using the methodology detailed in Chapter 2.

3.2.4 Soil Microbial Activity

Soil microbial activity was monitored by analysing the conversion of TTC to TPF using an adapted method of the one described by Tabatabai (1994). A full methodology for this assay can be found in Chapter 2.

3.2.5 Monitoring azoxystrobin concentration using high-performance liquid chromatography

Azoxystrobin recovery over the experimental period was monitored using HPLC as described in Chapter 2.

3.2.6 Extraction of soil DNA and RNA for Terminal Restriction Fragment Length Polymorphism analysis

DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis was carried out to analyse the overall soil bacterial, fungal, nematode, and general eukaryote community structures over the 4-month experimental period. RNA was extracted from the 1-month treatments to determine the impacts of azoxystrobin on active soil communities.

3.2.6.1 DNA Extraction

Total DNA extraction, sample preparation and analysis were carried out using the methods detailed in Chapter 2. The primer pairs used in these analyses were 63f-NED/1087r-VIC for bacteria, EF4f-FAM/EF3r for fungi, Nem 18Sf-VIC/Nem 18Sr for nematodes, and Euk20f-FAM/Euk516r for general eukaryotes. Further details of these primer pairs can be seen in Table 2.2 of Chapter 2.

3.2.6.2 RNA Extraction

To prepare soil samples for total RNA extraction, 10 g portions of soil were first placed in sterile 15ml plastic centrifuge tubes (VWR International Ltd., UK) and flash-frozen in liquid nitrogen. The tubes were then stored at -80°C until required.

The flash-frozen soils were then ground in a coffee grinder (Delonghi, UK) along with an equal quantity of dry ice. The grinder was cleaned with RNase Zap (Applied Biosystems, UK) before and after grinding each sample to ensure the removal of any RNases that may have been present. The ground samples were then transferred to sterile 50 ml plastic centrifuge tubes (Fisher Scientific, UK) and the lid loosely screwed on. The samples were stored at -80°C for 24 hours to allow the CO_2 to evaporate.

Total RNA extraction from the ground soils was carried out using the FastRNA® Pro Soil – Direct Kit (QBiogene, UK) with minor modifications to the manufacturer's instructions. Firstly, 150 mg soil was used per sample instead of the 500 mg – 1 g suggested in the protocol. This was done in order to reduce the presence of PCR-inhibitory compounds such as humic acids as much as possible whilst still allowing sufficient quantities of RNA to be extracted. Secondly, all room temperature incubation steps (steps 4, 11, 16, 18, 23 and 24 in the manufacturer's manual) were reduced from 5 to 4 minutes to minimise the possibility of RNA degradation whilst still allowing the step to work successfully.

Finally, in-between steps 16 and 17 in the manufacturer's protocol (see Appendix III), extra washes were carried out using 5.5 M guanidine thiocyanate (Fisher Scientific, UK) to remove any remaining contaminants. For each wash the RNA pellet was re-suspended in 1 ml of 5.5 M guanidine thiocyanate, centrifuged at 14,000 x *g* for 30 seconds, and the supernatant removed. These washes were repeated until the pellet returned to the original colour of the silica gel.

Several measures were taken to reduce the possibility of RNA sample contamination and/or degradation during extraction. All extractions were carried out on ice with salt added to reduce the melting temperature, apart from at the stages where room temperature incubations were specifically stated. Extractions were carried out in a fume hood with all surfaces being cleaned with RNase Away Wipes (MP Biomedicals, UK) before and after any work was carried out. All pipettes used were cleaned with RNase Away Wipes (MP Biomedicals, UK) before and after use, and new boxes of sterile, filter tip, plastic pipette tips were used for every set of extractions. Disposable nitrile gloves were changed on a regular basis and were cleaned with RNase Away Wipes (MP Biomedicals, UK) before handling any samples.

The concentration of RNA present in the extracts was determined using the NanoDrop® ND-1000 spectrophotometer (Labtech International Ltd., UK) in the same way as for extracted DNA as described in Chapter 2. RNA extracts were then purified using the RNeasy® MinElute™ Cleanup Kit (Qiagen, UK) following the manufacturers' instructions.

The concentration of RNA in the purified samples was quantified using the NanoDrop® ND-1000 spectrophotometer (Labtech International Ltd., UK) as previously, before being divided into 2 µl aliquots in sterile, 200 µl PCR tubes (Thermo Scientific, UK) for storage at -80°C until required.

Reverse transcriptase PCR (RT-PCR) was carried out using the Qiagen® OneStep RT-PCR Kit (Qiagen, UK) according to the manufacturers' instructions. Each 50 µl RT-PCR reaction contained: 17 µl RNase-free water, 10 µl, 5 x Qiagen OneStep RT-PCR Buffer, 2 µl dNTP Mix (Final concentration = 400 µM of each dNTP), 8 µl of each primer at a concentration of 0.1 µM μl^{-1}, 2 µl Qiagen OneStep RT-PCR Enzyme Mix, 1 µl RNase Inhibitor (5 U μl^{-1}), and 2 µl template RNA (1 ng μl^{-1}). The RT-PCR conditions consisted of a reverse transcription stage of 30 minutes at 50°C, followed by an initial PCR activation stage of 15 minutes at 95°C. Following this, the PCR

denaturation, annealing and extension phase conditions were specific to the primers used, and were as previously detailed in Chapter 2.

RT-PCR products were visualised on a 1% agarose gel to ensure that the fragment amplified was of the correct size, before being purified and prepared for T-RFLP analysis in the same way as for extracted DNA samples as detailed in Chapter 2. T-RFLP traces were analysed and the relevant statistical analyses carried out using the GeneMarker® (Softgenetics®, USA) and Primer6 (Primer-E Ltd., UK) software packages.

In addition to the analyses carried out as in Chapter 2, the DNA and RNA data from each treatment were also compared with each other to ascertain what percentage of the overall community TRFs represented active organisms.

3.2.7 Statistical Analyses

All statistical analyses carried out were the same as those carried out as detailed in Chapter 2. T-RFLP analysis for the samples derived from extracted RNA was carried out using Primer6 in the same way as for the soil DNA samples.

3.3 Results

3.3.1 Azoxystrobin recovery using HPLC

HPLC analysis showed a marked decrease in azoxystrobin within the first 2 weeks post-amendment (Figure 3.1). Following this, there was a minimal reduction in azoxystrobin recovery between the 2 and 16 week sampling points. After 2 weeks, 66% of the applied azoxystrobin was recovered. By 16 weeks, this value was 52%. These results showed lower compound degradation than the

corresponding treatment from the experiment in Chapter 2 where 46% of the compound was degraded in the first month and 63% after 4 months.

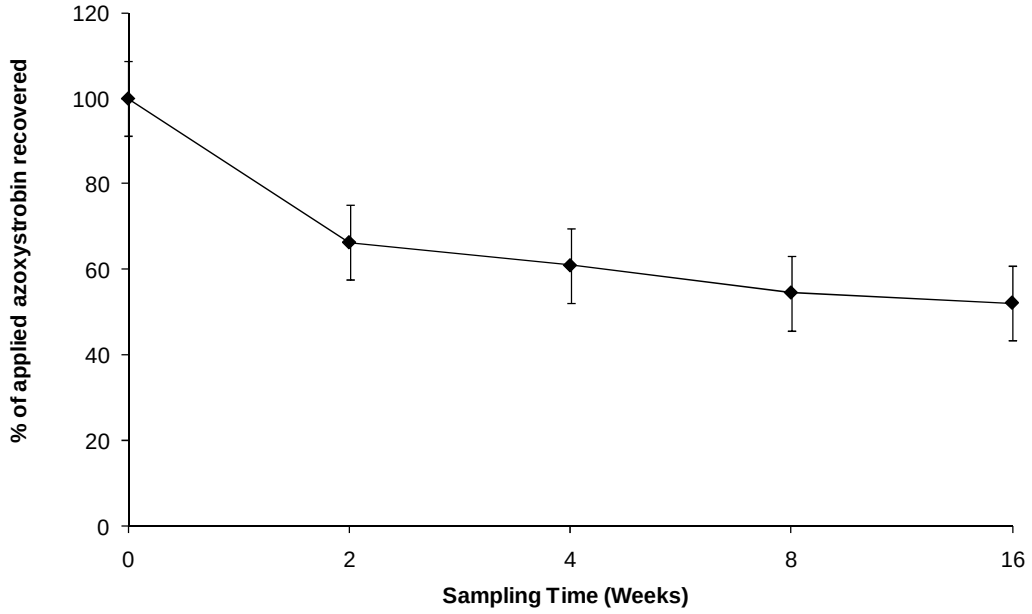


Figure 3.1. Percentage of initially applied azoxystrobin extracted using HPLC analysis. Error bars denote +/- the standard error of the mean. Each data point represents the mean of 5 experimental replicates.

3.3.2 Dehydrogenase Activity

2-way ANOVA analysis showed a significant impact of azoxystrobin application on dehydrogenase activity (p = 0.03). 2 weeks post-application, a reduction of 47% in TPF production was recorded in amended samples compared with the 0 mg kg^{-1} control (Figure 3.2). This percentage difference remained similar between the 2 and 8 week sampling points. After 16 weeks, TPF production in the amended samples was still only 77% that of the un-amended controls. This differed from the results observed in Chapter 2 which showed a recovery to control activity levels after 3 months. Indeed, after 4 months the TPF production in the 25 mg kg^{-1} treatment was 133% of the controls. Sampling time did not have a significant impact on TPF production (p = 0.500).

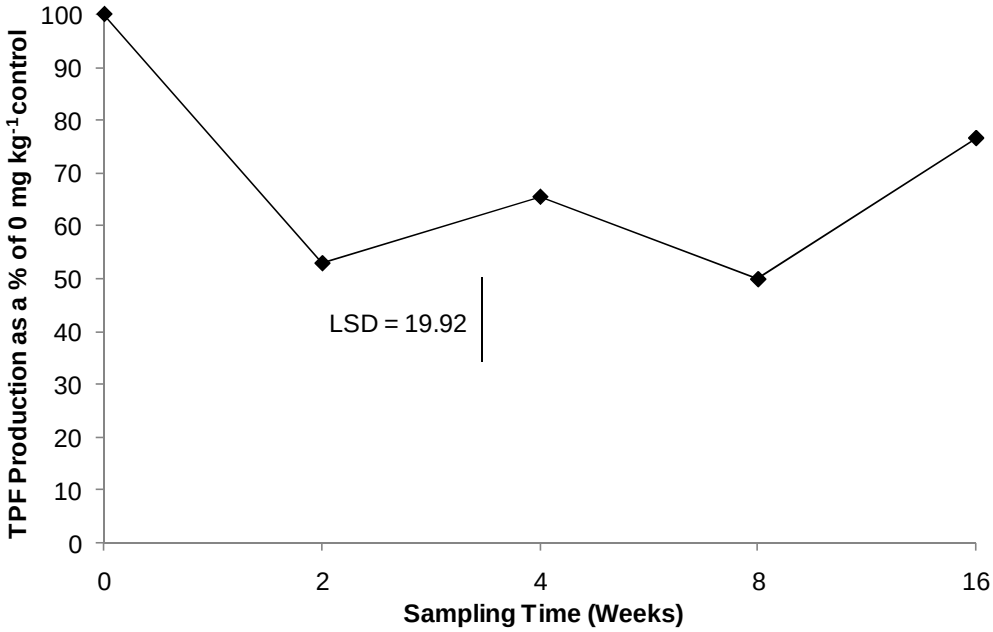


Figure 3.2. Percentage dehydrogenase activity in azoxystrobin-amended soils compared to 0 mg kg $^{-1}$controls. Error bars denote +/- the standard error of the mean. Each data point represents the mean of 5 experimental replicates.

3.3.3 Soil Microbial Biomass

There was no significant impact of azoxystrobin application on soil microbial biomass as determined by 2-way ANOVA analysis (p = 0.785) (Figure 3.3). Sampling time also did not significantly affect the microbial biomass (p = 0.405). The average biomass values ranged from 114.9 to 217.7 μg g^{-1} soil. These results were comparable to the results observed in Chapter 2 where the average biomass in the 25 mg kg^{-1} treatment ranged from 118.8 to 228 μg g^{-1} soil.

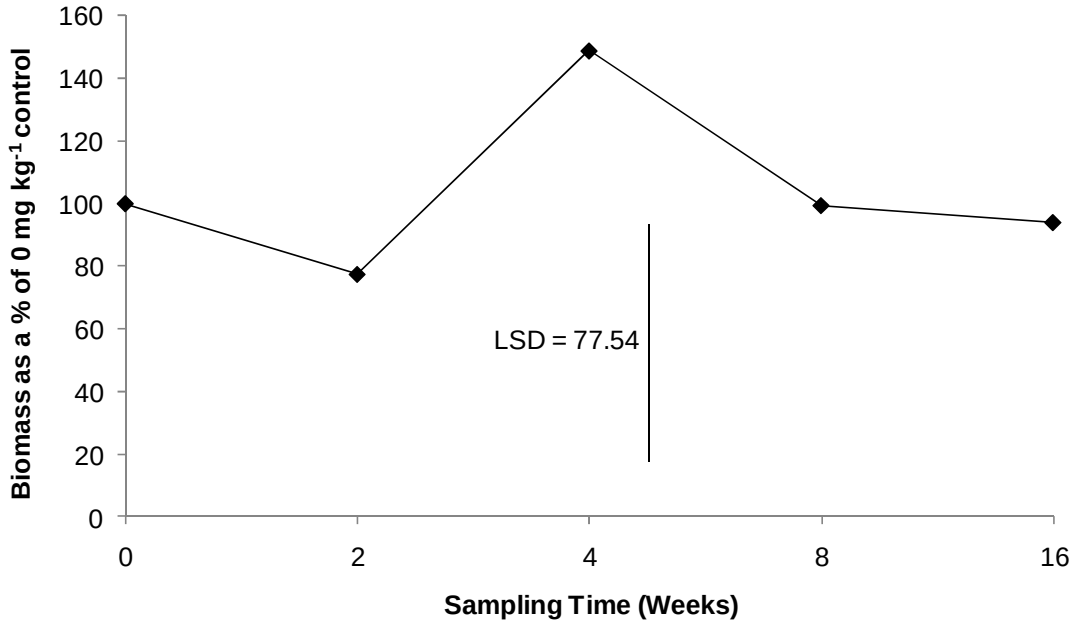


Figure 3.3 Microbial biomass in soils amended with azoxystrobin at a concentration of 25 mg kg^{-1}, recorded as a percentage of 0 mg kg^{-1} controls at each time point. Each data point represents the mean of 5 experimental replicates.

3.3.4 Impacts of azoxystrobin on overall and active microbial communities

3.3.4.1 Eukaryotes

3.3.4.1.1 Fungi

The numbers of fungal TRFs recorded 1-month post application ranged from 15 to 22 in the DNA-derived treatments, and 7 to 11 in the RNA-derived treatments. ANOVA analysis showed that there was a significant difference between TRF numbers in DNA and RNA-derived treatments (p = 0.04). There was no significant difference recorded between the 0 and 25 mg kg^{-1} replicates in samples produced from extracted DNA (p = 0.422). In contrast, there was a significant difference in TRF numbers (p = < 0.01) between un-amended and amended samples produced from extracted RNA. In the un-amended samples, an average of 67% of the TRFs present in the samples taken from extracted DNA (i.e. the overall community) were present in the samples taken from extracted RNA (i.e. the

active community). In amended samples, 53% of the TRFs that were observed in the DNA-derived samples were present in the RNA samples.

Shannon analysis also demonstrated a significant difference in fungal diversity between the un-amended and amended treatments in both DNA- and RNA-derived samples (Table 3.1).

Table 3.1 Average Shannon diversity (H') values for overall and active microbial communities in amended and un-amended treatments 1-month post-application.

	Azoxystrobin Concentration			
	0 mg kg^{-1} DNA	25 mg kg^{-1} DNA	0 mg kg^{-1} RNA	25 mg kg^{-1} RNA
Fungi	2.047^{a}	1.633^{b}	1.855^{ab}	1.152^{c}
Bacteria	2.767^{a}	2.691^{a}	2.621^{a}	2.660^{a}
Nematodes	1.936^{ab}	2.061^{a}	1.639^{b}	1.771^{ab}
General Eukaryotes	2.147^{ab}	2.319^{a}	1.564^{b}	1.924^{b}

LSD = 0.379. Each diversity value represents the mean of 5 experimental replicates. Significantly different values between treatments are shown by different letters.

ANOSIM analysis of fungal community structure backed up the TRF number analyses. There was a significant difference in community structure between amended and un-amended samples for both DNA- (p = 0.046) and RNA-derived samples (p = 0.024). There was also a highly significant difference in the community structure of samples produced using extracted DNA or RNA (p = < 0.01). This was supported by NMDS analysis (Figure 3.4) which showed an overlap in the two sample groups in the DNA-derived samples. However, there was no overlap in the RNA-derived samples, and the DNA and RNA-based samples formed clearly distinct groups.

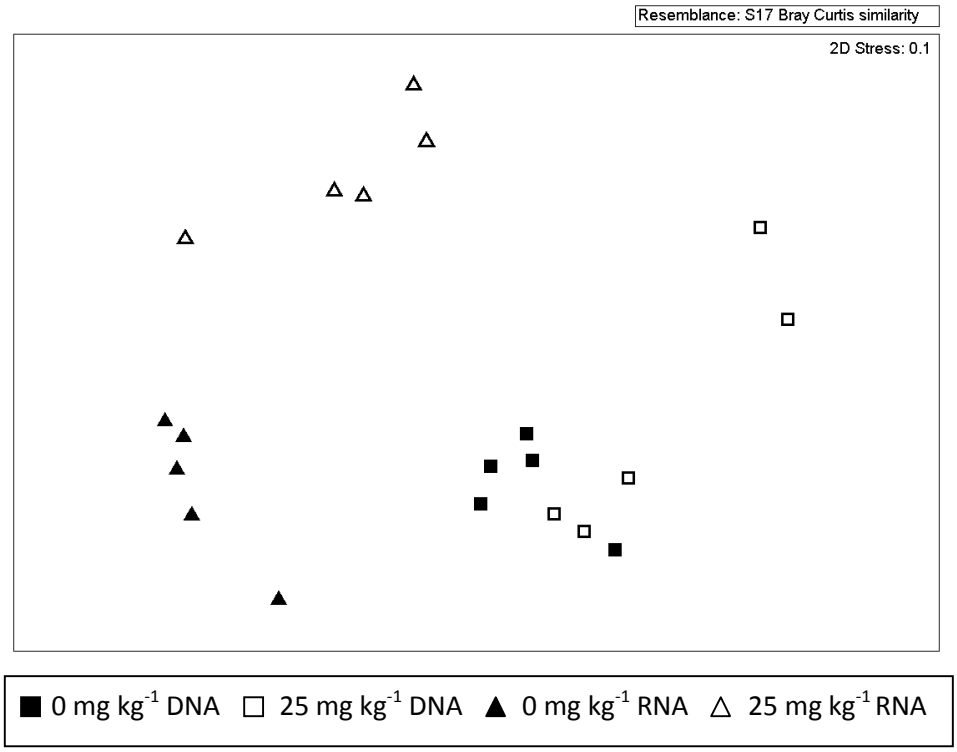


Figure 3.4 NMDS analysis of fungal community structures determined using extracted DNA and RNA 1-month post azoxystrobin application. Each data point represents an experimental replicate.

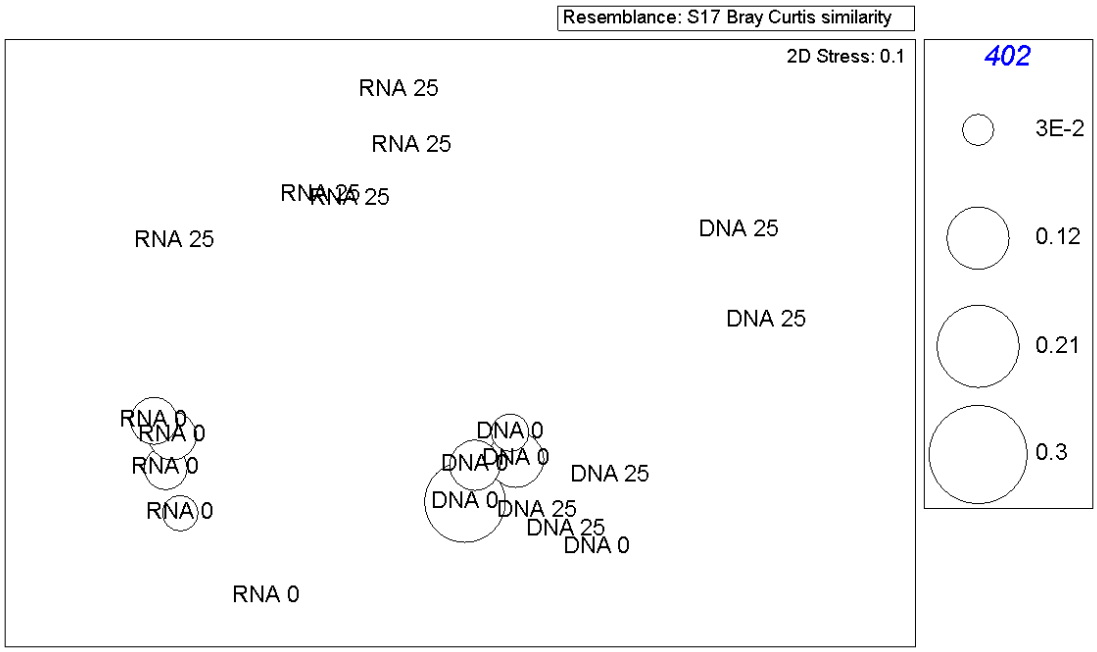
SIMPER analysis of the fungal community structure data showed that the TRF at 402 bp was particularly impacted by azoxystrobin amendment. Bubble plot analysis of this TRF (Figure 3.5a) showed that it was present within both the overall (DNA) and active (RNA) fungal communities in un-amended samples after 1 month. In contrast it was absent from both of these communities in azoxystrobin amended soils.

A comparison between the DNA and RNA-derived samples as a whole showed that this TRF was responsible for 14% of the total variation. TRF 402 bp was also responsible for 10% of the total variation when comparing the amended and un-amended treatments of the DNA-derived samples. When the RNA-derived samples were compared, this value was 16%.

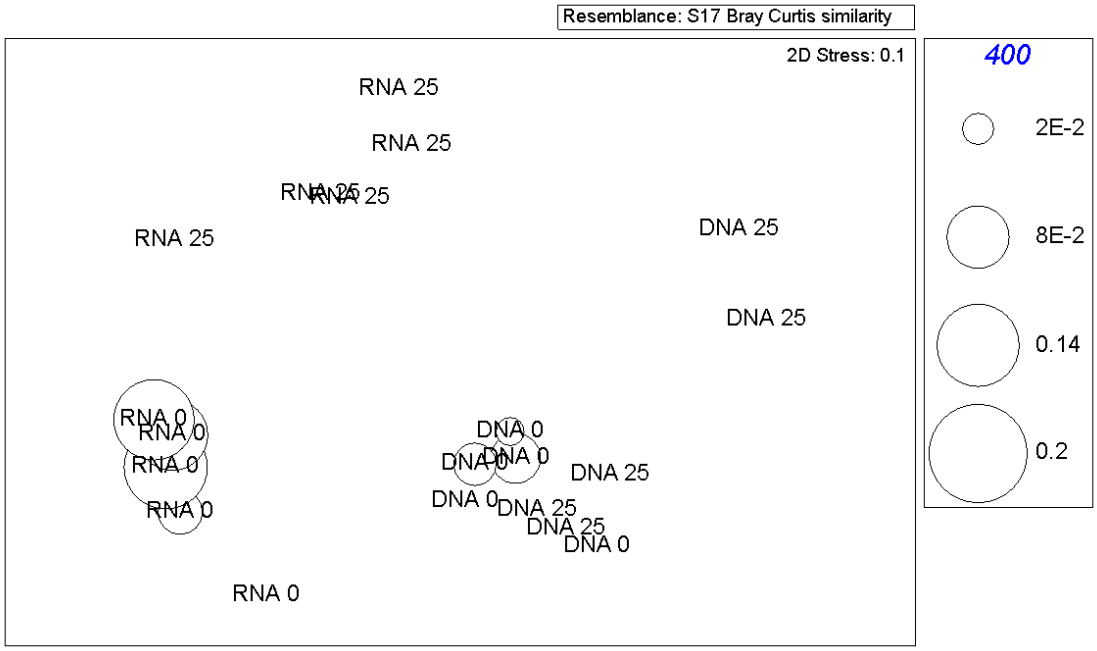
TRF 400 bp (Figure 3.5b) represented 6% of the total variation when DNA and RNA-derived T-RFLP traces were compared. It also represented 5% of the variation when the amended and un-amended treatments were compared for both DNA and RNA-derived samples. Bubble plot analysis of the un-amended treatments showed that although TRF 400 bp represented a relatively small proportion of the overall community as determined using extracted DNA, the RNA-derived data showed that the organism(s) represented by this TRF played a larger role in the active community. In contrast, TRF 400 bp was not present in either the DNA or RNA-derived samples taken from the 25 mg kg^{-1} treatment.

The TRF at 75 bp (Figure 3.5c) also had a noticeably higher prevalence in the active community of the un-amended treatment, compared with the amended treatment. Bubble plot analysis showed that this TRF was present at low levels in both the DNA- and RNA-derived samples for the un-amended treatment. For the 25 mg kg^{-1} treatment TRF 75 bp showed was not present in the DNA-derived samples but it was in the RNA-derived samples. This could mean that the organism(s) responsible for this TRF represented only a very small proportion (< 1%) of the overall community, but had a relatively high activity.

a)


Resemblance: S17 Bray Curtis similarity
2D Stress: 0.1
RNA 25
RNA 25
RNA 25
RNA 25
DNA 25
DNA 25
RNA 0
RNA 0
RNA 0
RNA 0
RNA 0
DNA 0
DNA 0
DNA 0
DNA 0
DNA 25
DNA 25
DNA 25
DNA 0
RNA 0
402
3E-2
0.12
0.21
0.3

b)


Resemblance: S17 Bray Curtis similarity
2D Stress: 0.1
RNA 25
RNA 25
RNA 25
RNA 25
DNA 25
DNA 25
RNA 0
RNA 0
RNA 0
RNA 0
RNA 0
DNA 0
DNA 0
DNA 0
DNA 0
DNA 25
DNA 25
DNA 25
DNA 0
RNA 0
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2E-2
8E-2
0.14
0.2

c)

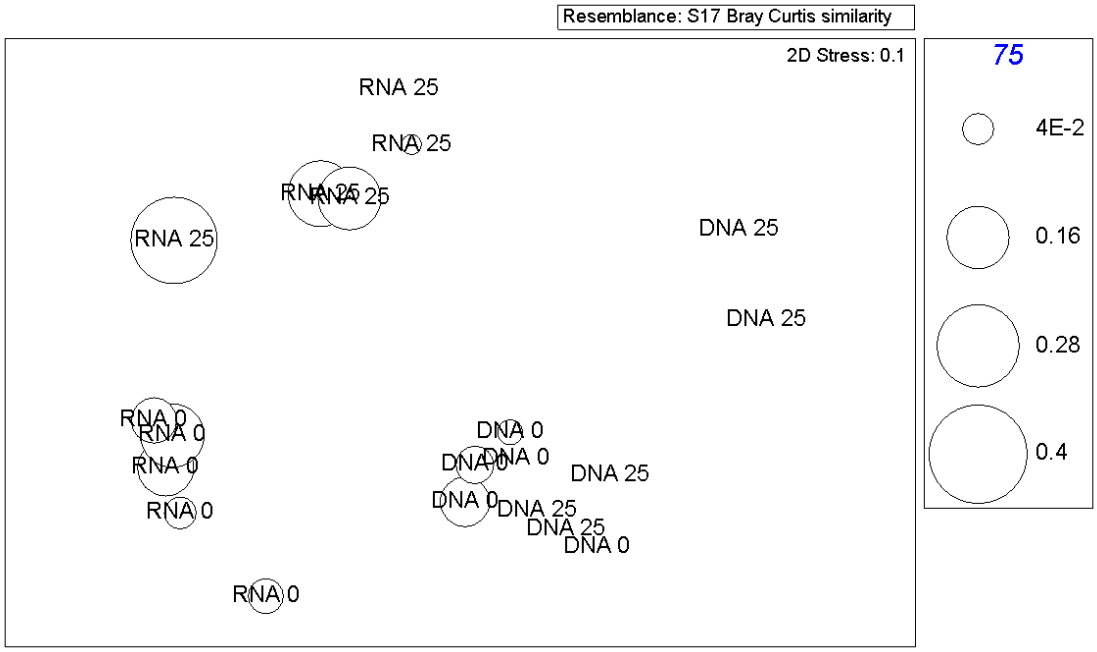


Figure 3.5. Bubble plot of TRF a) 402, b) 400, and c) 75 bp abundance in EF4f/EF3r fungal samples 1-month post application. Numbers in the bubbles denote pesticide concentration (mg kg^{-1}). 0 and 25 denote the azoxystrobin treatments. The numbers in the key denote the relative abundance of the TRF. Each sample point represents the average of 5 experimental replicates.

For the time-course experiment, the numbers of fungal TRFs recorded ranged from 14 to 28. 2-way ANOVA analysis showed that there was a significant difference in the number of TRFs in between amended and un-amended samples (p = 0.03). There was no significant impact of sampling time on fungal TRF numbers (p = 0.145). Azoxystrobin application also had a significant impact on fungal diversity as determined by Shannon diversity analysis (p = 0.02). Sampling time did not have a significant impact on diversity (p = 0.09). LSD analysis showed that fungal diversity was significantly different between the un-amended and amended samples at all sampling times apart from 8 weeks post-application (Figure 3.6).

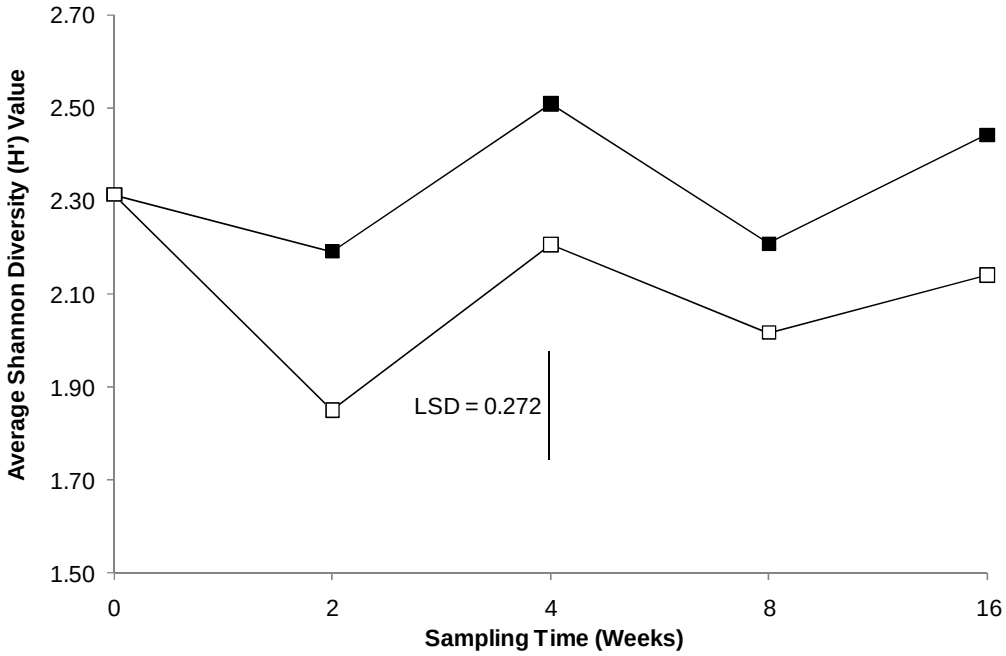
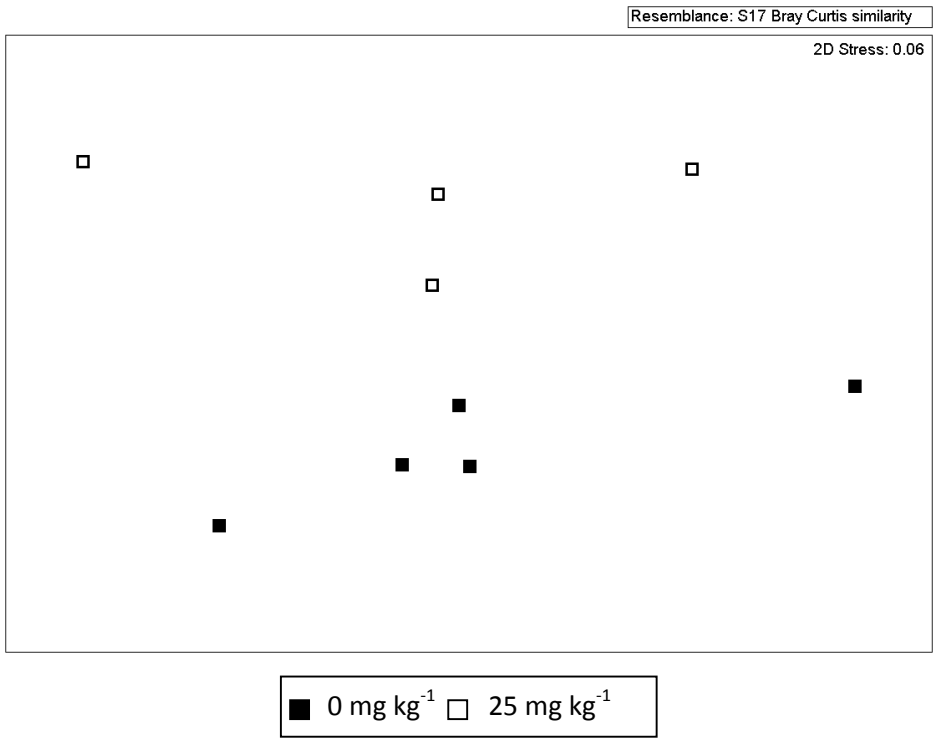


Figure 3.6. The average Shannon diversity (H') of total fungal communities recorded using the EF4f/EF3r primer pair. ■ 0 mg kg^{-1} □ 25 mg kg^{-1}. Each data point represents the mean of 5

ANOSIM analysis showed that azoxystrobin application at a concentration of 25 mg kg^{-1} did have a significant impact on fungal community structure (p = 0.032). Sampling time did not have a significant impact on community structure (p = 0.680). This was backed up by NMDS analysis where there was a noticeable grouping of points when the results were compared by pesticide concentration (Figure 3.7a). In contrast, NMDS analysis showed no grouping of points when the samples were compared by sampling time (Figure 3.7b).

a)



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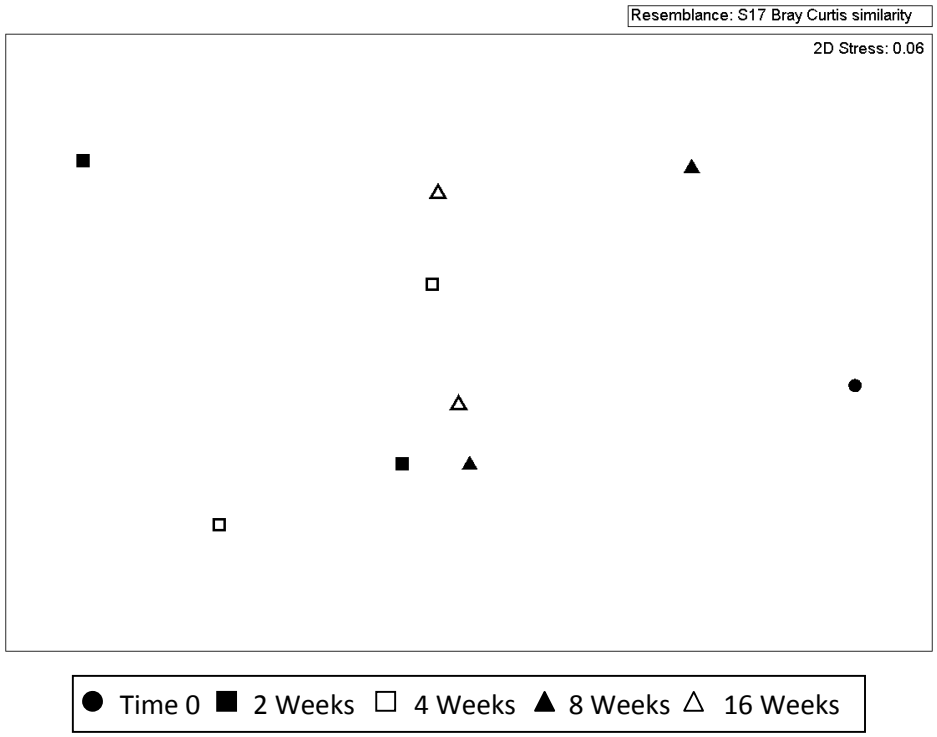
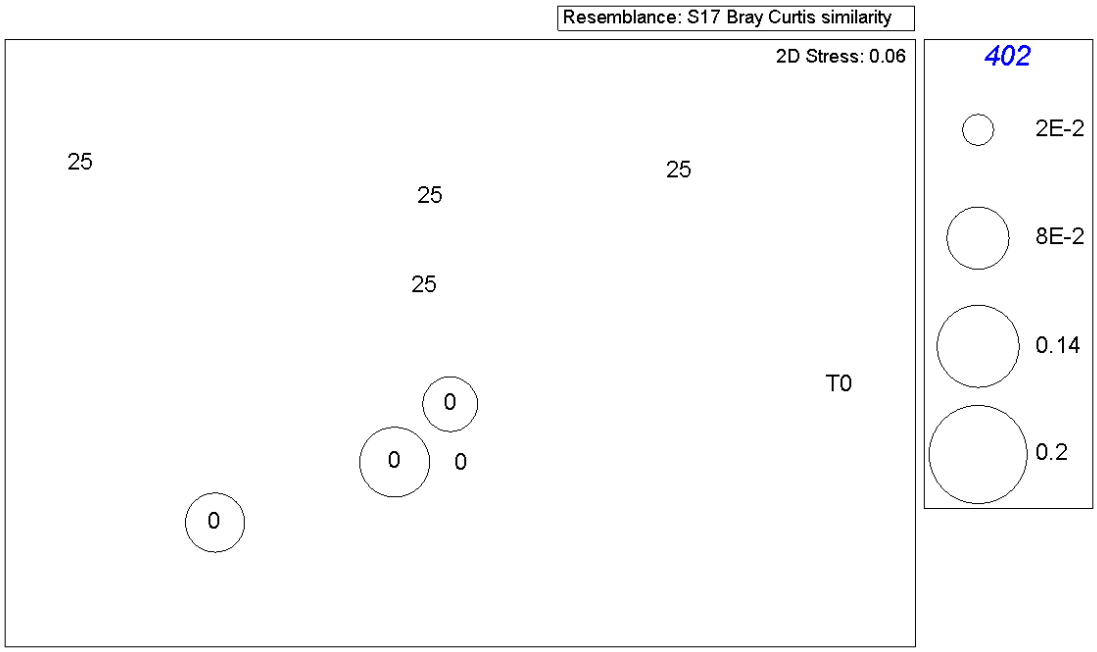


Figure 3.7 NMDS analysis of the total DNA-based fungal community structure produced using the primer pair EF4f and EF3r and grouped by a) azoxystrobin concentration and b) sampling time. Each data point represents the mean of 5 experimental replicates. In figure a) the time 0 point is included within the 0 mg kg^{-1} samples.

SIMPER analysis showed that over the course of the 4-month experiment period TRF 402 bp was responsible for 5% of the community variation between the 0 and 25 mg kg^{-1} treatments. Similar values were recorded for the TRFs at 400 and 75 bp with values of 5 and 3% being recorded, respectively.

Figure 3.8a illustrates that TRF 402 bp was prevalent in 3 of the 5 time points in the un-amended controls (2, 4, and 16 weeks), whereas it was not present at all in the amended treatment. TRF 75 bp (Figure 3.8b) was present at 3 time points in the un-amended control samples (0, 2, and 4 weeks), but was absent from the amended treatments.

a)



b)

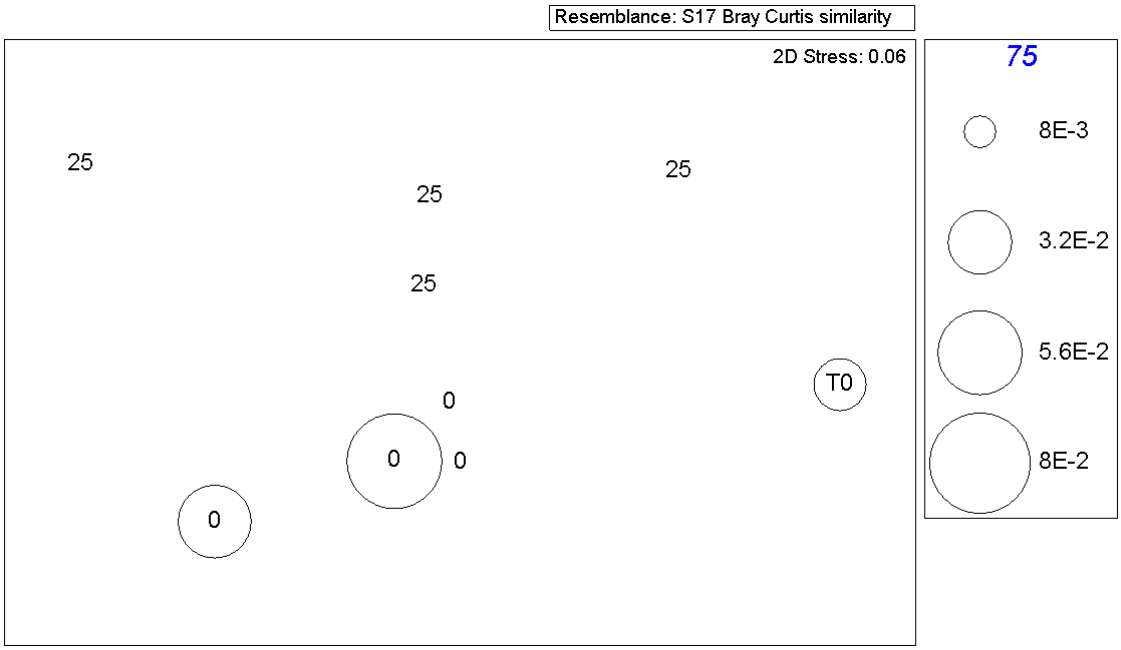


Figure 3.8. Bubble plot of TRF a) 402, and b) 75 bp abundance in EF4f/EF3r fungal samples over a 4-month experimental period. Numbers in the bubbles denote pesticide concentration (mg kg^{-1}). 0 and 25 denote the azoxystrobin treatments. The numbers in the key denote the relative abundance of the TRF. Each sample point represents the average of 5 experimental replicates. The Time 0 treatment is included in the 0 mg kg^{-1} treatments.

3.3.4.1.2 Nematodes

The number of nematode TRFs recorded after 1 month ranged from 19 to 7. There was a significant difference in the number of TRFs recorded in amended soil samples compared with un-amended controls, both in samples produced from extracted DNA (p = 0.015), and RNA (p = 0.018). There was a highly significant difference in the number of TRFs in samples produced using extracted DNA compared with the RNA samples (p = < 0.01). In the 0 mg kg^{-1} control samples, an average of 40% of the TRFs present in the DNA-derived were present in the RNA-derived samples. In the amended soils, RNA-derived samples contained an average of 42% of the number of TRFs present in the DNA-derived samples.

However, there was no significant impact of azoxystrobin application on active or overall nematode community diversity, as determined by LSD analysis (Table 3.1). There was no significant difference in nematode community structure in 0 and 25 mg kg^{-1} samples produced from extracted DNA (p = 0.211) after 1 month. However, there was a significant difference in structure when samples taken using extracted RNA were compared (p = < 0.01). There was also a significant difference in community structure between DNA and RNA-derived samples (p = < 0.01). These observations were backed up by NMDS analysis which showed no clear clustering of 0 and 25 mg kg^{-1} samples analysed using extracted DNA (Figure 3.9). Conversely, there was clear grouping observed in the RNA-derived samples. As with the fungal samples, there was a distinct grouping of DNA- and RNA-derived samples.

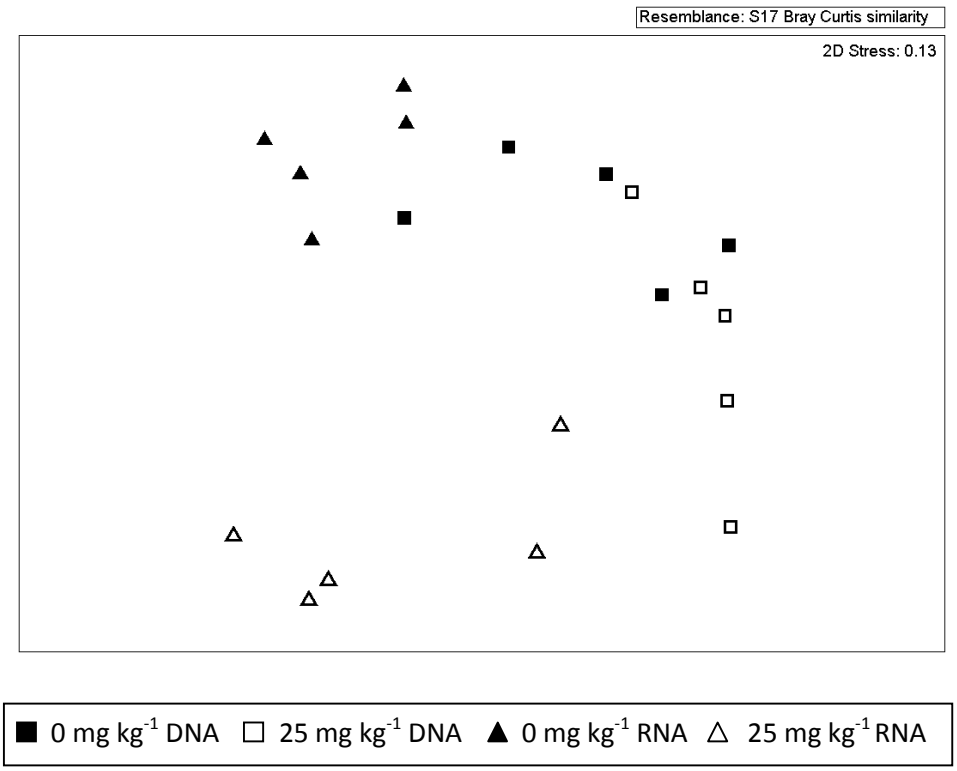
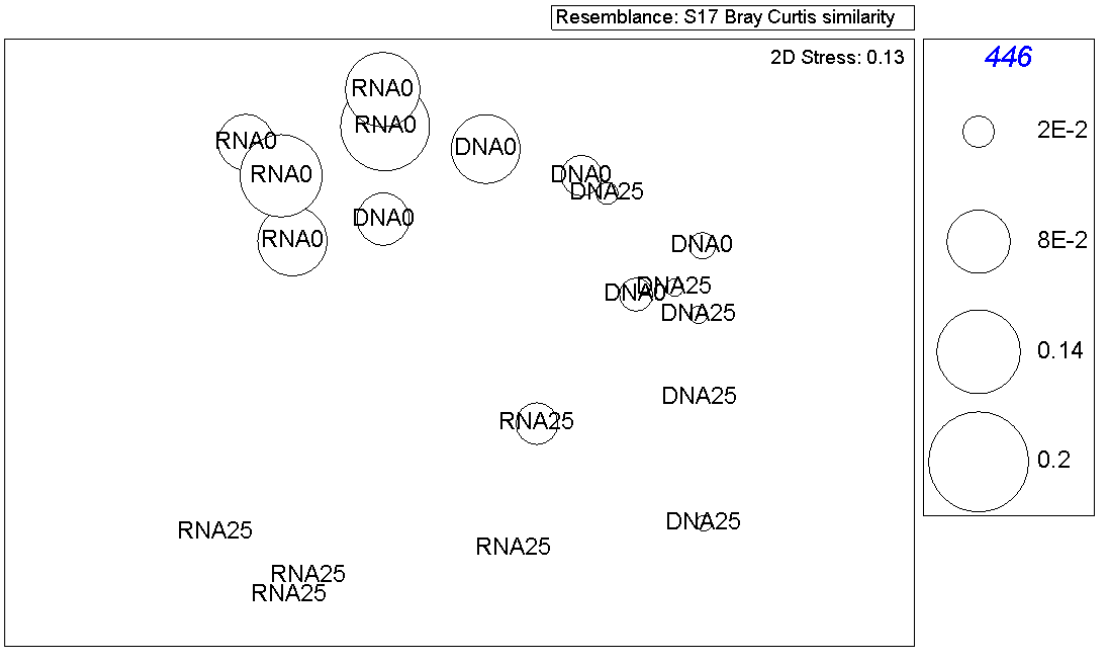


Figure 3.9. NMDS analysis of nematode community structures determined using extracted DNA and RNA 1-month post azoxystrobin application. Each data point represents an experimental replicate.

SIMPER analysis showed TRFs 446 and 423 bp were particularly sensitive to the application of azoxystrobin. TRF 446 bp (Figure 3.10a) was responsible for 14% of the total variation when the DNA- and RNA-derived results were compared as a whole. Further analysis showed that in the DNA-derived samples this TRF was responsible for 12% of the total variation. In the RNA-derived samples this value was 11%. Figure 3.10a illustrates that TRF 446 bp was far more prevalent in the un-amended treatments for both DNA- and RNA-derived samples.

TRF 423 bp (Figure 3.10b) accounted for 15% of the total variation when DNA- and RNA-derived treatments were compared. When the un-amended and amended treatments were compared this TRF also accounted for 18 and 19% of the variation in the DNA- and RNA-derived communities, respectively. This TRF was widely present in both the un-amended and amended treatments in the overall communities. However, it was more prevalent in the active community of the amended treatment.

a)



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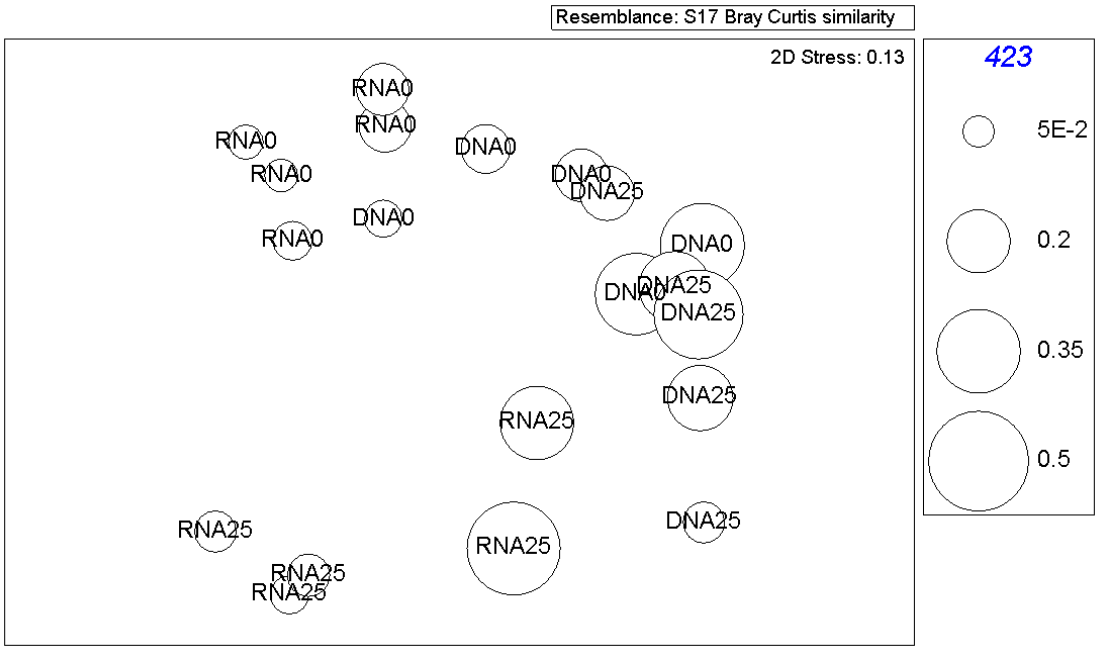


Figure 3.10. Bubble plots of TRF a) 446 bp b) 423 bp abundances in Nem18Sf/Nem18Sr nematode samples 1-month post application. Numbers in the bubbles denote pesticide concentration (mg kg^{-1}). The numbers in the key denote the relative abundance of the TRF. Each sample point represents the average of 5 experimental replicates.

During the 4-month time course experiment the numbers of DNA-based nematode TRFs recorded in this experiment ranged from 20 to 7. There was no significant impact of either azoxystrobin application (p = 0.225) or sampling time (p = 0.886) on the number of nematode TRFs as determined by 2-way ANOVA analysis.

However, 2-way ANOVA analysis of Shannon diversity data showed that azoxystrobin application had a significant impact on nematode diversity (p = 0.043). Sampling time, however, did not significantly impact upon diversity (p = 0.490). LSD analysis of this data showed that the significant differences in diversity occurred at the 4 and 8 week sampling points (Figure 3.11).

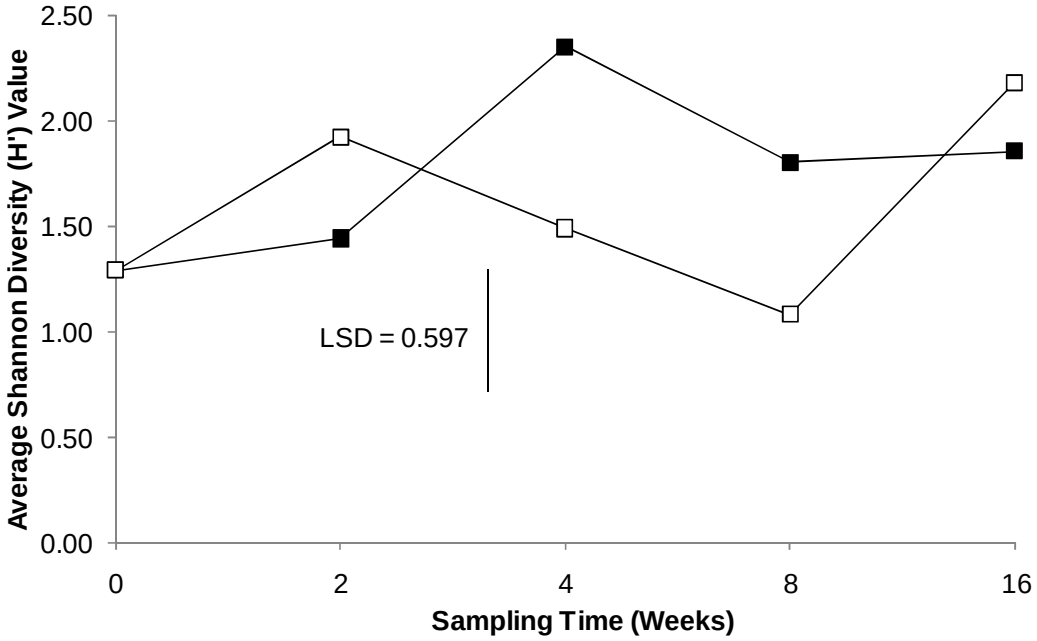


Figure 3.11. The average Shannon diversity (H') of total DNA-based nematode communities recorded using the Nem18Sf/Nem18Sr primer pair. ■ 0 mg kg^{-1} □ 25 mg kg^{-1}. Each data point represents the mean of 5 experimental replicates.

ANOSIM analysis showed that whilst azoxystrobin application had a significant impact on nematode community structure (p = 0.036), sampling time didn't (p = 0.813). This was supported by NMDS analysis which showed a grouping of points when comparing the impacts of azoxystrobin concentration on community structure (Figure 3.12a). Conversely, there was no discernable grouping of points in Figure 3.12b where community structures were compared based on sampling time.

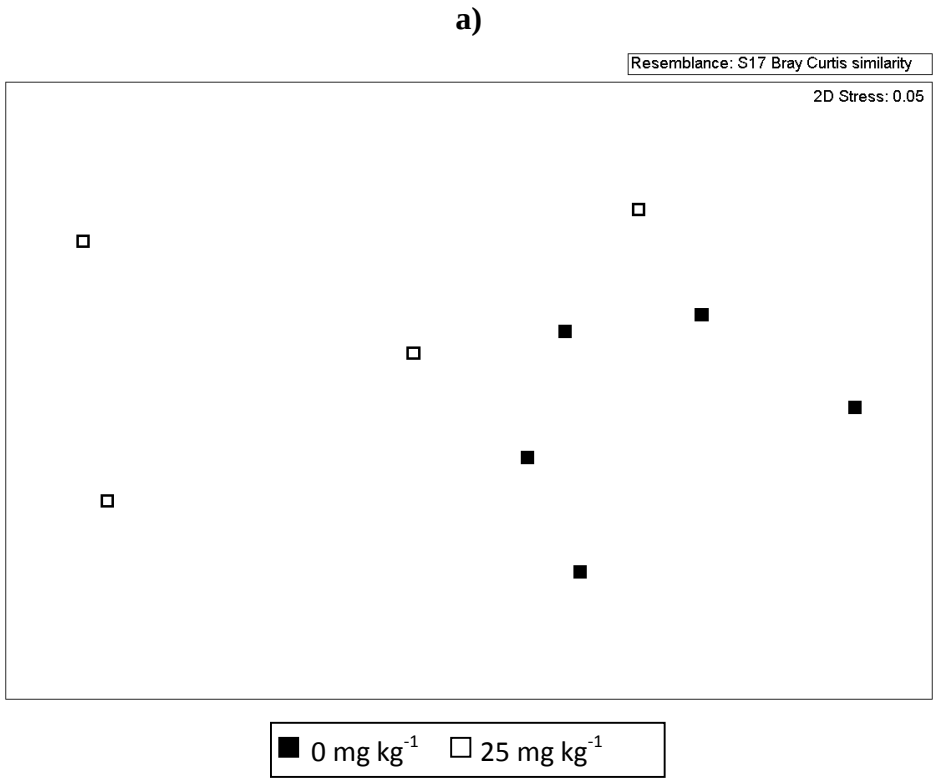
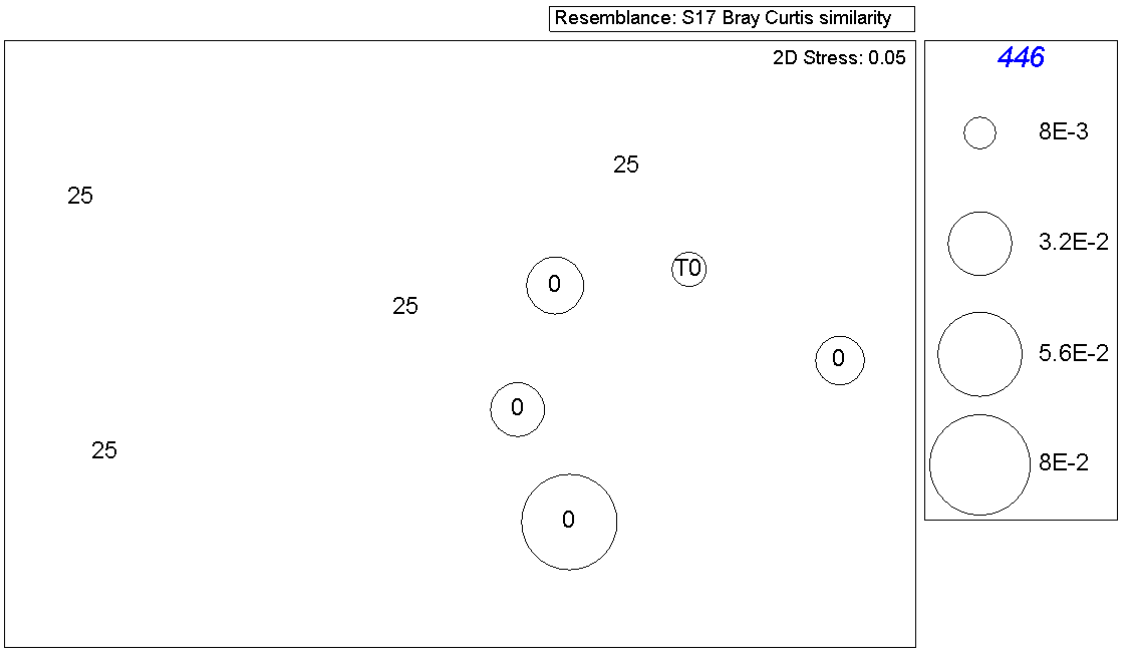


Figure 3.12. NMDS analysis of nematode community structure grouped by a) azoxystrobin concentration and b) sampling time. Each data point represents the mean of 5 experimental replicates. In figure a) the time 0 point is included within the 0 mg kg^{-1} samples.

Bubble plot analysis of individual TRFs showed that TRF 446 was highly impacted by azoxystrobin application (Figure 3.13a). Whilst this TRF was present to varying extents in each of the control samples, it was absent from all amended samples. TRF 423 (Figure 3.13b) was present in 4 of the 5 sampling points (Time 0, 4 weeks, 8 weeks, and 16 weeks) in the un-amended treatments. In contrast, it was only present at 2 sampling points (2 and 4 weeks).

a)



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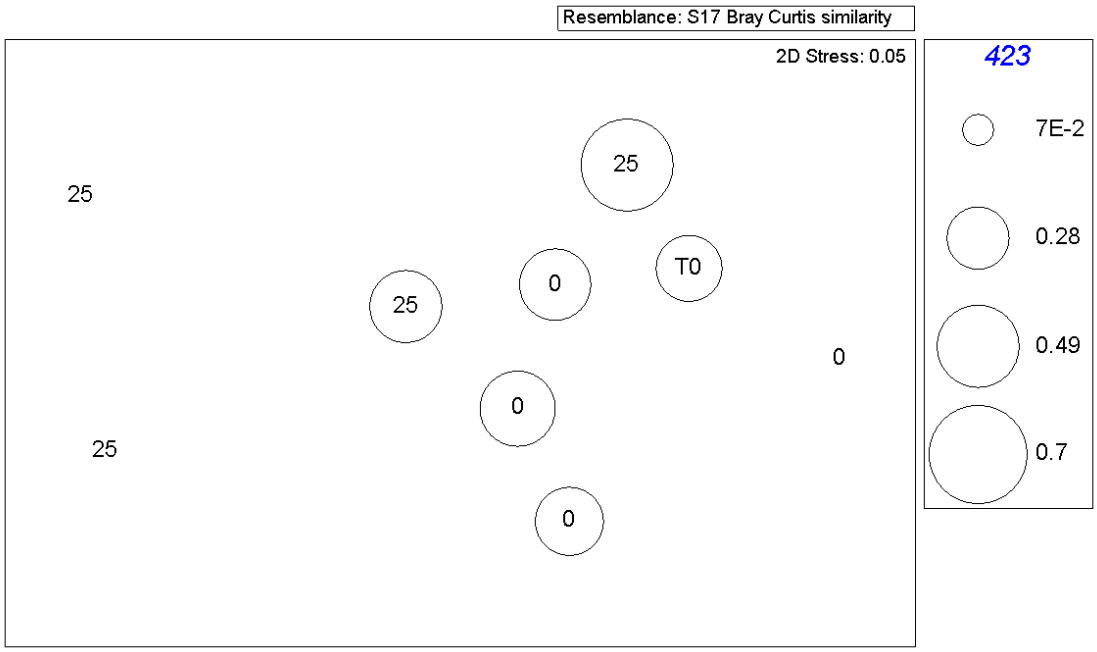


Figure 3.13. Bubble plots of TRF a) 446 bp b) 423 bp abundances in Nem18Sf/Nem18Sr nematode samples over a 4-month experimental period. Numbers in the bubbles denote pesticide concentration (mg kg^{-1}). The numbers in the key denote the relative abundance of the TRF. Each sample point represents the average of 5 experimental replicates. The time 0 point is included within the 0 mg kg^{-1} samples.

3.3.4.1.3 General Eukaryotes

The number of TRFs in the DNA-derived samples ranged from 37 to 14. In the RNA-derived samples there were between 17 and 12 TRFs. ANOVA analyses showed that there was no significant difference between the number of TRFs recorded in 0 and 25 mg kg^{-1} samples taken from extracted DNA (p = 0.491), but there was when samples taken using extracted RNA were analysed (p = < 0.01). On average, the 25 mg kg^{-1} RNA samples produced 81% of the TRF numbers produced by the un-amended treatment. As with the fungal and nematode samples, there was a significant difference in the number of TRFs observed in DNA-derived samples compared with RNA-derived ones.

There was no significant impact of azoxystrobin application on eukaryotic diversity in either the DNA or RNA-derived communities (Table 3.1, Page 143).

T-RFLP traces for the un-amended control samples showed that, on average, the traces derived from extracted RNA had 67% of the number of TRFs presented in the DNA-derived samples. For the amended soil samples, this value was 48%.

ANOSIM analysis of showed that amendment of soils with azoxystrobin did not significantly alter the eukaryote community structure, regardless of whether samples made using extracted DNA ($p = 0.968$) or RNA ($p = 0.300$) were analysed. As with the previous organism groups, however, the community structure in the DNA-derived samples was significantly different to that in the RNA-derived samples ($p = < 0.01$).

The results of the ANOSIM were backed up by NMDS analysis (Figure 3.14) which showed that there was no clear grouping of points based on whether the samples were amended or un-amended. The RNA-derived samples formed a distinct cluster compared with the samples made using extracted DNA.

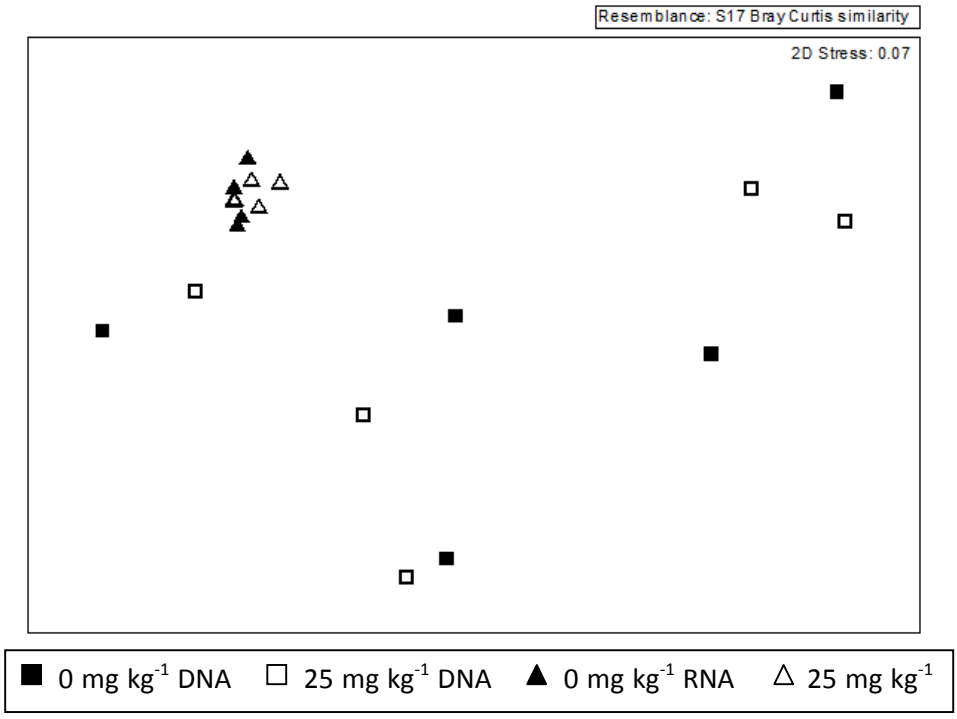


Figure 3.14. NMDS analysis of general eukaryote community structures determined using extracted DNA and RNA 1-month post azoxystrobin application. Each data point represents an experimental replicate.

Over the 4-month time course experiment the number of TRFs in the general eukaryote samples ranged from 38 to 12. Azoxystrobin application was found not to have a significant impact on general eukaryotic diversity as determined by Shannon diversity analysis (p = 0.752). Sampling time, however, did have a significant effect on diversity (p = 0.027). LSD analysis showed that this difference was caused by an increase in diversity in both sample sets 1-month post-application (Figure 3.15).

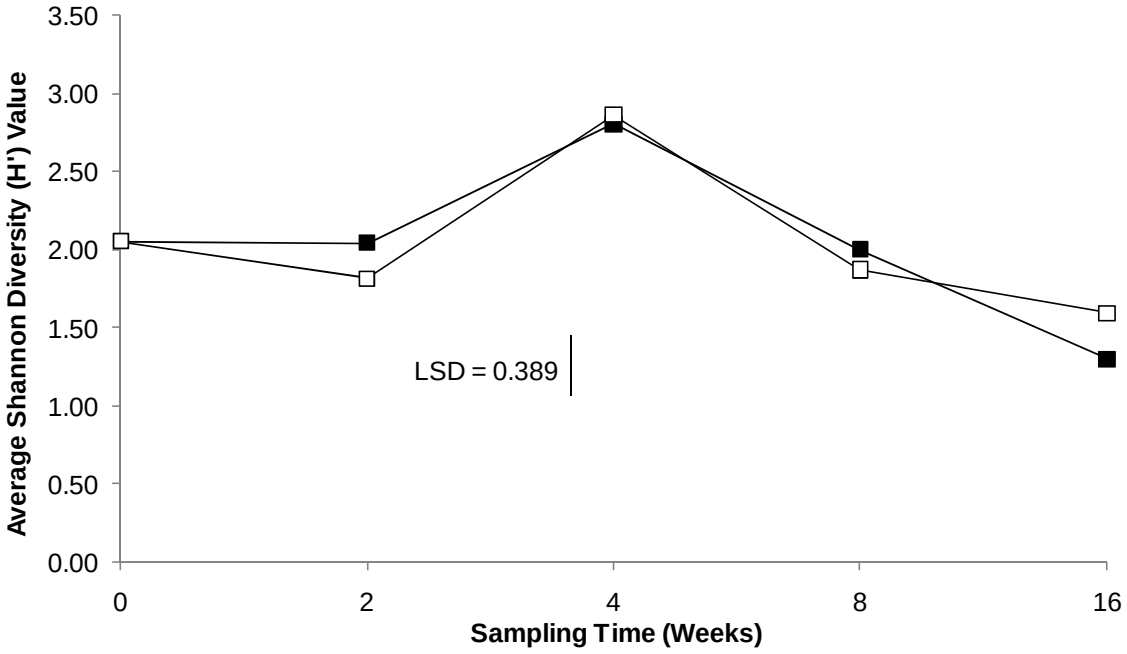
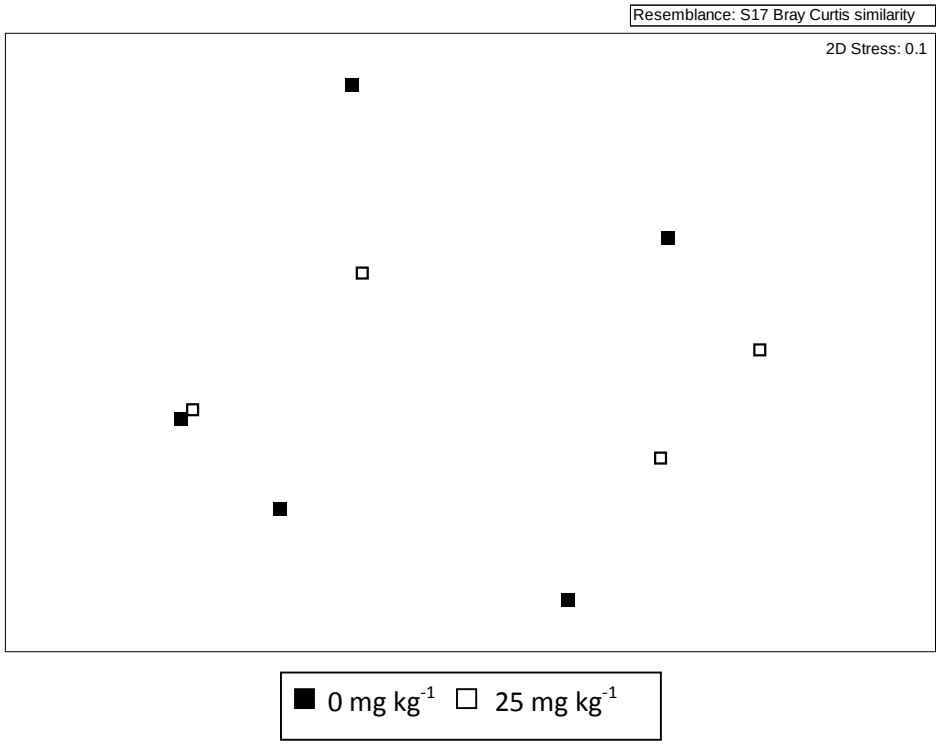


Figure 3.15. The average Shannon diversity (H') of general eukaryote communities recorded using the Euk20f/Euk516r primer pair. ■ 0 mg kg^{-1} □ 25 mg kg^{-1}. Each data point represents the mean of 5 experimental replicates.

ANOSIM analysis of the general eukaryote T-RFLP data showed that azoxystrobin application did not significantly impact community structure (p = 0.968). Conversely, sampling time did have a significant effect (p = <0.01). NMDS analysis supported these observations. There was no grouping of points when comparing amended and un-amended samples (Figure 3.16a), but there was when sampling times were compared (Figure 3.16b).

a)



b)

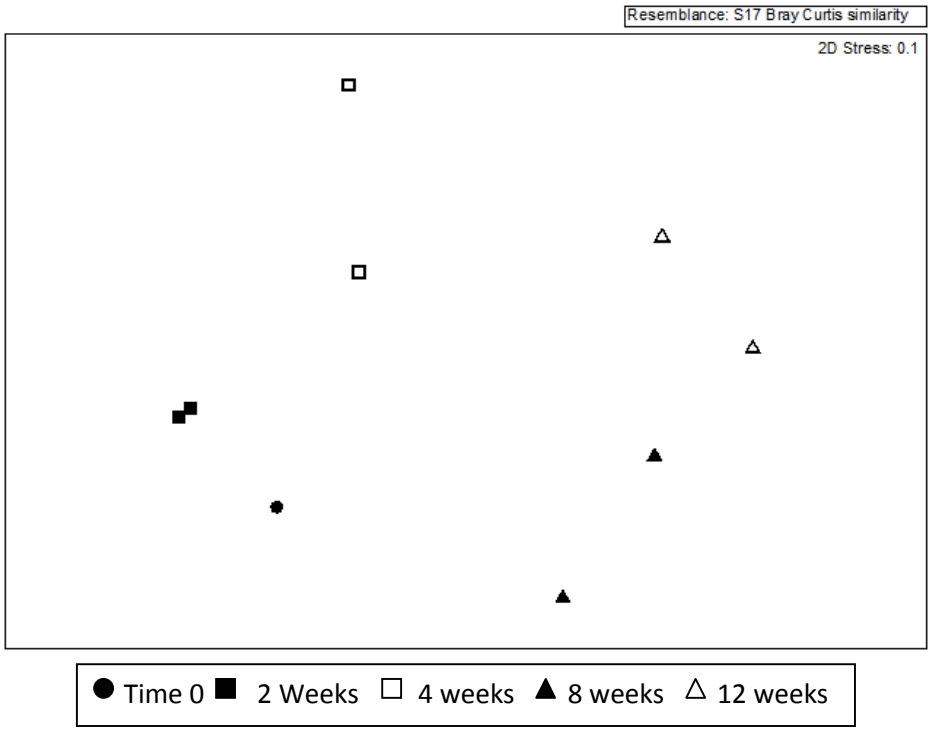


Figure 3.16. NMDS analysis of general eukaryote community structure grouped by a) azoxystrobin concentration and b) sampling time. Each data point represents the mean of 5 experimental replicates. In figure a) the time 0 point is included within the 0 mg kg^{-1} samples.

3.3.4.2 Prokaryotes

3.3.4.2.1 Bacteria

In the DNA-derived samples there were between 24 and 33 TRFs, and in the RNA-derived samples there were between 14 and 25. There was no significant impact of azoxystrobin application on the number of TRFs recorded, regardless of whether extracted DNA ($p = 0.199$) or RNA ($p = 0.099$) were analysed. However, there was a significant difference in TRF numbers between samples produced using extracted DNA, and those produced using RNA ($p = 0.038$).

Comparisons between DNA- and RNA-derived samples showed that in un-amended soils the total TRF numbers in the RNA-derived samples were 67% of those in samples produced using extracted DNA. In the amended samples this value was 78%.

Azoxystrobin application did not significantly impact the diversity of either the overall or active bacterial communities (Table 3.1).

There was no significant impact of azoxystrobin application on the structure of the bacterial community both when extracted DNA ($p = 0.341$) and RNA ($p = 0.651$) were used for analysis. The community structure was significantly different in DNA-derived samples compared with RNA-derived samples ($p = < 0.01$).

These observations were backed up by NMDS analysis (Figure 3.17). As with the general eukaryote samples no noticeable grouping was observed when amended and un-amended samples were compared. There was, however, clear grouping of samples when DNA- and RNA-derived samples were compared.

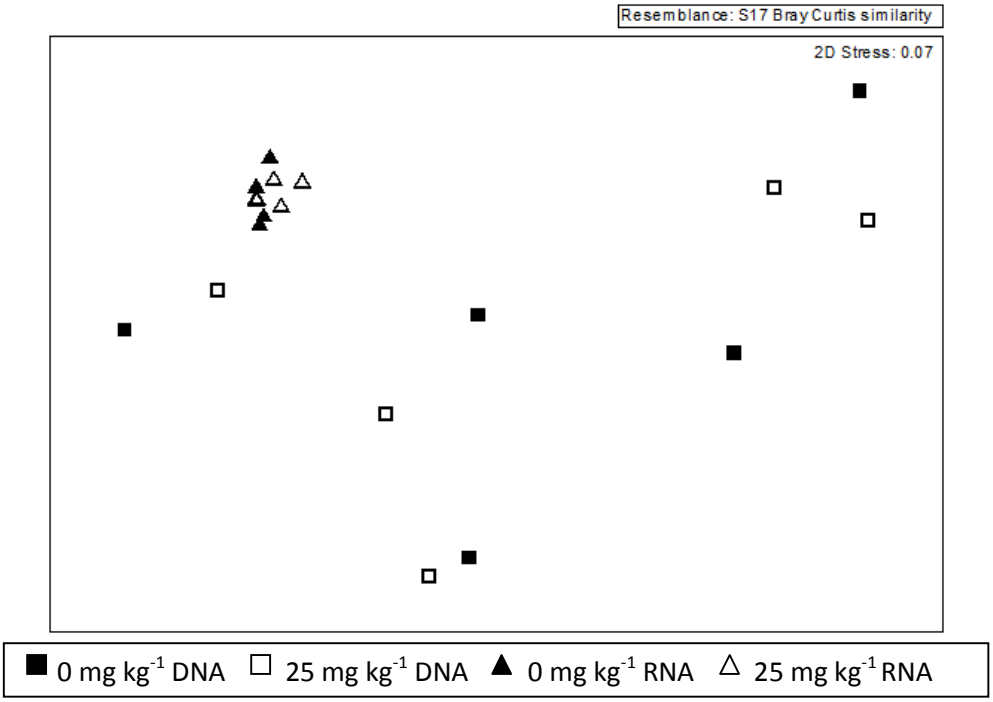


Figure 3.17. NMDS analysis of the bacterial community structures determined using extracted DNA and RNA 1-month post azoxystrobin application. Each data point represents an experimental replicate.

For the time-course experiment, the number of bacterial TRFs ranged from 55 to 23. 2-way ANOVA analyses demonstrated that neither azoxystrobin application (p = 0.949) or sampling time (p = 0.280) significantly affect TRF numbers.

Soil amendment with azoxystrobin did not significantly affect bacterial diversity as determined by Shannon diversity analysis (p = 0.705) (Figure 3.18). Additionally, there was no impact of sampling time on diversity (p = 0.308).

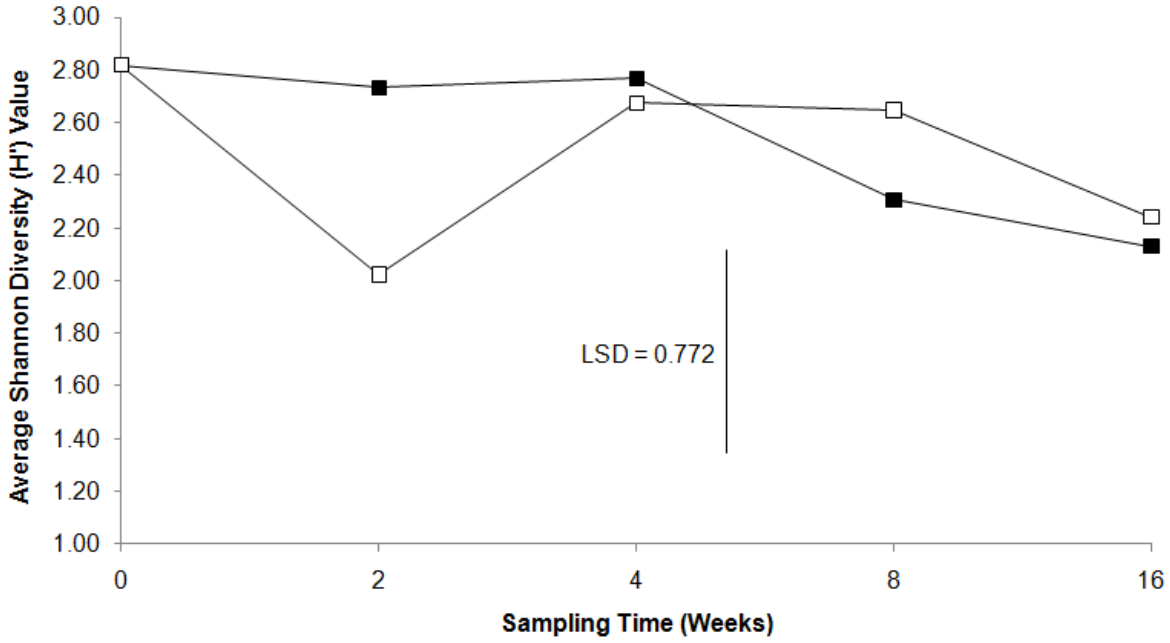
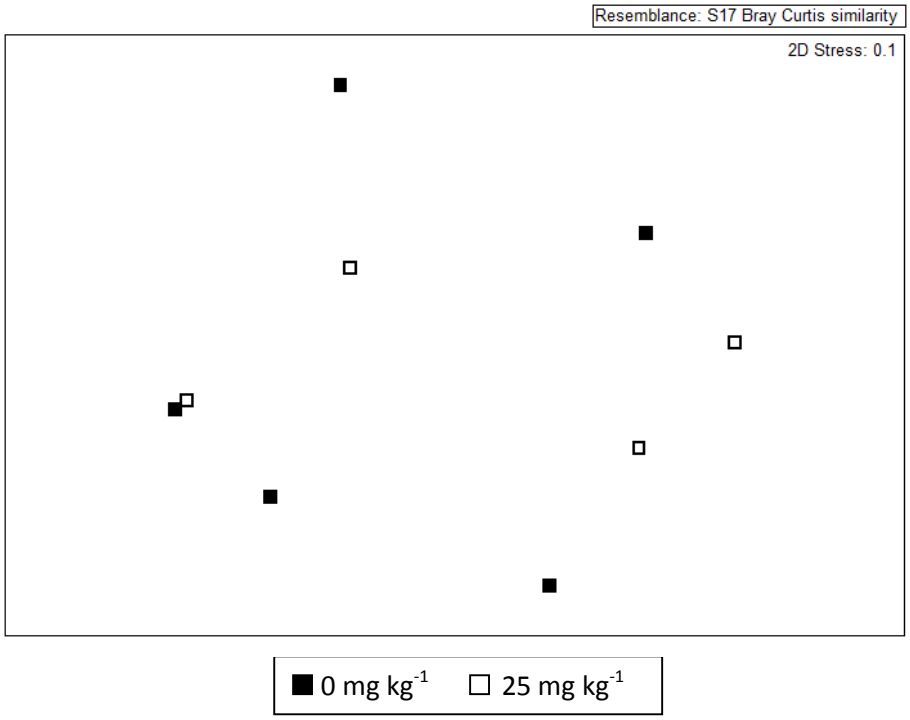


Figure 3.18. The average Shannon diversity (H') of bacterial communities recorded using the 63f/1087r primer pair comparing a) azoxystrobin concentration and b) sampling time. ■ 0 mg kg^{-1} □ 25 mg kg^{-1}. Each data point represents the average of 5 experimental replicates.

ANOSIM analysis showed that azoxystrobin application did not significantly affect the bacterial community structure (p = 0.532). There was a stronger influence of sampling time on community structure than pesticide amendment, but this was still not statistically significant (p = 0.060). This was supported by the NMDS analysis. There was no noticeable grouping of samples based on azoxystrobin concentration (Figure 3.19a). In contrast, there was some grouping of samples based on sampling time (Figure 3.19b). This was most notable in the 4 and 8 week samples. However, there was no grouping of the samples taken after 16 weeks.

a)



b)

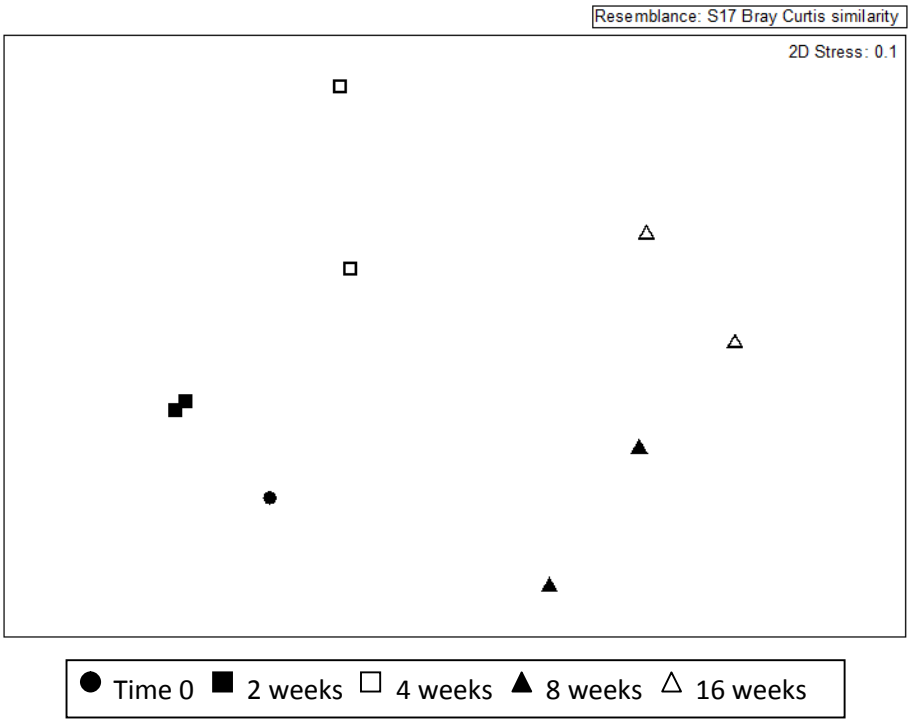


Figure 3.19. NMDS analysis of bacterial community structure grouped by a) azoxystrobin concentration and b) sampling time. Each data point represents the mean of 5 experimental replicates. In figure a) the time 0 point is included within the 0 mg kg^{-1} samples.

3.4 Discussion

In the previous chapter it was determined that azoxystrobin application (and the concentrations in which it was applied) had a significant impact on soil fungal and nematode communities as determined using T-RFLP analysis of DNA extracted from soil. The work carried out here aimed to determine the impacts of azoxystrobin amendment on active soil communities using T-RFLP analysis of samples derived from extracted soil RNA.

Soil dehydrogenase activity and microbial biomass, along with azoxystrobin degradation analysis by HPLC, were also carried out to determine the levels of reproducibility of the results obtained in Chapter 2.

3.4.1 HPLC, Biomass and Dehydrogenase analyses

The results of the assays in this chapter were comparable with those observed in Chapter 2. HPLC analysis showed that azoxystrobin degradation was highest within the first 2 weeks post-application with a total of 48% of the applied compound being degraded within the 4 month experimental period. This was lower than the 63% degradation recorded in Chapter 2 after 4 months. There was no significant impact of azoxystrobin application on soil microbial biomass, but dehydrogenase activity was significantly affected. The maximum inhibition was recorded after 8 weeks where the activity in the amended treatment was only 50% that of the control. One difference between the results was that in the experiment conducted in this chapter there was no recovery of dehydrogenase activity to the levels recorded in the un-amended treatment within 4 months. In contrast, the data in Chapter 2 showed a recovery to un-amended levels 3 months post-application in the 1 and 5 mg kg^{-1} (but not the 10 and 25 mg kg^{-1}) treatments. This could suggest that the microbial community in the soils used in Chapter 2 had a higher natural resilience to azoxystrobin application. Alternatively, this difference in community recovery could be attributed to differences in the amount of azoxystrobin degraded. In this scenario, the recovery in dehydrogenase activity could have been reduced compared to the Chapter 2

data because there was a higher percentage of the initial azoxystrobin remaining in the soil i.e. less of the applied stress had been removed by degradation.

3.4.2 T-RFLP Analysis of Overall and Active Community Impacts

3.4.2.1 Eukaryotes

3.4.2.1.1 Fungi

Shannon analysis also showed that azoxystrobin had a deleterious impact on fungal diversity in both DNA- and RNA-derived profiles. Overall diversity was lower in RNA-derived profiles than in DNA-derived ones. Indeed the 4 month time-course experiment showed that these impacts persisted over this period. On average, fungal diversity was 88% that of the control in this experiment as determined using extracted DNA. This is comparable with the 79% recorded for the same concentrations in Chapter 2.

The additional sampling point included in this experiment showed that this decrease occurred within the first 2 weeks post-application. After the first sampling point there was no further decrease in diversity relative to the control, although there was no recovery recorded either.

These results support those found in Chapter 2. This initial decrease in diversity with no recovery over the course of the 4-month time course experiment may be linked to the fact that not all of the applied azoxystrobin was degraded during the experimental period i.e. the stress had not been fully removed from the community. Indeed, for the experiment described in Chapter 2, 37% of the applied azoxystrobin remained after 4 months. This compares with 52% for the experiment described in this chapter.

However, the lack of any recorded recovery in diversity suggests that the fungal community had a low level of resilience to the compound. A similar result was obtained by Hoshino and Matsumoto (2007)

who analysed the impacts of the fumigant and nematocide chloropicrin (Pic) on soil bacterial diversity. Using Shannon (H') diversity analysis of DGGE profiles the authors observed that in treated plots there was a significant decrease in bacterial diversity 1 week post-application relative to the control. No evidence of a resilience response (i.e. a recovery of diversity) was observed over the 2-month experimental period (Hoshino and Matsumoto, 2007). Li *et al.* (2008) also observed that applications of the pesticide methamidophos had reduced total fungal diversity 1 week post application. However, in contrast to the results recorded in this chapter, an apparent resilience response was observed with diversity levels recovering to control levels within 8 weeks. The authors of these two papers did not monitor Pic degradation during the course of this work so possible links between diversity impacts and Pic presence/degradation could not be determined. A decrease in fungal diversity in response to azoxystrobin application was also observed in light and dark microcosms by Adetutu *et al.* (2008). As with the data presented in this chapter, Adetutu *et al.* (2008) observed no recovery in fungal diversity over the course of the experimental period (84 days). This could have been due to the fact that after 84 days 35 and 30% of the applied azoxystrobin still remained. This means that the fungal community would have still been under stress conditions even at the end of the experimental period.

The application of azoxystrobin also had a significant detrimental impact on the number of TRFs present in RNA-derived samples 1 month post-application. Additionally, comparisons between these samples and the equivalent DNA-derived samples showed that a lower percentage of TRFs present in DNA-derived samples (i.e. the overall community) were present in the RNA-derived samples (i.e. the active community) in the amended samples compared to the control.

NMDS and SIMPER analyses of fungal community structure 1-month post azoxystrobin application showed that differences between un-amended and amended treatments were more apparent in the RNA-derived samples, compared with DNA-derived ones.

Differences in DNA- and RNA-derived microbial community structures have been previously observed. For example, Moeseneder *et al.* (1999) observed that the RNA-derived marine bacterioplankton community structure was significantly different to the DNA-derived structure of the same samples. In this case the RNA-derived profiles showed a higher number of TRFs than the DNA-derived ones, which differed from the results presented in this chapter.

DNA- and RNA-derived community structure differences have also previously been observed when analysing the impacts of amendments. Mengoni *et al.* (2005) observed bacterial DNA- and RNA-derived community differences in soils subjected to amendment with chromate. The RNA-derived community structure was found to show more distinct changes as a result of chromate amendment. Research by Lillis *et al.* (2009) also showed that RNA-derived community profiles showed more pronounced changes compared with DNA-derived ones, this time when determining the impacts of 2,4-Dicholorophenol (2,4-DCP) on bacterial community structure. These studies correlate well with the azoxystrobin impacts on fungal community structure observed in this chapter.

In particular, 3 TRFs (402, 400, and 75 bp) were strongly impacted by azoxystrobin application. When the TRFs at 400 and 402 bp were compared with the clone libraries produced in Chapter 2 the nearest predicted TRF in the libraries was 397 bp (a predicted TRF which was only present in un-amended samples). BLAST searches showed that these sequences had 99% matches to the fungus *Penicillium sacculum* (*Eladia saccula*), an ascomycete capable of producing lignin-modifying enzymes (LME's) (Pangallo *et al.*, 2009). However, only one sequence was identified that would have produced a TRF close to 75 bp. This sequence was present in the azoxystrobin-amended library and had a 97% homology to a sequence identified as an "Uncultured Eimeriidae" in BLAST searches. This refers to a group of protozoa (Duszynski and Wilber, 1997), which suggests that there was some non-specific amplification of sequences by the EF4f/EF3r primer pair. This would differ from the results of Smit *et al.* (1999) who did not observe any non-fungal amplification using this primer pair. Another possibility could have been that this sequence had resulted from the production of a chimera

sequence. However, analysis using the Mothur programme (University of Michigan, USA) showed that this was not the case.

However, there were also significant community shifts that were observed in the fungal clone libraries from Chapter 2, but were not seen in either the DNA- or RNA-derived T-RFLP profiles. Most notably, sequences with high sequence homologies to the fungus Zygomycete sp. AM-2008a increased in prevalence from 9% of the library in the un-amended treatment to 41% of the library in the amended treatment.

These sequences would have produced TRFs of either 240 or 241 bp following digestion with *Hha*I. However, SIMPER analysis of the data obtained in this experiment showed that TRF 240 bp was only responsible for 1% of the variation between amended and un-amended treatments when the DNA-derived data was compared. When the RNA-derived data was compared TRF 240 bp was still only responsible for 2% of the total variation.

One possible reason for this could be that sequences that showed high homologies to a range of fungal species would have produced TRFs within the 240/241 bp. For example, sequences with high homologies to the plant pathogen *Fusarium oxysporum* (van Loon *et al.*, 1998) accounted for 6.5% of the sequences in the un-amended treatment library, but were completely absent from the azoxystrobin-amended library.

Furthermore, sequences showing high homologies to the nematopathogen *Pochonia suchlasporia* (Wu *et al.*, 2008) represented 4% of the total sequences in the un-amended treatment library and 0% in the amended treatment library. These sequences would have produced a TRF of 273 bp which, according to SIMPER analysis, was responsible for less than 1% of the community variation (in both DNA- and RNA-derived sample comparisons) in the experiment described in this chapter.

One possible reason for this could have been that the fungal clone libraries did not encompass the full community diversity within these samples. Indeed, Chao1 estimates of population carried out in Chapter 2 predicted that only 44 and 37% of the total community diversity had been accounted for within the 0 mg kg^{-1} and 25 mg kg^{-1} libraries, respectively. As a result, further research into this area would require much larger sequence libraries to be produced, probably using high-throughput techniques such as 454 sequencing.

3.4.2.1.2 Nematodes and Protozoa

Shannon diversity analysis showed that azoxystrobin application did not significantly impact nematode community diversity 1 month post-application, regardless of whether DNA- or RNA-derived communities were analysed. Additionally, community diversity was not significantly different in DNA-derived samples compared with RNA-derived samples across the different treatments.

However, the time-course experiment showed that azoxystrobin application did significantly affect nematode diversity over a 4-month period based on extracted DNA samples. Nematode diversity in the amended samples was, on average, 93% that of the un-amended soils. This correlated well with the results of the previous chapter where the diversity in the 25 mg kg^{-1} samples was 95% that of the controls. Ruess *et al.* (2001) showed that it was possible for fungicides to have a strong and direct deleterious effect on nematode community diversity. During their studies the authors showed that, on average, application of the fungicide benomyl (Methyl [1-[(butylamino)carbonyl]-1H-benzimidazol-2-yl] carbamate) reduced nematode diversity by 14% compared to the control (Ruess *et al.*, 2001).
More recently, Eisenhauer *et al.* (2010) observed that multiple applications of the pesticide fosthiazate significantly reduced nematode diversity in grassland sub-plots. There were also significantly fewer TRFs recorded in azoxystrobin-amended samples compared with the control, in samples derived from both extracted DNA and RNA. Active organisms accounted for a similar proportion of the total community in both un-amended and amended samples (40 and 42% respectively).

ANOSIM and NMDS analysis also showed that azoxystrobin application did not have a significant impact on the DNA-derived community structure but it did have one on the RNA-derived community. These results therefore are similar to those observed for fungi in this chapter. Additionally, these results match previous comparative analyses of amendment impacts on DNA- and RNA-derived bacterial community structures (Mengoni *et al.*, 2005; Lillis *et al.*, 2009).

Currently, there is a lack of published information on the use of molecular techniques to determine the impacts of pesticides or other amendments on nematode community structure. Indeed, to the authors' knowledge, this is the first time that DNA- and RNA-derived T-RFLP analyses have been used for this purpose. However, previous research using culture-dependent methods has indicated that amendments can significantly impact nematode communities.

Work by Ekelund (1999) and Ekelund *et al.* (2000) illustrated that bacterivorous nematodes were significantly impacted by the fungicides fenpropimorph and propiconazole even when the compounds were applied at the normal field rates. Ekelund (1999) showed that fenpropimorph inhibited the growth of bacterivorous nematodes by up to 67% compared to the un-amended control with no recovery observed within the experimental period. The effects of propiconazole were even more pronounced with no growth of bacterivorous nematodes observed at a high pesticide concentration (625 mg L^{-1}) and this effect persisted throughout the experiment (Ekelund *et al.*, 2000).

Additionally, Boucard *et al.* (2004) showed that 2 types of sheep dip (organophosphate and synthetic pyrethroid) had significant deleterious effects on soil protozoal survival (ciliates, flagellates, and amoebae) even when applied at low concentrations. Ramirez II *et al.* (2009) noted that mustard bio-fumigants which can be used to combat plant pathogenic nematodes also deleteriously affect potentially beneficial entomopathogenic nematodes. Of the 7 entomopathogenic species studied, only 1 species (*Steinernema feltiae*) was resistant to fumigation. More recently, Zhang *et al.* (2010) noted that the pesticides acetochlor and carbofuran had significant impacts on nematode communities over a

16-week experimental period. The highest impact was observed for plant-pathogenic nematodes 8 weeks after an acetochlor/carbofuran combination application. In this treatment nematode concentrations were only 47% that of the un-amended control. However, this appeared to be only a transient impact with a recovery being observed after 16 weeks (Zhang *et al.*, 2010).

The studies mentioned above indicate that soil nematode and protozoal communities can be significantly impacted by pesticide application. However, all of these studies used culture-dependent techniques to monitor these effects. This means that (as not all nematode species are culturable) the effects on the entire community could not be determined. Therefore, extensive further research is required using molecular techniques to determine the effects of pesticides on the structure and function of nematode communities. The relative lack of information about impacts on nematode communities is particularly surprising as nematodes form an integral part of soil food webs, and thus play essential roles in nutrient regulation and cycling (Mikola *et al.*, 2002).

The results in this chapter showed that the nematode TRFs at 446 and 423 bp were particularly strongly affected by azoxystrobin amendment.TRF 446 bp was far more prevalent in the un-amended treatment after 1-month in both the DNA- and RNA-derived treatments. Indeed, time-course experiment carried out in this chapter illustrated that this was also the case in the DNA-derived community throughout the 4 month experimental period. According to the SIMPER analysis, this TRF accounted for 12 and 11% of the variation in the DNA and RNA-derived communities, respectively. Unfortunately, there were no sequences within the nematode clone libraries produced in Chapter 2 that would have produced a predicted TRF of this size. As a result, no identification was able to be proposed.

In contrast, TRF 423 bp, which accounted for 18 and 19% of the total community variation in DNA- and RNA-derived samples respectively, was more prevalent in the amended RNA-derived community than in the un-amended one. There was little difference between the presence of TRF 423 bp between

amended and un-amended treatments in the DNA-derived samples. This may suggest that the organism or organisms represented by this TRF were ubiquitous within these soil communities, but were more active following azoxystrobin amendment. Analysis of the nematode clone libraries produced in Chapter 2 showed that the nearest predicted TRF to this was 418 bp for a sequence in the 25 mg kg^{-1} clone library that showed a 98% homology to the genus *Deontostoma* sp using the restriction enzyme *Aci*I. Interestingly, sequences that showed homologies to this genus were also found in the 0 mg kg^{-1} library but these would have produced a different TRF size using this restriction enzyme (482 bp).

Another possibility is that the organism or organisms represented by TRF 423 bp could have been related to the species *Plectus rhizophilus*. Sequences with close homologies to this species gave a predicted TRF of 417 bp with the restriction *Aci*I and increased in prevalence from 3.5% of the total sequences in the 0 mg kg^{-1} library to 12.5% in the 25 mg kg^{-1} library.

There were also some notable changes in the nematode clone libraries that were not apparent in either the DNA- or RNA-derived T-RFLP analyses. Firstly, sequences with a high homology to *Xiphinema rivesi* declined from 17% of the clone library total for the un-amended treatment, to 5% in the amended treatment library. Using *Aci*I these sequences would have produced a TRF of 102 bp. However, this TRF was not recorded at all during the T-RFLP analyses of either DNA- or RNA-derived communities.

Furthermore, sequences that showed high homologies to the genus *Achromadora* sp. represented 13.5% of the sequences obtained from the un-amended treatment. However, in the amendment treatment library there were no sequences that showed high homologies to this genus. Using the enzyme *Aci*I, these sequences would have produced TRFs of 556 bp. According to the SIMPER analysis carried out during this experiment, TRF 556 bp was responsible for less than 1% of the total community variation, regardless of whether DNA- or RNA-derived samples were analysed.

As with the fungal clone libraries, the nematode clone libraries indicated impacts of azoxystrobin that were not seen in the T-RFLP analyses (both DNA- and RNA-derived). Chao1 estimates of population size predicted that only 49 and 65% of the total nematode diversity was covered by the 0 mg kg^{-1} and 25 mg kg^{-1} clone libraries, respectively. These results further illustrate how the analysis of pesticide impacts on microbial communities across different trophic levels could benefit from the use of high throughput sequencing techniques.

3.4.2.1.3 General Eukaryotes

Azoxystrobin application did not significantly impact general eukaryote community structure or diversity, regardless of whether DNA- or RNA-derived profiles were analysed. There was no significant difference in eukaryotic diversity when DNA- and RNA-derived communities were compared for the un-amended control. For the amended treatment, the diversity recorded in the RNA-derived community was significantly lower than the equivalent DNA-derived profile.

The time course experiment showed that there was also a significant impact of azoxystrobin application on general eukaryote community structure and diversity across a 4 month experimental period. Indeed, the only significant impacts observed were attributable to sampling time. These results support those observed in the previous chapter with the exception that there was also no significant impact of sampling time in the Chapter 2 data.

This supported previous work in this area. Bending *et al.* (2007) analysed soils that had had one of three fungicides (azoxystrobin, chlorothalonil and tebuconazole) applied 3 months previously at the recommended maximum rates. Using DGGE analysis, the authors found that none of the pesticides had a significant impact on the soil eukaryote community structure in two soils with contrasting previous management histories (Bending *et al.*, 2007).

It is possible that there was sufficient functional redundancy and/or natural resistance within the eukaryotic community to enable its structure and diversity to remain unaltered following azoxystrobin application. Another possibility is that the community may not have been completely resistant to the pesticide, but it may however have been highly resilient. In this scenario the community would have recovered from any possibly impacts on its structure and/or diversity before the first samples were taken two weeks post-application.

3.4.2.2 Prokaryotes

3.4.2.2.1 Bacteria

In the bacterial T-RFLP samples there was no significant difference between the un-amended and amended samples. However, RNA analysis showed that a higher proportion of the TRFs in the amended samples were representative of active organisms (78%) compared with the control (67%).

Shannon diversity analysis also showed that there was no significant difference between un-amended and amended treatments, regardless of whether DNA- or RNA-derived treatments were compared. Additionally, there was no significant difference in community diversity between the DNA- and RNA- derived profiles of each treatment. The time course experiment also showed that there were no significant differences in diversity over a 4 month period which supported the findings of Chapter 2.

These results support the work of Nogales *et al.* (2001) who recorded no significant differences in DNA- and RNA-derived bacterial community structure in response to polychlorinated biphenyl (PCB) contamination. Further research has also illustrated the resistance and resilience of soil bacterial communities to perturbation with a range of compounds. Rodríguez and Toranzos (2003) analysed the impacts of a 100 mg kg^{-1} dose of the pesticide lindane on bacterial communities. The authors concluded that the bacterial communities exhibited high levels of both resistance and resilience (Rodríguez and Toranzos, 2003). Other studies have also shown that soil bacterial communities also

have high levels of resistance and resilience to mercury contamination (Müller *et al.*, 2001, Rasmussen and Sørensen, 2001).

3.4.3 Conclusions and Possible Future Advances

The results of the T-RFLP analyses presented here showed a significant impact of azoxystrobin application on the structure of RNA-derived fungal and nematode communities. These therefore backed up the findings of Chapter 2 that showed deleterious effects of azoxystrobin on fungal and nematode diversity, fungal 18S rRNA gene copy number, and soil microbial dehydrogenase activity.

Whilst these results provided a good overview of the impacts of azoxystrobin on microbial communities across different trophic levels, the techniques used do have limitations and drawbacks. Additionally, further work would be required to ascertain the finer-scale impacts of the compound on the microbial groups analysed.

Firstly, although the use of extracted rRNA for use in T-RFLP analyses instead of DNA showed greater impacts of azoxystrobin on microbial community structures (possibly because of a reduction in the genetic material extracted from dormant or dead organisms), rRNA can still potentially persist in the environment for months after the death of an organism (Hirsch *et al.*, 2010). Therefore, it is possible that some impacts of azoxystrobin application were still being masked.

Additionally, rRNA analysis could also be prone to biases resulting from the fact that the number of ribosomes (and also the rRNA gene copy number) differs between organism groups (Janssen, 2006). These differences can be quite marked. For example, in bacteria typical rRNA gene copy numbers can range from 10 copies per cell in bacilli to only 1 in pseudomonads (Lee *et al.*, 2009b). However, currently it is not known whether this difference is as apparent in, for example, fungi (Hirsch *et al.*, 2010). As a result, the potential exists that rRNA analyses could be inherently skewed towards organisms with higher numbers of ribosomes.

One possible solution to this would be to use T-RFLP analysis using cDNA derived from soil community messenger RNA (mRNA). Although mRNA is far less abundant within microbial cells (1-5% of the total RNA in cells (McGrath *et al.*, 2008)), it is also usually far more short-lived in the environment and therefore would provide a more accurate assessment of the active organisms within a community at the time of the analysis. Furthermore, it would also eliminate the ribosome biases associated with the use of rRNA.

Additionally, mRNA analysis can be used to study the activities of specific functional genes within a community. For example, Bürgmann *et al.* (2003) used mRNA extraction coupled with reverse transcriptase PCR (RT-PCR) to monitor changes in the expression of the nitrogen fixation marker gene *nifH* in *Azotobacter vinelandii* under different nitrogen abundance conditions in soil and liquid cultures.

Therefore, mRNA could be used to potentially pinpoint specific functional groups of organisms that are susceptible to azoxystrobin application. However, this approach in itself would also have its' limitations. Whilst it would enable impacts on a specific functional group to be analysed, microbial communities do not exist in isolation, they involve a multitude of inter-linked functional pathways across several trophic levels. Furthermore, mRNA analysis would not give the depth of phylogenetic information that could be obtained using rRNA.

An ideal technique for studying the impacts of azoxystrobin on microbial communities would therefore have the capacity to produce high volumes of sequence data and allow the activities of a plethora of functional genes to be analysed from the same sample set. Recent advances in deep sequencing techniques mean that this may soon be possible. Gifford *et al.* (2011) utilised total mRNA extraction and direct reverse transcription with random hexamers to analyse the bacterioplankton communities of coastal waters in the USA. The authors were able to produce a library of approximately 500,000 possible protein-coding sequences, and analyse the expression of 82 genes

involved in marine phosphorus, sulphur, and nitrogen cycles. Of these, 46 genes were found to be expressed at levels above the minimum threshold.

Techniques such as the ones employed by Gifford *et al.* (2011) could also potentially solve the problems encountered when comparing T-RFLP traces with clone library data. Most notably, in the case of both fungi and nematodes not all of the impacts seen in the T-RFLP data corresponded to those seen in the respective clone libraries and vice versa. Deep sequencing and metatranscriptomic analyses, however, would not only provide a far wider coverage of the microbial diversity within a community, but impact analyses based on functional gene expression would eliminate the need to compare 2 completely separate data sets.

Additionally, the lack of any PCR stages in these methods also serves to eliminate possible amplification biases associated with the use of gene specific primers, which have been contentious issues for many years. An example of this is the debate over possible biases of fungal primer pairs such as ITS1F/ITS4r and EF4f/EF3r (Smit *et al.*, 1999, Anderson *et al.*, 2003b).

Chapter 4

The Isolation and Identification of Azoxystrobin-Degrading Organisms

4.1 Introduction

4.1.1 The biodegradation kinetics of xenobiotic compounds

Soil microorganisms play a vital role in the degradation of and ultimate fate of xenobiotic compounds and their associated metabolites. They utilise the compounds as sources of essential elements (carbon, nitrogen, etc.) which can result in complete degradation to CO_2 and water. This process can potentially be carried out completely by a single strain of a species, or as the result of step-wise degradation processes involving a range of microbial consortia. Due to the importance of these degradation processes, their kinetics and the processes that affect them, have been widely studied for a range of compounds and environments.

Biodegradation can take place via two distinct processes. Growth-linked degradation kinetics have been widely observed for a range of compounds. These kinetics are characterised by an initial lag, followed by a rapid degradation phase which corresponds with a rapid increase in the biomass of the degrader organisms. Non growth linked (co-metabolic) degradation involves the degradation or partial degradation of a compound where the energy released is not directly used by the organism(s) for growth (Horvath, 1972). Co-metabolic degradation can involve complex pathways (Netzer *et al.*, 2004) and is generally slow because the biomass of the degrader organisms does not increase over time, meaning that the adaptation responses associated with growth-linked metabolism do not occur (Janke and Fritsche, 1985). Both growth-linked, and co-metabolic kinetics have previously been observed during biodegradation research using a range of chemical compounds and microbial species. Reardon *et al.* (2000) studied the biodegradation kinetics of benzene, toluene and phenol (amended both as single treatments and in combination) by monocultures of *Pseudomonas putida* F1. The

results showed growth-linked degradation for each of the compounds when applied as single compounds. For toluene and benzene, the complete degradation of the compounds correlated very strongly with a cessation in microbial growth. For phenol the kinetics were slightly different. The biomass continued to increase even after all of the parent compound had been removed. This was found to result from the continued degradation of the phenol metabolites. Degradation of the compounds by *Pseudomonas putida* F1 when applied in combination also showed growth linked kinetics, with toluene being preferentially degraded (Reardon *et al.*, 2000).

Similar kinetics were also observed by Carvalho *et al.* (2005) who monitored the degradation of fluorobenzene by the α-2 Proteobacteria strain F11. Following a short lag period (approximately 5 hours), there was a phase of rapid degradation, with complete degradation of the compound recorded after approximately 36 hours. A rapid increase in microbial biomass correlated well with the exponential degradation phase. There was no increase in biomass once the fluorobenzene had been degraded.

Greer and Shelton (1992) analysed the degradation of 2,4-Dicholorophenoxyacetic acid (2,4-D) by two bacterial strains (MI and 155). In order to determine whether the degradation of 2,4-D was growth linked the authors studied the changes in the numbers of colony forming units (cfu) per gram of soil over time. The results using strain MI showed that degradation was strongly growth-linked. After a lag phase of 18 hours there was a rapid increase in the numbers of cfus. This increase correlated well with the start of the exponential phase of 2,4-D degradation. Additionally, the increase in cfu numbers ceased once the 2,4-D had been fully degraded. In contrast, degradation by strain 155 did not appear to be growth-linked. There was a minimal lag phase in bacterial growth, and this growth was still continuing after 48 hours, even though the 2,4-D had been fully degraded after 24 hours. This indicates that individual chemical compounds can be subjected to both growth-linked and co-metabolic degradation in nature, with the strain MI utilising 2,4-D as a nutrient source, whilst strain 155 may be carrying out degradation using non-specific enzyme pathways. Furthermore, Cheah

et al. (1998) showed that the biodegradation kinetics of 2,4-D can also be affected by the soil structure and oxygen content. The authors studied the degradation in aerobic sand, an aerobic sediment, and an anaerobic muck soil. 2,4-D degradation showed kinetics in the aerobic sand that were more indicative of growth linked degradation, with a lag period followed by an exponential degradation phase. In contrast, the characteristic initial lag phase was not observed in the aerobic sediment and anaerobic muck soils.

Non-growth linked biodegradation of xenobiotic compounds has also been observed in previous research. Bending *et al.* (2006) studied the degradation of azoxystrobin (a strobilurin fungicide), isoproturon (a phenylurea herbicide), and diflufenican (an anilide herbicide) in two different agricultural soils. The degradation kinetics of these compounds showed a steady degradation over time with little or no initial lag phase. The authors concluded that this was indicative of co-metabolism of the compounds with no additional proliferation of the degraders (Bending *et al.*, 2006).

4.1.2 Factors Influencing the Degradation of Xenobiotic Compounds

There are many different factors that can affect the degradation of xenobiotic compounds in natural environments such as soil organic matter (SOM) content, pH, temperature, water content. SOM plays an essential role in the binding of pesticides and other chemicals to the soil matrix, and therefore in the bioavailability of compounds in the environment. The extent to which chemical compounds are bound to soils depends on their chemical structure. Compounds that become ionically bound to the SOM can become un-bound (and therefore bioavailable) as a result of pH fluctuations. The binding of chemicals to soils by adsorption results in a compound being initially bioavailable, with this availability decreasing over time as the compound becomes more strongly bound (Bollag *et al.*, 1992). Soil pH can also have a major influence on pesticide degradation. Singh *et al.* (2003) noted that increases in soil pH increased degradation of the insecticide chlorpyrifos over a 90-day experimental period. At pH 4.7 over 70% of the applied compound remained after 90 days, compared with approximately 2% at pH 8.4. This difference may have resulted because the higher pH had selected

for the proliferation of organisms capable of degrading the compound. Bending *et al.* (2003) also noted that isoproturon-degrading bacteria from the genus *Sphingomonas* sp. appeared to be more prevalent in soil samples with higher degradation rates. Furthermore, the abundance of the *Sphingomonas* sp. appeared to be dependent on soil pH. The results observed by Bending *et al.* (2003) may be an indicator of enhanced biodegradation in the soils with the larger *Sphingomonas* sp. populations. Indeed, Aislabie and Lloyd-Jones (1995) noted that the lag period between the application of a pesticide, and its degradation could represent the adaptation phase of soil community where organisms capable of degrading a compound are proliferating up to the levels required for degradation to proceed. This has lead to the development of the concept of enhanced biodegradation. This refers to the adaptation of microbial communities to repeated applications of a compound, which result in increased degradation rates over each successive application. Sanyal and Kulshrestha (1999) recorded non growth-linked kinetics when monitoring pesticide degradation (in this case the herbicide metolachlor) in soil systems. The authors also observed that repeated applications of the compound resulted to enhanced degradation, which is considered indicative of microbial community adaptation. The compound was applied in four treatments with DT_{50} values of 18, 6, 3.8, and 2.5 days being recorded for the first, second, third, and fourth enrichments, respectively. The capacity of microbial communities to adapt to, and increase their degradation of xenobiotic compounds has been a point of interest for many years. Abdelhafid *et al.* (2000) observed higher atrazine mineralisation rates in soils with a previous history of application, compared with non-adapted soils. In the control soil, approximately 90% of the applied atrazine was degraded in adapted soils after 50 days, whereas in the un-adapted soil less than 50% was degraded. Vischetti *et al.* (2008) observed that in biobed mixtures (40% urban garden compost, 40% vine branch, 20% soil v/v) the DT_{50} values for the degradation of the fungicide metalaxyl decreased significantly with repeated applications. The values decreased from 37 days after the first application, to 14 and then 4 days for the second and third amendments, respectively (Vischetti *et al.*, 2008). A similar pattern was observed by Fang *et al.* (2008) who observed that the DT_{50} value of the organophosphate insecticide chloropyrifos in an agricultural soil significantly decreased over the course of three 42-day enrichments. These analyses serve to illustrate

how, over time, the repeated application of pesticides can select for those organisms resistant to (and potentially involved in the degradation of) these compounds, which in turn would affect the structure (and possibly the diversity) of a community.

Biodegradation of the nematicide ethoprophos has been found to be enhanced following repeated applications. Karpouzas and Walker (2000) observed that the biodegradation of ethoprophos and isazofos was enhanced in soils previously treated with ethoprophos. For both compounds, some of the parent compound still remained in the previously un-treated samples after 50 days, whereas in the previously-treated soils the compounds has been fully degraded after 20 and 38 days for ethoprophos and isazofos, respectively. There was no enhanced degradation observed for cadusafos, fonofos, or oxamyl (Karpouzas and Walker, 2000). The enhanced degradation of isazofos recorded by Karpouzas and Walker (2000) may be indicative of cross-enhancement of degradation. This refers to the enhanced degradation of a compound, or compounds in systems that have previously been exposed to similar (but not the same) compound(s). Singh *et al.* (2005) also noted cross-enhanced degradation of diazinon, parathion, coumaphos, and isazofos in soils previously treated with chlopyrifos, but not in those previously treated with fenamiphos. The authors concluded that this was due to the higher level of structural similarity between chlorpyrifos and the pesticides tested which would allow for the same hydrolytic biodegradation processes to occur. Warton *et al.* (2003) showed that the pesticide fumigants 2-propenyl isothiocyanate (PrITC), benzyl isothiocyanate (BeITC) and 2-phenylethyl isothiocyanate (2-PeITC) degraded quicker in soils that had previously been treated with another fumigant, methyl isothiocyanate (MITC). This indicates that cross-enhancement of pesticide degradation could potentially occur for a wide variety of different compounds.

Although enhanced biodegradation has been observed for a range of pesticides, it doesn't appear to apply to all compounds. For example, repeated applications with fenamiphos have been found to increase the compound DT_{50} from 8.4 to 24 days over the course of three enrichments. Additionally,

three enrichment series of chlorothalonil have resulted in increases in DT_{50} from 12 to 21 days (Singh *et al.*, 2001).

However, when the required microbial species are present, factors such as compound chemical structure and nutrient availability can affect the extent of biodegradation. Many pesticides can be used as sources of carbon and/or nitrogen (and sometimes phosphorus) by microorganisms. During growth-linked metabolism, the presence of alternative sources of, for example, carbon may decrease the rate at which a pesticide is degraded. In contrast, during co-metabolic degradation the presence of an alternative carbon source may enhance degradation by providing a nutrient source for the degrader organisms (Aislabie and Lloyd-Jones, 1995). Specific chemical structures can also be important in determining the degradation of compounds in nature. The presence of, for example, side chains or chemical bonds that are rarely or never found in nature can affect the rate at which a compound is degraded as the indigenous organisms may not be adapted to degrade such compounds. Key *et al.* (1997) noted that trifluoromethyl bonds are very strong, and this allied to the fact that trifluoromethyl-containing compounds are rarely found in nature could lead to them having a higher persistence in the environment. Similar observations have also been made for compounds containing chlorine-based constituents. The herbicide 2,4,5-T has been found to be more persistent in the environment than a similar compound 2,4-D. This is thought to be due to 2,4,5-T having three chlorine molecules in its structure compared with two for 2,4-D (Atlas, 1988).

4.1.3 The use of culture-dependent and –independent methods to monitor the degradation of xenobiotic compounds

Many of the current research techniques used to determine the degradation of xenobiotic compounds by microbial communities are based around culture-dependent analyses of specific microbial groups or strains. However, only a small percentage of the total bacterial and fungal species within an ecosystem are currently culturable (Jansson *et al.*, 2002). As a result, recent research into

biodegradation has increasingly complemented culture-dependent methods with molecular culture-independent ones.

Lozada *et al.* (2004) studied bacterial community shifts in bacterial community diversity following the application of nonylphenol ethoxylate (NP*n*EO) surfactants to lab-scale activated sludge reactors. Culture-independent analyses were carried out in the form of fluorescent *in situ* hybridisation (FISH) and dot-blot hybridisation. The results showed that the amended treatments were dominated by members of the β-proteobacteria and the authors surmised that these may be important in the degradation of the surfactants. In contrast, culture-dependent methods identified mostly γ-proteobacteria from genera such as *Acinetobacter*, *Aeromonas*, *Shewanella*, and *Proteus*. Culture independent methods showed that γ-proteobacteria only represented a small proportion of the whole communities in the amended treatments (0.4-6.8%). This illustrates just how important culture independent methods are when monitoring whole microbial communities.

Travis *et al.* (2008) used DGGE to monitor overall bacterial community structure changes resulting from elevated TNT levels in soils. Culture-dependent methods showed a marked increase in the presence of bacteria from the genus *Pseudomonas*. DGGE analysis backed up this observation but was also able to compliment it by illustrating that non-culturable organisms from the genera such as *Burkholderia* and *Sphingomonas* also increased following TNT amendment. This is particularly notable as these genera have previously been associated with the degradation of xenobiotic compounds (Kanaly and Harayama, 2000). More recently, work by Chanika *et al.* (2010) using culture-dependent techniques indicated that two bacterial isolates (*Pseudomonas putida* and *Acinoetobacter rhizophaerae*) were the main species responsible for degrading the nematicide fenamiphos. However, DGGE analysis showed that, along with the two cultured species, the enrichment contained a mixture of un-cultured α-, β-, and γ-Proteobacteria along with some members of the phylum Bacteroidetes, suggesting that degradation could involve a larger microbial consortia.

Another possible way of using culture-independent methods alongside culture-dependent ones, is to analyse the activity of specific functional genes that are involved in the degradation of a compound of interest. These techniques when used alone, can lack the ability of the examples described previously to identify specific pesticide-degrading species, but they can give indications of the genes (and potentially the subsequent pathways) used in the biodegradation of these compounds. For example, Paulin *et al.* (2010) studied communities capable of degrading the chiral herbicide (*R,S*)-2-(2,4-dichlorophenoxy) propionate (*R,S* dichlorprop) by monitoring the expression levels of the dioxygenase genes *rdpA* and *sdpA*. These genes are involved in the initial stage of the degradation of the compound. The results showed that both of these genes were significantly more abundant within the amended soils compared with the un-amended controls. More recently, Udiković-Kolić *et al.* (2011) monitored the expression of *atz* and *trz* genes in atrazine-degrading cultures. The authors recorded that the genes *atzB* and *atzC* were abundant in degrading cultures containing low levels of atrazine and decreased by a factor of 3 to 4 when atrazine concentrations were increased. In contrast, the abundance of the *trzD* gene was approximately 400 times higher in the high atrazine concentration enrichment. These results indicated significant shifts in the atrazine degrading communities depending on the concentration of the compound.

Nielsen *et al.* (2011) used qPCR methods to monitor the abundances of the *tfd*A gene at different points during the degradation of the herbicide 2-methyl-4-chlorophenoxyacetic acid (MCPA). The *tfd*A gene is known to be involved in the first stage of MCPA degradation; therefore any increases would be indicative of microbial breakdown of the parent compound. The results showed that in soils where MCPA mineralisation occurred the abundances of class III *tfd*A genes increased markedly, and these increases correlated well with observed increases in mineralisation rates.

4.1.4 Bacterial and fungal degradation of xenobiotic compounds: A comparison

Xenobiotic compounds have been found to be degraded by a wide range of organisms within the natural environment. A high proportion of the current literature describes the degradation of

xenobiotics by microbial consortia or by single-strain bacterial cultures. However, there is also a large body of evidence showing that fungi are also important in degradation processes.

Bacteria and fungi have both been shown to either partially degrade, or fully mineralise aromatic compounds, albeit using different pathways. Figure 4.1 illustrates how, in bacteria, benzene is first hydroxylated as a result of dioxygenase enzymes to produce *cis*-dihydrobenzene. The aromatic ring is then re-formed to produce catechol. A second cleavage of this ring by dioxygenase enzymes then results in the formation of two potential intermediates which can subsequently be mineralised to CO_2 (Miller, 1996). Indeed, further research has shown that dioxygenase enzymes also play an essential role in the degradation of other poly-aromatic hydrocarbons (PAHs), including benzo(a)pyrene where they are involved in the cleavage of the 7,8,9,10-benzo ring during degradation by *Sphingomonas paucimobilis* (Haritash and Kaushik, 2009).

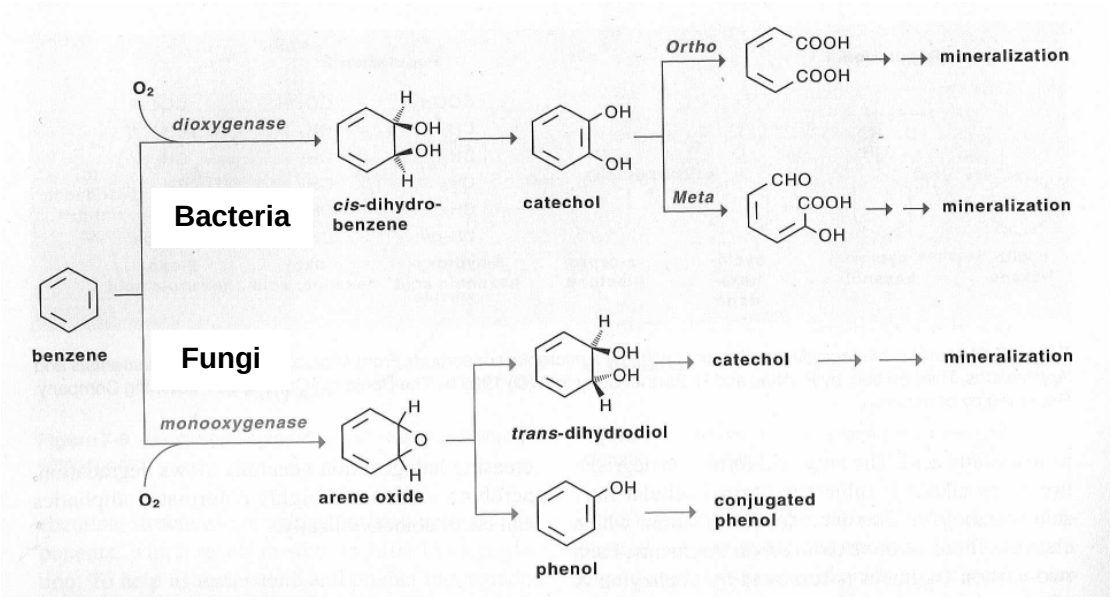


Figure 4.1 The aerobic biodegradation of benzene by bacteria and fungi. From: Miller (1996)

In contrast, fungal degradation of benzene by species such as *Cunninghamella echinulata* var. elegans (Cutright, 1995), first involves hydroxylation using monooxygenase enzymes to produce arene oxide. Following this the hydrolysis of arene oxide produces *trans*-dihydrodiol, which is subsequently converted to catechol and then mineralised to CO_2. Alternatively, arene oxide can be isomerised to

form phenols and, as a result of conjugation with other compounds such as sulphates, can form conjugated phenols. The formation of conjugated phenols is thought to be an important part of the detoxification of benzene and other PAHs (Miller 1996). Another area that has been the subject of much research is the role that lignolytic enzyme-producing fungi play in the degradation of PAHs (Haritash and Kaushik, 2009). One theory is that the irregular structure of lignin has led to the production of enzymes that have low substrate specificity, and therefore could have the capacity to facilitate the degradation of a wider variety of compounds including xenobiotics (Hofrichter *et al.*, 1998).

Spain (1995) reviewed the degradation of nitroaromatic compounds by bacteria and fungi. This group of compounds include trinitrotoluene (TNT) and other explosive compounds. Therefore, their degradation by microbial processes is a subject of much interest. For bacteria, nitroaromatic compounds can be degraded both aerobically and anaerobically. Anaerobic degradation is thought to be non-specific mainly involving the reduction of the nitro group by a range of bacterial species including *Viellonella alkalescens* along with members of the genera *Desulfovibrio* and *Clostridium*.

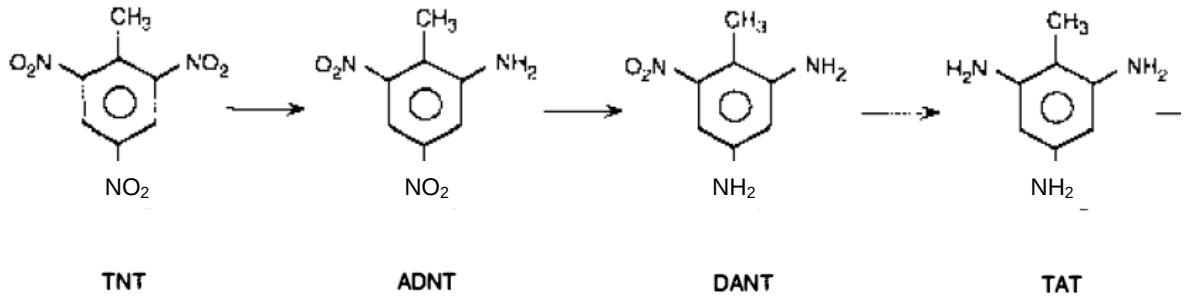


Figure 4.2. The reduction of TNT by anaerobic bacteria. TNT = Trinitrotoluene; ADNT = 2-amino4,6-dinitrotoulene; DANT = 2,4-diamino-6-nitrotoluene; TAT = 2,4,6-triaminotoluene. From: Spain (1995).

In contrast, anaerobic bacteria have been found to specifically degrade nitroaromatic compounds using a range of enzyme pathways. These involve the removal of the nitro group in the form of nitrite using monooxygenase or diooxygenase enzymes, the partial reduction of the nitrile group to hydroxylamine, or the partial reduction of the aromatic ring (Spain, 1995). *Comamonas* sp. strain

JS765 has previously been identified as being able to utilise TNT as a sole carbon, nitrogen and energy source. This pathway first involves the oxidation of TNT to 4-methylcatechol by nitrobenzene diooxygenase (NBDO) enzymes. Following this, the 4-methylcatechol is degraded using a *meta*-cleavage pathway. The end-products of this pathway are acetaldehyde (CH_3CHO) and pyruvate (CH_3COCOO^-) which can then feed into the tricarboxylic acid (TCA) cycle (Ju and Parales, 2010).

As with the degradation of PAHs, the degradation of nitroaromatic compounds by fungi is thought to involve the production of lignolytic enzymes (extracellular peroxidases) along with hydrogen peroxide. For example the white rot fungus *Phanerochaete chrysosporium* has previously been shown to fully mineralise TNT to CO_2 using these enzymes (Figure 4.3) (Spain, 1995). Additionally, other white rot fungi have been shown to partially degrade TNT to one of a number of intermediate compounds including 2-amino-4,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene, 2,6-diamino-4-nitrotoluene, and 4-amino-2,6-dinitrotoluene. In the absence of species capable of further degrading these intermediates, they may form dimers which have increased persistence within the environment (Pointing, 2001).

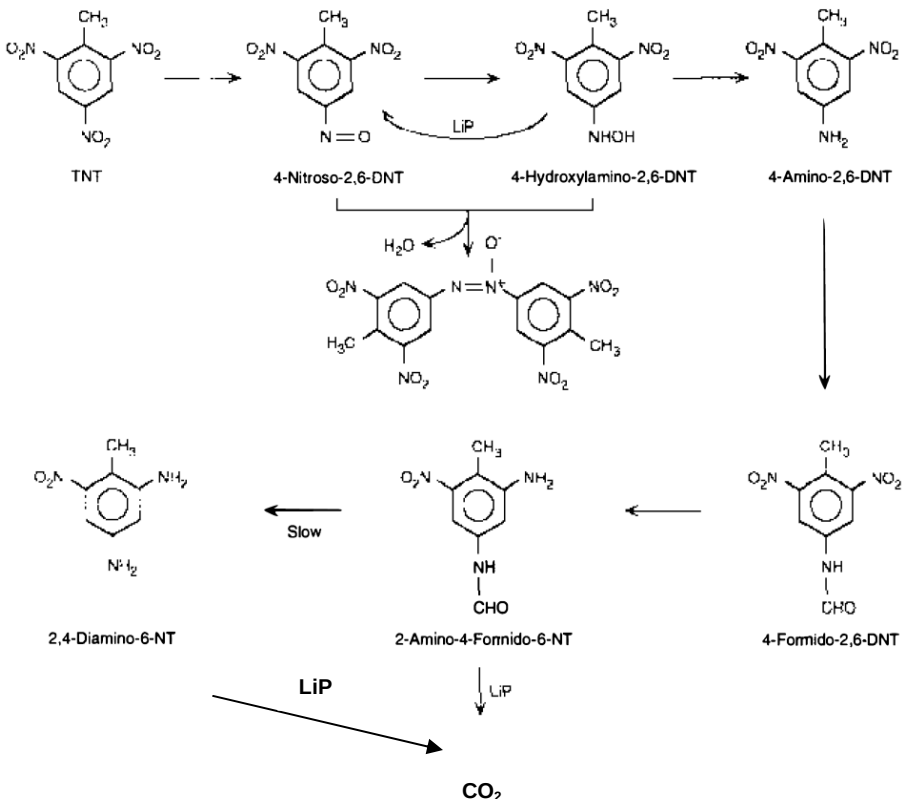


Figure 4.3 The transformation of TNT by *Phanerochaete chrysosporium*. TNT = Trinitrotoluene; DNT = Dinitrotoluene; NT = Nitrotoluene; LiP = Lignin peroxidase. From: Spain (1995)

Both bacteria and fungi have previously been identified as degraders of a wide range of pesticides. Levanon (1993) compared the roles of fungi and bacteria in the mineralisation of four pesticides: atrazine, alachlor, malathion, and carbofuran. The results showed that atrazine was only mineralised in soils containing both bacterial and fungal species. Fungal species were found to more commonly cleave the ethyl and isopropyl amino side-chains, whilst bacterial species were required for cleavage of the atrazine ring structure to occur. Similar results were also found for alachlor. The degradation of malathion and carbofuran was found to be mainly due to bacterial degradation alone and involved the action of carbofuran hydrolase (for carbofuran), and carboxylesterases (for malathion) (Levanon, 1993). Similarly, other organophosphorus pesticides including glyphosate, parathion, methyl parathion and chlorpyrifos are also known to be degraded by microbial action. Degradation of chlorpyrifos by *Enterobacter* sp. has been studied in detail. The bacterium first degrades that compound to diethylthiophosphoric acid (DETP) and 3,5,6-trichloro-2-pyridinol (TCP). Following this *Enterobacter* sp. degrades the DETP further, utilising the compound as a source of carbon and phosphorus in the process. In fungi, chlorpyrifos can be used as a sole carbon source (*Phanerochaete chrysosporium*) (Bumpus *et al.*, 1993) or phosphorus source (*Aspergillus* sp, *Trichoderma harzianum*, *Penicillium brevicompactum*) (Omar, 1998; Obojska *et al.*, 2002). *P. chrysosporium* first hydrolyses the chloropyrifos side chain, before cleaving the pyridinyl ring. Following this, the metabolites are then fully mineralised to CO_2 and water. Degradation by *Aspergillus* sp, *Trichoderma harzianum* and *Penicillium brevicompactum* is thought to involve a step-wise process involving a number of possible intermediates (Singh and Walker, 2006). Figure 4.4 shows some proposed pathways for the degradation of chloropyrifos by microbial action.

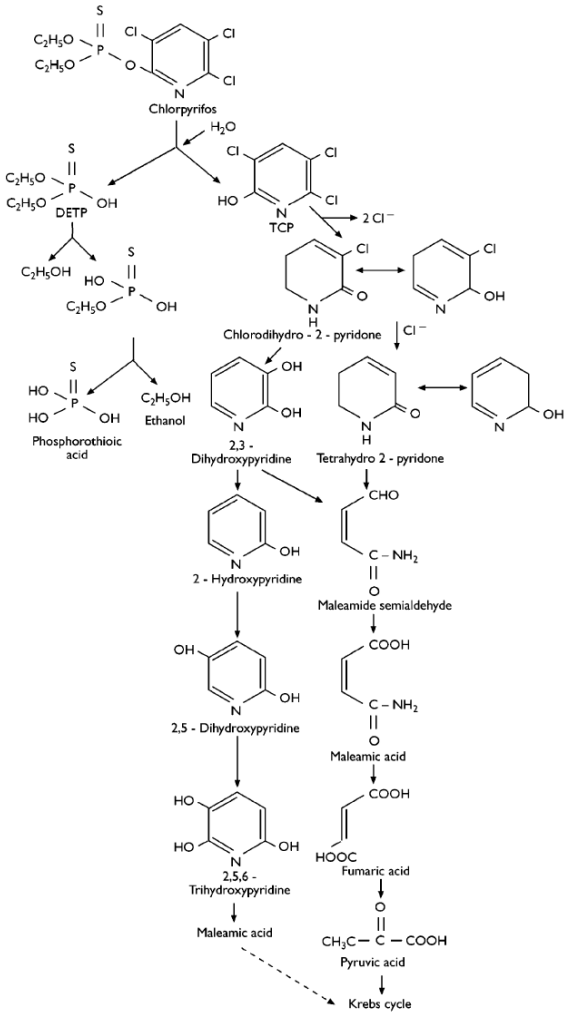


Figure 4.4 Proposed degradation pathways of chlorpyrifos by microbial action. DETP = Diethylthiophosphate; TCP = Trichloropyridinol. From Singh and Walker (2006).

4.1.5 The microbial degradation of azoxystrobin and other strobilurin fungicides

Despite the fact that they are used extensively worldwide, current knowledge about the microbial degradation of azoxystrobin and other strobilurin fungicides within soil environments is relatively minimal. Microbial degradation of azoxystrobin is known to involve the hydrolysis of the carboxyl ester bonds of the parent compound (Katagi, 2006). Recent research has suggested that the subtilisin-like carboxypeptidase group of enzymes may play an essential role in this process (Clinton *et al.*, 2011). Hydrolysis of one of these groups forms an acid metabolite, known as azoxystrobin acid (Ghosh and Singh, 2009a) (Figure 4.5).

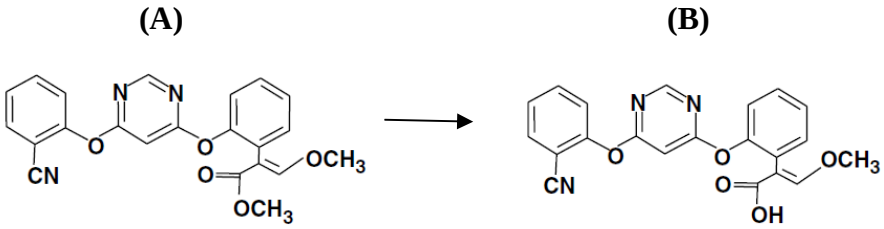


Figure 4.5 The chemical structure of (A) Azoxystrobin and its primary microbial breakdown product (B) Azoxystrobin acid. From: Ghosh and Singh (2009a)

Azoxystrobin acid is much more water soluble (860 mg L^{-1}) than its parent compound (6.7 mg L^{-1}) (Ghosh and Singh, 2009a) and has been previously known to persist in water courses (EU-New, 1998). Furthermore, Ghosh and Singh (2009b) noted that in agricultural soils azoxystrobin acid was first recorded on HPLC traces after 60 days when the azoxystrobin concentration had decreased to approximately 50% of the initial applied concentration, and was present in its highest concentrations after 120 days. This suggests that the metabolite had some level of persistence in the environment. Similar results were also found in water by Singh *et al.* (2010) where the concentrations of azoxystrobin acid present were highest at the end of the experiment (130 days).

Bending *et al.* (2006) observed that in two agricultural soils the degradation of azoxystrobin, as determined by HPLC analysis, appeared to show kinetics more commonly associated with co-metabolic degradation as opposed to growth-linked processes. Further work by Adetutu *et al.* (2008) noted that azoxystrobin was degraded readily in both light and dark conditions. Interestingly, degradation in the dark produced 1 unknown metabolite, whereas degradation under light conditions produced two known metabolites. Mineralisation of azoxystrobin was found to be very low in both treatments (less than 0.1% of the applied compound). The mineralisation of azoxystrobin in water systems has also been found to be very low (2.5 – 4.2% of the applied compound) (Singh *et al.*, 2010).

Similarly, kresoxim-methyl and trifloxystrobin are also degraded as a result of the hydrolysis of carboxyl ester groups to form more water-soluble acid metabolites. The acid metabolite of

trifloxystrobin is known to be present as one of 4 possible isomers, known as *EE*, *EZ*, *ZE*, and *ZZ* of which the *EE* isomer has been shown to be the major metabolite in soil systems (Banerjee *et al.*, 2007). As with the azoxystrobin acid metabolite, the trifloxystrobin acids have been recorded as persistent within the environment, and lack the fungicidal activity of the parent compound which has caused concerns about possible over-application in agricultural fields (Banerjee *et al.*, 2006). However, to the authors' knowledge, the nature of the microorganisms involved in the degradation of these three compounds has not been elucidated using either culture-dependent or –independent methods. This represents a significant knowledge gap that needs to be addressed in order to gain a fuller understanding of the fate of these compounds within the environment.

In contrast, pyraclostrobin has been found to be more readily degraded by demethoxylation reactions as opposed to ester hydrolysis (Katagi, 2006). To the authors' knowledge there is only one published example of pyraclostrobin-degrading organisms being cultured. Lopes *et al.* (2010) isolated a bacterium that showed a high sequence similarity to members of the genus *Klebsiella* sp. from soils capable of degrading pyraclostrobin and epoxyconazole. This strain was found to degrade 100% of the applied epoxyconazole after 120 hours, but only 31.7% of the pyraclostrobin. This suggests that the *Klebsiella* strain preferentially degraded epoxyconazle. There is currently no information from culture-independent analyses into the organisms involved in pyraclostrobin degradation, and therefore the extent to which non-culturable organisms are involved with this process remain unknown.

4.1.6 Experimental Hypotheses

Currently, there is nothing known about the organisms involved in the degradation of azoxystrobin in soils. Using culture-dependent and -independent methods, the work in this chapter involved the isolation and identification of bacteria capable of degrading azoxystrobin, and to determine their ability to degrade other strobilurins. Secondly, a bacterial clone library and T-RFLP analyses were used to identify bacteria which proliferated during azoxystrobin degradation *in situ*, and therefore inform about the likelihood that the degraders isolated were responsible for degradation in soil. The

hypotheses tested in this chapter were that: 1) sequential enrichments with azoxystrobin will result in adaptation within the microbial community leading to increased degradation rates; 2) any azoxystrobin degrading organisms that are cultured will also be active *in situ*;

4.2 Materials and Methods

4.2.1 Soil collection and preparation

Soil was collected from Hunts Mill Field, at the University of Warwick School of Life Sciences, Wellesbourne Campus, UK in January 2009. The characteristics of the soil, along with the methods by which it was collected are described in Chapter 2. Soils were sieved, stored, and re-wetted to a matric potential of -33 kPa, as described in Chapter 2.

4.2.2 Enrichment of soils with Azoxystrobin and Degradation Monitoring by HPLC

Previous research into microbial responses to pesticide amendment has indicated that microbial communities can adapt to the presence of a compound following repeated applications. An indicator of community adaptation is an increased capacity to degrade the particular compound. Therefore, the work carried out here aimed to determine whether soils that had previously been amended with azoxystrobin could degrade the compound better than a previously non-amended soil.

This assay used the 0 mg kg^{-1} and 25 mg kg^{-1} (4 month) treatment soils from the experiment described in Chapter 3. The 25 mg kg^{-1} treatment soil contained residual azoxystrobin with an average concentration of 13 mg kg^{-1}. Three 120 g soil portions of each treatment were amended with a 25 mg kg^{-1} dose of azoxystrobin, transferred to 250 ml sterile glass Duran bottles, and stored at 15ºC in the dark using the same methodologies as described in Chapter 2.The treatments were sampled every week for 4 weeks and were analysed for azoxystrobin degradation by HPLC using the methods described in Chapter 2.

4.2.3 Sequential Enrichments of Azoxystrobin-Degrading Cultures

Following the soil degradation assay, a series of sequential liquid culture enrichments were set up to try and select for organisms that were either resistant to, or involved in the degradation of azoxystrobin.

Liquid degradation cultures were also performed in sterile 250 ml glass Duran bottles. Three sample treatments were set up in triplicate. The first treatment contained the previously un-amended soil and the second contained the previously amended soil. Each of these treatments contained 15 g of soil sample and 25 ml of sterile minimal basal salts (MBS) media.

The MBS media (pH 6.8) contained (L^{-1}): Sodium chloride (NaCl) 0.3 g, ammonium sulphate $(NH_4)_2SO_4$ 0.6 g, potassium nitrate (KNO_3) 0.6 g, monopotassium phosphate (KH_2PO_4) 0.25 g, dipotassium phosphate (K_2HPO_4) 0.75 g, magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) 0.15 g (Skerman, 1967). 0.02 g of manganese sulphate tetrahydrate ($MnSO_4 \cdot 4H_2O$), and 0.005 g of filter sterilised iron sulphate ($FeSO_4$) were also added as trace elements (Roberts *et al.*, 1998). The $FeSO_4$ was dissolved in 20 ml of sterile distilled water; filter sterilised and added to the media once the media had cooled to room temperature following autoclaving. Once the stock had been prepared and divided into the individual Duran bottles, azoxystrobin was dissolved in 750 µl of methanol and added to each of the treatment samples to give a final pesticide concentration in each Duran bottles of 25 mg L^{-1}.

A negative control treatment was also set up in triplicate. This contained just the MBS media and the azoxystrobin solution and was used to determine whether non-biological degradation was occurring.

The Duran bottles were then wrapped in aluminium foil to minimise photo-degradation and shaken at 150 rpm at a temperature of 15°C. The treatments were then sampled on a weekly basis for 4 weeks. At each sampling point 2 ml of the culture liquid was removed to assess degradation by HPLC

analysis. The preparation of the samples and subsequent analysis by HPLC was the same as detailed in Chapter 2.

After 4 weeks, 5 ml of each culture was then transferred to new sterile Duran bottles. These cultures were then re-enriched with 20 ml of MBS and 15 ml of azoxystrobin solution (present as the sole carbon source) to the same concentration as previously, and shaken under the same conditions as previously. Samples were taken every week for 4 weeks as previously and degradation monitored by HPLC. Following this, a final enrichment and 4-week sampling cycle was performed.

4.2.4 Culture-Independent Analyses of Bacterial Community Structure and Diversity Changes during Sequential Enrichment

The aim of this analysis was to identify bacterial strains that increased in abundance during the degrader culture enrichment process, and to try and relate these strains to the isolates obtained from culture-dependent analyses.

At the end of each of the four 4-week enrichment series, 1 ml aliquots of each treatment were removed and stored at -80ºC in sterile 2 ml Eppendorf tubes for use in T-RFLP analysis to determine how the overall bacterial community structure and diversity had altered during the enrichment process.

DNA extraction from the samples was carried out using the FastDNA® Spin Kit (Qbiogene, UK) as in Chapter 2, with the exception that DNA was extracted from 500 µl of the culture solution as opposed to from 500 mg of soil.

The bacterial primer pair used for the PCR reactions was 63f-NED/1087r-VIC. Further details of these primer pairs can be seen in Table 2 of Chapter 2. The PCR conditions used were the same as those used in Chapter 2 for this primer pair, as were the procedures used for the preparation of the T-

RFLP samples. As previously, the T-RFLP samples were analysed by the Genome Centre at the University of Warwick, School of Life Sciences, Wellesbourne Campus. Details of the methods used to analyse the T-RFLP data can be seen in Chapter 2.

4.2.5 Detailing the Maximum Dilution for Azoxystrobin Degradation in the Liquid Cultures

Following the third enrichment cycle, the resulting cultures were monitored to determine the highest dilution at which their degradative performance remained un-affected.

For this analysis, 5 ml of each culture was taken and serially diluted with sterile distilled water to concentrations from 10^{-1} to 10^{-7} of the original culture. MBS medium and azoxystrobin solutions were added to the samples and the bottles were shaken under the same conditions as previously. In this assay the cultures were monitored for 16 days and samples were taken for HPLC analysis every 4 days.

Once the highest dilution to still record degradation had been determined, these cultures were used for culturing onto minimal basal salts agar (MBSA) containing azoxystrobin. The MBSA was made in the same way as the MBS except that 15 g L^{-1} Difco Bacto-Agar (Difco Laboratories, Detroit, USA) was dissolved in the media prior to autoclaving, as previously detailed by Roberts *et al.* (1998). Once the media had cooled following autoclaving, azoxystrobin was applied following the method of Rosenzweig *et al.* (2008) whereby the pesticide was dissolved in acetone and applied to the molten agar to give a final concentration of 10 mg L^{-1}. Before inoculating the plates, the chosen cultures were serially diluted further to give dilutions ranging from 10^{0} to 10^{-6} in order to account for a wide range of possible bacterial densities. For each plate 50 µl of culture was pipetted onto the centre of the plate and spread using a sterile, disposable plastic spreader. The lids of the plates were then put back on and the plate left for 30 minutes for the liquid to soak into the agar. Following this, the plates were inverted and stored at 15°C in the dark and were monitored for growth on a daily basis. Control plates

were also set up containing 50 µl of the control samples to ensure their sterility.

4.2.6 Monitoring Azoxystrobin in Liquid Cultures Containing Single Bacterial Colonies.

After 10 days of growth on the agar plates, single colonies were selected for further degradation analysis and their morphologies recorded. Selection was carried out to ensure the widest possible variation in colony types were analysed for degradation.

The single colony degradation assays were set up in Cellstar® sterile plastic 6-well TC-plates (Greiner Bio-One, Stonehouse, UK). Each well contained an initial 7 ml of MBS and azoxystrobin dissolved in methanol at a concentration of 25 mg L^{-1}, plus a single bacterial colony which was transferred from the plate to the culture using a sterile disposable plastic loop. As with the culture dilution assay, samples were taken every 4 days for a total of 16 days and were analysed using HPLC as previously, except that only 1 ml of culture was taken at each time point. As a result, the volume of acetonitrile used for the extraction process was also adjusted accordingly.

In total, 60 single colony cultures were set up, along with 5 un-inoculated control samples. An additional 10 x 200 µl plate wash samples were produced to determine whether azoxystrobin degradation required co-cultures to be present.

After 16 days, 50 µl aliquots were taken from the 21 cultures with the lowest percentage azoxystrobin (40 to 80%) recovery during the experimental period and were re-inoculated onto MBSA containing azoxystrobin. Following a 10-day incubation period, the colony morphologies on the plates were recorded to ensure that they matched those of the original colonies. The colony growth on these plates also served as a check to ensure that the single colony cultures were still monocultures.

Following this, a second round of degradation assays were set up using colonies/plate washes from

the 21 new plates plus 5 un-inoculated controls. The sampling procedure for this assay was the same as for the previous one.

4.2.7 Colony Sequencing, Identification and Clone Library Production

Two single colony samples were chosen for 16S rRNA gene sequencing and identification. These colonies were chosen for further analysis as they were morphologically distinct from each other, and had degraded between 2 and 3 times more of the azoxystrobin than any of the other single colony cultures, which suggested that these organisms were highly adapted for the degradation of this compound. 10 single colonies from each of the two plates were analysed.

Single colonies were first picked from the plate using a sterile disposable plastic loop and transferred to sterile Eppendorf tubes containing 100 µl of sterile distilled water. The colonies were re-suspended in the water by pipetting and gentle vortexing. 5 µl of the suspension was then used in a 50 µl PCR reaction. Each PCR reaction contained: 43 µl of MegaMix (Microzone Ltd, UK), 1 µl of 5 µM 63f bacterial primer, 1 µl of 5 µM 1087r bacterial primer, and 5 µl of colony suspension. The PCR conditions used were the same as those detailed for this primer set in Chapter 2.

A sequencing reaction was then performed using the bacterial PCR products. Each 10 µl sequencing reaction contained: 2 µl of BigDye, 2 µl of 5 x buffer, 1 µl of 3.2 µM primer (either M13R or M13F), 3 µl of PCR product (approximately 385 ng of DNA), and 2 µl of sterile distilled water. The sequencing reaction parameters and the methods by which the sequences were analysed were the same as detailed for the clone libraries in Chapter 2.

In order to compare the identification of the colony samples with the presence of possible azoxystrobin degraders in a soil environment, a clone library was produced using DNA samples from the 25 mg kg^{-1} (1 Month) treatment of the experiment detailed in Chapter 2.

This clone library was produced using the methods detailed in Chapter 2 with the primer pair 63f/1087r used for the initial PCR process. The methods used for sequence analysis were also the same as those described in Chapter 2.

The two different strains obtained were then maintained on MBSA for 10 days at 15ºC in the dark as previously, before being used in characterisation assays. These assays aimed to determine the effects of a) initial inoculum concentration, b) solid growth media type, and c) the presence of an additional carbon source and/or nitrogen limitation on azoxystrobin degradation. Additionally, cultures were set up to determine whether the isolated bacteria could degrade three other strobilurin fungicides (pyraclostrobin, trifloxystrobin, and kresoxim methyl).

4.2.8 The Effects of Initial Bacterial Inoculum Concentration on Azoxystrobin Degradation.

An assay was carried out to determine how an increase in the initial concentration of inoculation affected the degradation of azoxystrobin.

Prior to the start of the assay, inocula with specific cell concentrations had to be prepared. Firstly, degradation cultures of the two colony types were set up as previously, using a 5 ml aliquot of 10^{-4} dilutions from the original cultures. The new cultures were shaken at 150 rpm in the dark and at 15ºC, as previously. Following this, a ten-fold dilution series was prepared (10^{-1} to 10^{-10}) and 0.25 ml aliquots of these dilutions were transferred to 1.75 ml of sterile MBS media. Aliquots (50 µl) were then spread onto MBSA containing azoxystrobin. After 10 days, numbers of colonies on the plates were counted and the cell numbers in the dilution cultures determined. The optical densities (OD) of these samples were measured using a Unicam 5625 UV/VIS spectrometer (Unicam, UK) at a wavelength of 600 nm.

For the inoculation concentration assay, 3 cell concentrations (10^2, 10^6, and 10^{10} cells ml^{-1}) were used

for each of the two isolated bacteria. As previously, the cultures were set up in Cellstar® sterile plastic 6-well TC-plates. The volumes of MBS media and azoxystrobin concentration were the same as used previously. The cell cultures were applied to the media as a single 280 µl dose. This gave actual cell numbers in the test samples of 2.8×10^1, 2.8×10^5, and 2.8×10^9 for the 10^2, 10^6, and 10^{10} cells ml^{-1} dilutions, respectively. Three experimental replicates were set up for each treatment.

As with the single colony degradation assays, samples were taken every 4 days for 16 days, and 1 ml of culture was taken at each sampling point for HPLC analysis.

4.2.9 The Effects of Solid Growth Media Type on Azoxystrobin Degradation

This assay was carried out to determine whether the ability of the degraders to degrade azoxystrobin was altered when they were grown on different solid media prior to inoculation into liquid culture.

For this assay, the degrader cultures were grown in liquid culture as previously. Aliquots (50 µl) of each degrader (or negative control) were then plated onto 4 different types of agar. These were: MBSA, R2A agar (Oxoid, UK) nutrient agar (NA) (Oxoid, UK), and Lysogeny Broth agar (LB) (Sigma Aldrich, UK). Following 10 days of colony growth, cell count and OD measurements were carried out as previously. Based on the results of the previous assay, a cell concentration of 10^6 cells ml^{-1} was chosen for this, and subsequent, assays. The degradation cultures were set up, sampled, and analysed in the same manner as in the inoculation concentration assay with 3 replicates of each treatment being set up.

4.2.10 The Effects of Carbon and Nitrogen Availability on Azoxystrobin Degradation

The capacity of the degrader cultures to degrade azoxystrobin under a range of carbon and nitrogen source constraints was analysed. The four variations of the MBS liquid media set up were: 1) Azoxystrobin as the sole carbon and nitrogen source (pH 6.7); 2) Azoxystrobin as the sole carbon source but with 6 mM $(NH_4)_2SO_4$ as a nitrogen source (pH 6.8); 3) Azoxystrobin as the sole nitrogen

source but with 120 mM acetic acid ($C_2H_4O_2$) as an alternative carbon source (pH 6.4); 4) Both the alternative carbon and nitrogen sources present in the culture (pH 6.6). Acetic acid was chosen as the alternative carbon source because both *Cupriavidus* and *Rhodanobacter* sp. have previously been identified being able to utilise it (Wang and Yu, 2001; Huong *et al.*, 2008) whereas some species of *Cupriavidus* are known to be unable to utilise glucose (Sato *et al.*, 2006). The carbon and nitrogen source concentrations were based on concentrations used previously in liquid culture experiments as detailed in the scientific literature.

The assay was set up using the same methodology as the previous assay, with the sampling times, along with the sampling and analytical methods also remaining the same.

4.2.11 The Degradation of Three Alternative Strobilurin Fungicides by Azoxystrobin-Degrading Cultures

A liquid culture degradation assay was set up to determine whether the two azoxystrobin-degrading cultures were also capable of degrading other members of the strobilurin fungicide group. The alternative strobilurin fungicides tested alongside azoxystrobin were trifloxystrobin, pyraclostrobin, and kresoxim-methyl. These three compounds were chosen because they have similar levels of persistence within the environment, and although they are all from the same group of fungicides, they do have some structural differences as can be seen in Figure 1.6 of Chapter 1 (Page 24). All of the fungicides were obtained from Greyhound Chromatography, UK.

This assay was set up in 250 ml sterile glass Duran bottles. Each bottle contained: 25 ml of MBS media, 15 ml of pesticide solution at the same concentration as in previous assays, and 1 ml of degrader culture at a cell concentration of 10^6 cells ml^{-1}. For this assay there were 3 replicates of each treatment, plus 3 uninoculated controls for each fungicide.

Samples were taken every 2 days for 16 days with 2 ml of culture being removed at each sampling

time and prepared for HPLC analysis in the same manner as in the sequential enrichments mentioned earlier in this section. The HPLC equipment, column, flow rate and liquid phase used, were the same as those described in Chapter 2. The retention times of the compounds were approximately 3 minutes for azoxystrobin, 4 ½ minutes for pyraclostrobin, 5 minutes for trifloxystrobin, and 4 minutes for kresoxim-methyl. The recovery efficiency of the equipment was monitored using control samples containing the equivalent of 10 mg kg^{-1} each pesticide dissolved in acetonitrile. A control sample measurement was taken after every 10 experimental sample measurements. Recovery of control samples varied from 95.7% to 89.8% of the applied concentration.

4.2.12 Mineralisation of Azoxystrobin by Degrader Cultures

This assay was performed to determine the extent to which the azoxystrobin-degrading cultures were fully mineralising the compound to CO_2. The assay was set up and carried out in the Department of Environmental Sciences at Lancaster University, UK. The method used was based on that of Reid *et al.* (2001) and involved the use carbon-14 (^{14}C)-labelled azoxystrobin (>98% purity, Syngenta Crop Protection AG, Basel, Switzerland) labelled at the terminal ring with mineralisation being determined by the amount of $^{14}CO_2$ evolved into sodium hydroxide (NaOH) traps.

The design of the respirometers was similar to that of Reid *et al.* (2001) and can be seen in Figure 4.6. Samples were prepared in sterile 250 ml Duran bottles with modified lids. Each lid had a hole drilled through the centre into which a 30 mm stainless steel bolt was placed. This bolt was held in place using a washer and wing-nut. A stainless steel crocodile clip was then attached to the bottom of the bolt. The CO_2 trap consisted of a 7 ml glass scintillation vial containing 1 ml of 1 M NaOH solution applied onto a 20 x 20 mm filter paper. This vial was then attached to the crocodile clip.

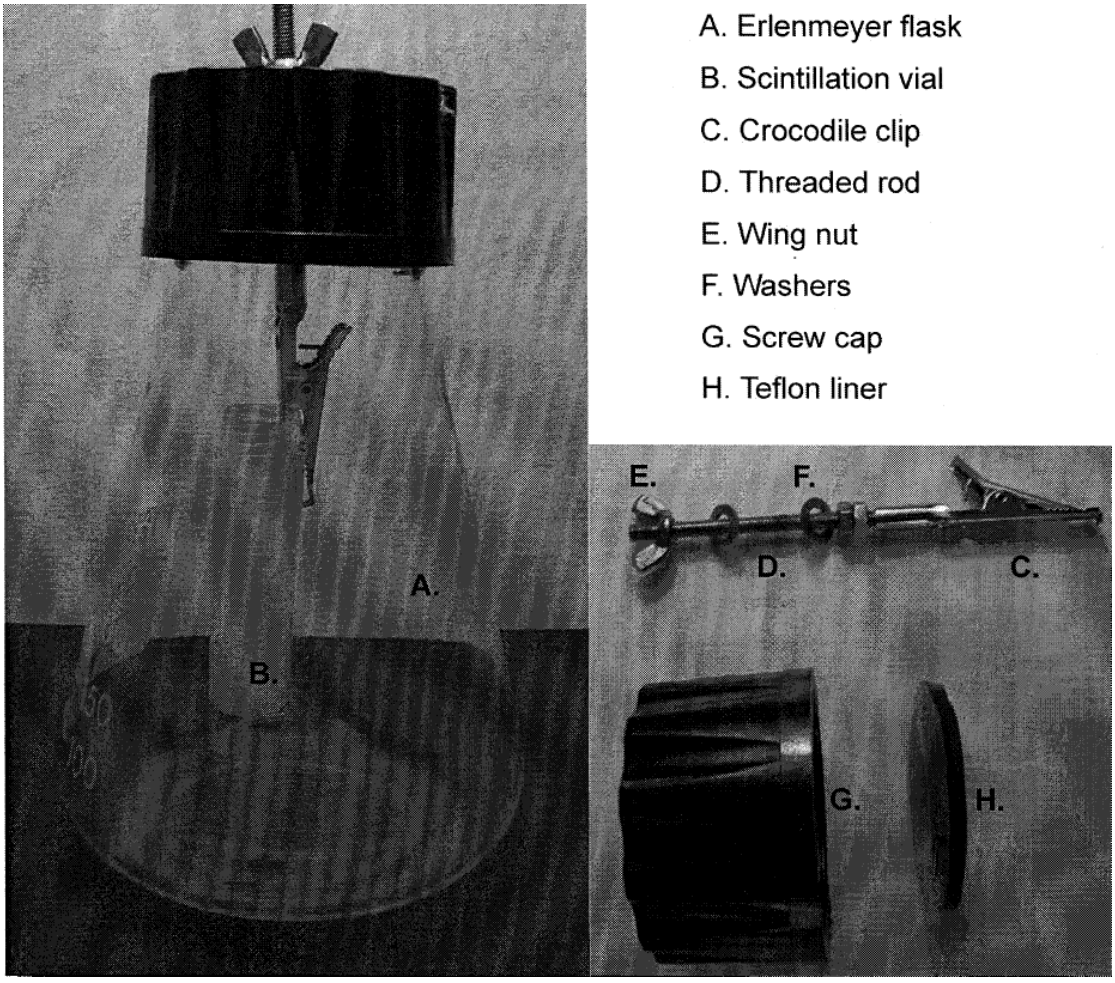


Figure 4.6. The design of the respirometers for the azoxystrobin mineralisation assay. Taken from Reid *et al.* (2001). Note that 250 ml Duran bottles were used in the experiment described in this chapter.

The experimental samples contained 25 ml of sterile MBS media, and 5 ml of the required degrading organism in MBS at a concentration of 10^6 cells ml^{-1}. The ^{14}C-labelled azoxystrobin was added in a solution containing 16 µl of ^{14}C-compound dissolved in 74 µl of toluene. This gave an equivalent azoxystrobin concentration of approximately 10 mg L^{-1} and a total activity of 7,500 Bq. Three degrader treatments were set up, two containing individual degraders and the third containing a mixture of both. The mixed culture contained 10^6 cells ml^{-1} of each degrader. In addition to the three different degrader treatments, an uninoculated negative control containing only the MBS and azoxystrobin solutions was also set up. 3 replicates of each treatment were set up. Samples were shaken at 150 rpm in the dark at room temperature. Analysis of the cultures for $^{14}CO_2$ evolution was

carried out every 24 hours.

At each sampling point, the scintillation vial was first removed from the Duran bottle, wiped with an acetone-soaked paper towel and replaced with a fresh vial containing 1 ml of 1 M NaOH. 5 ml of Ultima Gold liquid scintillation fluid (Perkin Elmer, UK) was then added to the removed vial. Following this, the lid was screwed tightly on, the vial shaken by hand and then stored in the dark at room temperature for 24 hours to allow the filter paper to become transparent. At the same time, the Duran bottles were returned to the shaker for a further 24 hours.

Three blank samples containing 1 ml of 1 M NaOH and 5 ml of liquid scintillation fluid were also prepared at every time point.

The concentration of evolved $^{14}CO_2$ activity trapped in the scintillation vial was determined by liquid scintillation counting using a Canberra Packard Tri-Carb 2250CA liquid scintillation analyser (Canberra Packard, UK). The degradations per minute (dpm) value for each sample was recorded and the data expressed as the percentage ^{14}C-azoxystrobin mineralised compared with the initial concentration present in the culture at Time 0.

4.2.13 Statistical Analyses

For the degradation assays DT_{50} values were calculated using the vinterpolate function in the GenStat Version 12 statistics programme (VSN International, UK) as detailed in Chapter 2. Least significant difference (LSD) analysis was used to determine significant differences between treatments at each time point. 2-way ANOVAs were used to analyse any significant differences over the entire time course of an assay. More details about the statistical methods used can be found in Chapter 2.

4.3 Results

4.3.1 The Degradation of Azoxystrobin in Once- and Twice-Amended Soils

HPLC analysis results showed that there was a significant difference in azoxystrobin degradation in the once- and twice-amended soils over the 4-week experimental period (p = 0.019). In the twice-amended soils 30% of the applied azoxystrobin had been degraded within the first week (Figure 4.7). This compared to 14% in the once-amended treatment. After 4 weeks, 32 and 43% of the applied compound had been degraded in the once- and twice-amended treatments, respectively. There was also a significant difference in the DT_{25} values for each treatment (p = < 0.01). The average DT_{25} values were 12 and 5.5 days for the once- and twice-amended soils, respectively.

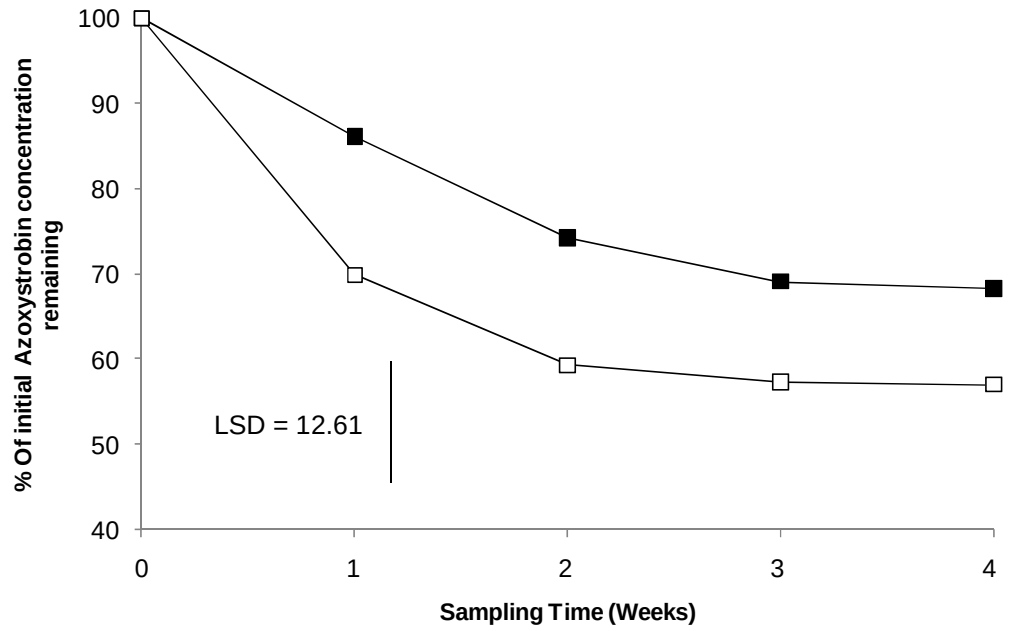


Figure 4.7.Percentages of initially applied azoxystrobin extracted using HPLC analysis. ■ Once-amended □ Twice-amended. Each data point represents the mean of 3 experimental replicates.

4.3.2 Sequential Enrichments of Azoxystrobin-Degrading Cultures

4.3.2.1 HPLC Analysis of Azoxystrobin Degradation

There was a significant difference in degradation between the once- and twice amended treatments over the course of the three liquid enrichments (p = 0.028). This was particularly noticeable at the 2-week sampling point of each enrichment (Figure 4.8). At this sampling time, the percentage of applied azoxystrobin recovered differed between the once- and twice amended treatments by 32, 22, and 20% for the first, second and third enrichments respectively. All three of these values represent significant differences as determined by LSD analysis.

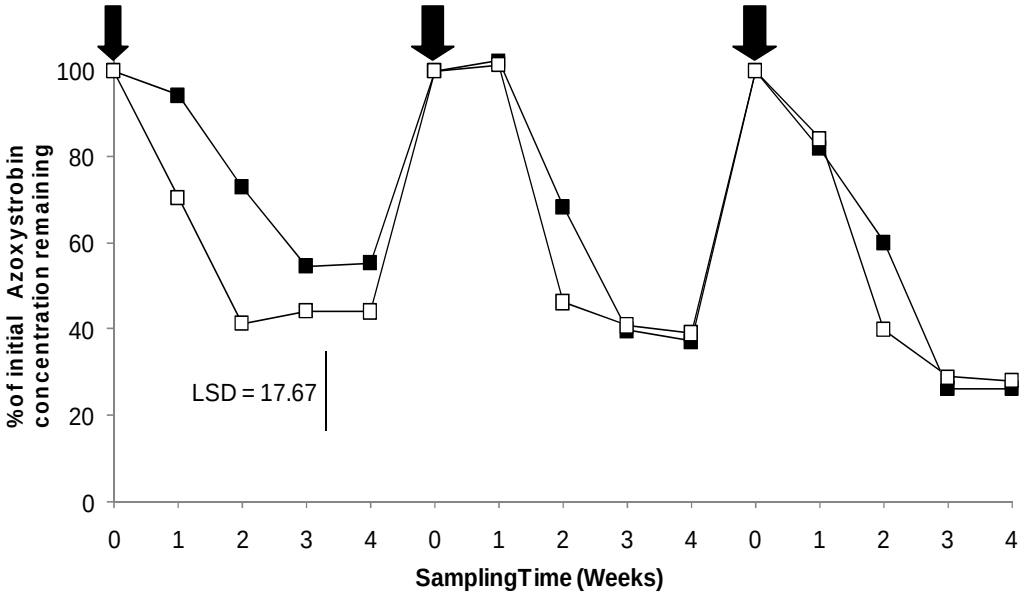


Figure 4.8. Percentages of applied azoxystrobin recovered from the once- and twice-amended samples over a series of 3 x 4 week liquid culture enrichments ■Once-enriched □Twice-enriched. The black arrows denote the points at which the cultures were enriched. Each data point represents the mean of 3 experimental replicates.

In the first enrichment, 55 and 44% of the applied azoxystrobin remained in the once- and twice-amended treatments respectively after 4 weeks. Azoxystrobin degradation increased in both the once- and twice-amended treatments over the subsequent 2 enrichments. The once-amended treatment degraded 63 and 74% of the compound during enrichments 2 and 3, respectively. In the twice-amended treatment, 61% of the azoxystrobin was degraded in enrichment 2, and 72% in enrichment 3.

There was a significant difference between the DT_{25} values for the once- and twice-amended treatments in enrichment 1 (p = 0.039). The values were 11 and 5 days for the once- and twice-amended treatments, respectively. In contrast, the DT_{25} values were not significantly different in enrichments 2 and 3. In enrichment 2, the DT_{25} values were 11 and 9 days for the once- and twice amended treatments respectively. Both treatments had a DT_{25} value of 8 days in enrichment 3.

The average DT_{50} values recorded for the twice-amended cultures were 1.78, 1.96, and 1.62 weeks for enrichments 1, 2, and 3, respectively. There was an insufficient reduction in azoxystrobin recovery for a DT_{50} value to be calculated for the first enrichment of the previously once-amended treatment. In enrichments 2 and 3 the DT_{50} values were 2.48 and 2.22 weeks, respectively. There was an insufficient decrease in azoxystrobin recovery in either treatment during any of the three enrichments for DT_{90} values to be calculated.

4.3.2.2. T-RFLP Analysis of Bacterial Community Structure and Diversity Changes

The average numbers of bacterial TRFs recorded after 4 weeks in the soil and three liquid enrichment series can be seen in Table 4.1. There was no significant difference in the number of TRFs recorded in the once-amended treatments compared with the twice-amended ones (p = 0.407). Comparisons between the different enrichments also showed no significant impact on TRF numbers (p = 0.075).

Table 4.1. Average bacterial TRF numbers for the once- and twice-amended soil treatments sampled 4 weeks post-amendment.

	Average Number of TRFs			
	Soil	**Liquid (1)**	**Liquid (2)**	**Liquid (3)**
Once-Amended	27[a]	16[a]	9[a]	6[a]
Twice-Amended	18[b]	12[a]	9[a]	9[a]

*Different letters denote significant differences between the two treatments. LSD = 10.69. Each TRF number represents the mean of 3 experimental replicates.

There was no significant difference between the bacterial community diversity of the once- and twice-amended treatments, over the 4 enrichments (p = 0.455) (Figure 4.9). However, there was a

significant difference in bacterial diversity when the enrichment number was compared (p = 0.037). The average bacterial diversity in the once-amended treatment was 2.58 in the soil enrichment compared with 1.08 after the third liquid culture enrichment. For the twice-amended treatment, these values were 2.24 and 1.47 after the soil and third liquid enrichments, respectively.

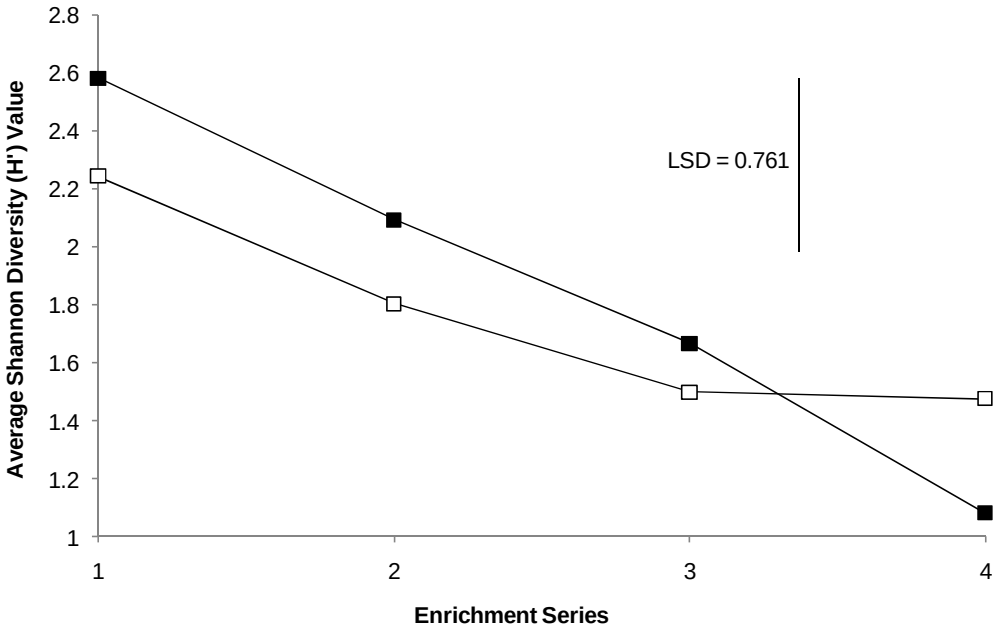


Figure 4.9. The average Shannon diversity (H') of bacterial communities from the once- and twice-amended treatments. ■ Once-amended □ Twice-amended. Enrichment series 1 refers to the soil degradation series; series 2-4 refer to the 3 liquid culture series. Each data point represents the mean of 3 experimental replicates.

ANOSIM analysis showed, however, that the bacterial community structure was significantly different between the once- and twice-amended treatments (p = < 0.01). Additionally, the community structure also differed significantly between the different enrichment series (p = < 0.01). This was backed up by NMDS analysis (Figure 4.10) which showed a grouping of sample points based both on the treatment (once- or twice-amended) and the enrichment number.

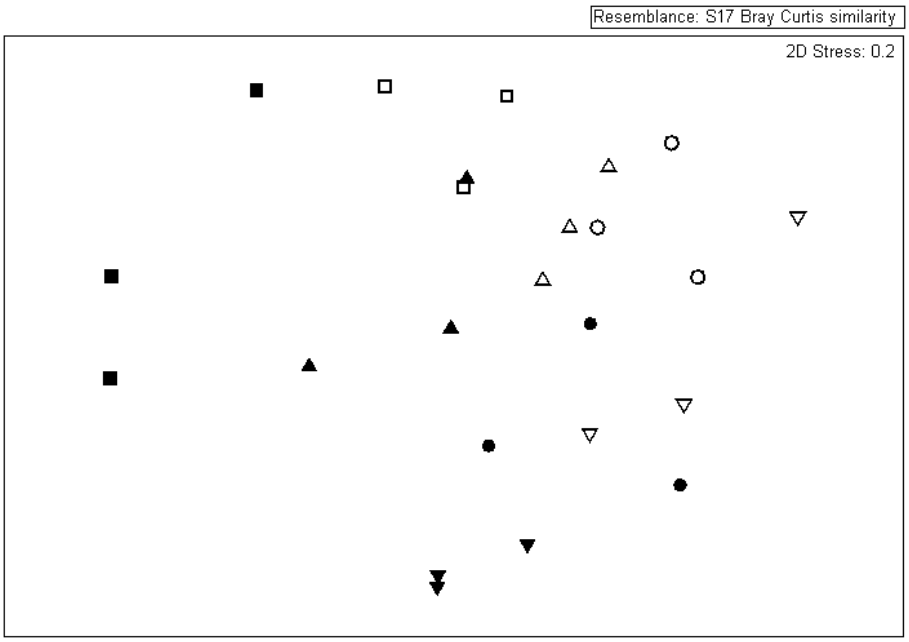
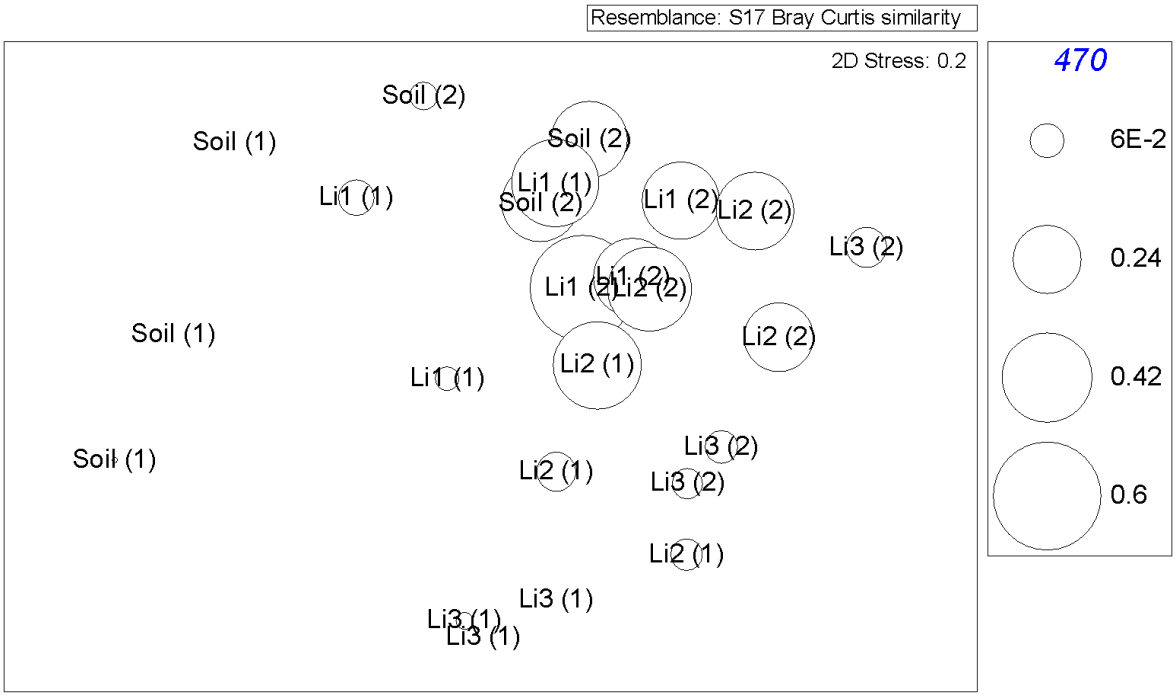


Figure 4.10. NMDS plot of the bacterial community structures in the once- and twice-amended treatments during 4 different enrichment series. Filled symbols denote the once-amended treatments and open symbols represent the twice-amended treatments. ■ □ Soil Enrichment; ▲ △ Liquid Enrichment 1; ● ○ Liquid Enrichment 2; ▼ ▽ Liquid Enrichment 3. Each data point represents an experimental replicate.

SIMPER analysis showed that the presence of the TRFs at 470 and 501 bp were responsible for a high level of the variation between the once- and twice-amended treatments. Each of these TRFs was responsible for approximately 12% of the total variation between the two treatment types. In the twice-amended treatment, TRF 470 bp was highly prevalent, particularly in the first three enrichments. In contrast, it had a relatively low prevalence in the once-amended treatments throughout the four enrichments (Figure 4.11a). Similarly, TRF 501 bp was more prevalent in the twice-amended treatment than in the once-amended treatment (Figure 4.11b). However, in contrast to TRF 470 bp, the presence of TRF 501 bp increased markedly in enrichments 3 and 4 compared with enrichments 1 and 2.

a)



b)

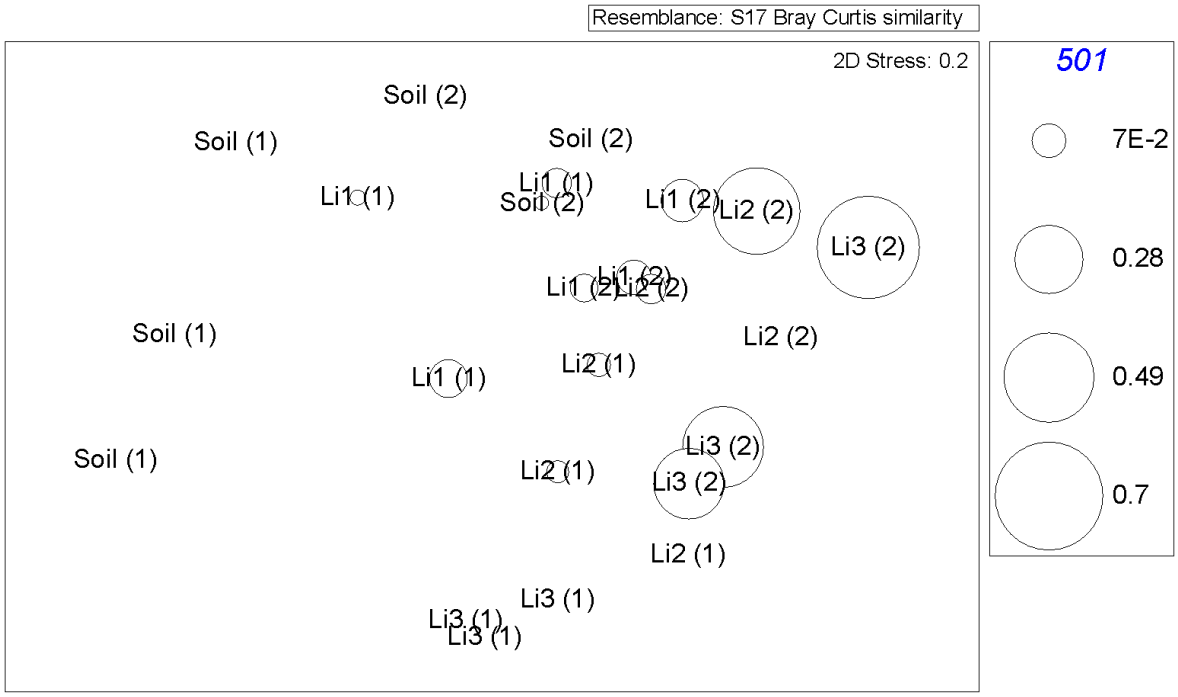


Figure 4.11. Bubble plot of TRF a) 470 and b) 501 bp relative abundance in bacterial samples taken from the 1 soil and 3 liquid enrichment series 1-month post application. Each data point represents and experimental replicate. Soil = Soil Enrichment; Li1 = Liquid Enrichment 1; Li2 = Liquid Enrichment 2; Li3 = Liquid Enrichment 3. Numbers in brackets denote the once- (1) and twice-amended (2) treatments. The numbers in the key denote the relative abundance of the TRF in a particular trace.

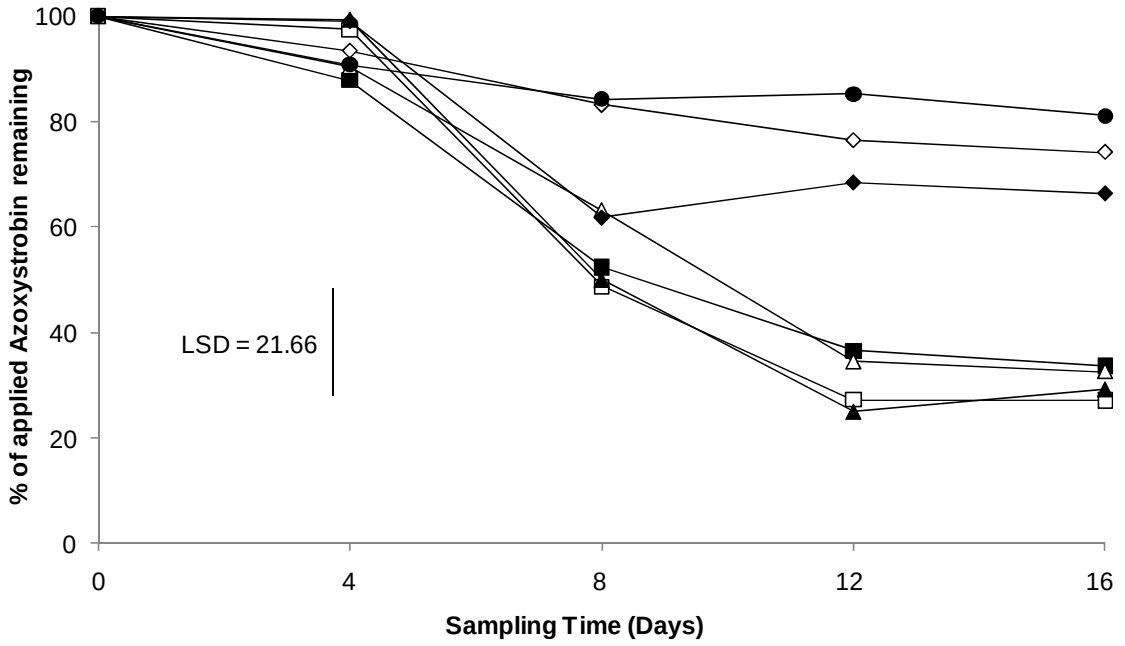
4.3.3 Detailing the Maximum Dilution for Azoxystrobin Degradation in Liquid Cultures

In order to investigate the effects of inoculum cell number on azoxystrobin degradation in the initial once- and twice amended treatments, aliquots taken from the third liquid cultures after 4 weeks were serially diluted ten-fold to concentrations from 1 x 10^{-1} to 1 x 10^{-7} that of the original culture. These were then used in a further liquid culture degradation assay which was sampled every 4 days for 16 days.

In the once-amended treatments (Figure 4.12a) there was a clear difference between the percentages of azoxystrobin recovered, particularly at the higher dilutions. Azoxystrobin recovery was not significantly different in the 1 x 10^{-1} to 1 x 10^{-4} dilutions throughout the experimental period. The percentage of the applied azoxystrobin recovered from these treatments after 16 days ranged from 27 to 34%. However, the percentage recovered was significantly higher (indicating that degradation was significantly lower) for the 1 x 10^{-5}, 1 x 10^{-6}, and 1 x 10^{-7} treatments with values of 56, 74, and 81% recorded, respectively.

For the twice-amended cultures (Figure 4.12b), there was no significant difference in the amount of azoxystrobin recovered after 16 days in the dilutions ranging from 1 x 10^{-1} to 1 x 10^{-5}. This therefore indicates that the microbial community in the twice-amended cultures had a ten-time greater degradation potential than the once-amended treatments. The percentage of azoxystrobin recovered from these cultures at the end of the experiment ranged from 15 to 37%. There was no discernable reduction in recovery in the 10^{-6} and 10^{-7} treatments within the 16-day experimental period.

a)



b)

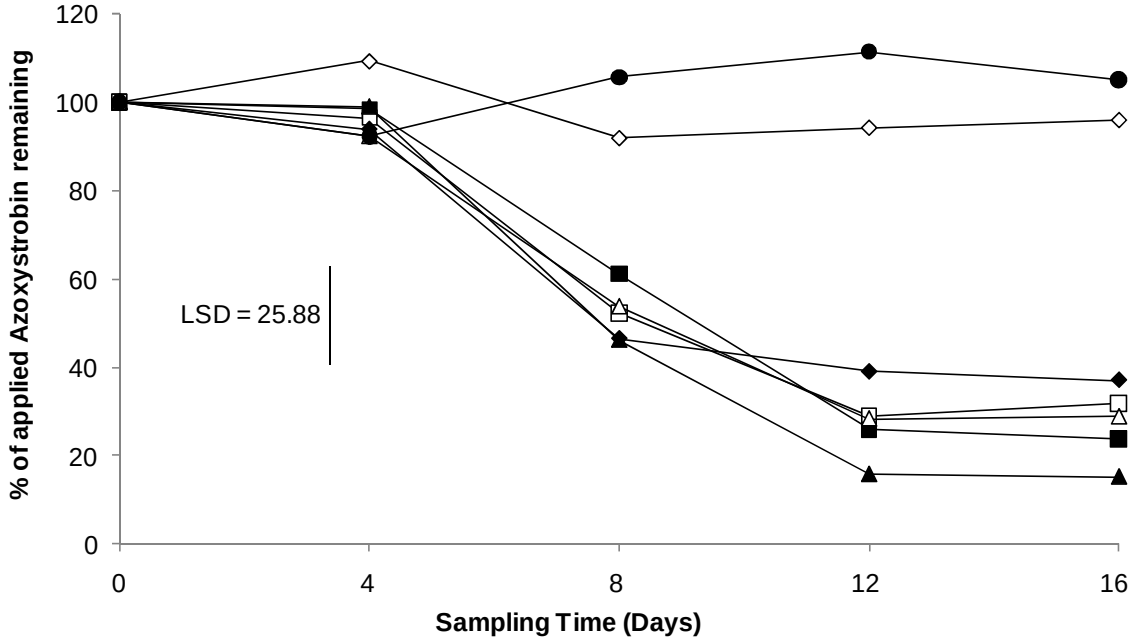


Figure 4.12. The percentage of azoxystrobin recovered from 10-fold dilutions of the initial (A) once- and (B) twice-amended liquid cultures. ■ 1 x 10^{-1} □ 1 x 10^{-2} ▲ 1 x 10^{-3} △ 1 x 10^{-4} ◆ 1 x 10^{-5} ◇ 1 x 10^{-6} ● 1 x 10^{-7}. Each data point represents the mean of 3 experimental replicates.

4.3.4 Single Colony and Plate Wash Degradation Assays

4.3.4.1 Assay 1

The results from the single colony degradation assays can be seen in Figure 4.14. There were a wide range of recovery percentages observed within the experimental samples. In 40 of the cultures, at least 90% of the applied azoxystrobin still remained after 1 month. Between 70 and 90% of the applied azoxystrobin was extracted from further 10 cultures. The remaining 15 cultures produced extracted azoxystrobin percentages ranging from 35% (colony 15) to 59% (colony 29) (Figure 4.13).

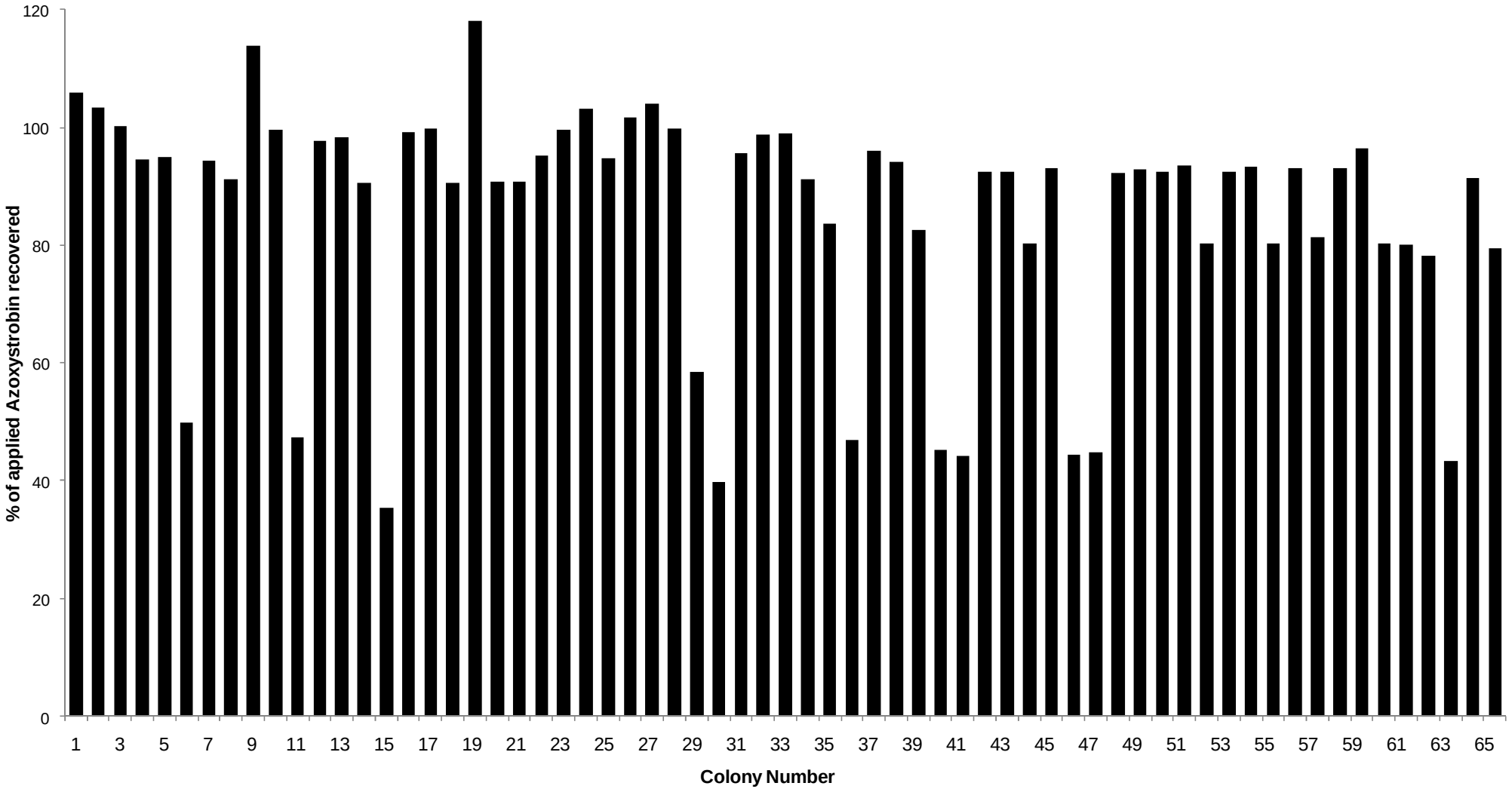


Figure 4.13. Percentages of azoxystrobin recovered after 1 month from the single colony assays. Samples 1-5 denote the un-inoculated controls and samples 6-65 denote the 60 single colony treatments.

The results of the plate wash treatments can be seen in Figure 4.14. Of the 10 different cultures, 5 had degraded between 56 and 58% of the applied azoxystrobin. The remaining 5 cultures degraded less than 10% of the compound each.

Recovery percentages for the negative control samples ranged from 95 to 106% of the applied azoxystrobin concentration.

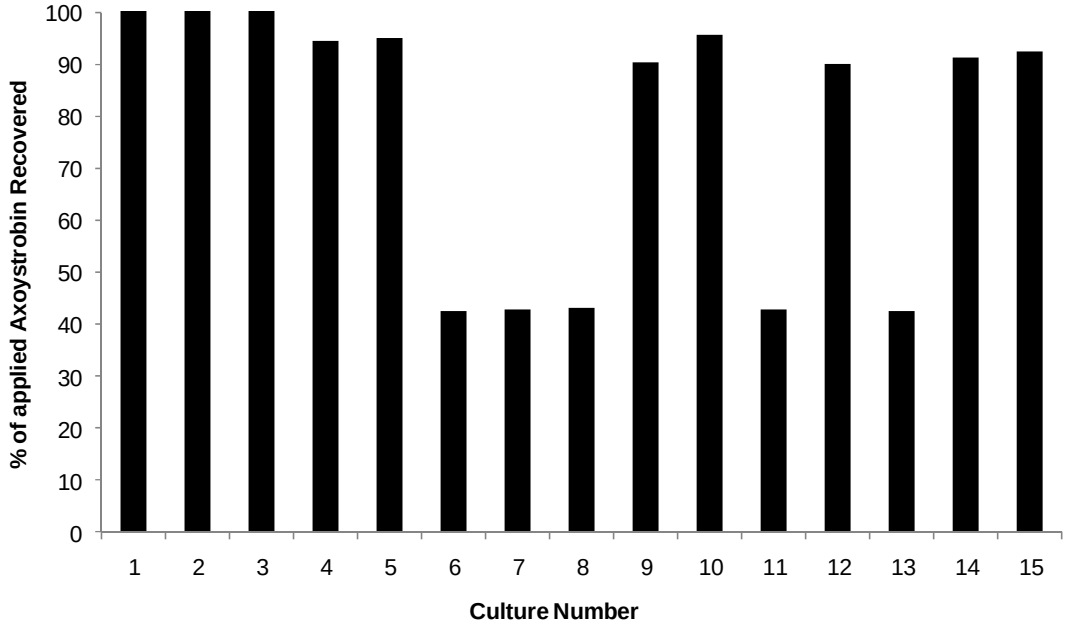


Figure 4.14. Percentages of azoxystrobin recovered after 1 month from the 10 plate wash cultures. Samples 1-5 refer to the uninoculated controls and samples 6-15 refer to the 10 plate wash cultures.

The 16 cultures with the lowest percentage recovery (11 single colonies and 5 plate washes), plus a further 5 selected from the 70 to 90% recovery range (all single colonies) were then re-plated onto MBSA + azoxystrobin for use in a follow-up colony degradation assay. The descriptions of these colonies can be seen in Table 4.2. Samples 1 to 16 refer to the single colony treatments, and samples 17 to 21 refer to the 5 plate wash treatments.

Table 4.2. The colony morphologies of the cultures used in Assay 2. Note that the colony number column refers to the original colony culture number from Assay

Colony Morphology						
Colony Number	**Colour**	**Form**	**Elevation**	**Margin Type**	**Halo Present**	**Colony Diameter (mm)**
1	Yellow	Circular	Raised	Entire	No	2.0
2	Yellow	Circular	Flat	Entire	No	5.0
3	White	Circular	Flat	Undulate	Yes	1.0
4	White/Grey	Circular	Raised	Entire	No	1.0
5	White/Grey	Circular	Raised	Entire	No	1.0
6	Yellow	Irregular	Raised	Entire	No	2.0
7	Grey	Circular	Raised	Entire	No	2.0
8	White	Irregular	Flat	Entire	No	2.0
9	White	Circular	Raised	Entire	Yes	0.5
10	Grey	Circular	Raised	Entire	No	0.5
11	White	Circular	Raised	Entire	Yes	0.5
12	White	Circular	Raised	Entire	No	0.5
13	Grey	Circular	Umbonate	Entire	No	0.5
14	White	Circular	Raised	Entire	No	0.5
15	White	Circular	Raised	Entire	No	0.5
16	Yellow	Circular	Raised	Entire	No	2.0
17	Mixed	Mixed	Mixed	Mixed	Mixed	Mixed
18	Mixed	Mixed	Mixed	Mixed	Mixed	Mixed
19	Mixed	Mixed	Mixed	Mixed	Mixed	Mixed
20	Mixed	Mixed	Mixed	Mixed	Mixed	Mixed
21	Mixed	Mixed	Mixed	Mixed	Mixed	Mixed

4.3.4.2 Assay 2

Overall, the percentages of azoxystrobin recovered were markedly lower in this assay than in the previous one (Figure 4.15).

Seven of the single colony cultures (culture numbers 10, 11, 13, 15, 16, 19 and 20) showed degradation of between 30 and 35% of the applied azoxystrobin. Between 50 and 60% of the compound was extracted from a further seven treatments (culture numbers 6, 8, 9, 14, 17, 18 and 21). The remaining two single colony cultures degraded 85 and 91% of the azoxystrobin during the experimental period (cultures 12 and 7, respectively).

For the plate wash cultures, three degraded between 55 and 60% of the azoxystrobin (cultures 22,23 and 24). Additionally, one culture (culture number 25) degraded 90% of the compound. This contrasts markedly with the fifth culture (culture number 26) which only degraded 40% of the azoxystrobin. The recovery of azoxystrobin from the uninoculated controls varied from 95 to 103% of the applied compound.

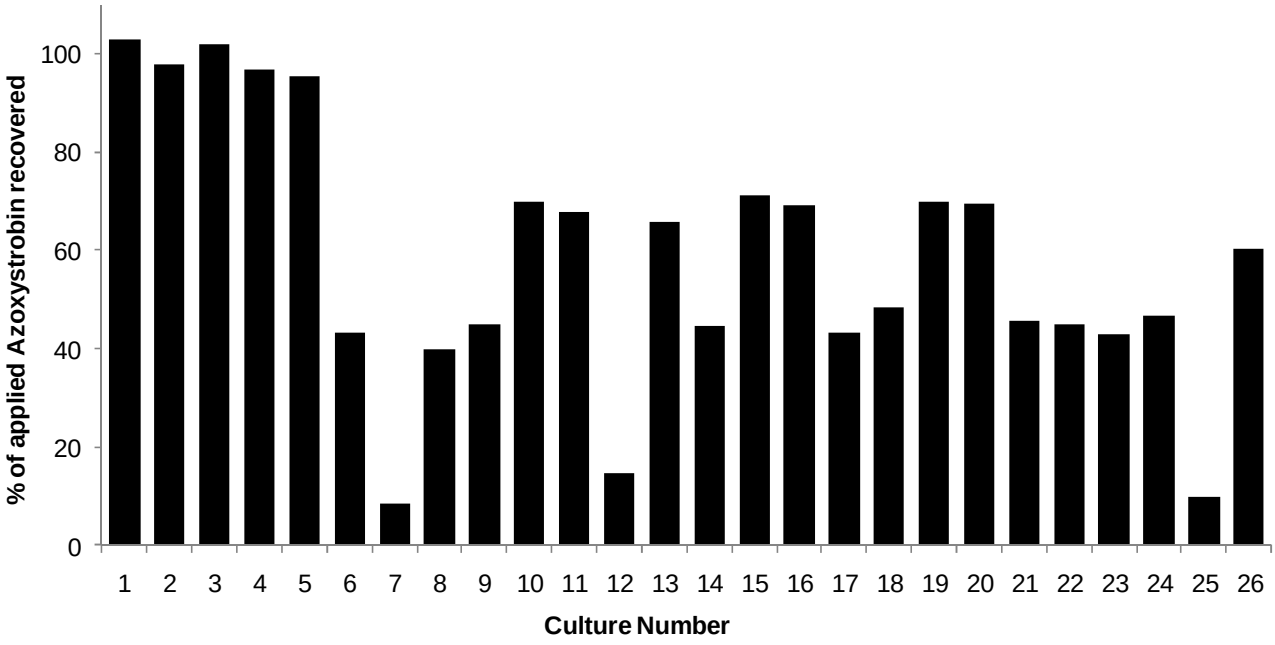
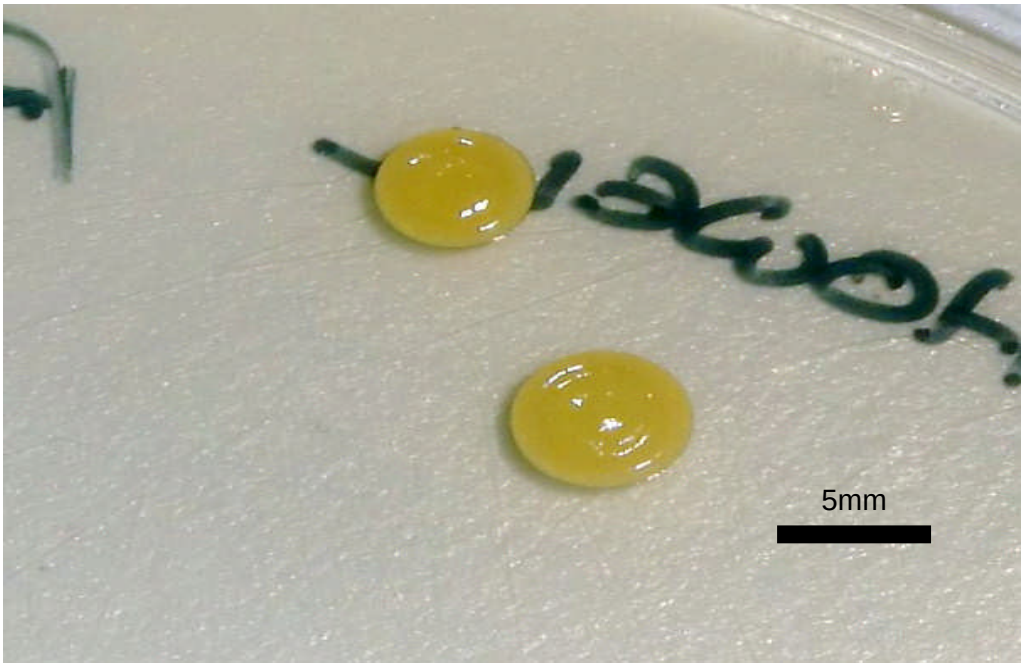


Figure 4.15. Percentages of azoxystrobin recovered after 1 month from the liquid culture degradation assays. Samples 1-5 represent the uninoculated controls; cultures 6-21 represent the single colony treatments; cultures 22-26 represent the plate wash treatments.

4.3.5 Colony Sequencing and Identification

Photographs of colonies grown for 10 days from cultures 7 and 12 can be seen in Figures 4.16a and 4.16b, respectively. The colonies from culture 7 were relatively large (5 mm in diameter). They were circular, yellow in colour and had a ridged surface. In contrast, the colonies from culture 12 were smaller, and white/grey in colour. The surface of the colonies was smooth and raised. Neither of the two colony types produced any kind of halo in the agar.

a)



b)

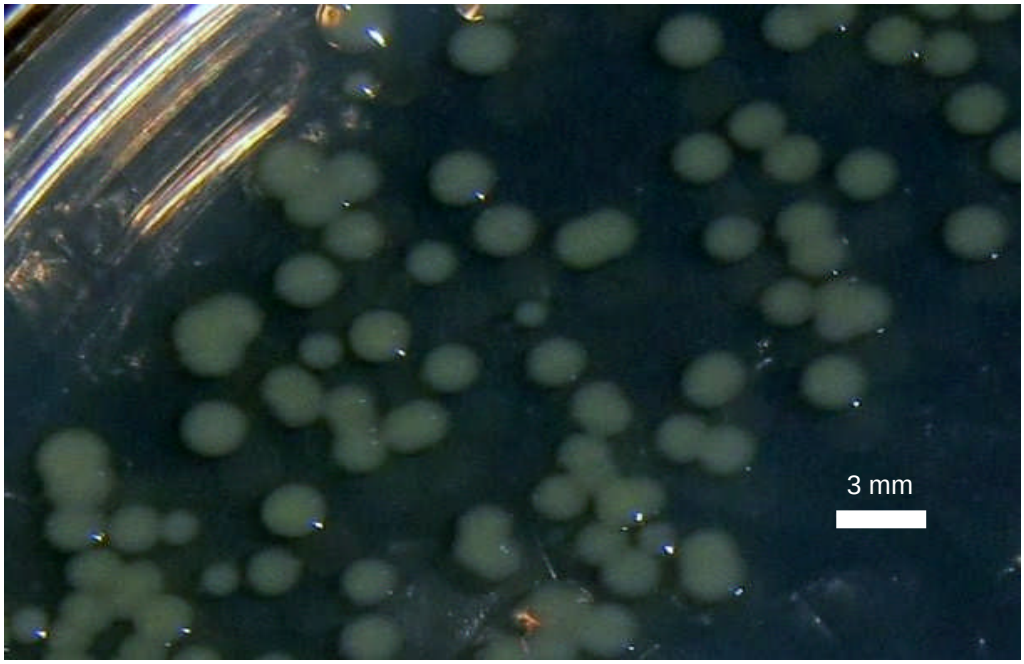


Figure 4.16. Colonies from degradation culture numbers 7 a) and 12 b) grown on MBSA with azoxystrobin as the sole carbon source. The plates were inoculated on the 4th of March 2011, and were photographed on the 14th of March 2011

BLAST searches of the 16S rRNA gene sequence determined that the colonies from culture sample 7 showed 98% sequence homology to its closest hit from the search, an uncultured *Rhodanobacter* sp. (JF968486.1). Colonies from sample 12 had a 99% sequence similarity to its top hit, *Cupriavidus* sp. B-8(2011) (JN128831.1). The sequences were submitted to the EMBL database under the accession numbers HE576771.1 and HE598561.1 for the *Cupriavidus*, and *Rhodanobacter*, respectively. Figure 4.17 compares the sequence similarities of the two strains isolated in this chapter with the 10 most similar sequences from BLAST searches. The *Cupriavidus* sp. isolate grouped closely with *Cupriavidus* sp. B-8 (2011) (JN128831.1) whilst the *Rhodanobacter* sp. isolate grouped closest with the uncultured *Rhodanobacter* sp. clone IAFIL35 (DQ145582.2) (Figure 4.17).

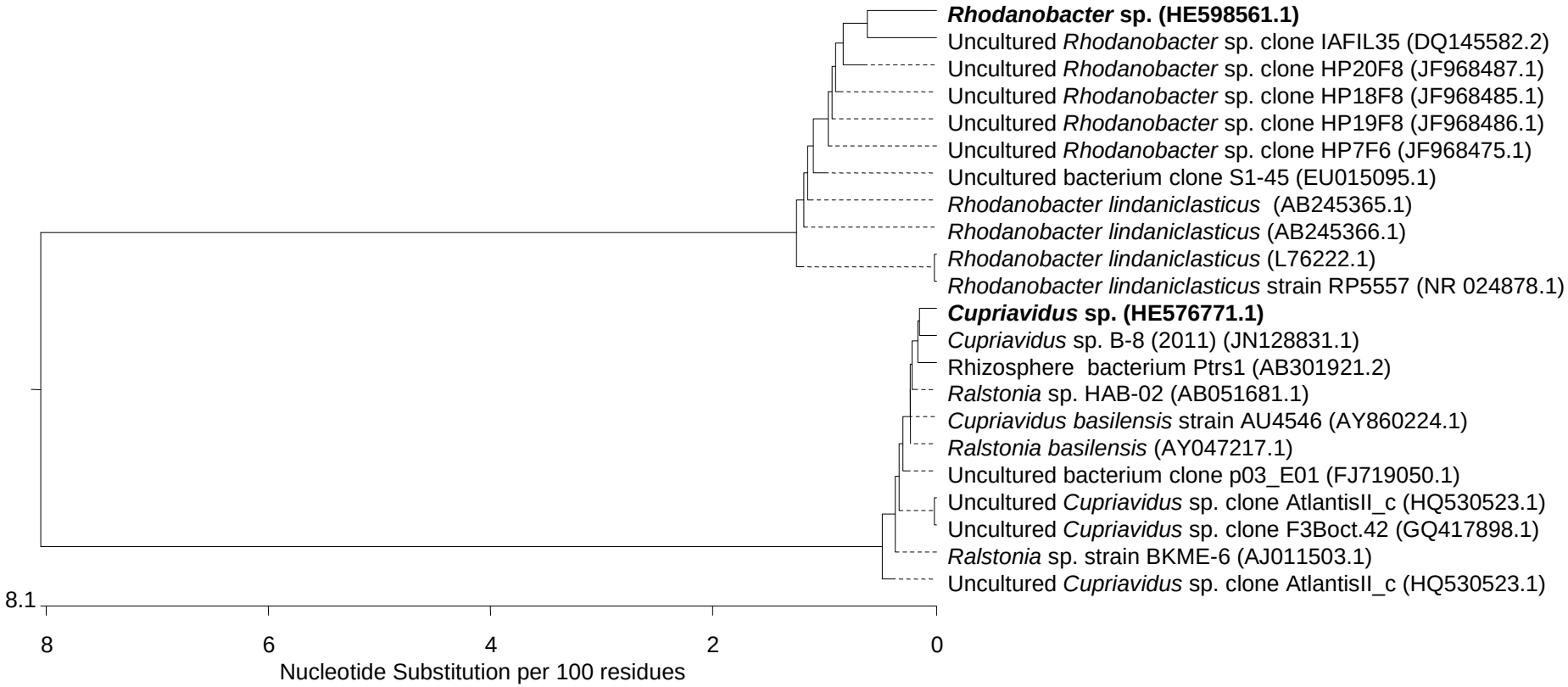


Figure 4.17. A phylogenetic tree showing 16S rRNA gene sequence similarities between the *Cupriavidus* sp. and *Rhodanobacter* sp. strains isolated in this experiment, and their 10 closest matches from the BLAST search database. Sequences were aligned using the Clustal V Method and bootstrapping was performed using 1000 trials. Numbers in brackets denote the sequence accession number from the BLAST search database. The two entries marked in **bold** type denote the two strains isolated in this chapter.

4.3.6. Bacterial Clone Library Production

A 16S rRNA bacterial clone library was produced to identify the bacterial genera responsible for the TRFs that increased following azoxystrobin application in this chapter (470 and 501 bp), and in Chapter 2 (470, 472, and 814 bp). The library was produced using samples from the 25 mg kg^{-1} (1 month) treatment of the experiment described in Chapter 2. The clone library consisted of a total of 88 individual sequences.

Figure 4.18 shows the collector's curve obtained from the bacterial clone library. There was a steady increase in the number of unique clones found in the library. None of the final 12 sequences obtained represented unique clones suggesting that the majority of the community diversity had been accounted for within the library. However, analysis using the Chao1 estimate of population of size suggested that only 43% of the total community diversity had been accounted for in the clone library.

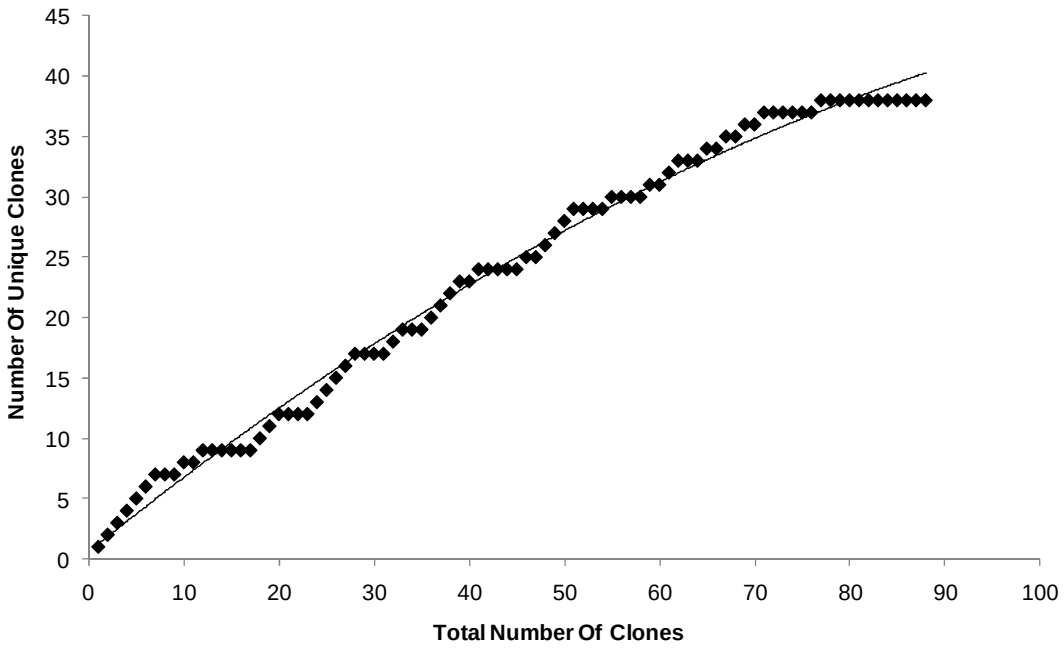


Figure 4.18. A Collector's curve for the bacterial clone library produced using the 25 mg kg^{-1} (1 Month) DNA samples from the experiment described in Chapter 2. The library was produced using the bacterial primer pair 63f/1087r.

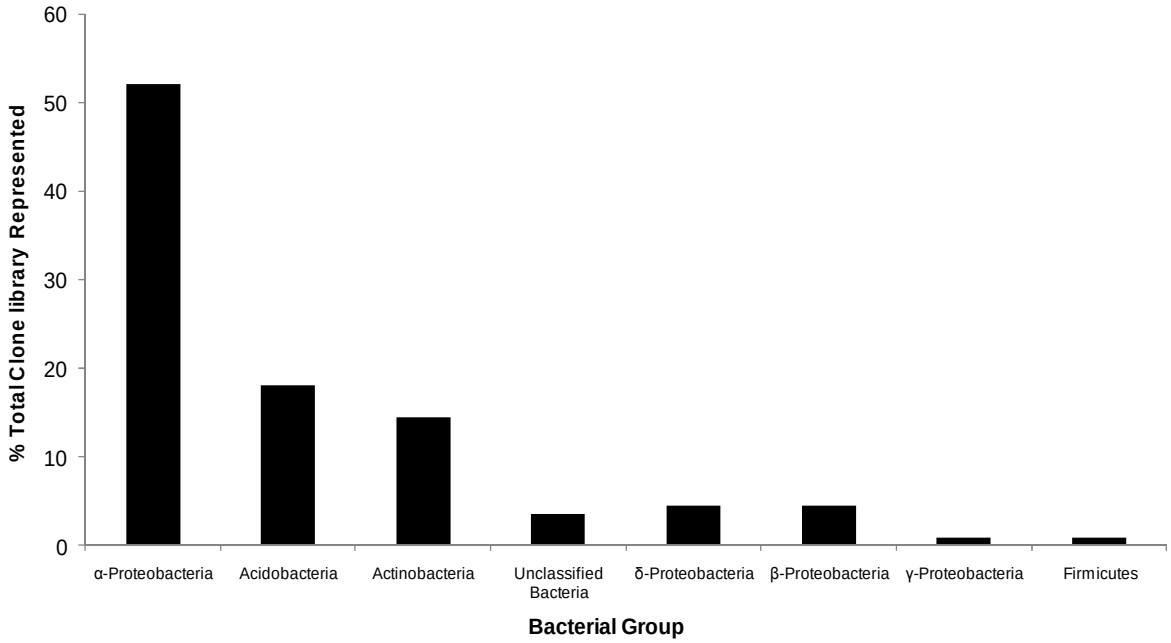


Figure 4.19. The structure of the bacterial clone library produced from the using the 25 mg kg^{-1} (1 month) DNA samples from the experiment detailed in Chapter 2.

Blast searches showed that 19.5% of these were unclassified Rhizobiales (α-proteobacteria), 13.5% Acidobacteria Gp_6, and 7% unclassified α-proteobacteria. The most abundant named genera were *Rhodococcus* sp., *Pseudolabrys* sp., *Streptomyces* sp, *Sphingomonas* sp., and *Bradyrhizobium* sp. each of which represented 3.5% of the total clone library. α-proteobacteria represented 52% of the sequences in the cone library, with acidobacteria and actinobacteria also being prevalent (18.0% and 14.5%, respectively) (Figure 4.19). No sequences that showed a high homology to either *Cupriavidus* sp. or *Rhodanobacter* sp. were found within the library.

In order to link the isolates obtained from the liquid culture assays to the bacterial T-RFLP and clone library data two types of analyses were carried out. Firstly, the clone library sequences were analysed to identify which would have produced TRFs that were the same size as the TRFs that increased in relative abundance following azoxystrobin application using the restriction enzymes *Hha*I. Secondly, the sequences of the *Cupriavidus* sp. and *Rhodanobacter* sp. isolates were analysed to produce predicted TRF sizes. These predicted TRF sizes were then compared with the obtained T-RFLP data.

Three of the sequences would have produced TRFs within the 470-472 bp size range using the *Hha*I restriction enzyme. Two of these sequences showed a 100% sequence homology to the genus *Rhodococcus* sp. and would have both produced a TRFs at 470 bp. The third sequence had a 95% sequence homology to the genus *Methylibium* sp. and would have produced a TRF of 471 bp. Using the restriction enzyme *Msp*I, one of the sequences would have produced a TRF of 499 bp. This sequence had a 100% homology to the gamma proteobacterial genus *Haliea*. None of the sequences obtained in the clone library would have given a TRF of 814 bp using either of the restriction enzymes. TRFs of 418 and 322 bp would have been produced by the *Cupriavidus* sp. and *Rhodanobacter* sp. isolates, respectively. However, the relative abundances of these TRFs in the T-RFLP data did not correlate with an increased presence of azoxystrobin.

4.3.7. The Effects of Initial Bacterial Inoculum Concentration on Azoxystrobin Degradation.

The two azoxystrobin degrading cultures were analysed to determine the impacts of the initial cell concentration on the degradation of the compound. Two-way ANOVAs showed that azoxystrobin recovery was not significantly different for the two organisms tested (p = 0.797). Conversely, the initial concentration of cells added to the culture did significantly impact upon compound recovery (p = < 0.01).

After 4 days; only 31% of the applied azoxystrobin was recovered from the 10^{10} cells ml^{-1} *Rhodanobacter* sp. cultures (Figure 4.20). This was significantly lower than in any of the other cultures as determined by LSD analysis. From 8 days onwards, there was no significant difference in azoxystrobin recovery for the 10^6 and 10^{10} treatments for either organism.

Compound recovery was significantly higher, however in the 10^2 cells ml^{-1} samples after 8 and 12 days for both organisms compared with the 10^6 and 10^{10} treatments. The average DT_{50} values for the *Cupriavidus* sp. cultures were 9.4, 5.6, and 4.4 days for the 10^2, 10^6, and 10^{10} treatments, respectively.

An average DT_{50} value of 9.0 days was recorded for the 10^2 cells ml^{-1} *Rhodanobacter* sp. cultures. This compared with 5.4 and 2.9 days for the 10^6 and 10^{10} cells ml^{-1} treatments. There was no significant difference between the average DT_{50} values recorded for the *Cupriavidus* sp. and *Rhodanobacter* sp. culture.

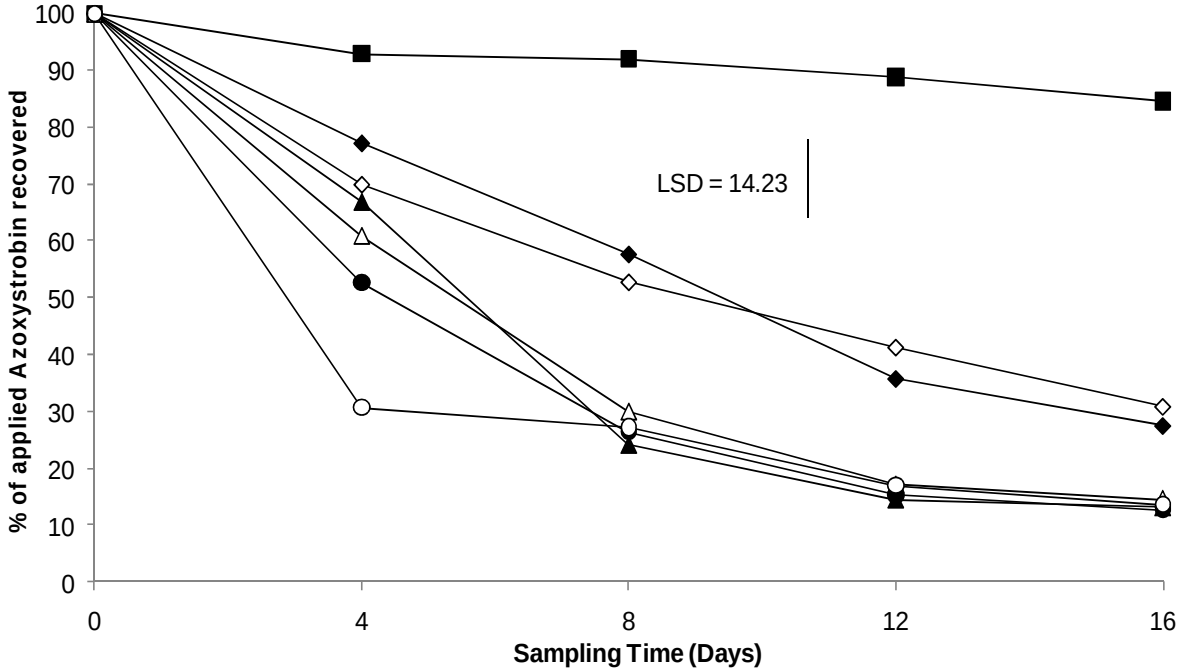


Figure 4.20. Percentages of applied azoxystrobin recovered from *Cupriavidus* sp. and *Rhodanobacter* sp. liquid cultures inoculated with 3 different starting cell concentrations. ■ Control ◆ *Cupriavidus* sp. 10^2 ◇ *Rhodanobacter* sp. 10^2 ▲ *Cupriavidus* sp. 10^6 △ *Rhodanobacter* sp. 10^6 ● *Cupriavidus* sp. 10^{10} ○ *Rhodanobacter* sp. 10^{10}. Control = uninoculated control samples. Each data point represents the average of 3 experimental replicates.

4.3.8. The Effects of Solid Growth Media Type on Azoxystrobin Degradation

The impact of solid growth media type on the recovery of azoxystrobin from *Cupriavidus* sp. and *Rhodanobacter* sp. cultures were analysed in a subsequent degradation assay.

2-way ANOVA analyses showed that the bacterial strain significantly affected the percentage of azoxystrobin recovered (p = 0.01), but the growth media type did not (p = 0.100). The significant difference recorded when comparing the two strains could be attributed to the *Rhodanobacter* sp.

samples that were grown on LB agar (Figure 4.21). There were significantly higher percentages of the applied azoxystrobin recovered after 8, 12, and 16 days with this treatment relative to the other media. There were no significant differences between any of the other treatments.

The average DT_{50} values recorded for the *Cupriavidus* sp. treatments ranged from 2.7 to 3.1 days. This compared with 3.0 to 4.5 days for the *Rhodanobacter* sp. cultures.

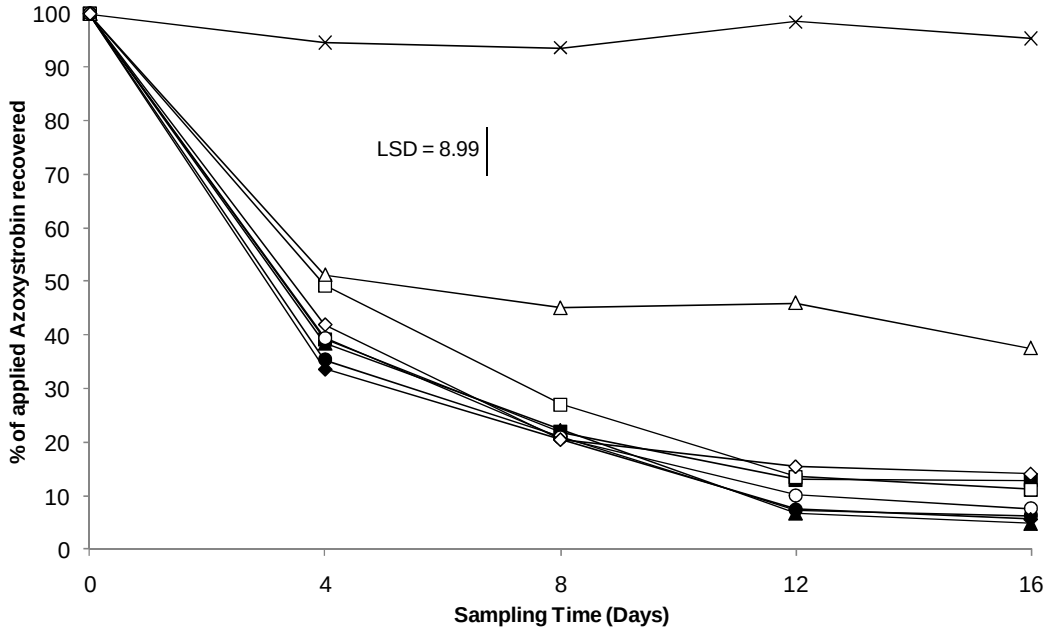


Figure 4.21. Percentages of applied azoxystrobin recovered from liquid cultures of *Cupriavidus* sp. and *Rhodanobacter* sp. initially grown on 4 different types of solid media. ■ *Cupriavidus* sp. MSM □ *Rhodanobacter* sp. MSM ▲ *Cupriavidus* sp. LB △ *Rhodanobacter* sp. LB ● *Cupriavidus* sp. NA ○ *Rhodanobacter* sp. NA ◆ *Cupriavidus* sp. R2A ◇ *Rhodanobacter* sp. R2A.Each data point represents the average of 3 experimental replicates.

4.3.9 The Effects of Carbon and Nitrogen Availability on Azoxystrobin Degradation

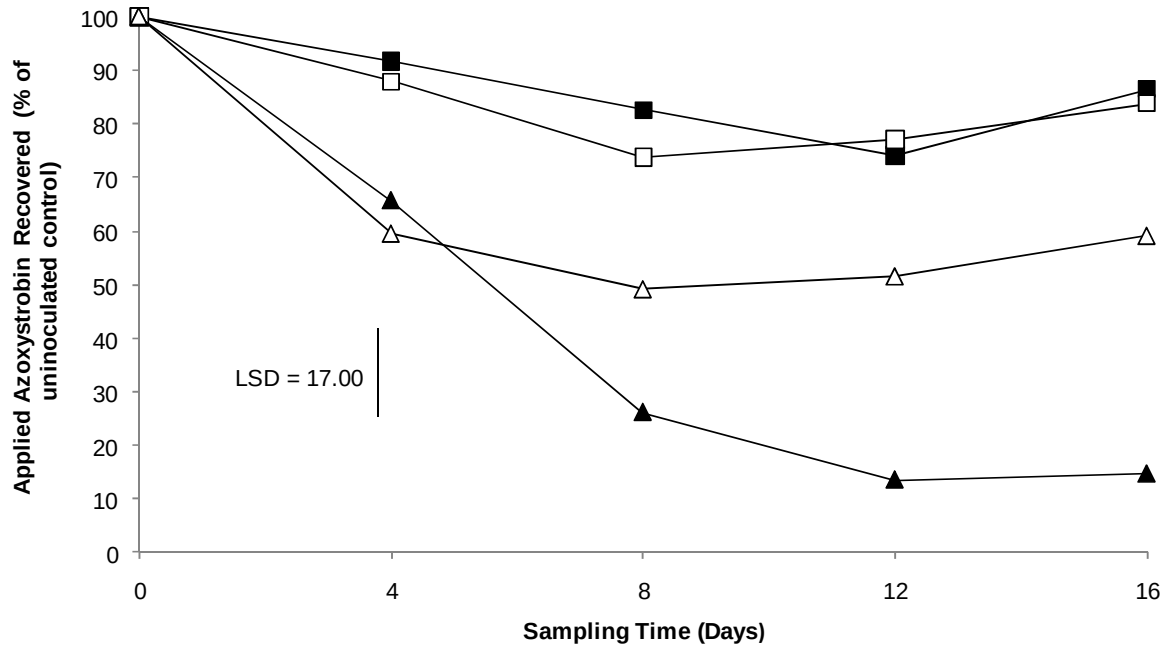
The effects of carbon and nitrogen source availability on the recovery of azoxystrobin from *Cupriavidus* sp. and *Rhodanobacter* sp. cultures were determined.

The bacterial strain did not have a significant impact on the percentage of azoxystrobin recovered ($p = 0.907$) but the presence or absence of carbon or nitrogen sources did ($p = < 0.01$).

Figures 4.22a and 4.22b illustrate this strong similarity in azoxystrobin recovery between the two strains. In both cases the lack of an additional nitrogen source resulted in the minimal loss of the parent compound regardless of whether an additional carbon source was present or not. Azoxystrobin loss was greatest in the samples where the pesticide was present as the sole carbon source and an additional nitrogen source was supplied. In the *Cupriavidus* sp. cultures (Figure 4.22a) only 14.5% of the applied compound was recovered after 16 days. In the *Rhodanobacter* sp. cultures this percentage was even lower at 11.5% (Figure 4.22b).

The addition of an alternative carbon source to the cultures significantly reduced the loss of the parent compound within the experimental period. In the *Cupriavidus* sp. cultures 59.0% of the applied azoxystrobin was recovered in the presence of an alternative carbon source. This compared with 61.0% for the *Rhodanobacter* sp. samples.

a)



b)

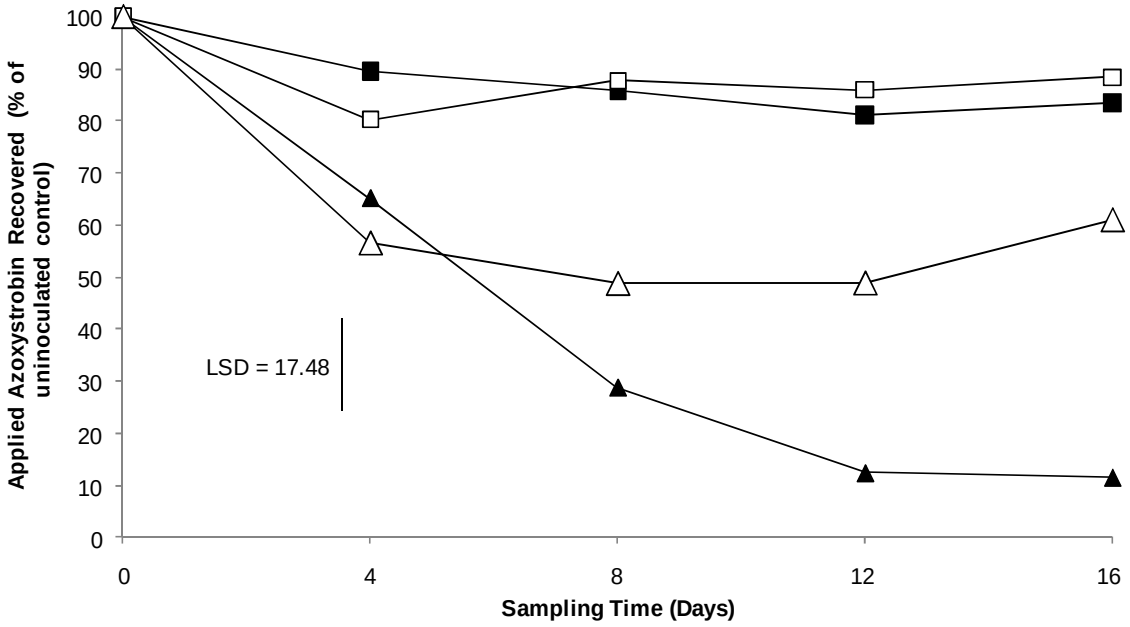


Figure 4.22. The percentage of azoxystrobin recovered from a) *Cupriavidus* sp. and b) *Rhodanobacter* sp. liquid cultures under different carbon and nitrogen source treatments. ■ – C – N □ + C – N ▲ - C + N △ + C + N. Each data point represents the mean of 3 experimental replicates.

4.3.10 The Degradation of Three Alternative Strobilurin Fungicides by Azoxystrobin-Degrading Cultures

The ability of the *Cupriavidus* sp. and *Rhodanobacter* sp. to remove azoxystrobin from liquid cultures was compared with three other strobilurin fungicides (pyraclostrobin, trifloxystrobin, and kresoxim methyl).

As with the previous experiment, the results when the two organisms were compared were not significantly different (p = 0.235). However, pesticide dissipation was significantly impacted by the particular compound applied (p = < 0.01).

The degradation kinetics for all four of the compounds were similar for both of the bacterial strains (Figure 4.23). There was a lag in degradation in the first 4 days post-application. This was followed

by a rapid degradation phase between 4 and 10 days. Following this, the degradation rate decreased markedly and had stopped by 14 days.

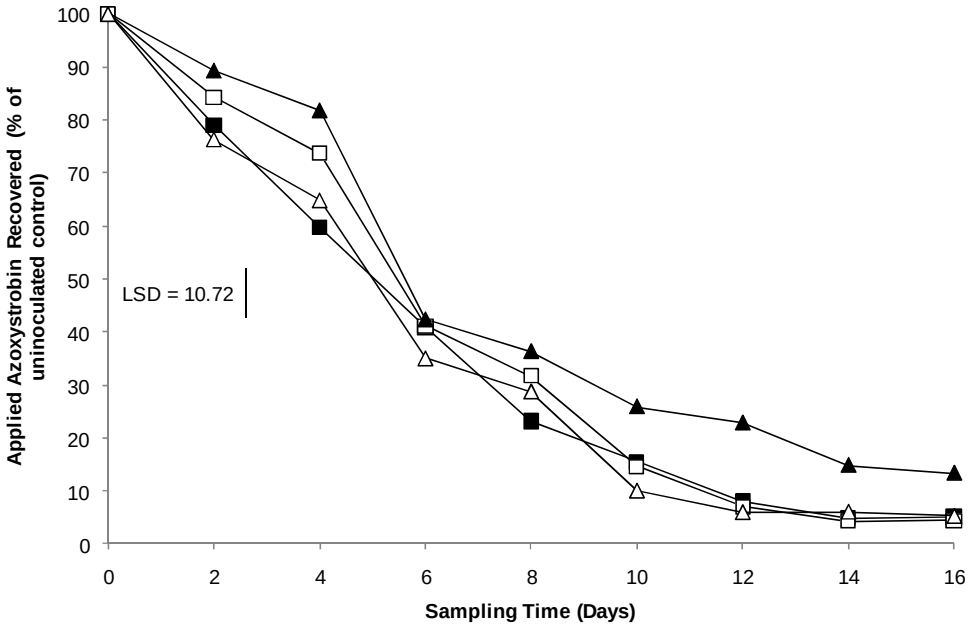
More specifically, for the *Cupriavidus* sp. cultures (Figure 4.23a) there was a significantly higher degradation of pyraclostrobin and trifloxystrobin 4 days post-amendment compared with azoxystrobin and kresoxim methyl. Additionally, from 10 to 14 days post-amendment there were significantly greater percentages of trifloxystrobin remaining compared with the other three compounds. The average DT_{50} values were 4.7, 5.1, 6.0, and 5.1 days for the azoxystrobin, pyraclostrobin, trifloxystrobin, and kresoxim methyl, respectively.

There were also some significant differences between the pesticide treatments in the *Rhodanobacter* sp. cultures (Figure 4.23b). At the 4 day sampling point the percentage of trifloxystrobin (77%) recovered was significantly higher than that recorded for azoxystrobin (54%). The percentage of trifloxystrobin recovered was also significantly higher than the other three treatments at the 12 day sampling point. In contrast to the *Cupriavidus* sp. treatments, no significant differences were observed after 10 or 14 days.

Average DT_{50} values of 4.5 and 5.3 days were recorded for the azoxystrobin and pyraclostrobin, respectively. These compared with values of 5.4 days for trifloxystrobin and 4.8 days for kresoxim methyl.

In the uninoculated control cultures the amounts of pesticide remaining after 16 days range from 96 to 90% for azoxystrobin, 103 to 91% for pyraclostrobin, 94 to 92% for trifloxystrobin, and 102 to 92% for kresoxim-methyl (data not shown).

a)



b)

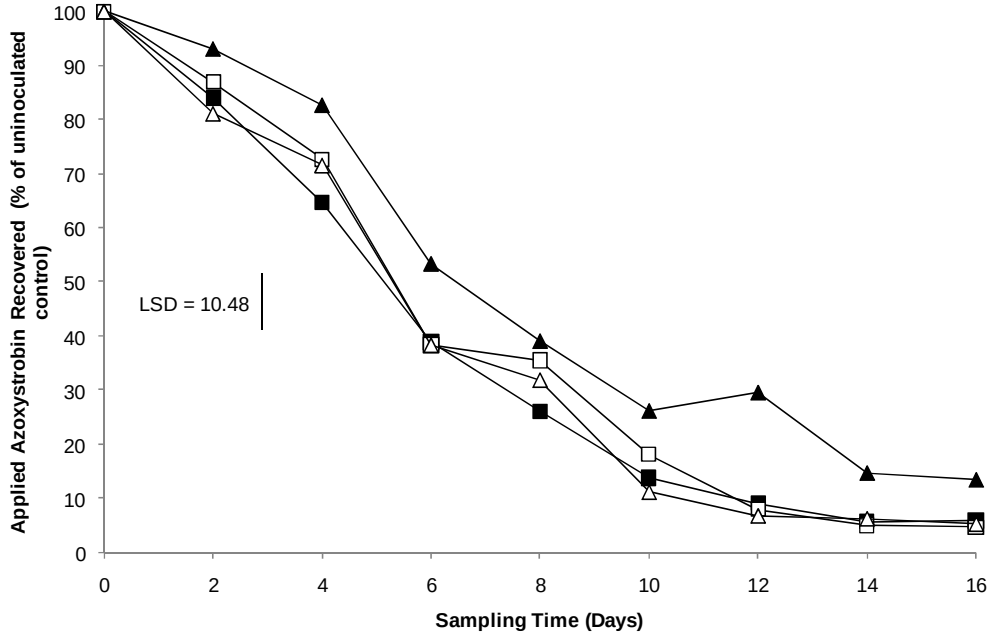


Figure 4.23. The percentage of 4 different strobilurin fungicides from individual (A) *Cupriavidus* sp. and (B) *Rhodanobacter* sp. liquid cultures. ■ Azoxystrobin □ Pyraclostrobin ▲ Trifloxystrobin △ Kresoxim-methyl

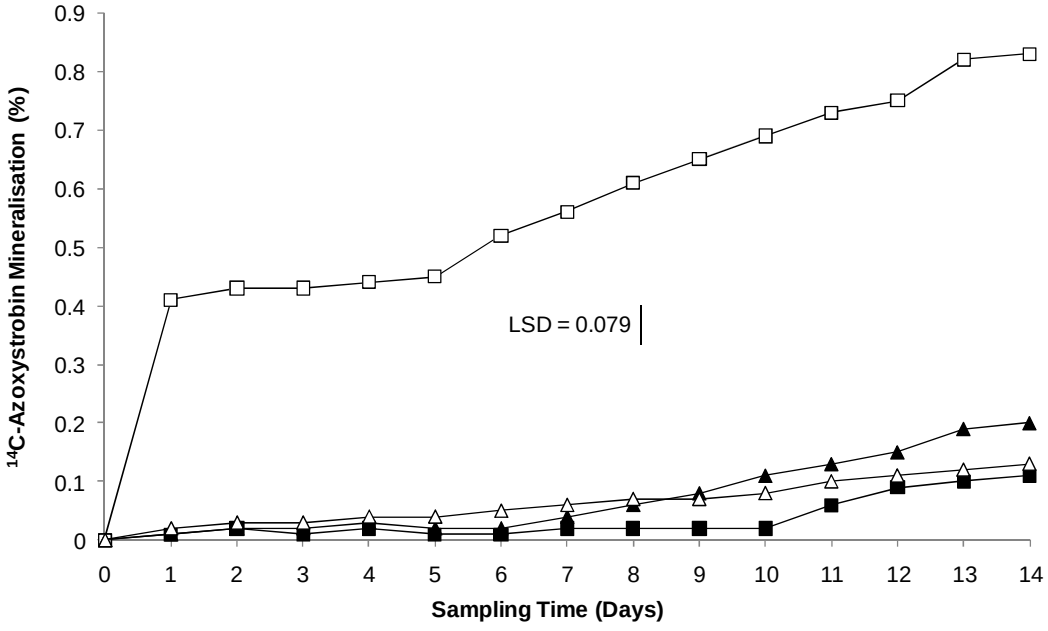
4.3.11. The Mineralisation of Azoxystrobin by Two Degrader Cultures

The abilities of liquid monocultures of *Cupriavidus* sp. and *Rhodanobacter* sp., along with a mixed culture, to fully mineralise ^{14}C-labelled azoxystrobin to $^{14}CO_2$ were monitored using liquid scintillation counting.

The results showed only very low levels of mineralisation in all of the treatments (Figure 4.24a). The percentage of azoxystrobin mineralised in the *Cupriavidus* sp. cultures after 14 days was 0.83% compared with 0.20% and 0.13% for the *Rhodanobacter* sp. and mixed cultures respectively. Indeed, as the purity of the azoxystrobin used was only > 98% purity, it could not be confirmed that the $^{14}CO_2$ evolved resulted from the mineralisation of azoxystrobin.

HPLC analysis of azoxystrobin degradation from the three cultures showed similar kinetics to the previous experiment (Figure 4.24b). There was no significant difference in azoxystrobin degradation between the three culture types (p = 0.847). The total amount of azoxystrobin degraded after 14 days ranged from 88.5 to 90%.

a)



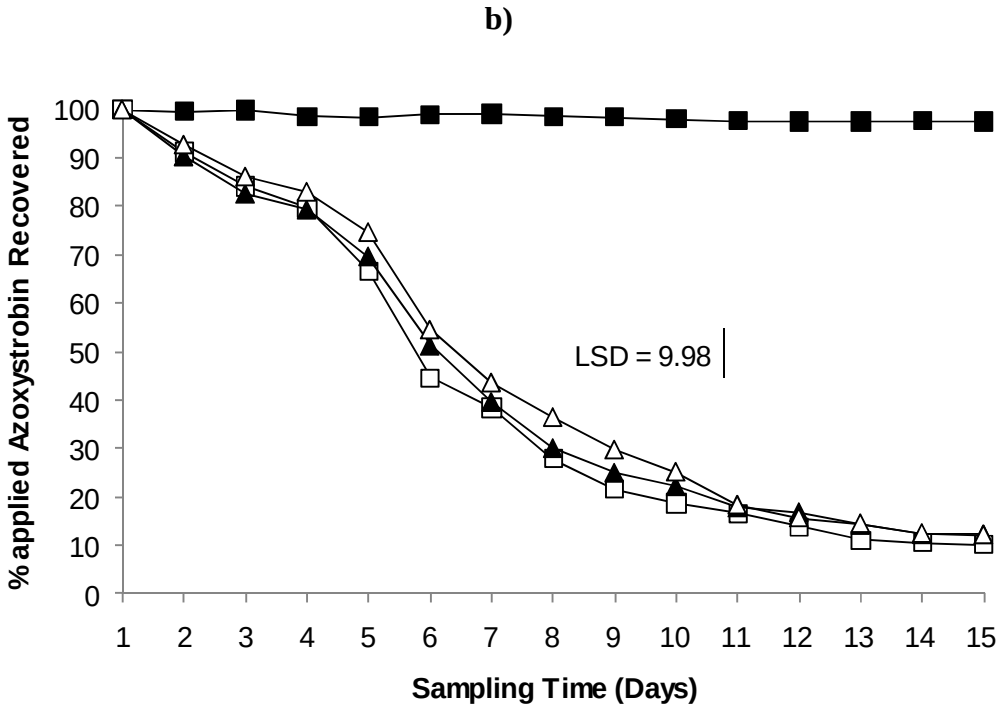


Figure 4.24. The percentage of (A) ^{14}C-azoxystrobin mineralised to $^{14}CO_2$ and (B) the degradation of azoxystrobin as determined using HPLC by 3 different degrader cultures. ■ Uninoculated control □ *Cupriavidus* sp. ▲ *Rhodanobacter* sp. △ *Cupriavidus* sp. + *Rhodanobacter* sp. Each data point represents the average of 3 experimental replicates.

4.4 Discussion

4.4.1 Sequential Enrichments of Azoxystrobin-Degrading Cultures

In the previous amended soil cultures, twice as much azoxystrobin had been degraded after 1 week, compared with the previously un-amended treatment. The degradation rates and total amounts of azoxystrobin degraded increased over the course of the three liquid enrichments. In enrichments 2 and 3 DT_{50} values of 2.48 and 2.22 weeks respectively were recorded. Degradation up to this point was quicker in the previously amended cultures with values of 1.78, 1.96, and 1.62 weeks recorded for the three enrichments.

These values are significantly lower than the values previously recorded for azoxystrobin within soil environments. In the work of Bending *et al.* (2007) the DT_{50} of a 5 mg kg^{-1} azoxystrobin dose within microcosms of Hunts Mill soil was between 8 and 12 weeks. Joseph (1999) also previously identified the DT_{50} value of the compound as being between 8 and 12 weeks in non-sterile, aerobic soils. In 2009, Ghosh and Singh observed the DT_{50} values of azoxystrobin in an Indian sandy loam soil as being between 9 and 15 weeks in non-composted soils, and between 5 and 10 weeks in soils amended with 5% compost. However, the results of work carried out by Adetutu *et al.* (2008) would have put the DT_{50} value for azoxystrobin as being between 3 and 7 days in both light and dark microcosms.

In the initial soil enrichment, there was a significantly higher degradation of azoxystrobin in the twice-amended treatment compared with soils that had only received one amendment. In the first liquid enrichment there was a clear lag in degradation before an increase between 1 and 2 weeks post-application. These degradation kinetics are characteristic of growth-linked degradation with the lag representing a growth/adaptation phase for the degrader organisms, which was followed by a period of rapid degradation as the biomass of the degrader organisms continued to increase The lack of a lag phase in the twice-amended treatment suggests that in these samples the organisms responsible for azoxystrobin were present in higher concentrations. This appears to indicate that these soils were more highly adapted to degrade the compound than the once-amended treatment. Both treatments exhibited a lag phase at the start of the second liquid, but neither did at the start of the third. This suggests that by the third liquid enrichment the azoxystrobin-degrading organisms were sufficiently enriched to enable degradation to occur immediately, without the lag phase normally associated with growth-linked metabolism. Furthermore, following the sequential enrichments it was observed that the twice-amended cultures could maintain their degradative ability at a ten times higher dilution (10^{-5}) than the once-amended treatment (10^{-4}). This suggests that the twice-ameded cultures were more highly adapted for azoxystrobin degradation than the once-amended treatements.This would indicate that the community had adapted to the presence of the compound. These results also backed

up the observation that the twice-amended treatments had lower DT_{50} values than the once-amended cultures.

In the first liquid culture enrichment cycle, there were significant differences in the percentages of azoxystrobin recovered in previously un-amended and amended soils. However, over the course of the subsequent two enrichments these differences noticeably reduced. This suggests that the sequential enrichments with azoxystrobin were selecting for organisms within the community that were involved in the degradation of the compound. Previous research has also illustrated the adaptation of xenobiotic compound-degrading communities as a result of sequential enrichment. The work of Felsot *et al.* (1981) illustrated that degradation of the insecticide carbofuran was enhanced in soils with a previous history of application. Similar results were also found for other compounds such as PAHs (Abuhamed *et al.*, 2004), the fungicide metalaxyl (Vischetti *et al.*, 2008), and the insecticide chloropyrifos (Fang *et al.*, 2008). This work, along with the results obtained in this chapter illustrate that microbial communities can adapt to the addition of a xenobiotic compound to a particular environment and select for species associated with its degradation.

4.4.2 The Isolation of Azoxystrobin-Degrading Bacteria from Liquid Cultures

The two bacterial strains isolated from liquid cultures showed 98 and 99% sequence homologies to members of the genera *Rhodanobacter* and *Cupriavidus*, respectively.

The genus *Rhodanobacter* was first proposed by Nalin *et al.* (1999) who identified a novel bacterium that they named *Rhodanobacter lindaniclasticus*. This organism is a gamma proteobacterium that phylogenetically groups close to genera such as *Xanthomonas* and *Xyella*.This organism was found to be capable of degrading the insecticide lindane (Nalin *et al.* 1999). Since 1999, a further 9 individual species have been added to this genus (*R. fulvus*, *R. thiooxydans*, *R. spathiphylli*, *R. ginsengisoli*, *R. terrae*, *R. ginsenosidimutans*, *R. soli*, *R. panaciterrae*, and *R. xiangquanii*) (Bui *et al.*, 2010; Wang *et al.* 2011b; Zhang *et al.*, 2011). In addition to *R. lindaniclasticus*, *R. xiangquanii* has also been found

to be capable of degrading xenobiotic compounds. Zhang *et al.* (2011) noted how this organism was capable of degrading the herbicide anilofos within a wastewater treatment system.

Cupriavidus is a genus of beta proteobacteria and encompasses some of the former members of the genus *Wautersia*. Members of this genus have been associated with the degradation of a wide range of xenobiotic compounds. Streber *et al.* (1987) observed that *Cupriavidus necator* JMP134 was capable of degrading the herbicide 2-methyl-4-chlorophenoxyacetic acid (MCPA). The organisms' degradative ability was found to be due to the presence of an 80 kb conjugative plasmid (pjP4) that carries the required genes. Additionally, the authors found that the gene *tfd*A is involved in the first stage of MCPA degradation (Streber *et al.,* 1987). This research was supported by Nielsen *et al.* (2011) who observed that class III *tfd*A gene expression was significantly increased in MCPA-degrading soils. Pérez-Pantoja *et al.* (2008) also described how *C. necator* JMP134 was capable of mineralising 2,4-D, halobenzoates, chlorophenols and nitrophenols. The authors explained how the organism was able to use 60 out of 140 aromatic compounds tested as a sole carbon source, and that it was capable of performing the majority of the common ring cleavage metabolic pathways for aromatic compounds (Pérez-Pantoja *et al.*, 2008). In addition to this, Chen *et al.* (2008) were able to use *Cupriavidus taiwanensis* to degrade phenol within an aqueous fed-batch system. The authors observed a maximum degradation rate of 0.213 g phenol h^{-1} of a 15-hour sampling period (Chen *et al.*, 2008). Other members of this genus capable of degrading xenobiotic compounds include 2,3-dihydroxypropane-1-sulfonate (DHPS) degrader *Cupriavidus pinatubonensis* (Mayer *et al.*, 2010) and *Cupriavidus pampae* which was found to be capable of degrading 22% of the applied concentration of the herbicide 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB) over the course of a 25-day assay (Cuadrado *et al.*, 2010).

4.4.3 Characterizing the degradative abilities of the isolated *Cupriavidus* and *Rhodanobacter* sp. strains

The degradation of azoxystrobin in liquid cultures was dependent on the initial concentration of the isolates added. When the isolates were applied at a concentration of 10^2 cells ml^{-1} azoxystrobin degradation was reduced compared with the two other treatments (10^6 and 10^{10} cells ml^{-1}). Indeed, the results showed that the 10^2 treatments required approximately twice as long to degrade 50% of the applied azoxystrobin compared with the 10^6 and 10^{10} treatments. In the 10^2 cells ml^{-1} treatments, almost linear degradation kinetics were observed over the experimental period. This differs from the kinetics for the 10^6 and 10^{10} cells ml^{-1} treatments which showed a rapid degradation phase between 0 and 8 days, after which the rate of degradation decreased markedly. These results indicated that in the liquid cultures the degradation was not growth-linked i.e. there was little or no proliferation of the degraders following inoculation. This, however, contradicts some of the other observations made during the different liquid culture experiments.

Degradation was strongly affected by the presence or absence of alternative carbon and/or nitrogen sources. The addition of an alternative carbon source reduced the degradation of azoxystrobin by approximately 50%. This indicates that some of the bacteria were metabolizing the applied acetic acid in preference to azoxystrobin. Previous research by Reardon *et al.* (2000) showed that when toluene was applied along with either benzene or phenol to liquid degrader cultures containing *Pseudomonas putida* F1, degradation of the benzene or phenol was markedly reduced. When benzene and phenol were added as a combination, the degradation of phenol was significantly reduced. Further research has shown that the addition of succinate to batch cultures inhibited the degradation of benzene (Rüegg *et al.*, 2007).

Lee and Cho (2009) studied the degradation of methyl *tert*-butyl ether, benzene, toluene, ethylbenzene, and xylene in liquid degrader cultures containing *Rhodococcus* sp. EH831. The application of ethylbenzene was found to significantly inhibit the degradation of the other compounds.

In contrast, the application of methyl *tert*-butyl ether to a culture did not significantly decrease the degradation of any of the other compounds. Work by Zhou *et al.* (2011) showed that microbial degradation of the manufacturing reagent tetrahydrofuran (THF) by *Pseudomonas oleovorans* DT4 could be inhibited by the presence of xylene, ethylbenzene, toluene or benzene. Xylene was found to have the strongest inhibitory effects, and benzene the weakest.

For both organisms, the absence of an additional nitrogen source dramatically reduced the degradation of azoxystrobin. This suggests that whilst the compound was used as a carbon source, the isolates were unable to utilise it as a source of nitrogen. Previous research has also shown that in cultures where a xenobiotic compound has been provided as a sole carbon and nitrogen source, degradation of the compound can be affected. Chirnside *et al.* (2007) found that the herbicide alachlor provided an insufficient nitrogen source to enable degradation by a microbial consortium to occur. Indeed, the authors discovered that the presence of a second herbicide, atrazine, as a nutrient source was required before degradation of alachlor could begin. Additionally, a cessation in the degradation of alachlor was found to strongly correlate with the complete degradation of atrazine (Chirnside *et al.*, 2007).

Rehman *et al.* (2007) observed that the addition of an alternative nitrogen source (ammonium sulphate, ammonium nitrate, ammonium dihydrogen phosphate, or ammonium chloride) increased the degradation of *para*-nitrophenol (PNP) by *Pseudomonas pseudomallei* EBN-10. Additionally, Zhang *et al.* (2009b) also reported that the addition of ammonium nitrate (NH_4NO_3) enhanced the degradation of PNP by *Rhodococcus* sp. CN6 in liquid culture experiments. These suggest that, as with azoxystrobin, the microbial communities analysed were better adapted for using xenobiotic amendments as sources of carbon rather than nitrogen.

There was no significant mineralisation of azoxystrobin to CO_2 by either of the two isolates. These observations back up previous studies into the mineralisation of the compound in soil. Adetutu *et al.* (2008) observed less than 1% azoxystrobin mineralisation in coarse loamy sand samples over a 21-

day experimental period. In addition, a longer-term experiment by Singh *et al.* (2010) found that only between 2.5-4.2% of the applied compound was mineralised in water samples with different pH values after 130 days. The authors also concluded that azoxystrobin mineralisation rates were not dependent on water pH (Singh *et al.*, 2010).

Overall, the degradation of azoxystrobin appeared to be growth-linked. The strongest evidence for this was that, for both isolates, an additional source of nitrogen was required for degradation to occur whilst the presence of a second carbon source reduced degradation. This suggests that the isolates used azoxystrobin as a carbon (but not nitrogen) source. The most likely transformation steps required for degradation (but not complete mineralisation) to occur would be the removal of carbon from the side-chains, whilst keeping the three ring structures intact. Previous research has identified an azoxystrobin metabolite resulting from biological action (known as azoxystrobin acid) (Ghosh and Singh, 2009a) (Figure 4.5) which would support this theory and represent the first stage of the degradation process.

Both *Cupriavidus* sp. and *Rhodanobacter* sp. were also able to degrade three other strobilurin fungicides (trifloxystrobin, pyraclostrobin, and kresoxim methyl) along with azoxystrobin. This suggests that previous applications with azoxystrobin conferred the cross-enhanced degradation of trifloxystrobin, pyraclostrobin and kresoxim methyl, probably due to the structural similarity of the different compounds to azoxystrobin. There were slightly lower degradation rates recorded in trifloxystrobin-amended for both organisms. One possible reason for this could be the presence of the trifluoromethyl group on one of the side chain of this molecule which is not present in the other three. Trifluoromethyl compounds are rarely found in nature and so microorganisms may either not be adapted to degrade them (Key *et al.*, 1997) or may degrade them more slowly as observed in the work described in this chapter. This is supported by the fact that natural carbon-fluorine bonds are known to be very strong and therefore highly resistant to cleavage (Key *et al.*, 1997).

However, there have been mixed reports on the effects that trifluoromethyl groups could have on compound degradation. For example, Abernethy and Walker (1993) studied the degradation of the insecticide hydramethylnon by the basidiomycete fungus *Phanerochaete chrysosporium*. The authors observed no release of fluoride and concluded that this may have been due to the trifluoromethyl groups being slowly mineralised (Abernethy and Walker, 1993). Locke *et al.*, (2007) observed mixed results when analysing the degradation of the herbicide fluometuron. Although, DT_{50} values of between 11 and 16 days were recorded for the parent compound, complete mineralisation was only 3% after 42 days (Locke *et al.*, 2007). At the other end of the spectrum, Liang *et al.* (2010) recorded 80% degradation of the herbicide lactofen by the alpha proteobacterium *Brevundimonas* sp. LY-2 within 5 days, despite the presence of a trifluoromethyl group in its side chain.

At the present time, available information on the microbial degradation of trifloxystrobin is minimal. Indeed, to the authors' knowledge the data presented here represents the first example of specific organisms that may be capable of carrying out these processes under laboratory conditions. Additionally, this would also appear to be the case for kresoxim-methyl.

4.4.4 Culture-Independent Analysis of Azoxystrobin-Degrading Cultures

T-RFLP analysis of the initial four enrichment cultures showed that the bacterial community structure was significantly altered over the course of these enrichments. Additionally, there was a significant difference in community structure between the once- and twice-amended treatments. Bacterial diversity declined significantly over the course of the four enrichments for both treatments. However, there was no significant difference in diversity between the treatments.

The TRF at 470 bp was responsible for 12% of the community structure variation and was more prevalent in the twice-amended treatment. This correlates well with the T-RFLP data obtained for bacterial community structure using samples from the experiment described in Chapter 2 where TRFs at 470 and 472 bp were only present in amended treatments, and represented a higher proportion of

the population in higher azoxystrobin concentration treatments. Clone library analysis showed that sequences with a high homology to the genera *Rhodococcus* sp. and *Methylbium* sp. would have produced TRFs within this size range following restriction digests with *Hha*I. Therefore, these organisms may have been involved in the degradation of azoxystrobin in these systems, and suggests that the liquid culture enrichments had selected for an adapted azoxystrobin-degrading community i.e. the community present was different to in the original soil. TRF 501 bp also increased in abundance in the later azoxystrobin enrichments and clone library analysis showed that a TRF at 499 bp was produced by a sequence showing a 100% homology to the genus *Haliea* sp. These findings are supported by previous research that the genus *Rhodococcus* sp. contains species capable of degrading a range of different compounds from pyrene (Walter *et al.*, 1991), to polychlorinated biphenyls (PCBs) (Seto *et al.*, 1995), to the benzimidazole fungicide, carbendazim (Lin *et al.*, 2011). The work of Walter *et al.* (1991) and Seto *et al.* (1995) indicated that the isolated organisms utilised the contaminant as a sole carbon source whereas the isolate characterised by Lin *et al.* (2011) was capable of using carbendazim as both a sole carbon and nitrogen source.

Additionally, the genus *Methylibium* sp. (a genus of methylotrophic beta proteobacteria) was only proposed in 2006 with the discovery of the methyl *tert*-butyl ether-degrading species *Methylibium petroleiphilum* which utilised the compound as a sole carbon source (Nakatsu *et al.*, 2006).

Sequence analysis of the *Cupriavidus* sp. and *Rhodanobacter* sp. strains isolated in this chapter showed that they would have produced TRFs of 154 and 175 bp using the restriction enzyme *Hha*I, and 377 and 104 bp using the enzyme *Msp*I. These TRFs were not noticeably affected by the sequential enrichment process. This suggests that, although the culture-dependent analysis indicated that *Cupriavidus* and *Rhodanobacter* sp. were the main genera involved in azoxystrobin degradation, in reality additional genera that weren't cultured using these methods such as *Rhodococcus* and *Methylibium* sp. may play also play essential roles in this process.

Previous research has also illustrated the possible importance of non-culturable bacterial species in the degradation of xenobiotic compounds. Lozada *et al.* (2004) recorded that γ-Proteobacteria were most commonly isolated from activated sludge communities adapted to degrade nonylphenol ethoxylate (NP*n*EO). In contrast, culture-independent methods illustrated that the community was in fact dominated by β-Proteobacteria with the γ-Proteobacteria only representing a small proportion of the community. Travis *et al.* (2008) further emphasised this point by recording (using DGGE analysis) that bacterial communities in soils adapted for TNT degradation were a mixture of the genera *Pseudomonas, Methylocapsa*, *Sphingomonas*, *Burkholderia*, *Bradyrhizobium*, *Mycobacterium*, and *Azoarcus* sp. However, using culture-dependent methods the *Pseudomonas* sp. was seen to be the dominant genus (Travis *et al.*, 2008).

Work by Bending *et al.* (2003) isolated a bacterial strain with a 100% sequence homology to the genus *Porphyrobacter* sp. from isoproturon-degrading liquid cultures. However, culture-independent analysis using DGGE showed two bands that increased in intensity in isoproturon-degrading cultures compared with un-amended controls. Sequencing of the DGGE bands showed that neither of them represented the *Porphyrobacter* sp. isolated from the liquid cultures. Instead the first band had a 98.8% sequence homology to *Sphingomonas terrae*, and the second had a 100% homology to *Sphingomonas* sp. strain SRS2.

More recently, Chanika *et al.* (2011) observed that two bacteria which were subsequently identified as *Pseudomonas putida* and *Acinetobacter rhizophaerae* were dominant in cultures enriched for the degradation of the nematacide, fenamiphos. These two species were also identified in clone library analyses, but these techniques also identified that a number of non-culturable bacterial species belonging to the α-, β-, and γ-Proteobacteria and Bacteriodetes phyla were also present. This suggests that in this research the degradation of fenamiphos may have involved a larger and more diverse consortia of bacteria than was indicated using the culture-dependent analysis.

The work detailed above, plus the results of the experiments described in this chapter illustrate the need to combine the use of both culture-dependent and –independent methods for monitoring the community dynamics in environmental samples contaminated with xenobiotic compounds, and for the identification of possible microbial genera responsible for degrading them in nature.

Chapter 5

Final Discussion and Future Work

5.1 Major Findings of the Work Performed

The major findings of the work described in this thesis are discussed below:

1) The application of azoxystrobin had significant impacts on both target and non-target microbial communities across different trophic levels

The work performed in Chapters 2 and 3 of this thesis illustrated that azoxystrobin, can have significant impacts on fungal community structure and diversity. In the case of the 25 mg kg^{-1} treatment, the significant reduction in diversity was first observed 1 month post-application and persisted for the 4 month experimental period. This suggests that fungal community diversity had a low resilience to azoxystrobin application. Such impacts could be expected due to the fact that azoxystrobin is a broad-spectrum fungicide. However, significant impacts were also observed on non-target organisms. The application of azoxystrobin also significantly impacted nematode community structure, and general eukaryotic diversity. Currently, the OECD regulations for monitoring the possible direct toxicity effects of new compounds on non-target species are restricted to larger vertebrates (such as rats) and macroinvertebrates (such as *Daphnia magna*). However, the mode of action of azoxystrobin suggests that the compound may be able to exert direct toxic effects on a far wider range of non-target organisms. The compound acts by binding to the Q_o site of the cytochrome bc_1 complex within fungal mitochondria which disrupts the production of ATP within the fungal cells (Bartlett *et al.*, 2002). Fungi, however, are not the only organism group to possess such a system. Indeed, the mitochondrial system is present in all eukaryotes (such as nematodes) which could lead to the possibility that these organisms too could be susceptible to direct toxicity. Nematodes (along with other eukaryotic microorganisms) form an essential part of soil ecosystems. They are central to soil food webs as both predators and prey organisms, and perform essential roles in soil nutrient cycling. Therefore, direct impacts on these organism groups (as with other soil microorganisms) could

significantly affect the soil community as a whole as a result of indirect, knock-on effects. For example, nematodes and other microbial species are known to be essential components in the processes that provide nitrogen to crops and other plant species in the form of ammonia and other similar compounds (Sprent, 1987). Therefore, if the application of a pesticide was to significantly impact organisms responsible for these processes, this could potentially have a detrimental impact on the growth of crops and subsequently reduce the capacity of a field to provide ecosystem goods in the form of food for humans and/or cattle (Costanza *et al.*, 1997). Furthermore, the alteration of the community structure of a soil ecosystem could also significantly impact its ability to perform ecosystem services such as contaminant bioremediation (Costanza *et al.*, 1997). This therefore suggests that the OECD testing procedures could benefit from the introduction of direct toxicity tests for some non-target microbial species to compliment the current carbon and nitrogen mineralisation tests that are currently used to determine impacts on soil microorganisms (OECD, 2011).

Soil microbial biomass (SMB) analysis failed to pick up the impacts on the microbial communities that were indicated by the T-RFLP analyses detailed in chapters 2 and 3. Previous research has suggested that the use of broad-scale techniques could give mis-leading indications of pesticide impacts on microbial communities by masking more subtle, but still ecologically-significant impacts (Bending *et al.*, 2007). Such incidences have previously been observed for pesticides such as chlorothalonil. This compound is known to significantly impact other broad-scale aspects such as soil dehydrogenase activity (Bending *et al.*, 2007) whilst biomass assays have indicated no significant impact of the compound (Chen *et al.*, 2001; Bending *et al.*, 2007). Currently, fine-scale molecular analyses are not part of the OECD pesticide testing procedures. However, the results obtained in this thesis suggest that the use of broad-scale methods may not be sufficient to satisfactorily determine the impacts of pesticides on specific microbial communities. Therefore, the results described in this thesis suggest that the introduction of fine-scale molecular methods to the testing procedures for new pesticide compounds could represent a significant step forward. The molecular techniques used in this thesis, however, were not without their inherent drawbacks. For example, the individual TRFs could

not be ascribed to single genera or species and the clone libraries produced were unable to cover the entire diversity of the fungal and nematode communities as determined by Chao1 analyses. Furthermore, the T-RFLP analysis did not give any indications of potential impacts on microbial function. As a result, the current OECD tests may benefit more from the use of high-throughput pyrosequencing methods to monitor impacts on microbial communities. Additionally, the use of functional gene analyses could potentially support or replace the carbon and nitrogen mineralisation assays and allow impacts on specific essential ecosystem functions to be monitored.

2) Azoxystrobin degradation in both soil and liquid cultures was enhanced following repeated enrichments. Previous enrichments of liquid cultures with azoxystrobin also enabled the cross-enhanced degradation of other strobilurin fungicides.

The degradation of azoxystrobin in soils was found to be significantly enhanced in treatments that had previously been exposed to the compound. Furthermore, the enrichment of liquid cultures with azoxystrobin was found to confer cross-enhanced degradation of three other strobilurin fungicides, pyraclostrobin, trifloxystrobin, and kresoxim methyl. Enhanced and cross-enhanced degradation of pesticides has previously been identified for other pesticide compounds. It is debatable whether enhanced and cross-enhanced degradation of pesticides within an environment would represent a positive or negative change. One possible positive effect could be a reduction in possible direct toxic effects on (particularly non-target) soil microorganisms, and this could increase the resistance and resilience of the community. However, if a community became too well adapted to the presence of a compound, the indigenous community could potentially degrade it before it had performed its intended function, for example, the removal of fungal plant pathogens. In an agricultural context this could deleteriously affect crop yields and as a result reduce the capacity of a field to provide ecosystem goods. Indeed, Felsot *et al.* (1981) recorded that carbofuran degradation in corn fields in the USA was enhanced in soils that had previously been amended with the compound. This enhanced

degradation was also linked to increased incidences of rootworm infections resulting from the reduced persistence of the compound in the environment. Work by Karpouzas and Walker (2000) showed that degradation of the nematicide, ethoprophos was enhanced with repeated applications. This was considered a cause for concern as this compound was widely used in Greece to protect potato crops from plant-pathogenic nematodes (Karpouzas and Walker, 2000). Furthermore, it has also been observed that enhanced conditions for ethoprophos degradation could persist for three years (Smelt *et al.*, 1996). Karpouzas and Walker (2000) also noted that initial applications of ethoprophos also enabled the future cross-enhanced degradation of a related compound, isazofos. Research using the insecticide chlorpyrifos has shown that repeated applications of this compound can also result in enhanced degradation after only one previous application (Singh *et al.*, 2003). Amendment of soils with chlopyrifos has also been found to enable the cross-enhanced degradation of other compounds with similar community structures such as diazinon, parathion, coumaphos and isazofos. Under laboratory conditions, this enhanced degradation was found to persist for a period of one year (Singh *et al.*, 2005).

However, the concept of enhanced degradation does not apply to all pesticide compounds. The half lives of the widely-used compounds fenamiphos and chlorothalonil have been found to increase following repeated applications. In the case of chlorothalonil, the half life increased from 12 days after the first enrichment to 21 days after a third enrichment. For fenamiphos the half life was 8.4 days after the first and second enrichments, but this increased to 24 days after the third enrichment (Singh *et al.*, 2001). This scenario raises the potential problem that the increased persistence of some pesticides in the environment could exacerbate their impacts on soil communities. This problem could apply to chlorothalonil as the compound has previously been shown to have significant deleterious impacts on soil dehydrogenase enzyme activity (Bending *et al.*, 2007).

Therefore, the results described in Chapter 4 serve to illustrate the need to develop compounds that do not persist too long in the environment that they deleteriously impact indigenous non-target

organisms, whilst still ensuring that a compound persists long enough to perform its required function. One possibility could be the production of more highly active compounds that persist in the environment for only short periods, or compounds that are less active but are more specific and so will persist for longer without severely impacting non-target organisms. This is of particular interest for widely used compounds such as azoxystrobin that was applied to over 1 million hectares of land in the UK alone in 2009 (FERA, 2009).

The degradation of azoxystrobin was found to be dependent on the presence/absence of alternative carbon or nitrogen sources. Azoxystrobin degradation required the presence of an alternative nitrogen source to proceed efficiently, whilst the presence of an alternative carbon source reduced azoxystrobin degradation by approximately 50%. Similarly, Aislabie and Lloyd-Jones (1995) noted that the degradation rates of the pesticides 2,4-D and carbofuran can also decrease in the presence of alternative carbon sources. Abdelhafid *et al.* (2000) noted that the presence of an alternative nitrogen source significantly decreased the degradation of the herbicide, atrazine. However, on each occasion some of the compound still remained at the end of each experiment. One possible reason for this could have been that the bioavailability of the compound decreased over time (pesticide ageing). This may be the case for the assays performed in soil in Chapters 2,3 and 4. However, the liquid culture experiments detailed in Chapter 4 also showed incomplete degradation despite the reduced amount of material available for the compound to bind to. Therefore, there may have also been other reasons for the incomplete degradation of the compound. These may include: the loss of co-factors required for degradation, the accumulation degradation-inhibiting metabolites. Furthermore, the degradative abilities of the two isolates could have been limited to the side-chains of the compound, whilst the triple ring structure remained intact. This last point is supported by the fact that although degradation of the ring-labelled ^{14}C-azoxystrobin was recorded by HPLC, complete mineralisation to $^{14}CO_2$ was minimal.

Currently, the OECD ready test for pesticide biodegradation (OECD 301) does not take into account the potential impacts of other compounds within the environment (OECD, 2011). The ease with which such assays such as those described in Chapter 4 can be performed suggests that the OECD regulations could benefit from the use of alternative carbon and nitrogen sources without placing too much of a strain on time and resources.

5.2 Final Conclusions

The work carried out in this thesis aimed to describe how the application of a model compound, azoxystrobin, to soils could affect the structure, diversity, and function of microbial communities from different trophic levels. Culture-independent analyses illustrated how azoxystrobin application could affect fungal community structure and diversity, along with the structure of nematode communities, and the diversity of general eukaryote communities. Owing to the widespread use of pesticides worldwide, and the growing body of evidence that broad-scale analyses such as those used in the OECD regulations for new pesticide compounds can fail to pick up pesticide impacts on specific (particularly non-target) microbial groups, it seems clear that these regulations would benefit from the introduction of molecular methods to the procedures. These would then allow a clearer picture of pesticide impacts on microbial communities to be determined in a level of detail that was not possible at the time at which the current OECD tests were devised. Degradation of azoxystrobin by the isolated degraders *Cupriavidus* sp. and *Rhodanobacter* sp. highlighted the potential pitfalls associated with the repeated application of the same compound to an environment, particularly the possible development of enhanced and cross-enhanced degradation. This emphasises the importance of developing compounds that have higher activities and so require fewer repeat applications, or alternatively encourage the use of structurally different pesticides with the same target organisms on a rotation basis to try and minimise the development of excessive enhanced biodegradation. This could then subsequently reduce some of the crop yield losses that result from the wide range of known phytopathogens.

However, the research described in this thesis was all performed in soil or liquid cultures in the absence of plants. The presence of plants and the effects of a rhizosphere may alter the impacts of azoxystrobin and other similar compounds on microbial communities and on the fate of the compounds in soils. Abdel-Nasser *et al.* (1979) noted that pesticide impacts could vary in different plant rhizopheres and following the application of different compounds. In contrast, work by Lin *et al.* (2007) showed that the presence of vegetation reduced the negative effects of 2,4-Dichlorophenol and pentachlorophenol on soil microbial biomass. Additionally, Shaw and Burns (2004) recorded that the [U-14C]2,4-dichlorophenoxyacetic acid (2,4-D) mineralisation increased in the presence of a plant rhizosphere, and this increase was dependent on the species and age of the plant(s) present. Therefore, further work would be required to determine the fate and impacts of azoxystrobin in a field setting.

5.3 Possible Future Work

The results obtained in this thesis leave plenty of scope for further research in the future. This future research could potentially be performed to answer questions such as:

- **Does azoxystrobin application in combination with another fungicide (chlorothalonil, cyproconazole, difenconazole, or fenpropimorph) have significant impacts on soil microbial communities? Also, how do any impacts of combination applications compare with single compound applications?** The research carried out in this thesis focussed on the impacts of azoxystrobin only on soil microbial communities. However, azoxystrobin is licensed for use in the UK in combination with either chlorothalonil, cyproconazole, difenconazole, or fenpropimorph (British Crop Protection Council, 2008) and therefore it is important to consider what effects such combinations may have on the resistance and resilience of soil microbial communities i.e. are the impacts observed for azoxystrobin exacerbated by the presence of a second compound?

- **How does azoxystrobin application affect specific microbial functional groups within the soil community?** Whilst the work here presents the impact of azoxystrobin on overall, and active community structures as a whole, further research is still required to determine impacts on specific functional groups. An example of a target gene could be the *nifH* gene which is involved in nitrogen fixation (Tyson *et al.*, 2004). Alternatively, another target could be the *tfdA* group of genes whose expression has previously been linked with the degradation of xenobiotic compounds (Bælum *et al.*, 2008).

- **To what extent does soil type affect the impacts of azoxystrobin on soil microbial communities?** Soil type can be a major factor in the fate of pesticides in the environment as it affects the sorption (and therefore the bioavailability) of a compound. The work presented in this thesis analysed the impacts of azoxystrobin on microbial communities in a sandy loam soil. Further studies could therefore compare the impacts of azoxystrobin on microbial communities in a sandy loam soil with those in soils with high clay and/or silt contents.

- **Can azoxystrobin application have direct toxic effects on soil nematodes?** The work presented in chapters 2 and 3 of this thesis illustrated that azoxystrobin application had significant impacts on soil nematode communities. However, the work was unable to determine whether any impacts on nematodes were due to direct or indirect effects. This work would involve the use of culture-dependent techniques that have previously been used to determine direct toxicity effects of fungicides on eukaryotic soil microorganisms (Ekelund 1999, Ekelund *et al.*, 2000) to study the possible impacts of azoxystrobin application at different concentrations on defined nematode species.

- **How do the results for azoxystrobin impacts on soil microbial community structure and diversity obtained in lab experiments compare with those in a field environment? How do the degradation kinetics of azoxystrobin in the field compare with those in the lab?** The results of the analyses carried out in this thesis showed that azoxystrobin application

significantly impacted fungal and nematode communities, and that compound degradation was initially fast but that degradation rates decreased in soils after the first 2 weeks post-application. However, how these results relate to what happens in nature is currently unknown. Schuster and Schröder (1990ab) studied the impacts of pesticides on non-target organisms using broad-scale analyses under lab and field conditions. The authors recorded more distinct impacts of the pesticides in the lab experiments (Schuster and Schröder, 1990b) and noted that in the field experiments the results appeared to be highly influenced by weather conditions (Schuster and Schröder, 1990a). Therefore, further work could focus on the use of culture-independent techniques to focus on the impacts of azoxystrobin on specific microbial communities in field and lab environments.

- **How do the results of the culture-dependent azoxystrobin degrader analyses recorded in Chapter 4 compare to stable isotope probing (SIP) analysis of degraders *in situ*?** The culture-dependent analyses described in Chapter 4 were able to isolate two bacteria capable of partially-degradaing azoxystrobin and three related compounds. However, only a small percentage of the total microbial communities within natural environments are culturable. This means that there may be many other organisms present in the natural environment, but that are currently non-culturable. The use of SIP could solve this problem by identifying degraders *in situ*. Previous research using SIP and with 2,4-D as a model compound to successfully monitor *in situ* degradation in both natural mixed populations (Cupples and Sims, 2007; Lerch *et al.*, 2009) and in mono-cultures (Lerch *et al*., 2007). Therefore, these well-established techniques could be adapted to monitor azoxystrobin-degrading communities.

- **What metabolite(s) were formed during azoxystrobin degradation? Could the presence of the metabolite(s) explain the incomplete azoxystrobin degradation observed in Chapter 4?** Previous research by Ghosh and Singh (2009a) identified one biological

degradation product of azoxystrobin (azoxystrobin acid), whilst Adetutu *et al.* (2008) recorded that one metabolite was produced under dark conditions, and two under light conditions. Ghosh and Singh (2009a) were able to use HPLC combined with a diode array detector (DAD) to detect azoxystrobin acid. Therefore, an adapted method of the HPLC procedures used in this thesis could potentially be used to monitor azoxystrobin metabolite production. This would also allow comparisons to be made between the concentration of metabolite present, and the decreases (and eventual incomplete degradation) of azoxystrobin recorded in Chapters 2,3 and 4.

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Appendices

Appendix I

Genstat Code for Determining Azoxystrobin DT_{50} Values

```
job

read [serial=yes] resp[1...21]

100.000    96.570    102.648    89.461    90.449:
100.000    94.578    88.842     87.460    88.953:
100.000    87.469    84.242     89.575    74.192:
100.000    70.575    52.554     50.230    32.796:
100.000    70.281    54.014     35.249    28.857:
100.000    68.937    51.665     38.251    30.783:
100.000    60.725    28.271     13.516    15.068:
100.000    62.442    30.695     16.870    12.807:
100.000    59.373    30.599     20.580    15.493:
100.000    31.509    27.054     14.415    15.070:
100.000    31.114    27.483     15.173    15.996:
100.000    29.308    26.988     21.118    9.664:
100.000    78.538    42.656     35.610    28.034:
100.000    78.056    58.688     34.040    27.125:
100.000    75.087    71.621     37.626    27.271:
100.000    69.840    26.075     13.776    12.160:
100.000    67.506    23.468     13.826    14.482:
100.000    63.317    22.636     15.706    12.463:
100.000    58.983    25.636     15.539    12.814:
100.000    58.847    24.993     15.228    13.342:
100.000    39.896    28.401     15.125    11.806:


vari time;values=!(0,4,8,12,16)

vari [nvalues=21]DT50_T

for i=1...21

        interpolate [method=value]\
        time;\
        oldint=resp[i];\
        newint=!(50);\
        newval=DT50_T$[i]
print DT50_T$[i]
endfor
 print DT50_T
```

Appendix II

Example Genstat Code for Producing a Modified ANOVA with Contrast Analysis

```
factor [levels=2] contrast
calc contrast=newlevels(conc;!(1,2,2,2,2))
"General Analysis of Variance."
BLOCK Rep
TREATMENTS Sample*(contrast/conc)
COVARIATE "No Covariate"
ANOVA [PRINT=aovtable,information,means; FACT=32; CONTRASTS=7; PCONTRASTS=7; FPROB=yes;\
 PSE=diff,lsd; LSDLEVEL=5] diversity
APLOT [RMETHOD=simple] fitted,normal,halfnormal,histogram
AGRAPH [METHOD=means; PSE=differences] X=
```

Appendix III

Extraction of RNA from soil using FastRNA pro soil direct kit (MP Biomedicals)

1. Mix 10g freeze-dried soil with an equal volume of dry-ice.

2. Grind in a coffee grinder and/or using a Fast Prep machine adding a 3mm tungsten carbide bead to each tube.

3. Transfer the mixture to a loosely closed 50ml centrifuge tube and placed at -70°C overnight to allow the evaporation of CO_2.

After this, the protocol for the FastRNA pro soil direct kit is as follows:

1. Weigh 100-150mg of soil and place in a purple-cap sample tube containing Lysing Matrix E.

2. Add 1ml of RNApro™ Soil Lysis Solution to the tube.

3. Invert several times to resuspend the soil and lysing matrix in the solution.
   - Ensure the cap is securely closed to prevent leakage in the next step

4. Process tubes in a FastPrep® machine for 20 seconds at a setting of 4.0

5. Remove the tube and centrifuge at ≥ 14,000 x g for 5 minutes at room temperature.

6. Transfer the liquid to a new microcentrifuge tube

7. Add 750µl of Phenol:Chloroform (1:1) and vortex for 10 seconds

8. Incubate for 4 minutes at room temperature to permit nucleoprotein dissociation and increase RNA purity.

9. Centrifuge at ≥ 14,000 x g for 5 minutes at 4°C

10. Remove upper aqueous phase to a new centrifuge tube without disturbing the interphase.
    - Note: if a portion of the interphase is accidentally transferred, repeat the centrifugation in step 9 with the contaminated upper phase and transfer the new upper phase to a clean microcentrifuge tube.

11. Add 200µl of Inhibitor Removal Solution.

12. Invert 5 times to completely mix

13. Centrifuge at ≥ 14,000 x g for 5 minutes at room temperature.

14. Remove the liquid above the pellet to a new microcentrifuge tube.
    - Note: following centrifugation, a 10-25µl "bubble" may appear over a debris pellet. If a bubble appears, transfer only the liquid above the bubble to a new RNase-free microcentrifuge tube.

15. Add 660µl of cold 100% isopropanol to the sample, invert 5 times and place at -20°C for at least 30 minutes.
    - Note: White stands may be observed in some samples. The strands, which include DNA and humic substances, will be removed in subsequent steps.

16. Centrifuge at ≥ 14,000 x g for 15 minutes at 4°C and discard the supernatant
    - Note: The RNA pellet may appear as chocolate-coloured or "dirty" due to the presence of humic substances contamination. The amount of colour will vary between soils and will be removed in subsequent steps.

17. Carefully wash the pellet with 500µl of cold 70% ethanol (made with DEPC-H_2O).

18. Remove the ethanol and air dry the pellet for 5 minutes at room temperature.
    - Note: DO NOT completely dry the RNA as this will increase the difficulty of resuspending the RNA in the next step.

19. Resuspend the RNA in 200µl of DEPC-H_2O.
    - Note: The RNA may be pipetted to enhance resuspension.

20. Add 600µl of RNAMATRIX® Binding Solution and 10µl of RNAMATRIX® Slurry to the RNA. Incubate at room temperature on a shaker table, a rotator, or with frequent inversion for 4 minutes.

21. Microcentrifuge (pulse-spin) for approx 10 seconds to pellet the RNAMATRIX®-bound RNA and discard the supernatant. Use caution not to remove the RNAMATRIX®.

22. Completely resuspend the RNAMATRIX®-bound RNA in 500µl of prepared RNAMATRIX® Wash Solution.
    - Note: Ensure that 15ml of ethanol has been added to the RNAMATRIX® Wash Solution Concentrate prior to use.

23. Microcentrifuge (pulse-spin) for approx 10 seconds and discard the supernatant. Use caution not to remove the RNAMATRIX®.

24. Microcentrofuge (pulse-spin) a second time for approx 10 seconds and carefully remove any residual wash solution with a pipette. Use caution not to remove the RNAMATRIX®.

25. Air dry for 4 minutes at room temperature .

26. Add 50µl of DEPC-H_2O and completely resuspend the RNAMATRIX® by vortexing. Incubate 4 minutes at room temperature to elute the RNA. Finger-tap the tube bottom frequently to provide gentle mixing.

27. Microcentrifuge (pulse-spin) for approx 10 seconds and transfer the supernatant containing eluted RNA to a new tube.
    - Note: Do not discard the RNAMATRIX® pellet!

28. Repeat step 26 and 27 to provide a final RNA volume of 100µl.
    - Note: If matrix carryover occurs, remove the matrix by pulse-spinning the microcentrifuge tube for approx 10 seconds to pellet the matrix. Carefully transfer only the supernatant to a new RNase-free microcentrifuge tube.

Need:

- ❖ FastPrep® machine
- ❖ 100% ethanol
- ❖ Chilled 70% Ethanol (prepared with DEPC-treated H_2O)
- ❖ Chilled Isopropanol
- ❖ 1.5 or 2.0ml RNase-free Microcentrifuge tubes