The Use of Human Cell Lines to Monitor the Cytotoxicity of Environmental Mixtures in Soil

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
ANOVA	Analysis of variance
BABS	Bioaugmentation and biostimulation
CO_2	Carbon dioxide
DAD	Diode array detector
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FBS	Foetal Bovine Serum
FDA	Fluorescein diacetate
GC	Gas Chromatography
HCl	Hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
HVA	Modified humic acid vitamin B agar
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
LOD	Limit of detection
MC	Medium only control
MEA	Malt extract agar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NA	Natural attenuation
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
PBS	Phosphate buffered saline
PI	Propidium iodide
psi	Pounds per square inch

RIPK	Receptor-interacting serine/threonine protein kinases
RNase	Ribonuclease A
RO	Reverse osmosis
RSD	Relative standard deviation
RTW	Retention time window
SC	Solvent control
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of the mean
TPH	Total petroleum hydrocarbons
TSA	Tryptone soy agar
VV	Vitavax 200FF
WHC	Water holding capacity

ABSTRACT

Humans are typically exposed to a multitude of chemical mixtures in the environment with varying impacts on human health. Current guidelines for assessing the human health hazard of environmental mixtures such as agricultural chemicals and bioremediated soil allow for predictions based solely on chemical analysis. However chemical analysis may not detect interactions between chemical compounds or degradation products which may contribute to the toxicity of the soil.

The use of *in vitro* bioassays may provide a more comprehensive assessment of the risk posed by the mixture to human health. Therefore, the overall aim of this study was to use human cell lines to monitor the cytotoxicity of various environmental mixtures as they degraded in soil. This was achieved by using a range of human cell lines including HepG2 (liver), HaCaT (skin), MRC-5 (lung), JAr (placenta) and WIL2NS (lymphocytes) to monitor the cytotoxicity of environmental mixtures. This thesis also aimed to determine if *in vitro* cytotoxicity monitoring of environmental mixtures using human cell lines was more sensitive for detecting changes in toxicity compared to chemical analysis. Specific environmental mixtures that were investigated in this thesis included the commercial agricultural product Vitavax 200FF and its active ingredients, carboxin and thiram. The other environmental mixture investigated in this thesis was soil contaminated with total petroleum hydrocarbons (TPH), which was subjected to bioremediation using two different methods.

*C*ytotoxicity of Vitavax 200FF to cell lines was in the following order; WIL2NS cells > JAr cells > HaCaT cells > MRC-5 cells > HepG2 cells. The cytotoxicity was mainly due to the effects of thiram in the formulation.

The cytotoxicity of Vitavax 200FF and its active ingredients was then monitored during their degradation in soil in simulated laboratory experiments. Both sterile and non-sterile soil was employed to investigate the impact of the presence of microbes on the degradation and cytotoxicity of the product formulation Vitavax 200FF in soil. It was found that high concentrations of Vitavax 200FF could alter the population of soil microorganisms. It was also demonstrated that degradation of the active ingredients of Vitavax 200FF, carboxin and thiram could occur via abiotic catalytic processes as well as via biotic transformation. Degradation products generated from both abiotic and biotic processes were likely to be less toxic than their parent compounds. However, it was found that the soil could remain toxic to human cells for a longer period of time than predicted from chemical testing.

The cytotoxicity of soil contaminated with TPH during bioremediation was then monitored. It was found that the reduction of TPH levels detected via chemical analysis over time did not correlate with a reduction of cytotoxicity over time. It was also found that bioremediation of TPH contaminated soil via the addition of nutrients and microorganisms could potentially be more harmful than allowing the contaminants to degrade in the soil unassisted.

The results of this thesis reinforced the view that assessment using chemical analysis alone is insufficient to determine potential hazards to human health. It was found that a combined approach which includes both chemical and *in vitro* cytotoxicity testing of chemical mixtures using human cells provided a more comprehensive evaluation of the toxicity posed by complex environmental mixtures.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Dao Hong Thi Swain

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1 CHAPTER 1: Introduction

1.1 Environmental Mixtures

Humans are typically exposed to a multitude of chemicals mixtures in the environment with varying impacts on human health. In addition, the composition and concentration of chemicals in environmental mixtures can vary greatly between sources (Brack, 2003, Donnelly *et al.*, 2004). These mixtures may be classified as simple or complex mixtures. Simple mixtures are generally classified as mixtures with ten or less constituents of which the identity and concentration of each chemical is known (Groten *et al.*, 2001). Examples of simple chemical mixtures include commercial chemical formulations such as pharmaceutical and agricultural products or other mixtures in which the chemical constituents are well defined and can be easily replicated.

On the other hand, complex mixtures are defined as mixtures with greater than ten chemical constituents of which the identity and concentration of all the chemicals may not be known (Groten *et al.*, 2001). Complex mixtures can contain over thousands of chemicals which may be too time-consuming and expensive to quantify using chemical analysis. In addition, particular compounds within the mixture may be difficult to elucidate using current chemical methods due its low concentration in the mixture or molecular structure (Dévier *et al.*, 2011). Other environmental factors such as light, heat or the presence of organisms may also alter the mixture over time and thus add to the complexity of the mixture (Dévier *et al.*, 2011). Examples of complex mixtures include industrial waste, cigarette smoke and contaminated soil or water.

The toxic potential of a chemical mixture is generally estimated from the effects of individual chemical compounds that are known to be in the mixture (Brack, 2003). However, in complex environmental mixtures, identification of all compounds within the mixture may not be possible. Therefore, the mixture may contain compounds or

breakdown products which are not detected during chemical analysis but are toxicologically significant (Rice *et al.*, 2008). Indeed, numerous studies have shown that the degradation products of various agricultural chemicals and petroleum hydrocarbons have similar or greater toxicity when compared to the parent compound itself (Boyd *et al.*, 1997, Tixier *et al.*, 2001, Zhang *et al.*, 2011, Donner *et al.*, 2013). Furthermore, many degradation products often have a greater persistence in the environment than their parent compounds (Boxall *et al.*, 2004) and may accumulate over time.

The toxicity of a compound may also be altered by the presence of other compounds in the mixture (Donnelly *et al.*, 2004). This may be due to the interactions between the chemicals causing changes in the absorption, metabolism or excretion of the mixture (Heys *et al.*, 2016). The chemical interactions can be additive, synergistic, potentiated, or antagonistic. However, prediction of chemical interactions from the known chemical constituents in simple mixtures and complex mixtures in particular is difficult as the interaction may only occur at particular concentrations or environmental conditions (Groten *et al.*, 2001, Jonker *et al.*, 2005)

Different biological systems may also show different interactions with the same combination of chemicals. The toxic effect of glyphosate and cypermethrin in combination were found to be synergistic in tadpoles (Brodeur *et al.*, 2014) but antagonistic in fish (Brodeur *et al.*, 2016). Therefore, in order to adequately assess the toxicity of an environmental mixture, the effects of all chemical constituents, degradation products and chemical interactions within the mixture as well as the biological system used must be taken into account. Toxicity analysis of the chemical mixture as a whole would allow a more relevant assessment of the risk posed by the mixture.

1.1.1 Chemical mixture effects

Prediction of the risk posed by human exposure to chemical mixtures remains a challenge. This is because compounds within mixtures can interact with each other, resulting in different toxicological responses compared to the individual compounds (Feron *et al.*, 2002, Heys *et al.*, 2016). The type of interactions present within a complex environmental mixture can be difficult to predict, particularly if the identity and concentration of all compounds within the mixture have not been elucidated (Donnelly *et al.*, 2004). Even if all compounds within a complex mixture were quantified, it is unlikely that toxicity data for all possible chemical combinations within the mixture will be available. This is because complex environmental mixtures can contain over thousands of different compounds, many of which have not been well characterised (Heys *et al.*, 2016).

1.1.1.1 Additive effects

An additive effect is when the observed toxicity of the mixture is equal to the sum of the effects of its individual constituents (Heys *et al*., 2016; Figure 1-1). A chemical mixture for which the effects are considered to be additive would indicate no interaction between the chemical constituents in the mixture. Additive effects have well established models for predicting the toxicity of mixtures from their known chemical constituents and are describedin section 1.1.2.

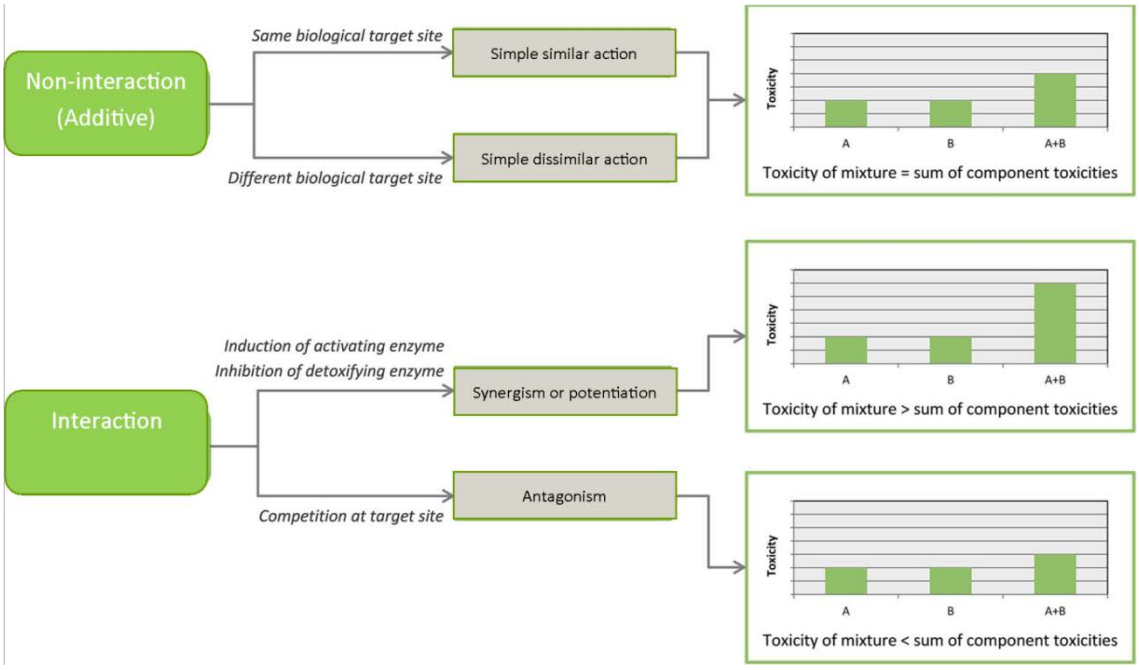


Figure 1-1. Effects arising from non-interaction or interaction of chemical constituents within a chemical mixture.
Modified from Heys *et al.* (2016)

1.1.1.2 Greater than additive effects (synergism or potentiation)

Some chemical mixtures may produce a greater toxicological effect than that predicted from an additive model. In this case, the interaction is either due to synergy or potentiation (Heys *et al.,* 2016; Figure 1-1). Mixtures which show synergy or potentiation are of great toxicological concern as they have the potential to be more hazardous to human health than predicted.

A synergistic interaction refers to when chemicals which are known to exert toxic effects individually, enhance each other's toxicity when applied together (Feron *et al.*, 2002, Heys *et al.*, 2016). Many chemical combinations commonly used in agricultural and petroleum products have been found to have synergistic effects when applied together in biological systems and some examples are provided in Table 1-1.

Potentiation refers to when a chemical has no significant toxicity normally, but exerts a toxic effect when it is present in combination with another chemical (Heys *et al.*, 2016). This also includes chemicals with known toxic effects that induce a greater than additive effect when applied at a non-toxic concentration with other chemicals (Heys *et al.*, 2016). Many chemical combinations commonly used in agricultural and petroleum products have been found to show potentiation when applied together in biological systems and some examples are provided in Table 1-2.

Although there are many studies which show evidence of synergy or potentiation between chemical combinations, the mechanism by which it occurs is not always identified. However, some possible mechanisms include alterations of activating or detoxification enzymes, cellular uptake or excretion (Chadwick *et al.*, 1991, James *et al.*, 2004, Kim *et al.*, 2013).

Table 1-1. Examples of chemical combinations that have synergistic effects.

N.D = not determined.

Chemical combination	Effect	Mechanism for synergy	Reference(s)
Atrazine + Chlorpyrifos	Increased toxicity in fish	Inhibition of acetylcholinesterase	(Pérez *et al.*, 2013)
Benzo(a)pyrene + Polychlorinated Biphenyls	Increased genotoxicity in fish	Inhibition of detoxification enzymes	(James *et al.*, 2004)
Chlorpyrifos + Pyrethrum	Increased toxicity in neuronal cells	N.D.	(Axelrad *et al.*, 2002)
Deltamethrin + Fenitrothion + Fipronil + Lambda-cyalothrine +Teflubenzuron	Increased toxicity in human Caco-2 cells	Increased oxidative stress	(Ilboudo *et al.*, 2014)
Diazinon + Nonylphenol	Increased toxicity in amphibians	N.D.	(Aronzon *et al.*, 2016)
Glyphosate + Cypermethrin	Increased toxicity in tadpoles	N.D.	(Brodeur *et al.*, 2014)
Permethrin + Propoxur	Increased toxicity in mosquito larvae	N.D.	(Corbel *et al.*, 2006)
Polycyclic Aromatic Hydrocarbons + Arsenic	Increased immunotoxicity in mice	N.D.	(Li *et al.*, 2010)
Prochloraz + Esfenvalerate	Increased toxicity in aquatic organisms	N.D.	(Bjergager *et al.*, 2012)

Table 1-2 Examples of chemical combinations that have potentiating effects.

N.D = not determined.

Chemical combination	Effect	Mechanism for potentiation	Reference(s)
2,6-Dinitrotoluene + Pentachlorophenol	Increased genotoxicity in rats	Increased metabolism of 2,6-dinitrotoluene to genotoxic metabolites	(Chadwick *et al.*, 1991)
Chlorfluazuron + Tween 80	Increased toxicity in human HepG2 cells	N.D.	(Li *et al.*, 2015a)
Cypermethrin + Cadmiun	Increased toxicity in zebrafish	Inhibition of CYP1A1 biotransformation of cypermethrin	(Yang *et al.*, 2016)
Glyphosate + TN-20	Increased toxicity in rat heart cells	Increased cellular uptake of glyphosate	(Kim *et al.*, 2013)
Maneb + Mancozeb	Increased toxicity in rat pheochromocytoma cells	Activation of NF-κB	(Williams *et al.*, 2013)
Metolachlor + Endosulfan	Increased toxicity in earthworms	Inhibition of acetylcholinesterase	(Stepic *et al.*, 2013)
Thiram + Endosulfan	Increased toxicity in Ehrlich ascites tumor cells	Increased oxidative stress	(Rana *et al.*, 2010)

1.1.1.3 Less than additive (antagonism)

Antagonism occurs when the toxicity of the mixture is less than the sum of the effects of its individual constituents (Heys *et al.*, 2016). This would imply the interference of the action of one or more chemicals by other chemicals within the mixture. Many chemical combinations commonly used in agricultural and petroleum products have been found to have antagonistic effects when applied together in biological systems and some examples are provided in Table 1-3.

Table 1-3 Examples of chemical combinations that have antagonistic effects.

N.D = not determined.

Chemical combination	Effect	Mechanism	Reference(s)
Avermectin + Tween 80 or PEG6000	Decreased toxicity in HepG2 cells	N.D.	(Li *et al.*, 2015a)
Chlorothalonil + Atrazine	Decreased toxicity to aquatic organisms	N.D.	(Phyu *et al.*, 2011)
Diazinon + Deltamethrin	Decreased toxicity in rats	Decreased lipid peroxidation	(Elhalwagy *et al.*, 2009)
Dichlorvos + Pirimicarb	Decreased toxicity in mouse liver	N.D.	(Wang *et al.*, 2014)
Permethrin + Atrazine	Decreased toxicity to aquatic organisms	N.D.	(Phyu *et al.*, 2011)
Toluene and p-xylene	Decreased lipid peroxidation in human placental mitochondria	N.D.	(Sawicka *et al.*, 2008)

1.1.2 Models used to assess chemical mixtures

There are two main models which are used to assess the toxicity of a chemical mixture namely the simple similar action model and the simple dissimilar action model (Feron *et al.*, 2002). Simple similar action, also known as concentration addition, is defined as when the chemicals have the same mechanism of action but may differ in their potency. In this case, prediction of the toxicity of a mixture is based on the assumption that the combined effect of chemicals with simple similar action in a mixture will be proportional to their concentrations within the mixture (Altenburger *et al.*, 2000, Feron *et al.*, 2002). Therefore, chemical constituents within the mixture can be substituted with other chemicals at an equivalent effective concentration without altering the toxicity of the mixture as a whole. Previous studies have found that the model shows a good correlation between the predicted and actual toxicities of chemical mixtures containing similarly acting chemicals in various biological systems (Altenburger *et al.*, 2000, Feron *et al.*, 2002, Junghans *et al.*, 2003, Arrhenius *et al.*, 2004, Porsbring *et al.*, 2010)

The simple dissimilar action model, also known as response addition, is used when the chemicals within the mixture have different mechanisms of action (Feron *et al.*, 2002). The simple dissimilar action model estimates toxicity using the sum of the toxic response of each individual chemical in the mixture. Previous studies have found that the model shows a good correlation between the predicted and actual toxicities of chemical mixtures containing chemicals with different mechanisms of action in various biological systems (Backhaus *et al.*, 2000, Faust *et al.*, 2003)

The problem with both models used to predict toxicity is that they rely on the identity, concentration, potency and mechanism of action of all individual constituents in the mixture being known (Feron *et al.*, 2002). However, environmental mixtures may contain many unknown constituents whose potential effects would not be able to be

taken into account during toxicity prediction using the models. Even if the identity and concentration of all chemical constituents in the mixture were known, it is possible that the potency or mechanism of action of at least one of the chemicals has not yet been fully characterised.

Experiments which used the simple similar action model to predict the toxicity of mixtures containing dissimilarly acting chemicals generally overestimated the effects (Backhaus *et al.*, 2000, Phyu *et al.*, 2011). On the other hand, experiments which used the simple dissimilar action model to predict the toxicity of mixtures containing similarly acting chemicals generally underestimated the effects (Arrhenius *et al.*, 2004, Syberg *et al.*, 2008). However, experiments which compared the ability of each model to predict the toxicity of mixtures containing both similary and dissimilary acting chemicals generally showed a good correlation between predicted and actual toxicity for both models (Syberg *et al.*, 2008, Huang *et al.*, 2011).

Both simple similar action and simple dissimilar action models assume that the toxicity of the chemicals in the mixture do not affect one another (Feron *et al.*, 2002). Therefore, prediction of the toxicity of the mixture is generally accurate when only additive effects are present within the mixture.

Deviations from the predicted and actual toxicity of a chemical mixture may indicate the presence of non-additive effects such as synergistic or antagonistic interactions (Heys *et al.*, 2016). It is these non-additive effects that present a major challenge in estimating the toxicity of a chemical mixture as their presence is difficult to predict even when all chemical constituents and concentrations are known (Groten *et al.*, 2001). Hence, this thesis focuses on the evaluation of the toxicity of complex mixtures as a whole, rather than their individual components.

1.1.3 Environmental transformation of chemical mixtures

Mixtures may show changes in their chemical profiles over time in different environments. This is because chemicals can be degraded by biotic and abiotic processes which may alter their toxicity.

Abiotic processes occur in the absence of biological organisms and are the result of chemical reactions such as photolysis via light, hydrolysis via water, oxidation, thermal decomposition, or other redox reactions with metals and other chemicals within the mixture (Boxall *et al.*, 2004). In contrast, biotic degradation of chemicals is due to their metabolism by biological organisms (Olivares *et al.*, 2016). Different types of organisms may have different combinations of metabolic enzymes. Therefore, there is the potential for the degradation products of any given parent compound to vary greatly between organisms.

The mechanism by which a chemical in a mixture is degraded may influence its toxicity. In many cases, environmental transformation/degradation of chemicals would result in degradation products with lower toxicity (Sinclair *et al.*, 2003, Baran *et al.*, 2006, Abramović *et al.*, 2013, Paul *et al.*, 2013). However it is generally the case that only the parent compounds and their concentration are identified during routine chemical testing. This could be a problem in the context of assessing the risk of environmental mixtures as the mixture may still have toxic potential even if levels of the parent compounds are very low or absent.

Studies have also shown the potential for degradation of chemicals to generate products with equal or higher toxicity than the parent compound. Solutions containing capecitabine were found to increase in toxicity to aquatic organisms when degraded abiotically via UV light, whilst biotic degradation via green algae and sludge was found to be less toxic compared to the parent compound (Guo *et al.*, 2015). Photolysis of the

chloroacetamide herbicide acetochlor resulted in degradation products with equal toxicity to the parent compound (Souissi *et al.*, 2013). In another study, known biodegradation products of 2,4-Dinitroanisole were found to have similar or greater toxicity compared to the parent compound in bacteria (Olivares *et al.*, 2016). Metabolism of geniposide, a compound commonly used to prepare blue food colouring, by microbes found in the human intestine was also found to produce metabolites that were more toxic to human hepatocytes (Khanal *et al.*, 2012)

Identification and characterisation of all degradation products within a complex mixture may not be achievable. Therefore, there is a need for a method of assessing the toxicity of complex mixtures that focuses on a biological endpoint and is not reliant on chemical analysis. This thesis uses human cell lines to assess the toxicity of chemical mixtures and utilises cell viability as the biological endpoint.

1.1.3.1 Biotransformation in human cells

Parent compounds and degradation products present in complex mixtures may also undergo further biotransformation in human cells following exposure. Biotransformation in human cells generally involves the conversion of lipid soluble compounds into water soluble metabolites through the action of cellular enzymes (Nassar, 2010). In whole organisms, the conversion of the compound to a more water soluble form enables more efficient excretion of the compound in the urine and is an important feature in cells as it prevents the accumulation of toxic metabolites (Eisenbrand *et al.*, 2002).

Biotransformation of chemicals in cells involves two major pathways. The first pathway involves phase I enzymes which enable modification of the chemical via dealkylation, hydroxylation, oxidation, and deamination (Omiecinski *et al.*, 2011). The second pathway involves phase II enzymes which enable conjugation of the compound via

glucuronidation, sulfation, methylation, acetylation, glutathione conjugation, or amino acid conjugation (Omiecinski *et al.*, 2011).

The function of biotransformation in cells is to reduce the hazardous effect of chemical compounds. However, in some cases biotransformation may result in a metabolite that has increased toxicity to cells. A classic example is the chemotherapeutic drug 5-Fluorouracil (5-FU) which is metabolised in cells to 5-fluorodeoxyuradine monophosphate (FdUMP), 5-fluorodeoxyuradine triphosphate (FdUTP) and fluorouradine triphosphate (FUTP) (Longley *et al.*, 2003). These metabolites are able to damage cellular DNA and cause subsequent cell death (Kunz *et al.*, 2009).

The rate of conversion of 5-FU to its metabolites is dependent on the metabolic activity in cells (Longley *et al.*, 2003). It is noted that different cell types within the human body may have different metabolic capacities (Krämer *et al.*, 2009). Therefore, the level of toxicity caused by biotransformation of different compounds or their toxic metabolites may be influenced by the type of cell in which exposure occurs. Therefore, this thesis utilised a range of human cell types.

1.2 Selected environmental mixtures

The range of mixtures present in the environment is diverse and therefore evaluation of all possible types of environmental mixtures was beyond the scope of this thesis. Instead, this thesis focuses on two classes of environmental mixtures and their degradation in soil.

1.2.1 Agricultural chemicals

Nearly all commercial formulations of agricultural chemicals such as pesticides and fungicides are complex mixtures of active other non-active ingredients which are designed to increase the efficacy of the product. In Australia, toxicological evaluation of the commercial formulation to determine potential risks to human health is permitted to be extrapolated from the effects of the individual active and non-active ingredients (Australian Pesticides and Veterinary Medicines Authority, 2009a, Australian Pesticides and Veterinary Medicines Authority, 2009b). Although toxicological testing on the actual product formulation is encouraged, it is not necessarily required in order to gain approval for use, particularly when the active ingredient has already been approved for use in a similar product.

This has implications when evaluating the toxic potential of a product as non-additive interactions (e.g. synergistic, potentiating or antagonistic interactions) between the active or other non-active ingredients in the product formulation may increase or decrease its toxicity when compared to the expected additive effects of the individual ingredients themselves. Indeed, many studies have shown synergistic or antagonistic interactions between mixtures of agricultural chemicals commonly used together as active ingredients (Pape-Lindstrom *et al.*, 1997, Surgan *et al.*, 2010, Bjergager *et al.*, 2012, Coleman *et al.*, 2012, Brodeur *et al.*, 2014). Other studies have also shown synergistic or antagonistic interactions between active and non-active ingredients within a product formulation (González *et al.*, 2007, Pereira *et al.*, 2009, Beggel *et al.*, 2010, Kim *et al.*, 2013, Mesnage *et al.*, 2014).

Due to the high frequency of the use of agricultural chemicals in the environment, the potential for human exposure is high. Exposure to agricultural chemicals may be a direct consequence of manufacturing, handling, or use of the chemicals (Grey *et al.*, 1983, Li *et al.*, 2011). Alternatively, exposure to agricultural chemicals may be an indirect consequence of unintentional exposure to residues in food, soil or water

(Crépet *et al.*, 2013, Enault *et al.*, 2015). Therefore, agricultural chemicals represent a class of chemicals suitable for assessing toxicity related to their exposure to humans. This thesis focuses on the toxicity of a commercial product formulation Vitavax 200FF, containing known active (carboxin and thiram) and unknown non-active ingredients; and degradation of the product formulation or its active ingredients in soil.

1.2.1.1 Carboxin

Carboxin (Figure 1-2) is a widely used agricultural fungicide that is well known as an inhibitor of succinate dehydrogenase (Mowery *et al.*, 1977, Shima *et al.*, 2011, Yang *et al.*, 2011). Limited studies have evaluated the toxicity of carboxin in bacterial, aquatic and animal models (DellaGreca *et al.*, 2004, World Health Organization, 2009, Milenkovski *et al.*, 2010, Aydin *et al.*, 2012), however there is very little information on the toxicity of carboxin in humans.

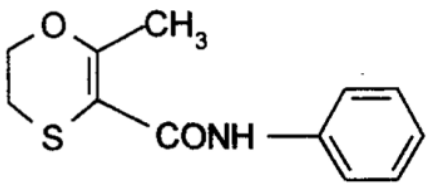


Figure 1-2. Chemical structure of carboxin
Image sourced from Hustert *et al.* (1999)

1.2.1.2 Thiram

Thiram (Figure 1-3) is another widely used fungicide with limited information on its effects to human health. A number of previous studies have investigated the toxicity of thiram in bacterial (Dive *et al.*, 1984, Milenkovski *et al.*, 2010) and animal models (Dalvi *et al.*, 1986, Maita *et al.*, 1991, Shukla *et al.*, 1996, Agrawal *et al.*, 1997, Greene *et al.*, 1997, Dalvi *et al.*, 2002, Sook Han *et al.*, 2003, Grosicka *et al.*, 2005). Some

studies have shown the toxicity of thiram in human cell cultures including in skin fibroblasts (Cereser *et al.*, 2001a), microvascular endothelial cells (Kurpios-Piec *et al.*, 2015) and lymphocytes (Perocco *et al.*, 1989, Li *et al.*, 2015b). However the toxicity of compounds to cells has been shown to be influenced by various factors including cell type (Kim *et al.*, 2014, Tang *et al.*, 2014), xenobiotic metabolism capability (Autrup, 2000, Chang *et al.*, 2007, Bains *et al.*, 2013), and loss or gain of function mutations (Bunz *et al.*, 1999, Poele *et al.*, 1999, Shield *et al.*, 2001, Clodfelter *et al.*, 2005, Vilar *et al.*, 2008). Therefore, more studies using different human cell types are required to gain a comprehensive understanding of the human health risk posed by thiram.

The type of cell death induced by thiram is also unclear (see Chapter 4 for a description of the types of cell death). One study showed that thiram-induced activation of caspase 3 and release of mitochondrial cytochrome-c in human T lymphocytes, indicating apoptosis (Li *et al.*, 2015b). Other studies in rat pheochromocytoma PC12 cells and V79 Chinese hamster fibroblasts also showed apoptotic cell death (Sook Han *et al.*, 2003, Grosicka *et al.*, 2005). In contrast, thiram induced necrotic cell death in human fibroblasts (Cereser *et al.*, 2001a) and Ehrlich ascites tumor cells (Rana *et al.*, 2010). Analysis of liver, kidney, heart, pancreas, and brain tissues of thiram-fed chickens also showed morphological features consistent with necrosis (Subapriya *et al.*, 2007). Therefore more studies are required to determine the mode of cell death induced by thiram.

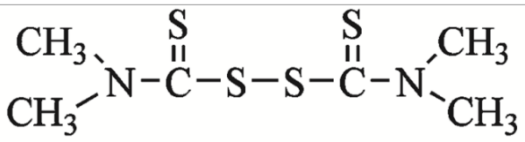


Figure 1-3. Chemical structure of thiram
Image sourced from Sharma *et al.* (2003)

1.2.1.3 Vitavax 200FF

Vitavax 200FF is a commercially available formulated product containing equal amounts of the fungicides carboxin and thiram as active ingredients (200g/L each) as well as proprietary surfactants, additives and emulsifiers of undisclosed molecular nature. The product is widely used as a commercial seed dressing for a variety of grains such as wheat, barley, oats, triticale, maize, sweet corn, and cottonseed. Vitavax 200FF is applied undiluted directly to seed or can be diluted to improve seed coverage by mixing 4 parts water to 1 part of product prior to application, as directed on the label. As a seed dressing, there is a high risk for direct human exposure to Vitavax 200FF during manufacture and application. In addition, there is also a high potential for frequent secondary exposure resulting from handling of the treated seed such as during planting or transferring seed between containers (White *et al.*, 2004). Although the risk of human exposure to Vitavax 200FF is high, no studies have been performed to date that evaluate the toxicity of Vitavax 200FF in relation to human exposure.

Furthermore, no studies have examined potential synergistic effects of the active ingredients of Vitavax 200FF, carboxin and thiram when used in combination. Thiram has been shown to interact synergistically in mammalian cells with a number of compounds including endosulfan (Rana *et al.*, 2010), and cadmium (Iwahashi *et al.*, 2007). As carboxin and thiram are commonly used together in commercial product formulations, there is a need to evaluate potential synergistic effects between carboxin and thiram in relation to human cytotoxicity.

1.2.2 Total Petroleum Hydrocarbons (TPH)

Total petroleum hydrocarbons are complex mixtures that contain a variety of hydrocarbons from crude oil (Agency for Toxic Substances and Disease Registry, 1999). Total petroleum hydrocarbons are commonly used in the modern world and are

critical in a number of combustion processes such as fossil fuel combustion, production of aluminium, iron and steel, and petroleum refining (Mao *et al.*, 2009, Vrabie *et al.*, 2009). Due to the ubiquitous nature of petroleum hydrocarbons in the environment, exposure to them is unavoidable and they are therefore a good representative of unintentional environmental exposure (Jacob, 1996).

The exact composition of total petroleum hydrocarbons in crude oil vary greatly depending on geographic region (Petersen *et al.*, 2010, Jung *et al.*, 2013). In addition, degradation of petroleum hydrocarbons in the environment also increases the complexity of the mixture. TPH mixtures are considered to be complex mixtures with toxic and carcinogenic potential (Agency for Toxic Substances and Disease Registry, 1999). Polycyclic aromatic hydrocarbons which are commonly found in TPH mixtures are known to form DNA binding reactive intermediates after being metabolically activated by cytochrome P450 enzymes (Sevastyanova *et al.*, 2007)

1.2.2.1 Bioremediation of petroleum hydrocarbon contaminated soil

Bioremediation is considered to be an environmentally friendly and cost effective process which utilises microorganisms to degrade contaminating petroleum hydrocarbons in soil (Boopathy, 2000). The end point of bioremediation is usually evaluated by chemical analysis to determine when the residual concentration of total petroleum hydrocarbons in the soil has decreased to levels deemed to be safe by regulatory authorities (Vidali, 2001). Although many studies focus on demonstrating that bioremediation degrades a variety of contaminants in the environment, there is very little data that demonstrate that the toxicity of the soils to humans decrease as a result of bioremediation (Alexander *et al.*, 2002).

Current studies examining the effect of bioremediation on the toxicity of soil are mainly performed using ecotoxicity studies with plant and earthworm models or on bacterial

models. Some of these studies show that the toxicity of the soil does indeed decrease during the course of bioremediation. Bioremediation of coke oven soil contaminated with PAH's was found to decrease the toxicity of the soil to bacteria and algae (Mendonça *et al.*, 2002). In another study, diesel contaminated soil bioremediated using biostimulation showed decreased toxicity to *Daphnia magna* and soil nematodes (Molina-Barahona *et al.*, 2005).

Although many studies show that bioremediation is able to reduce the toxicity of contaminated soil, many others have shown that bioremediation increased soil toxicity. In one study, bioremediation of diesel-spiked soil showed decreased seedling emergence and root length over the course of bioremediation, indicating increased toxicity (Marwood *et al.*, 1998). A different study which examined the toxicity of soil contaminated with polycyclic aromatic hydrocarbons found an increase in toxicity to chicken DT40 B-lymphocytes following bioremediation (Hu *et al.*, 2012). These studies demonstrate the need to assess the risk posed by a complex environmental mixture using a combined approach of both chemical and biological testing.

1.3 Toxicity screening methods

1.3.1 Chemical analysis

Toxicity screening using chemical analysis involves the elucidation of the identity and concentration of all individual constituents within a chemical mixture. The toxicity of the chemical mixture is then extrapolated based on the known toxicity and mechanism of action of the individual constituents (Allan *et al.*, 2012, Heys *et al.*, 2016). Models used for extrapolation assume only additive effects as described in section 1.1.1.1 (Additive effects). Therefore interactions between compounds in the environmental mixture which may alter its cytotoxicity may be missed (Paton *et al.*, 2005).

For chemical analysis, reliable quantification requires the use of a validated method in conjunction with internal standards containing known concentrations of a compound with a similar chemical profile to the target compound (Shannon *et al.*, 1993). The analysis of different classes of compounds within the mixture may also require different equipment and sample preparation (Dévier *et al.*, 2011).This generally does not present any major issues when only simple mixtures are examined. However due to the high number of different chemicals that may be present in complex mixtures, quantification of all compounds within the mixture may not be possible due to interference from other compounds or due to time and resource constraints. In addition, some chemicals within complex environmental mixtures are likely to be unknown or poorly characterised. Although chemical methods are constantly updated to include more accurate and sensitive determination of new and existing chemicals, it is likely that at least one compound within the complex mixture will lack a suitable method for its elucidation.

While chemical analyses provide useful information on the composition and concentration of toxic compounds in an environmental sample, for complex mixtures not all compounds can be quantified or accurately identified. Therefore for many complex mixtures such as contaminated soils, risk assessment is based only on the proportion of its known chemical constituents (Schinner, 2005, Vasseur *et al.*, 2008). At best, complex mixtures are screened for the presence of all currently known hazardous compounds. This would present a problem as the unknown chemicals within the mixture have the potential to have toxic effects that are not taken into account, or they may have the potential to interact with the known chemicals to alter toxicity of the mixture.

1.3.2 Bioassays

Bioassays measure the response after exposure of a biological model to a mixture and therefore enable a direct measurement of toxicity. As bioassays analyse the mixture as a whole, they enable the integration of all additive, synergistic, potentiating and additive effects in the one analysis (Nirmalakhandan *et al.*, 1994, Brack, 2003, Allan *et al.*, 2012).

One limitation of the exclusive use of bioassays to assess the toxicity of mixtures is that although they allow assessment of the mixture as a whole, they do not provide information on the chemical constituents. Therefore, identification of chemicals responsible for the toxic effect or groups of chemicals contributing to interactive effects may not be practicable.

In order to adequately assess the hazard posed by an environmental mixture, many studies now employ an integrated approach comprising both chemical analysis and bioassays to analyse the complete mixture (Booth *et al.*, 2008, Shaw *et al.*, 2009, Allan *et al.*, 2012, Larsson *et al.*, 2013, Heys *et al.*, 2016).

1.3.2.1 Ecotoxicity assays

The majority of studies which employ bioassays to study the toxicity of environmental mixtures generally focus on ecotoxicity. Ecotoxicity bioassays are designed to assess the toxicity of environmental mixtures to non-target biota and use biological models such as plants, microbes, invertebrates or aquatic organisms (Ågerstrand *et al.*, 2015). Differences between the biological activities of these models and human cells mean that extrapolation of the results may not accurately predict the potential risks to human health (Perreault *et al.*, 2011).

1.3.2.2 In vivo animal models

The traditional model for assessing the toxicity of chemical for human risk assessment involves the use of *in vivo* animal models. In this model, whole animals are exposed to the chemicals and the response is then extrapolated to humans. The objective of acute toxicity testing in animals is to establish the lowest dose which induces a toxic effect and to identify major target organs of toxicity (Barlow *et al.*, 2002).

Ethical concerns regarding the use of animal based methods have been raised over the years due to the suffering of the animals involved and the reliability of data to enable extrapolation to humans. This is because different species may show different responses to chemicals due to different toxicokinetics involving absorption, distribution, metabolism or excretion (Dybing *et al.*, 2002). This may result in some chemicals eliciting high toxicity in animal models but low or negligible effects in humans. Conversely, chemicals may exert low or negligible toxic effects in animal models but induce a highly toxic effect in humans.

There are many other limitations of animal models for toxicity testing including that they are expensive and time consuming. There is also a high amount of variability in the results, even within the same species and therefore a high level of expertise is required to design and interpret the data. This intra-species variability may be due to differences in sex, age, weight or diet of the individual animals (Barlow *et al.*, 2002).

The limitations and ethical concerns of animal testing have led to the concept of the three R's (reduction, refinement and replacement). The principles of the three R's involve developing new experimental procedures which reduce the total number of animals necessary for the study; refining existing experimental procedures to minimise animal pain and distress, and the development of alternative non-animal experimental

procedures which can replace existing animal models (Soldatow *et al.*, 2013, Doke *et al.*, 2015)

Given that each complex environmental mixture may have unique properties and the high cost of animal testing, the use of animal models to assess their toxicity may not be feasible as the data would only be relevant for the particular mixture and may not be applicable to any other case. As a consequence, the use of animal models to assess the toxicity of complex environmental mixtures would contradict the principles of the three R's and would be considered as an unacceptable approach.

1.3.2.3 In vitro bioassays

In vitro bioassays utilising cultured cells are recognised as a good alternative to *in vivo* models for routine screening for cytotoxicity. They allow significant advantages over *in vivo* animal models in that they provide a rapid, high throughput and cost effective method for assessing toxicity. As a result, *in vitro* bioassays are routinely used instead of in *vivo models* in the initial cytotoxicity screening stages of drug compounds to determine their toxicity and mode of action (Tesei *et al.*, 2005).

By using human cell lines in *in vitro* bioassays, any species specific effects as found in in vivo animal testing is minimised. Therefore the use of human cell lines in *in vitro* bioassays may enable a more relevant toxicological assessment of the chemical to humans compared to animal models. *In vitro* bioassays also offer lower variability of data which can be partly attributed to the genetic homogeneity of the population (Eisenbrand *et al.*, 2002). Cell culture conditions are tightly controlled and allow high numbers of cells to be exposed to the chemical mixture simultaneously.

A wide range of human cell lines are commercially available originating from various tissues, disease types or with well characterised genetic modifications. Therefore, the choice of cell line may also allow investigation of particular areas of interest such as tissue specific cytotoxicity or if the cytotoxic effect could be influenced by particular genetic dispositions or metabolic pathways (Eisenbrand *et al.*, 2002, Soldatow *et al.*, 2013). Indeed, the use of a battery of well characterised cell lines to examine the cytotoxic potential of compounds is common practice in toxicology (Congiu *et al.*, 2008, Xia *et al.*, 2008, Hearn *et al.*, 2013)

In addition, *in vitro* methods may also allow for easier elucidation of the specific mechanism of cytotoxicity on a sub-cellular level. Cells can be easily manipulated and

the use of different endpoints for analysis would enable valuable insight into cellular changes that may occur following exposure to a chemical mixture.

1.3.2.3.1 In vitro bioassay endpoints

A plethora of *in vitro* bioassays which measure different biological end points are available to test for cytotoxicity. Examples of biological endpoints that are suitable for detection of cytotoxicity include cell morphology, membrane permeability, cell proliferation, cell adhesion or cell metabolism.

It is noted that the extent of cytotoxicity measured using one end-point may not correlate with the same level of cytotoxicity when measured using a different end point. This is because the mechanism by which chemical compounds exert their cytotoxicity may cause greater effects in one endpoint at the time of analysis compared to another. For example, the level of cell death detected following hyper thermic exposure of human cell lines was greater when using cell adherence as an endpoint compared to membrane permeability (Elengoe *et al.*, 2014). In another example, a greater level of cytotoxicity was detected using cell metabolism (via the MTT assay) as an endpoint compared to cell proliferation and membrane permeability endpoints (via the neutral red and LDH leakage assays respectively) following exposure of HepG2 cells to cadmium chloride (Fotakis *et al.*, 2006).

Alternatively, some compounds may interfere with the detection method used. For example, compounds that increase mitochondrial activity may overestimate the number of viable cells using cell metabolism as an end point (Pagliacci *et al.*, 1993). Therefore to minimise the risk of a false positive or negative result, it is desirable to use multiple endpoints to confirm the effect of chemical compounds on cell viability (Chiba *et al.*, 1998, Edwards *et al.*, 2012, Gliga *et al.*, 2014). This thesis used the following three methods.

1.3.2.3.2 Selected In vitro bioassays

1.3.2.3.2.1 The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay is used extensively as a rapid colorimetric method for determining cellular growth and viability (Marks *et al.*, 1992, Schappacher *et al.*, 2010, Scherließ, 2011, Lone *et al.*, 2013, Seidl *et al.*, 2013). The assay uses the yellow water-soluble tetrazolium salt MTT, which is reduced via the cleavage of the tetrazolium ring by mitochondrial dehydrogenases to insoluble purple formazan crystals in metabolically active cells. The formazan crystals are then solubilised with organic solvents such as dimethyl sulfoxide (DMSO) or SDS to enable quantification using a spectrophotometer (Young *et al.*, 2005, Fotakis *et al.*, 2006). The amount of formazan crystals formed has been shown to be directly proportional to the number of living cells in culture (Mosmann, 1983).

Advantages of the MTT assay include the ability to detect cells which are not dividing but are still metabolically active. In addition, the assay provides a faster, more cost effective and non-radioactive alternative for assessing cell viability compared to other assays such as the [3H]thymidine incorporation assay (Gieni *et al.*, 1995).

Although the MTT assay is widely used to determine cell viability, studies have shown the potential of the assay to overestimate or underestimate the population of viable cells in the presence of compounds which directly interfere with MTT reduction in cells or by affecting cell metabolism of viable cells (Bernhard *et al.*, 2003, Wang *et al.*, 2010, Wang *et al.*, 2011, Stepanenko *et al.*, 2015).

1.3.2.3.2.2 Crystal violet assay

The crystal violet assay uses cell adherence as an endpoint with cell number determined as a function of the dye taken up by cells (Gillies *et al.*, 1986a). The assay is based on the assumption that adherent cells detach from culture plates during cell death and the remaining cells are quantified by staining cell nuclei with crystal violet.

Advantages of the assay are that it is rapid and easy to perform. In addition, the assay enables a measurement of cell number that is not affected by intracellular activities such as cell metabolism. A limitation of the assay is that it can only be performed on adherent cells. In addition, cells which adhere weakly may tend to wash off and cause high variation of the results.

1.3.2.3.2.3 Trypan blue exclusion assay

The trypan blue assay uses membrane permeability as an endpoint and relies on the ability of viability cells to exclude the trypan blue dye (Niles *et al.*, 2013). One advantage of using the trypan blue exclusion assay is that it does not rely on interaction of intracellular components (e.g. conversion of MTT by cellular enzymes) for the result. However the assay is not without its limitations and may cause a false positive result for chemicals that induce membrane permeability without affecting cell viability. For example, high levels of cell death were detected using the trypan blue exclusion assay following incubation of cells with a pore forming compound, even though the cells were still metabolically active and viable (Tran *et al.*, 2011).

1.4 Human cell lines

In order to investigate a range of potential routes of exposure, a selection of cell lines were used that cover liver, skin, lung, reproductive and peripheral blood cells.Cell line characteristics are detailed in Table 1-4.

Table 1-4. Cell line characteristics

Cell line	Tissue	Cell type	Organism	Culture Properties
HepG2	Liver	Hepatocyte	*Homo sapiens*, human	Adherent
HaCaT	Skin	Keratinocyte	*Homo sapiens*, human	Adherent
JAr	Placenta	Trophoblast	*Homo sapiens*, human	Adherent
MRC-5	Lung	Fibroblast	*Homo sapiens*, human	Adherent
WIL2NS	Spleen	B lymphocyte	*Homo sapiens*, human	Suspension

1.1.1 HepG2

The liver is the major organ in which biotransformation of xenobiotics takes place in the body (Wilkening *et al.*, 2003).The human hepatocellular cell line HepG2 has been reported to be a suitable model system for hepatotoxicity testing (Knasmuller *et al.*, 1998, Sevastyanova *et al.*, 2007) and is thus widely used in cell based assays for cytotoxicity. The cells were isolated from a primary hepatoblastoma and are known to retain wildtype p53 function as well as many of the morphological characteristics of liver parenchymal cells (Knasmuller *et al.*, 1998).

In addition HepG2 cells are metabolically competent, having retained the activities of a number of phase I and phase II enzymes of human liver required for biotransformation

and can thus be used to detect both direct and indirect acting toxicants (Knasmuller *et al.*, 1998, Valentin-Severin *et al.*, 2003).

Although HepG2 cells provide a suitable model system for toxicity testing, it is recognized that the xenobiotic metabolizing enzymes of the cell line are lower compared to primary human hepatocytes and have been shown to be less sensitive when assessing genotoxicity when using the COMET assay (Wilkening *et al.*, 2003). The use of primary hepatocytes for toxicity screening however is limited due to a shortage of available human liver material of which any primary cells obtained undergo only a limited number of cell divisions (Knasmuller *et al.*, 1998).

1.4.1 HaCaT

The human keratinocyte cell line HaCaT was established from the skin of the upper back of a sixty two year old male (Boukamp *et al.*, 1988). The cell line is immortal, allowing for easy culturing and retains characteristics of normal keratinocytes including normal differentiation to epidermal tissue following transplantation in mice (Boukamp *et al.*, 1988).

In addition, activities of xenobiotic phase II enzymes (glutathione S-transferase, UDP-glucuronosyltransferase and N-acetyltransferase) in HaCaT cells have been shown to be similar to that in *ex vivo* human skin (Götz *et al.*, 2012a). As a result, the cell line would represent a suitable model for assessing the cytotoxic risk posed by chemical compounds. Indeed, HaCaT cells have been used in previous studies to evaluate the toxic potential of various compounds and chemical mixtures to human skin (Bae *et al.*, 2001, Hecker *et al.*, 2002, Gehin *et al.*, 2005, Pelin *et al.*, 2011, Cortés-Eslava *et al.*, 2013, Liang *et al.*, 2014a, Jha *et al.*, 2016). Therefore HaCaT cells represent a suitable model for assessing the toxic potential of environmental mixtures in human skin in the present study.

1.4.2 MRC-5

The human fibroblast cell line MRC-5 was established from lung tissue from a male foetus (Jacobs *et al.*, 1970). The cell line grows in a uniform adherent monolayer with the morphological characteristics of normal fibroblasts (Jacobs *et al.*, 1970, Liu *et al.*, 2013b).

MRC-5 cells have been used previously in studies to evaluate the toxic potential of compounds to human lung tissue (Hadjur *et al.*, 1995, Altaf *et al.*, 2015, Kolundžić *et al.*, 2016). Therefore MRC-5 cells would represent a suitable model for assessing the toxic potential of environmental mixtures to human lung tissue in the present study.

1.4.3 JAr

The human JAr cell line was established from a trophoblastic tumour of the placenta (Pattillo *et al.*, 1971). The cell line is known to proliferate rapidly in culture and exhibit invasive properties (Plessinger *et al.*, 1999). Other characteristics of the placenta that are present in the JAr cell line include secretion of steroidal hormones and cytokines (Pattillo *et al.*, 1971, Roth *et al.*, 1996, Plessinger *et al.*, 1999).

JAr cells have been used previously in studies to evaluate the toxic potential of compounds to human reproduction and development (Plessinger *et al.*, 1999, Chen *et al.*, 2010, Zhou *et al.*, 2015). Therefore JAr cells would represent a suitable model for assessing the toxic potential of environmental mixtures to human reproduction and development in the present study.

1.4.4 WIL2NS

Lymphocytes are important components of the immune system in humans and are found in the blood, bone marrow and lymphatic tissues (Salazar *et al.*, 2012). As a consequence, chemicals that induce toxic effects in lymphocytes have the potential to have adverse effects on the immune system (Salazar *et al.*, 2012). In addition, chemicals that cause toxic effects in lymphocytes may also have the potential to be toxic to other peripheral blood cells.

The human B-lymphocyte WIL2NS cell line is a suspension cell line that is commonly used for studies that investigate the toxic potential of compounds and chemical mixtures to human lymphocytes (Saito *et al.*, 2004, Sharif *et al.*, 2011, Yin *et al.*, 2015). It is noted that WIL2NS cells have been found to be deficient in glutathione-S-transferase M1, a phase II xenobiotic metabolising enzyme (Shield *et al.*, 2004b). This feature of WIL2NS cells may be useful for investigating compounds whose toxic effects are impacted by the metabolic capacity of cells.

1.5 General research objectives

The overall aim of this thesis was to assess the suitability of human cell lines to detect the cytotoxicity of environmental mixtures. The following chapters include more specific aims relevant to the chapter however the general aims of the thesis were:

- To determine the cytotoxicity of environmental chemical mixtures using human cell lines
- To determine if human cell lines provide a more sensitive method for determining the toxicity of soil contaminated with chemical mixtures as it degrades over time compared to chemical analysis

The experimental chapters in this thesis were designed to explore these aims and are summarised below:

Chapter 3 is a method development chapter which aimed to optimise a HPLC method for simultaneous determination of carboxin and thiram from soil. The optimised HPLC method in Chapter 3 was used for comparison to *in vitro* toxicity testing using human cell lines in Chapter 5. Chapter 3 also confirmed the concentration of carboxin and thiram in the product formulation, Vitavax 200FF, which was used in subsequent Chapters 4 and 5.

Chapter 4 examined the cytotoxicity of carboxin and thiram individually, or in mixtures where they were applied in combination, or within a product formulation Vitavax 200FF containing other undisclosed ingredients. The main aim of Chapter 4 was to determine any potential non-additive effects within the mixtures using human cell lines. The type of cell death induced by carboxin and thiram individually or in their mixtures was also investigated.

Mixtures of greater complexity compared to Chapter 4 were investigated in Chapter 5. This was achieved by using human cell lines to monitor the cytotoxicity of carboxin and thiram individually, in combination, or within the product formulation Vitavax 200FF as they degraded over time in soil. Experiments in Chapter 5 used both sterile and non-sterile soil, in order to investigate the impact of the presence of microbes on the degradation and cytotoxicity of the product formulation Vitavax 200FF in soil.

Degradation experiments in Chapter 5 were performed using controlled laboratory settings to simulate environmental conditions. Therefore, Chapter 6 investigated the cytotoxicity of bioremediated soil, which represented an actual environmental mixture. The purpose of this chapter was to compare the effectiveness of two different bioremediation methods to degrade contaminants and reduce the cytotoxicity of soil to human cells.

2 CHAPTER 2: Materials and Methods

2.1 Chemical reagents and solutions

All chemicals used were of analytical reagent grade and were obtained from Sigma, St Louis, USA unless otherwise stated. Carboxin and Thiram were of analytical standard grade and were obtained from Sigma-Aldrich (Castle Hill, Australia). The commercial formulation Vitavax 200FF containing 200g/L each of carboxin and thiram as active ingredients was obtained from the Department of Agriculture, Fisheries and Forestry, Queensland, Australia. Acetonitrile, methanol, heptane and acetone were of high performance liquid chromatography (HPLC) grade and were obtained from Merck Millipore (Darmstadt, Germany).

Reagents are grouped according to the experimental categories in which they are used.

2.1.1 General reagents

2.1.1.1 0.01M Calcium Chloride solution

Calcium chloride dihydrate (1.47g) was first dissolved in 300ml RO water. The solution was them made up to 1L using RO water and stored at room temperature until required.

2.1.1.2 5-Fluorouracil stock solution

5-Fluorouracil (5-FU) was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 100mM. The 100mM stock 5-FU was then diluted further in DMSO to a working concentration of 40mM 5-FU and stored in the dark at 4°C.

2.1.1.3 20x Phosphate Buffered Saline stock solution

20x Phosphate Buffered Saline stock solution was prepared by dissolving 160g NaCl, 4g KCl, 28.8g Na_2HPO_4, 4.8g KH_2PO_4 in 900ml RO water. The solution was made up to 1L with RO water and stored at room temperature.

2.1.1.4 Phosphate Buffered Saline (PBS)

Phosphate buffered saline (1X) was prepared by adding 50ml 20x Phosphate Buffered Saline stock solution to 950ml RO water. The solution was sterilised by filtering through a 0.22µM membrane (Millipore, USA) and stored at room temperature.

2.1.1 Cell viability assay reagents

2.1.1.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) stock solution

A stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was prepared by dissolving MTT in PBS to a stock concentration of 5mg/ml. The stock solution was then filter sterilized through a 0.22 µM membrane (Millipore, USA) and stored at -20°C in 1ml aliquots until required.

2.1.1.2 20mM Hydrogen Chloride (HCl)

12M HCl (1.66ml) was added to 998.33ml RO water to a final concentration of 20mM HCl. The solution was stored at room temperature until required.

2.1.1.3 20% Sodium Dodecyl Sulphate (SDS) in 20mM Hydrogen Chloride (HCl)

SDS (40g) was added to 100ml 20mM HCl with stirring and heat to dissolve the powder. 20mM HCl was added to the solution to achieve a final volume of 200ml. The solution was stored at room temperature until required.

2.1.1.4 Crystal Violet Stain (0.5% Crystal Violet in 50% Methanol)

Crystal violet (0.5g) was dissolved in 100ml 50% methanol. The solution was stored at room temperature until required.

2.1.1.5 Sodium chloride solution 0.9% (NaCl)

NaCl (0.9g) was first dissolved in 90 ml of RO water. RO water was added to achieve a final volume of 100ml. The solution was stored at room temperature until required.

2.1.1.6 33% Acetic Acid Solution

33 ml of acetic acid solution was added to 67 ml of RO water and stored at room temperature until required.

2.1.1.7 Trypan Blue Staining Solution

Trypan blue (0.2g) was dissolved in 100ml 0.9% NaCl saline solution (section 2.1.1.5). The solution was then filter sterilized through a 0.22 µM membrane (Millipore, USA) and stored at 4°C in 1ml aliquots until required.

2.1.2 Reagents for analysis of cell cycle and apoptosis

2.1.2.1 0.1% Sodium azide

Sodium azide (0.1g) was dissolved in 100 ml PBS and stored at room temperature until required.

2.1.2.2 Propidium iodide stock solution (1mg/ml)

The stock solution of propidium iodide was prepared by dissolving 1mg of propidium iodide in 1ml PBS. The stock solution was stored in the dark at 4°C.

2.1.2.3 RNAse stock solution (10mg/ml)

The stock solution of RNAse was prepared by dissolving 100mg RNAse in 10ml PBS. The solution was filter sterilised through a 0.20µM syringe filter (Sarstedt, Germany) and stored at 4°C in 1ml aliquots.

2.1.2.4 0.1% Triton X-100 in PBS

Triton X-100 (1ml) was made up to 1L using PBS. The solution was stored at room temperature until required.

2.1.2.5 PI mixture

PI mixture was prepared by combining 5ml 0.1% Triton X-100 in PBS (section 2.1.2.4), 100µl RNAse stock solution (section 2.1.2.3) and 100µl propidium iodide stock solution (section 2.1.2.2). The solution was used immediately after preparation.

2.1.3 Reagents for microbial activity and enumeration

2.1.3.1 60mM sodium phosphate buffer pH 7.6

Sodium phosphate (8.5g) was first dissolved in 800ml RO water. The pH was adjusted to 7.6 using HCl and the solution was then made up to 1L using RO water. The solution was sterilised by filtering through a 0.22µM membrane (Millipore, USA) and stored at 4°C

2.1.3.2 4.8mM fluorescein diacetate (FDA) in acetone

Fluorescein diacetate (100mg) was dissolved in 50ml acetone and stored at -20°C in the dark.

2.1.3.3 602mM fluorescein stock solution

Fluorescein sodium salt (22.6mg) was dissolved in 20ml acetone and made up to 100ml using 60mM phosphate buffer pH 7.6 (section 2.1.3.1). The solution was stored at room temperature in the dark.

2.1.3.4 50g/L benomyl solution

Benomyl (2.5g) was dissolved in 50ml RO water and stored at 4°C until required.

2.1.3.5 Vitamin B 100x stock solution

Vitamin B 100x stock solution was prepared by adding 5mg thiamine-hydrochloride, 5mg riboflavin, 5mg niacin, 5mg pyridoxine-hydrochloride, 5mg inositol, 5mg Ca-panthotenate, 25mg p-aminobenzoic acid, and 25mg biotin to 95ml RO water. The pH was adjusted to 4.5 and the volume made up to 1L. The solution was sterilised by filtering through a 0.2 µM membrane and stored at -20°C.

2.1.3.6 100mg/ml streptomycin

Streptomycin sulfate (1g) was dissolved in 10ml RO water. The solution was filter sterilised through a 0.20µM syringe filter (Sarstedt, Germany) and stored at -20°C in 1.1ml aliquots.

2.1.3.7 15mg/ml tetracycline

Tetracycline (0.75g) was dissolved in 50ml 70% ethanol and stored in the dark at -20°C in 1.1ml aliquots.

2.1.3.8 0.85% Saline solution

Sodium chloride (8.5g) was made up to 1L using RO water. The solution was autoclaved to sterilise (121°C at 15 psi for 35 min) and stored at room temperature until required.

2.2 Soil samples

2.2.1 Soil for experiments involving carboxin, thiram, carboxin and thiram in combination or Vitavax 200FF (Chapters 3 &5)

Soil was obtained from a wheat field site in Lameroo, South Australia with no history of chemical application in the prior twelve months. The soil was collected from the surface layer of the soil up to a depth of 10cm and sieved through 4 mm mesh to remove debris such as stones, sticks, seeds and chaff. The soil was air-dried at room temperature by spreading soil 5 cm in depth in trays and allowed to dry until a constant weight was achieved. For sterile soil experiments, the soil was autoclaved twice in 500g lots (121°C at 15 psi for 35 min), with a period of 24h between each autoclave cycle. The autoclaved soil was air-dried again prior to use in experiments by spreading

soil 5cm in depth in sterile trays and dried in a laminar flow until a constant weight was achieved.

2.2.2 Soil for experiments involving soil contaminated with total petroleum hydrocarbons (Chapter 6)

Soil was obtained from Flinders Bioremediation that had been subjected to bioremediation using monitored natural attenuation (NA) or a combination of bioaugmentation and biostimulation (BABS). Briefly, soil from a bioremediated biopile (previously contaminated with petroleum hydrocarbons and bioremediated to below 10000 mg/kg) was sieved to remove any unwanted material such as stones and concrete. Two separate piles of the bioremediated soil, each containing 30 kg of soil were prepared and spiked with 3 kg oil sludge from a crude tank bottom of an oil refinery based in Sydney, Australia. The biopiles were then subjected to either bioremediation via NA or BABS. Soil samples were collected at 0, 4, 8 and 12 weeks after initiation of bioremediation and were stored at -20°C. Soil samples were homogenized by air-drying at room temperature until a constant weight was achieved prior to extraction.

2.3 Soil physicochemical properties

2.3.1 Soil pH

Soil pH was determined using a standard protocol according to Rayment *et al.* (2011) using method 4B1 pH of 1:5 soil/0.01M calcium chloride extract. Briefly, 1g of soil was added to 5ml 0.01M calcium chloride solution in a 10ml centrifuge tube and shaken for 1h at 25°C. The solution was allowed to sit for 30min to allow the soil to settle to the bottom of the tube. A calibrated pH meter was then used to measure the pH of the aqueous layer.

2.3.2 Air-dry moisture content

The air-dry moisture of soil was determined using a standard protocol according to Rayment *et al.* (2011) using method 2A1 air-dry moisture content. Briefly, 10g of soil was weighed in a weighing container and dried at 105°C overnight to a constant weight. The container was then allowed to cool overnight in a dry dessicator (no dessicant) and reweighed to determine the weight of moisture. The air dry moisture content was calculated using Equation 1 below:

Equation 1

$$Air\ dry\ moisture\ content\ (\%) = \left(\frac{weight\ of\ air\ dry\ soil - weight\ of\ oven\ dry\ soil}{weight\ of\ air\ dry\ soil\ (g)}\right) x\ 100$$

2.3.3 Water holding capacity (WHC)

Soil (50g) previously dried overnight at 105°C was saturated with 25ml RO water and allowed to drain for 24h. The soil was then weighed and dried in an oven at 105°C for 24h to remove the water from the soil. The dried soil was weighed again to determine the weight of water that was present in the soil. The weight of water in the soil was taken as the 100% water holding capacity of 50g of soil (Guerin, 2000).

The volume of water to add to 25g air-dried soil to achieve a 60% water holding capacity was determined using Equation 2.The weight of water was converted to volume using the specific gravity of water at 20°C with the assumption of a room temperature of 20°C.

Equation 2

$$Volume\ of\ water\ (ml) = \frac{\left(\frac{100\%\ WHC(g) x\ 60\%}{2}\right) - \left(\frac{25g\ x\ air\ dry\ moisture\ content}{100}\right)}{Specific\ gravity\ of\ water\ at\ 20°C}$$

2.4 Soil treatments for experiments involving carboxin, thiram, carboxin and thiram in combination or Vitavax 200FF (Chapters 3 & 5)

25g lots of air-dried soil were added to 100ml Erlenmeyer flasks and wetted to 60% water holding capacity by the addition of 3.07 ml sterile RO water. The soil was then spiked with 100 µl of the treatments dissolved in acetone so that the final concentration of acetone in all soil samples was constant (3.3%). Specific treatments and their concentrations are described in the methods section of each chapter. A solvent control consisting of wetted soil spiked with 100 µl acetone and a soil only control was also included. Spiked soil samples were then mixed thoroughly using a spatula.

For extraction recovery experiments (Chapter 3), the soil was sampled immediately after mixing. For degradation experiments (Chapter 5), the soil was incubated in the dark at 27°C and sampled after 0, 1, 3, 7, 14, 21 or 28 days. Soil samples were weighed every three days during the incubation period to enable maintenance of soil moisture. This involved replacing the amount of water that had evaporated from the sample using sterile RO water.

2.5 Extraction of carboxin and thiram from soil

Two extraction methods were used to extract carboxin and thiram from soil and their suitability judged based on percent recovery of both compounds. Results for the extraction of soil using hexane:acetone are shown in Appendix I. Acetonitrile was deemed to be the most suitable extractant for soil and therefore all extractions of carboxin and thiram from soil in this thesis were performed using a modified method from Sherif *et al.* (2011).

Acetonitrile (30ml) was added to Erylenmyer flasks containing 25g soil (section 2.4) and sonicated in a chilled sonicating water bath for 15 min. The extract was centrifuged at 8000 g for 10 min and the supernatant was transferred to a new tube. To collect the remaining residue, the remaining soil was vortexed with 20 ml acetonitrile for 10 sec, centrifuged at 8000 g for 10 min and the supernatant added to the tube. The pooled extract was centrifuged at 8000 g for 10 min to remove debris and a rotary evaporator was used to evaporate the acetonitrile. The dried extract was dissolved in 750 µl methanol to produce a 33.3 g/ml extract. The extracts were centrifuged at 10000 g for 10 min to remove the remaining debris and the supernatant was used for HPLC analysis or cell culture experiments.

2.6 Preparation of Vitavax 200FF for HPLC analysis

A 7.5% Vitavax200FF solution was prepared by dissolving 0.75 ml Vitavax200FF in 9.25 ml acetone. 100 µl of 7.5% Vitavax200FF solution was added to 30 ml acetonitrile and sonicated in a chilled sonicating water bath for 15 min. A rotary evaporator was used to evaporate the acetonitrile and the dried extract was dissolved in 750 µl methanol. The extract was centrifuged at 10000 g for 10 min to remove debris. Prior to injection into HPLC, the extracts which were dissolved in methanol were diluted with filtered RO water to a methanol:water ratio of 40:60 (v/v). Further dilutions of the extract were prepared by diluting in methanol:water (40:60 v/v).

2.7 Total petroleum hydrocarbons (TPH) extractions of soil samples

Total petroleum hydrocarbons were extracted from soil or oil sludge using a modified standard protocol of determining hydrocarbon content in soil according to International Organisation for Standardisation (International Organisation for Standardisation, 2004), ISO/DIS 16703 GC method. Briefly, 40 ml acetone and 20 ml heptane were added to 10 g of homogenized soil or 1 g oil sludge followed by sonication for one hour in a sonicating waterbath. Soil particles were separated from the extract by centrifugation at 2660 g and the supernatant was decanted into a glass separating funnel. The supernatant was washed twice to remove the acetone via the addition of 100 ml distilled water, shaken vigorously for 5 min and the bottom layer discarded. The top layer was collected and a 1 ml aliquot of the extract was taken for TPH analysis via gas chromatography (GC). Prior to analysis, samples were diluted using an internal standard solution consisting of n-decane and n-tetracontane at 20 µg/ml and 30 µg/ml respectively in heptane.

The remaining volume was weighed to calculate the exact volume using the specific gravity of heptane (0.681g/L at 25°C). The extract was then evaporated to 1-2 ml using a rotary evaporator and the remaining heptane in the sample evaporated under a gentle stream of nitrogen. The soil extracts were then dissolved in a solution of heptane:DMSO (1:9) to a stock concentration of 9g soil per ml. The oil sludge extract was dissolved to a stock concentration of 200 mg/ml of TPH in a solution of heptane:DMSO (1:9) The final extracts were stored in the dark at room temperature. A blank extraction was also performed without soil to determine any potential contamination from solvents or insufficient washing of glassware. As only a limited amount of sample was available for this study, it is noted that only one extraction was performed for each soil sample (n=1).

2.8 Quantification of carboxin and thiram using HPLC

2.8.1 Dilution of soil extracts

Prior to injection into HPLC, soil extracts which were dissolved in methanol (section 2.5), were diluted with filtered RO water to a methanol:water ratio of 40:60 (v/v). Further dilutions of the extract were prepared by diluting in methanol:water (40:60 v/v).

2.8.2 Preparation of standards

Carboxin (25mg) or thiram (25mg) was dissolved in methanol and made up to 25 ml in a volumetric flask to obtain a stock solution containing 1000µg/ml of carboxin or thiram. A solution of 200 µg/mL in methanol:water (40:60 v/v) of each chemical was prepared by adding 0.3 mL of the 1000 µg/ml stock solution to 0.3 ml methanol and 0.9 ml filtered RO water. A standard stock solution of 100 µg/mL of carboxin and thiram in methanol:water (40:60 v/v) was prepared by adding 1 ml of 200 µg/mL carboxin in methanol:water (40:60 v/v) to 1 ml of 200 µg/mL thiram in methanol:water (40:60 v/v). The 8 standard solutions ranging from 0.391 – 50 µg/mL carboxin and thiram were then prepared from the standard stock solution of 100 µg/mL of carboxin and thiram in methanol:water (40:60 v/v) by 1:2 serial dilutions in methanol:water (40:60 v/v).

2.8.3 Preparation of the mobile phase

The mobile phase was prepared by mixing volumes of methanol with volumes of filtered RO water at the following ratios: 80:20, 70:30, 60:40 or 45:55 v/v (methanol:water). The mobile phase was filtered through a 0.45 µm membrane prior to use in HPLC. The optimal mobile phase of 45:55 v/v (methanol:water) as determined in Chapter 3 was used for subsequent experiments in Chapter 5.

2.8.4 HPLC analysis

HPLC analysis was performed using a modified method from Gopal *et al.* (2006). Chromatography utilised an Agilent 1100 HPLC apparatus equipped with a degasser, quarternary pump and diode array detector (DAD). A Luna C18 reversed phase column (5 µm, 250 mm x 4.6 mm i.d.) was used as the stationary phase. Methanol:water was used as the mobile phase (refer to section 2.8.3) with an isocratic flow rate of 1 ml/min and column temperature of 30ºC. All samples were centrifuged at 10000 g for 10 min to remove debris prior to injection. 50 µl aliquots of standards (section 2.8.2) or samples were injected and the response detected at 254 nm. A calibration curve was generated by plotting the peak area against the concentration of carboxin or thiram and linear regression was used to determine the equation of the line. Standard solutions of 25 µg/mL carboxin or 25 µg/mL thiram were also analysed periodically in each run to confirm the retention time of the compound.

2.9 Quantification of Total Petroleum Hydrocarbons using Gas chromatography (GC)

TPH content of soil extracts was performed by Dr. Daniel Jardine at Flinders Analytical, Bedford Park, Australia according to International Organisation for Standardisation (International Organisation for Standardisation, 2004), ISO/DIS 16703 GC method. A Varian 3800 Gas Chromatograph equipped with a Varian 8200 Autosampler, Flame Ionization Detector, and splitless injection valve. An Alltech EC-5 capillary column (30 m × 0.25 mm with 0.25 µm film thickness) was used with helium as a carrier gas flowing at a rate of 2 ml/minutes in a constant flow mode.

2.10 Cell lines and cell culture

2.10.1 Cell lines

HaCaT, WIL2NS, JAr and MRC-5 cell lines were purchased from American Type Culture Collection (ATCC). HepG2 cells were kindly provided by Professor Greg Barritt (Department of Medical Biochemistry, Flinders University, Bedford Park, Australia).

2.10.2 Cell culture

2.10.2.1 Cell culture medium

The medium used to culture each cell line is outlined in Table 2-2. All cell culture media were sterilised by filtering through a 0.22µM membrane and stored at 4°C. Prior to use, the medium was equilibrated to 37°C in a water bath.

Table 2-1. Cell culture media

Cell line	Culture Medium
HepG2	Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated FBS, 4mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10mM HEPES and 1.5g/L sodium bicarbonate
HaCaT	RPMI-1640 with 10% heat-inactivated FBS, 2mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 1.5g/L sodium bicarbonate
JAr	RPMI-1640 with 10% heat-inactivated FBS, 2mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 4.5g/L glucose, 1mM sodium pyruvate, 10mM HEPES and 1.5g/L sodium bicarbonate
MRC-5	Eagle's Minimum Essential Medium (EMEM) with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 1.5g/L sodium bicarbonate
WIL2NS	RPMI-1640 with 5% heat-inactivated FBS, 2mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 1.5g/L sodium bicarbonate

2.10.2.2 Subculture of adherent cell lines

Cells (HepG2, HaCaT, JAr and MRC-5) were grown to 80% confluence in 75cm^2 cell culture flasks in a humidified atmosphere at 37°C with 5% CO_2. Cells were washed with phosphate-buffered saline (PBS) and detached with 1 ml 0.25% trypsin-EDTA (~5-10min). 9ml of fresh culture medium was then added and the cell suspension was centrifuged at 312g for 5min. The medium was removed and the cell pellet was resuspended in fresh culture medium. The number of viable cells harvested was calculated via the Trypan Blue exclusion assay (section 2.11.3) using a haemocytometer. To subculture, 1x10^6 - 2x10^6 cells were added to new 75cm^2 cell culture flasks containing 20ml culture medium.

2.10.2.3 Subculture of suspension cell lines

Cells (WIL2NS) were grown in 25cm^2 cell culture flasks until they reached a density of 1x10^6 cells/ml. The cell suspension was centrifuged at 312g for 5min and the cell pellet was resuspended in fresh culture medium. The number of viable cells harvested was calculated via the Trypan Blue exclusion assay (section 2.11.3) using a haemocytometer. To subculture, 1x10^5 cells were added to new 25cm^2 cell culture flasks containing culture medium so that the final volume in the flask was 10ml.

2.11 Cell viability assays

2.11.1 MTT assay

Specific parameters used for each cell line to conduct the MTT assay are shown in Table 2-3. Briefly, 200µl of cells were seeded onto 96-well plates in four replicate wells and allowed to attach in a humidified atmosphere at 37°C with 5% CO_2. The plate format, seeding density and adherence times used for each cell line are shown in Table 2-3. Cells were then exposed to treatments diluted in culture medium (specific treatments and exposure times used are outlined in the methods section of each experimental chapter). Following exposure of cells to treatments, cells were washed twice using PBS and the MTT assay was performed.

To perform the MTT assay, the stock 5mg/ml MTT solution was diluted in media and added to wells so that the final concentration of MTT in the wells was as specified in Table 2-3 in a total volume of 200µl. Cells were incubated with MTT in a humidified atmosphere at 37°C with 5% CO_2 for the specified amount of time (Table 2-3). In order to dissolve the formazan crystals, 80µl of 20% SDS in 20mM HCl was added and plates were kept in the dark at room temperature for 18h. The absorbance was measured using an automatic plate reader (Biotek Instruments Inc.) at 570nm with a reference wavelength of 630nm.

A standard curve plate was included for each experiment, consisting of ten serial dilutions with ranges as specified in Table 2-3, with each concentration examined in four replicate wells on a 96-well plate. The absorbance values from the treatment plates were converted to cells per well using the equation from the standard curve run for each experiment. An example of an MTT standard curve is shown in Appendix II. Results were expressed as percent viability relative to the solvent control.

Table 2-2. Specific MTT assay parameters used for each cell line

Cell line	96-well plate format	Standard curve range (cells/well)	Seeding density (cells/well)	Adherence time (hours)	Final MTT concentration in wells (mg/ml)	MTT incubation period (hours)
HepG2	Flat bottom	0-80000	20000	24	2.5	4
HaCaT	Flat bottom	0-40000	10000	24	0.5	4
JAr	Flat bottom	0-80000	20000	2	0.5	1
MRC-5	Flat bottom	0-40000	10000	24	0.5	4
WIL2NS	Round bottom	0-80000	10000	N/A	0.5	4

2.11.2 Crystal violet assay

Briefly, 200µl of HepG2, HaCaT, JAr or MRC-5 cells were seeded onto 96-well flat bottom plates in six replicate wells and allowed to adhere in a humidified atmosphere at 37°C with 5% CO_2. The seeding density and adherence time for each of the cell lines were the same as that used for the MTT assay (Table 2-3). Cells were then exposed to treatments diluted in culture medium (specific treatments and exposure times used are outlined in the methods section of each experimental chapter). Following exposure of cells to treatments, cells were washed twice using PBS and the crystal violet assay was performed.

To perform the crystal violet assay, cells were stained by the addition of 50µl crystal violet stain (section 2.1.1.4) to each well and incubating at room temperature for 10 min. Excess stain and dead cells were washed off using RO water and the plate was allowed to dry overnight. Cells were then destained by the addition of 50µl 33% acetic acid solution (section 2.1.1.6) to all wells for 10 min. The absorbance was measured using an automatic plate reader (Biotek Instruments Inc.) at 570nm.

A standard curve plate was included for each experiment, consisting of ten serial dilutions with ranges as specified in Table 2-3, with each concentration examined in six replicate wells on a 96-well plate. The absorbance values from the treatment plates were converted to cells per well using the equation from the standard curve run for each experiment. An example of a crystal violet standard curve is shown in Appendix II. Results were expressed as percent cell number relative to the solvent control.

2.11.3 Trypan blue exclusion assay

The trypan blue exclusion assay was performed by diluting 50µl of cell suspension with 50µl trypan blue staining solution (section 2.1.1.7). 20µl of the mixture was then loaded onto a haemocytometer in duplicate for counting. Dead cells are stained blue due to their membrane being permeable to the dye, while viable cells are able to exclude the dye and appear yellow (Niles *et al.*, 2013). The number of viable cells in suspension was calculated using Equation 3. For cytotoxicity experiments, results were expressed as percent cell number relative to the solvent control.

Equation 3. Calculation of the number of viable cells using the trypan blue exclusion assay

$$\textit{Number of viable cells (cells/ml)} = \textit{mean cell number per } 0.1\text{mm}^3 \textit{ square} \times 20000$$

2.12 Cell cycle analysis

2.12.1 Cell treatments for cell cycle analysis

HepG2 cells and HaCaT cells were seeded at 5 x 10^5 cells/well and 3 x 10^5 cells/well respectively into 6-well plates and allowed to adhere for 24h at 37°C in a humidified atmosphere. After the adherence time, the culture medium was removed and cells were exposed to 2ml of treatment in culture medium for 4h or 24h at 37°C in a humidified atmosphere. Following treatment, cells were harvested by trypsinisation and processed for cell cycle analysis.

For WIL2NS cells, 6-well plates were seeded at 5 x 10^5 cells/well. Cells were then exposed to 1ml of treatment in culture medium for 4h or 24h at 37°C in a humidified atmosphere. Cells were processed for cell cycle analysis following treatment.

2.12.2 Cell cycle analysis by flow cytometry

Following treatment of cells as described in section 2.12.1, cells were fixed with 3ml ice cold 70% ethanol overnight at -20°C. Cells were centrifuged at 312g for 5min and the cell pellet resuspended in 0.5 ml PI mixture (section 2.1.2.5) to stain cellular DNA. The suspension was incubated in the dark for 30 min and subsequently analysed using Accuri C6 flow cytometry equipped with CFlow software. A total of 10000 events were analysed for each sample. Examples of histograms used to cell cycle analysis are included in Appendix III.

2.13 Analysis of apoptotic events using flow cytometry

Apoptotic events were detected using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA). Treatment and harvesting of HepG2, HaCaT and WIL2NS cells were the same as for cell cycle analysis (section 2.12.1). Cells were counted using the trypan blue exclusion assay (section 2.11.3) following treatment and harvesting. Cells were then washed twice using 1ml 0.1% sodium azide in PBS and resuspended in binding buffer at 10^6 cells/ml. 50µl of the suspension was transferred to a culture tube and double stained with 2.5µl Annexin V-FITC and 2.5µl PI for 15 min in the dark. 200µl of binding buffer was then added to the mixture and the sample was analysed using Accuri C6 flow cytometry equipped with CFlow software. A total of 10000 events were analysed for each sample.

Viable cells are negative for both Annexin V-FITC and PI. Early apoptotic cells are positive for Annexin V-FITC and negative for PI. Late apoptotic or necrotic cells are positive for both Annexin V-FITC and PI (Pietkiewicz *et al.*, 2015). Examples of histograms used for analysis of apoptosis are included in Appendix IV.

2.14 Measurement of microbial activity in soil using fluorescein diacetate (FDA) hydrolysis

Microbial activity in soil was analysed using the FDA hydrolysis method as described by Green *et al.* (2006). 1g of soil was added in duplicate to 125 ml Erlenmeyer flasks containing 50ml of 60mM sodium phosphate buffer pH 7.6 (section 2.1.3.1) and 0.5ml of 4.8mM FDA (section 2.1.3.2). The flask was then swirled for 10 seconds to mix and incubated at 37°C for 3h. 2ml acetone was then added to terminate FDA hydrolysis and the suspension was then centrifuged twice at 9000g for 5min to remove the soil. The absorbance of the supernatant was measured at 490nm.

A no soil blank control and a no FDA blank control were also included in the analysis and their values subtracted from the test sample. The blanked absorbance value of the test sample was then converted to the amount of fluorescein released through the use of a standard curve.

To generate the standard curve, 0.03, 0.1, 0.3 and 0.5 mg fluorescein standards were prepared by pipetting 0.15, 0.5, 1.5 and 2.5ml of the 602mM fluorescein stock solution (section 2.1.3.3) into separate 50ml volumetric flasks. The volume of each flask was then made up to 50ml using 60mM sodium phosphate buffer pH 7.6. 2.5ml acetone was added to the flask and the absorbance of the solution was measured at 490nm.

2.15 Enumeration of microorganisms in soil

2.15.1 Media for enumerating microorganisms

2.15.1.1 Tryptone soy agar (TSA)

Tryptone soy agar was used to enumerate the number of total bacteria in soil. 30g tryptone soy (Oxoid, Australia) and 15g agar (Oxoid, Australia) were made up to 1L using RO water and sterilised by autoclaving at 121°C at 15 psi for 35 min. 1ml of 50g/L benomyl solution (section 2.1.3.4) was added to the medium prior to pouring of plates to inhibit the growth of fungus.

2.15.1.2 Modified Humic Acid Vitamin B Agar (HVA)

Modified Humic Acid Vitamin B Agar was used to enumerate the number of actinobacteria in soil. 1g humic acid, 0.25g disodium phosphate (Na_2HPO_4), 0.85g Potassium chloride (KCl), 0.025g magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 0.05g iron(II) sulfate heptahydrate (FeSO4.7H2O), 0.01g calcium carbonate (CaCO3) and 18g agar (Oxoid, Australia) was made up to 1L using RO water. pH was adjusted to 7.2 and the medium sterilised by autoclaving at 121°C at 15 psi for 35 min. 1ml of Vitamin B 100x stock solution (section 2.1.3.5) and 1ml 50g/L benomyl solution (section 2.1.3.4) was added to medium prior to pouring of plates.

2.15.1.3 Malt Extract Agar (MEA)

Malt Extract Agar was used to enumerate the number of fungi in soil. 20g malt extract (Oxoid, Australia) and 15g agar (Oxoid, Australia) was made up to 1L using RO water and sterilised by autoclaving at 121°C at 15 psi for 35 min. 1ml of 100mg/ml streptomycin (section 0) and 1ml of 15mg/ml tetracycline (section 2.1.3.7) was added to the medium prior to pouring of plates.

2.15.2 Enumeration of microorganisms

Enumeration of microbes in soil was performed according to Weaver *et al.* (1994). Briefly, a 10^{-1} dilution was performed by adding 10g of soil to 0.85% saline solution (section 2.1.3.8). The suspension was shaken vigorously by hand for 30s and sonicated in an ice cold waterbath for 30s. The suspension was then mixed on a rotary mixer for 20min at 200rpm. The solution was allowed to stand for 30s to allow the soil to settle to the bottom on the flask. 1ml of the 10^{-1} dilution was removed and used to prepare further 10 fold dilutions in 0.85% saline solution.

10µl of the dilutions were seeded in triplicate on TSA, HVA or MEA medium for determination of total bacteria, actinobacteria and fungi respectively, according to the Miles and Misra method (Hedges, 2002). 0.85% saline solution was also included as a negative control. The plates were incubated for up to 3 days for total bacteria counts and up to 6 days for actinobacteria and fungi counts.

3 CHAPTER 3: Optimisation of HPLC method for simultaneous determination of carboxin and thiram

3.1 Introduction

Carboxin and thiram are widely used agricultural fungicides that are commonly used together in commercial product formulations. Common product formulations that have carboxin and thiram as active ingredients are seed treatments, where the product is applied to seed to protect it from pathogenic fungi during storage and subsequent germination of the seed in soil. Therefore, both carboxin and thiram can be present in commercial products and in field sown with treated seeds.

A method which used HPLC to simultaneously quantify carboxin and thiram was reported by Gopal *et al.* (2006), however their method was only validated for extractions of carboxin and thiram from wheat seed. Soils are considered to be complex mixtures and as a result, the extract could contain other compounds that may interfere with accurate quantification of carboxin and thiram. In addition, the optimal conditions for HPLC may vary between laboratories due to differences in HPLC systems (Chan *et al.*, 2004).

Quantification of carboxin or thiram from soil using high performance liquid chromatography (HPLC) have been described in previous studies (Gupta et al., 2012, Sherif et al., 2011, Isidori et al., 2012, Hustert et al., 1999). However, the methods used for quantification in each study were based on the separate extraction and quantification of each compound. As the studies do not simultaneously extract both carboxin and thiram from soil, their extraction methods could potentially yield high recovery of carboxin from soil but low recovery of thiram, or vice versa. Consequently, it is important to validate the extraction method for both carboxin and thiram to ensure

that the recovery of each compound from soil is suitable to enable accurate quantification.

The aim of the present study was to develop a HPLC method for simultaneous determination of carboxin and thiram from the product formulation Vitavax 200FF and from soil. The optimal method was determined by evaluating HPLC system suitability parameters, linearity of the calibration curve and recovery of carboxin and thiram from soil.

3.2 Methods

3.2.1 Spiking of soil

25 g of air-dried soil was wetted to 60% water holding capacity by the addition of 3.07 ml RO water. The soil was then spiked with 100 µl of carboxin and thiram in acetone so that the final concentration of carboxin and thiram in the soil was 3, 7.5, 15 or 30 mg/kg. Another two soil samples were spiked with 100 µl of carboxin or 100 µl thiram in acetone to achieve a final concentration of 30 mg/kg carboxin or thiram respectively. The final concentration of acetone in all soil samples was constant (3.3%). A solvent control consisting of wetted soil spiked with 100 µl acetone and a soil only control was also included. Spiked soil samples were then mixed thoroughly using a spatula.

3.2.2 Extraction of carboxin and thiram from soil or Vitavax 200FF

Extractions of carboxin and thiram from soil or from the product formulation, Vitavax 200FF, were performed as described in section 2.5 and section 2.6 respectively. All extractions were performed immediately after mixing.

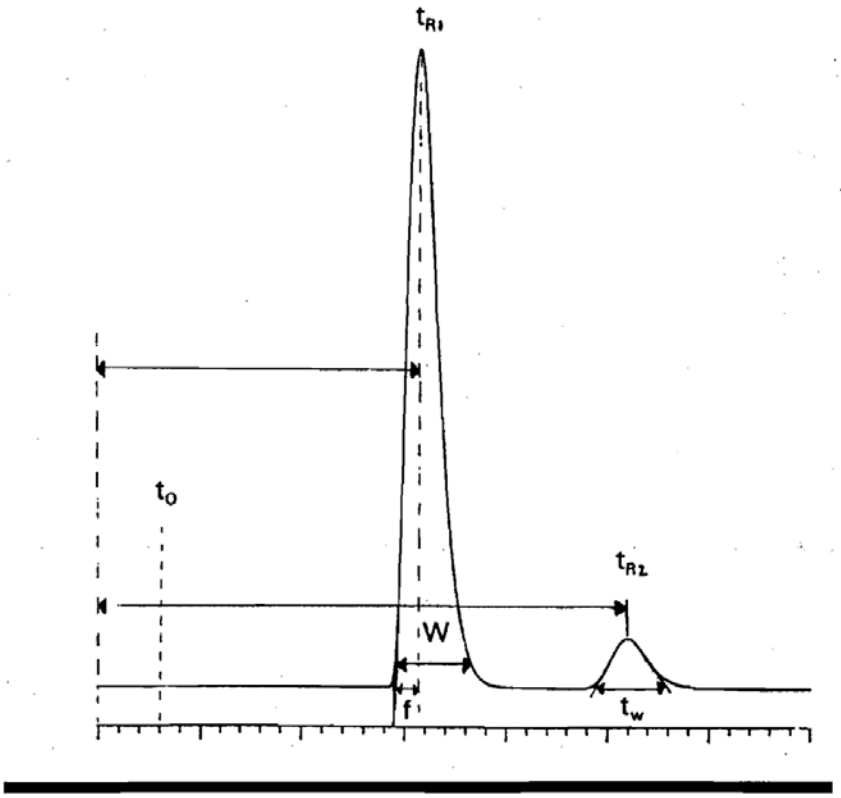
3.2.3 HPLC analysis

HPLC analysis was performed as described in section 2.8.

3.2.4 Calculation of HPLC parameters

All calculations were performed as according to recommended guidelines from FDA/CDER (1994). Calculation of system suitability parameters were performed using the definition of terms shown in Figure 3-1 and Equations 4-6. Precision was calculated from five replicate injections and the results are expressed as the percent relative standard deviation of the peak area or retention time.

Linearity of the calibration curve was determined using linear regression as described in section 2.8.4. The limit of detection (LOD) was defined as the lowest concentration of the standards that could be detected. Recovery of carboxin or thiram from soil was expressed as the extracted amount of the compound as a percentage of the spiked amount.



Where
W = width of the peak determined at 10% (0.10) from the baseline of the peak height
f = distance between peak maximum and peak front at W
t_o = elution time of the void volume or non-retained components
t_R = retention time of the analyte
t_W = peak width measured at baseline of the extrapolated straight sides to baseline

Figure 3-1. Definition of terms used for the calculation of HPLC parameters.
Image sourced from FDA/CDER (1994)

Equation 4. Capacity factor (k')

$$k' = (t_R - t_O) / t_O$$

Equation 5. Tailing Factor (T)

$$\mathbf{T = W / 2f}$$

Equation 6. Resolution (R_s)

$$R_s = (t_{R2}-t_{R1}) / ((1/2)*(t_{w1} + t_{w2}))$$

3.3 Results

3.3.1 Optimisation of HPLC mobile phase

A decrease in methanol concentration in the mobile phase from 80:20 to 45:55 (v/v) resulted in better separation of carboxin and thiram peaks, with no visual overlap of peaks observed for methanol:water at 45:55 v/v (Figure 3-2). Resolution between peaks was also improved with a lower methanol concentration in the mobile phase with the highest resolution between peaks achieved using methanol:water at 45:55 v/v (Table 3-1) The use of lower methanol:water ratios resulted in longer retention times of carboxin and thiram of 15.90 min and 12.25 min respectively for 45:55 v/v compared to 10.53 min and 8.66 min respectively for 60:40 v/v (Table 3-1). Of the mobile phases tested, only the parameters calculated for 45:55 v/v were all within FDA/CDER (1994) recommended guidelines (Table 3-1).

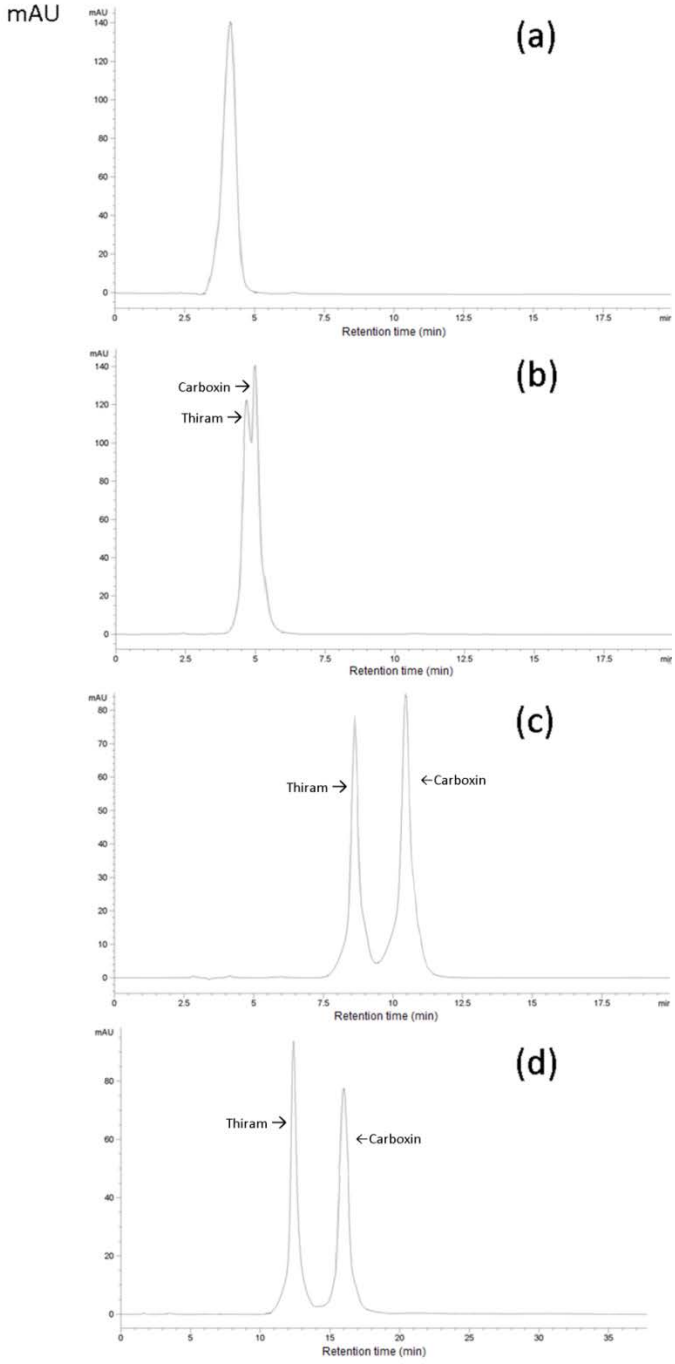


Figure 3-2. The choice of mobile phase affects the retention time and separation of carboxin and thiram.

HPLC chromatograms of 25µg/ml carboxin and thiram standards using methanol:water mobile phase mixtures of (a) 80:20 (v/v), (b) 70:30 (v/v), (c) 60:40 (v/v) or (d) 45:55 (v/v). The peaks corresponding to carboxin and thiram are indicated on the chromatograms.

Table 3-1. HPLC system suitability parameters determined for each mobile phase.

FDA/CDER recommended guidelines (FDA/CDER, 1994) are indicated for each parameter. Parameters were calculated by injecting 25µg/ml carboxin and thiram standards. ND = could not be determined

Parameter	Methanol:water (v/v)								FDA/CDER Recommended Guidelines
	80:20		70:30		60:40		45:55		
	carboxin	thiram	carboxin	thiram	carboxin	thiram	carboxin	thiram	
Retention time (min)	ND	ND	4.98	4.68	10.53	8.66	15.89	12.29	N/A
Capacity factor (k')	ND	ND	0.70	0.61	2.60	1.96	4.52	3.25	≥ 2
Tailing factor (T)	ND	ND	ND	ND	0.93	0.80	0.84	0.91	≤ 2
Resolution (R_s)	ND (overlap)		ND (overlap)		1.79		2.11		≥ 2
Precision - Peak Area (% RSD)	ND	ND	ND	ND	ND	ND	0.64	0.73	≤ 1%
Precision – Retention time (% RSD)	ND	ND	ND	ND	ND	ND	0.88	0.71	≤ 1%

3.3.2 Linearity and range

Both carboxin and thiram showed a linear correlation ($R^2 > 0.99$) between peak area and concentration in the range of 1.56-50 µg/ml when using methanol:water (45:55 v/v) as the mobile phase (Figure 3-3). The limit of detection (LOD) for both carboxin and thiram was 1.56 µg/ml.

3.3.3 HPLC analysis of Vitavax200FF

HPLC analysis of Vitavax200FF revealed the presence of carboxin and thiram at 216.7±26.9 and 213.3±22.1 g/L respectively (Table 3-2). The concentrations of carboxin and thiram detected in Vitavax200FF were both within 10% of that reported by the manufacturer of 200 g/L each of carboxin and thiram.

3.3.4 Recovery of carboxin and thiram from soil

Analysis of the soil only control revealed all peaks had retention times of less than 6 min (Figure 3-4), with similar results found for the solvent control (Figure 3-4). However, additional small peaks with retention times of 6.5 and 6.9 min were seen in some chromatograms of soil spiked with carboxin and thiram (Figure 3-4). The peak at 6.5 min was also seen in carboxin spiked soil, whilst the peak at 6.9 min was seen in thiram spiked soil (Figure 3-4).

Both carboxin and thiram show > 95% recovery from soil when spiked at concentrations greater than 7.5 mg/kg (Figure 3-5). The RSD was shown to decrease as the spiked concentration of carboxin and thiram increased. Soil spiked with 3 mg/kg of carboxin or thiram showed the lowest recovery and greatest variability between replicates of 89.8±24.5% and 76.1±26.5% respectively (Figure 3-5).

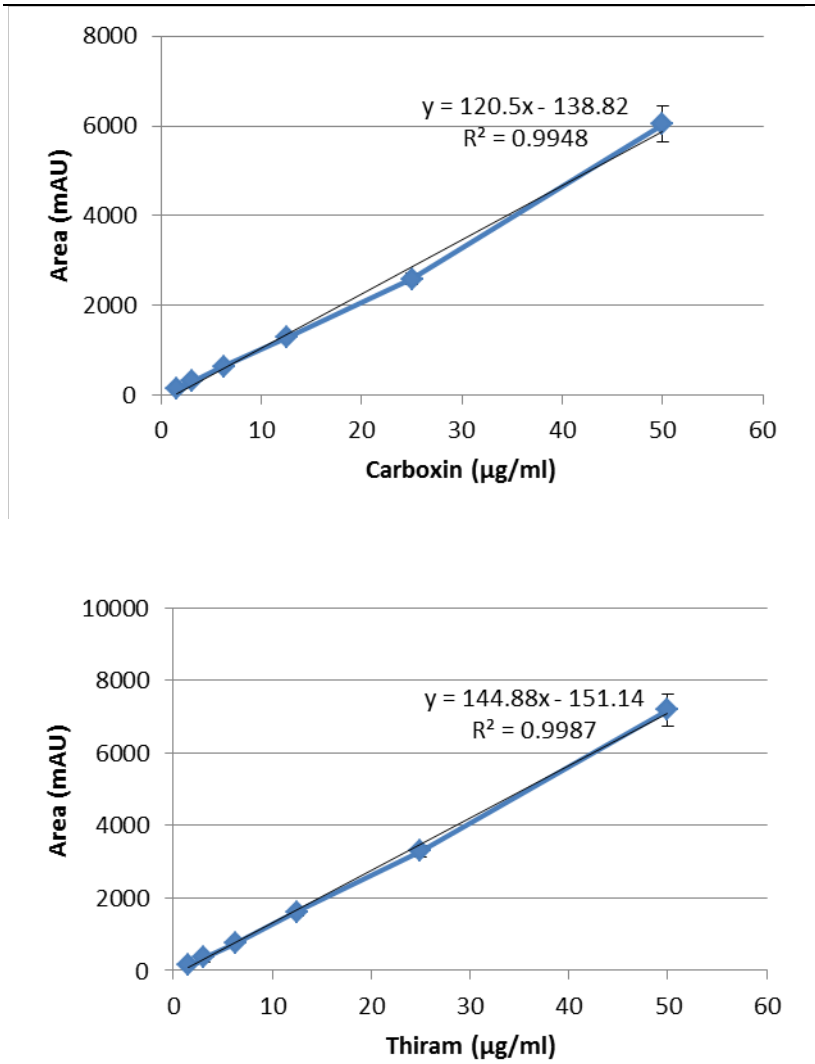


Figure 3-3. Standard curve of carboxin or thiram obtained by HPLC using methanol:water (45:55 v/v) mobile phase.

Results are shown as the mean±SD of three replicate samples (n = 3).

Table 3-2. Quantification of carboxin and thiram in the product formulation Vitavax200FF using HPLC.

The concentration of carboxin or thiram detected by HPLC shown as the mean±SD of three separate experiments (n = 3). The concentration of each compound reported on the label by the manufacturer is also shown for comparison.

Compound	**Concentration (g/L)**	
	Detected	Reported
Carboxin	216.7±26.9	200
Thiram	213.3±22.1	200

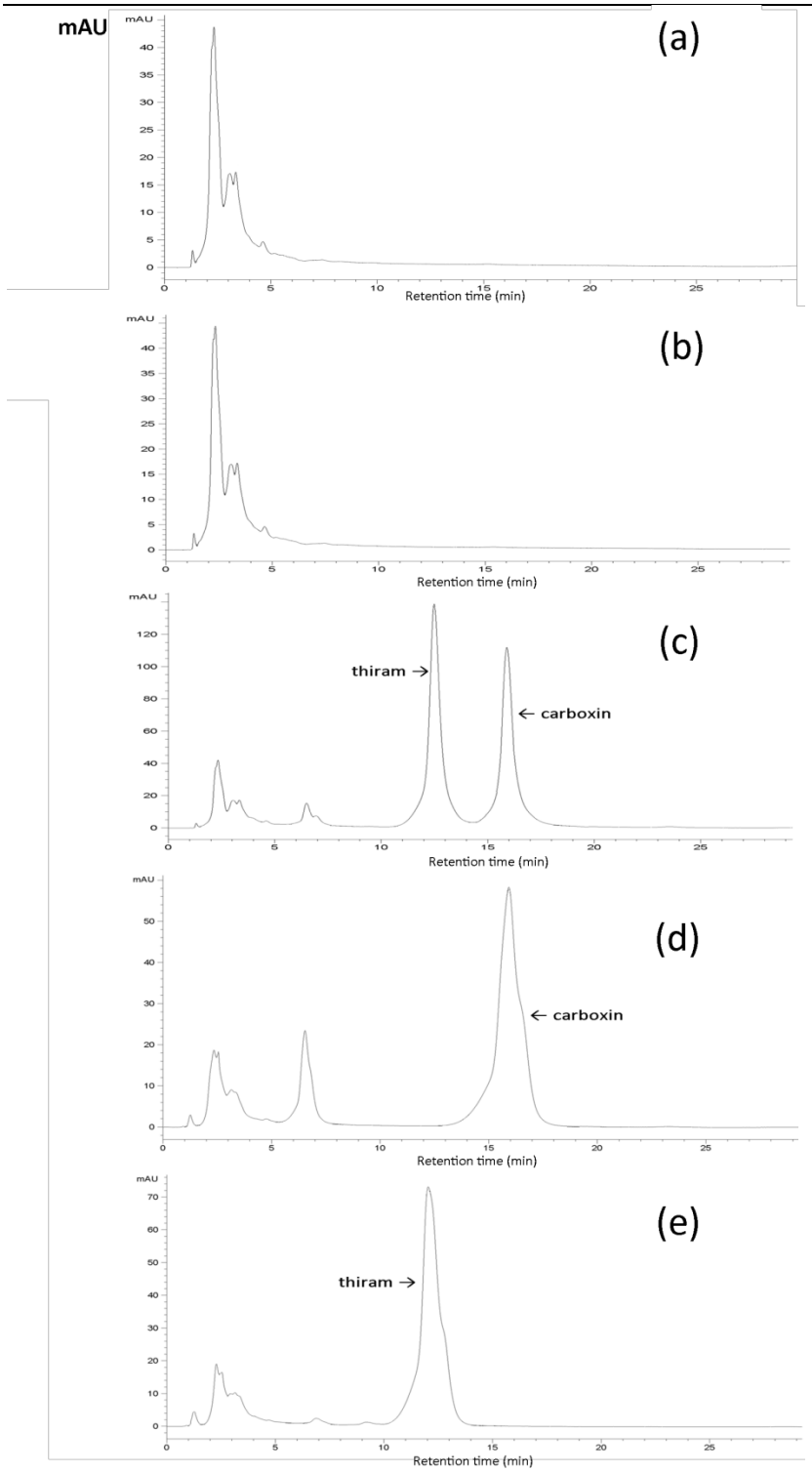


Figure 3-4. HPLC chromatograms of (a) soil only control, (b) solvent control, (c) carboxin and thiram, (d) carboxin, or (e) thiram spiked soil using methanol:water (45:55 v/v) mobile phase.

The peaks corresponding to carboxin and thiram are indicated on the chromatograms.

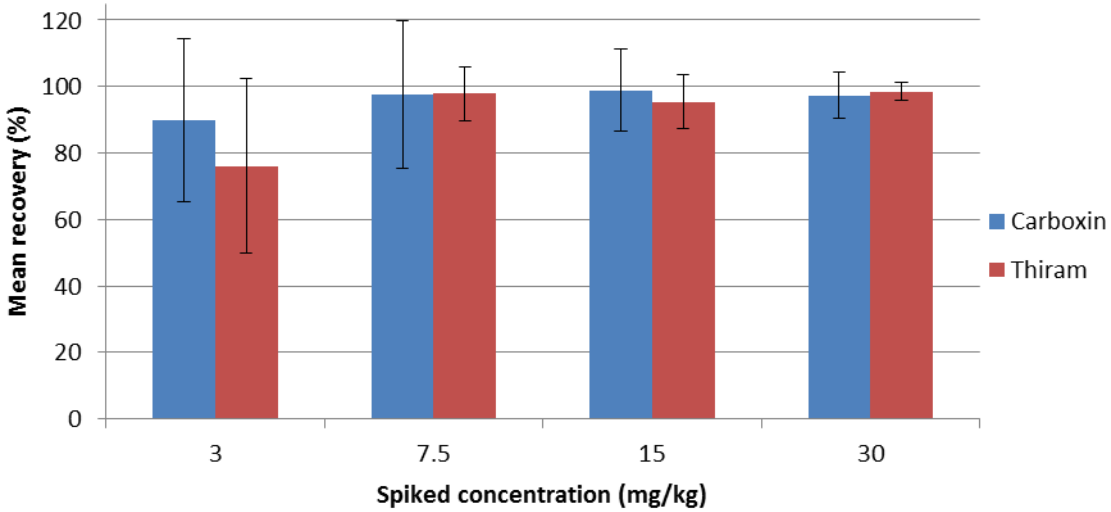


Figure 3-5. Recovery of carboxin and thiram from soil.

Results are presented as a percentage of the spiked concentration and is presented as the mean±relative standard deviation (RSD) of three separate experiments (n=3)

3.4 Discussion

The present study was undertaken to optimise and validate a HPLC method for simultaneous determination of carboxin and thiram in soil. Initial experiments were carried out to determine the optimal methanol:water ratio for the mobile phase. Methanol:water (45:55 v/v) was the only mobile phase tested that produced parameters within the FDA recommended guidelines (FDA/CDER, 1994). It was also anticipated that degradation of carboxin and thiram in soil may produce other peaks between the carboxin and thiram peaks and potentially interfere with quantification. Therefore, although the retention times of carboxin and thiram were relatively long for methanol:water (45:55 v/v) compared to the other mobile phases, it produced a resolution of 2.11 with minimal overlapping of peaks and was deemed to be preferable. Therefore, a mobile phase of methanol:water (45:55 v/v) was used for all subsequent HPLC analyses.

Carboxin and thiram standards were then analysed by HPLC to determine linearity and range. The high R^2 value (> 0.99) obtained for both carboxin and thiram indicate a good correlation between peak area and concentration. Therefore, the concentration of carboxin or thiram in a sample could be accurately determined from the equation of the line.

The optimal ratio of methanol:water in the mobile phase found in this study (45:55 v/v) for simultaneous determination of carboxin and thiram was much lower than that found in a previous study by Gopal *et. al.* (2006) in which the authors used 75:25 v/v with a flow rate of 0.7 ml/min, and a column of identical type and size (C18; 5 µm, 250 mm x 4.6 mm i.d.). Differences in the optimal ratio of methanol:water in the mobile phase may be due to differences in flow rate, temperature or injection volume of which the latter two were not reported by the authors. The method described by *Gopal et. al.* (2006) was also more sensitive with a reported LOD of 0.1 µg/ml for both carboxin and

thiram compared to the LOD of 1.56 µg/ml in this study. It was noted that the authors did not publish their chromatograms or report system suitability parameters as was done in this study. Therefore, it is unclear whether the method reported by *Gopal et. al.* (2006) produced parameters that were within the FDA recommended guidelines and could account for the differences in the optimal ratio of methanol:water and LOD found in this study. As such, this study provides a more comprehensive validation of simultaneous determination of carboxin and thiram.

The concentration of carboxin and thiram detected in Vitavax200FF was similar to that reported by the manufacturer (200 g/L each). The slightly higher levels detected in this study may be due to some evaporation of Vitavax200FF during storage, systematic error during extraction and preparation for HPLC, or variation during manufacturing. Detected levels of carboxin and thiram were within 10% of the manufacturers' specifications, indicating that the method was suitable for determining the concentration of the compounds in Vitavax200FF. Vitavax200FF is a relatively simple formulation with only two declared active ingredients. Other product formulations containing carboxin and thiram may also have additional active and non-active ingredients which have the potential to interfere with the accurate extraction and quantification of carboxin and thiram. Therefore, studies using different combinations of other active ingredients commonly used with carboxin and thiram would be required to determine the suitability of the method used in this study to quantify carboxin and thiram in other product formulations.

Recovery of carboxin and thiram from soil was greater than 95% when spiked at concentrations greater than 7.5 mg/kg, indicating efficient extraction of the compounds from soil. Although recovery of carboxin and thiram from soil spiked at 3 mg/kg was less than 95%, a recovery of 89.8±24.5% and 76.1±26.5% respectively was determined to be sufficient enough to allow quantification. A less than 100% recovery

was likely to be due to some degradation of carboxin and thiram during extraction and would account for additional peaks seen at 6.5 and 6.9 min. It is also possible that some residual carboxin and thiram remained in the soil after extraction and would contribute to a lower than 100% recovery.

The peak at 6.5 min was only observed in chromatograms of soil spiked with carboxin and thiram, or carboxin only and thus is likely a degradation product of carboxin. Similarly, the peak observed at 6.9 min only observed in chromatograms of soil spiked with carboxin and thiram, or thiram only and thus is likely a degradation product of thiram.

Partial degradation of carboxin and thiram during extraction can be due to a variety of factors. Sonication may cause partial degradation of compounds within a sample (Dükkancı *et al.*, 2006, Wang *et al.*, 2013). As a consequence, sonication of the soil in this study may have caused partial degradation of carboxin and thiram. Both carboxin and thiram have also been shown to degrade in water, soil or via exposure to sunlight (Chin *et al.*, 1970, Sharma *et al.*, 2003, Sherif *et al.*, 2011, Gupta *et al.*, 2012a). Therefore, partial degradation may also have occurred once the compounds came into contact with the soil or water during spiking and subsequent extraction. It is noted that the soil samples in this Chapter were extracted immediately after treatment. Therefore, a greater percentage degradation of carboxin and thiram may be observed after a longer period between spiking and extraction. Further studies are required to identify the cause of carboxin and thiram degradation during extraction.

4 CHAPTER 4: Evaluation of the *in vitro* cytotoxicity of Vitavax 200FF and its active ingredients in human cell lines

4.1 Introduction

Environmental mixtures can be classified as simple or complex mixtures as previously described in section 1.1. Agricultural chemicals represent an environmental mixture than can be both simple and complex. An agricultural chemical is classified as a simple mixture when the identity and concentration of all constituents within the mixture are known (Groten *et al.*, 2001). However, for many agricultural chemicals, only the active ingredients are disclosed on the product label whilst the remaining non-active ingredients remain proprietary information of the manufacturer and are not disclosed to the public. Unless the manufacturer disclosed all the ingredients of the product formulation for toxicological testing, the mixture is considered to be complex.

Toxicological evaluation of the commercial formulation to determine potential risks to human health in Australia is permitted to be extrapolated from the effects of the individual active and non-active ingredients (Australian Pesticides and Veterinary Medicines Authority, 2009a, Australian Pesticides and Veterinary Medicines Authority, 2009b). However, toxicological evaluation by a third party may not be possible if the proprietary ingredients are not disclosed. In addition, compounds within the product formulation may interact within the mixture to cause additive, synergistic, potentiated or antagonistic effects (Heys *et al.*, 2016). Potential interactions within chemical mixtures were previously outlined in section 1.1.1 and subsections 1.1.1.1 to 1.1.1.3.

Vitavax 200FF is an agricultural product containing carboxin and thiram as its active ingredients. As outlined in sections 1.2.1.1 to 1.2.1.3, limited studies are available which assess the toxicity of Vitavax 200FF or its active ingredients in human cells.

Therefore, this Chapter examined the effects of Vitavax 200FF and its active ingredients on cell viability in a range of human cell lines using the MTT, crystal violet and trypan blue cytotoxicity assays.

Cell cycle progression and cell death are highly controlled processes that control tissue homeostasis in multicellular organisms (Zhivotovsky *et al.*, 2010). It is acknowledged that observed reductions of cell viability in the MTT, crystal violet or trypan bue exclusion cytotoxicity assays compared to the untreated control may be due cell cycle arrest and/or cell death (Luo *et al.*, 1999, Lüpertz *et al.*, 2010, Ramezanpour *et al.*, 2014, Navanesan *et al.*, 2015). Therefore, it is important to investigate which mechanism is responsible for the reduction of cell viability.

The cell cycle consists of four main phases, namely G_1, S, G_2 and M phases as shown in Figure 4-1 (Owa *et al.*, 2001, Vermeulen *et al.*, 2003). The gap G_1 phase involves the synthesis of proteins in preparation for DNA replication and is activated in response to mitogenic stimuli (Lukas *et al.*, 1996). In response to deprivation of nutrients or growth factors, cells in G_1 phase can also enter a resting phase G0, where cell metabolism is dampened (Owa *et al.*, 2001).

Following G_1 phase, cells enter S-phase where DNA replication occurs. The gap G_2 phase follows S-phase which involves the synthesis of proteins in preparation for mitosis. Finally, M-phase involves mitosis where cell division occurs (Vermeulen *et al.*, 2003).

As shown in Figure 4-1, three major checkpoints are involved in the regulation of cell cycle. Regulation of the three checkpoints is mainly controlled by activation and inhibition of a range of cyclin-dependent kinases (CDK)(Pucci *et al.*, 2000). CDK is activated by binding to cyclin to form a CDK-cyclin complex. The activated complex is

then able to phosphorylate specific proteins to induce a signalling cascade, resulting in cell cycle progression (Owa *et al.*, 2001).

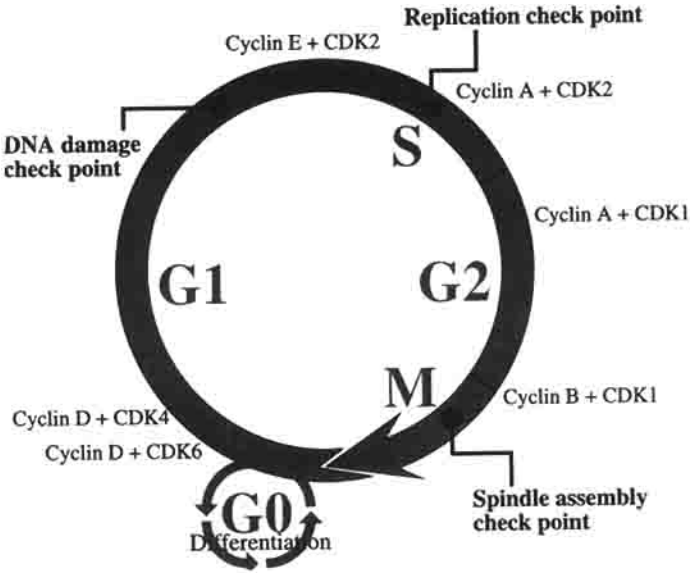


Figure 4-1. Cell cycle phases and check points.
Image sourced from (Owa *et al.*, 2001).

Apoptosis is a highly regulated form of cell death which is known to be activated by three main pathways; the extrinsic pathway, the intrinsic pathway and the granzyme pathway as shown in Figure 4-2 (Elmore, 2007). Each pathway is activated by specific cellular stimuli resulting in an intracellular signalling cascade involving pathway specific caspases. The pathways share a common downstream execution pathway involving activation of effector caspases 3, 6 and 7, leading to the irreversible commitment of the cell to apoptotic cell death (Elmore, 2007, Ouyang *et al.*, 2012). Activation of the execution pathway initiates a series of events which are characteristic of apoptotic cell death including DNA degradation, chromatin condensation, nuclear fragmentation, externalization of phosphatidylserine, cell shrinkage, membrane blebbing and the formation of apoptotic bodies (Hetz *et al.*, 2005, Elmore, 2007, Orrenius *et al.*, 2011).

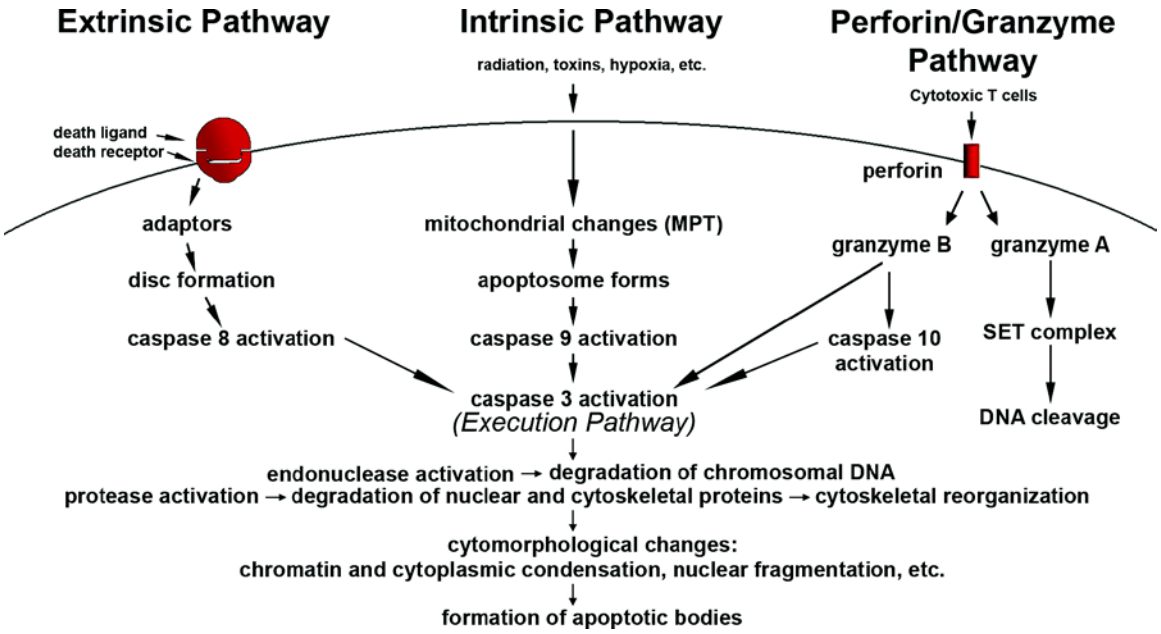


Figure 4-2. Apoptosis pathways.
Image sourced from (Elmore, 2007).

In contrast, necrotic cell death is characterised by ATP depletion and loss of membrane integrity leading to an influx of water, extracellular ions and cell swelling (Dive *et al.*, 1992, Hetz *et al.*, 2005). Subsequent rupture of the plasma membrane results in the release of cellular contents including lysosomal enzymes into the surrounding tissue lead to tissue injury and inflammation (Dive *et al.*, 1992, Hetz *et al.*, 2005). Although necrosis has been traditionally viewed as an accidental and uncontrolled form of cell death (Ouyang *et al.*, 2012), there is increasing evidence that cells can die through a programmed form of necrosis, which is regulated by key mediators including receptor-interacting serine/threonine protein kinases (RIPK;(Cho *et al.*, 2010, Moquin *et al.*, 2013, Newton *et al.*, 2014, Newton, 2015).

Loss of normal regulation of cell cycle or cell death can lead to a number of human disease states due to accumulation of DNA damage, uncontrolled cell proliferation, or

excessive cell death (Zhivotovsky *et al.*, 2010). No studies are available which examine the effects of Vitavax 200FF or its active ingredients carboxin and thiram on cell cycle. In addition, no studies have examined the type of cell death induced by carboxin in human cells. As outlined in section 1.2.1.1, the type of cell death induced by thiram in human cells is also unclear. In addition, there is the potential for interaction between carboxin, thiram, or other inactive ingredients within the product formulation Vitavax 200FF to cause a change in cell cycle progression or cell death.

Therefore the specific aims of this study were:

1. To determine the cytotoxic effects of carboxin and thiram individually or in a mixture in five different human cell lines.
2. To investigate the type of cell death and effects on cell cycle induced by carboxin and thiram individually and when in a mixture.
3. To determine if the cytotoxicity of the product formulation Vitavax 200FF can be estimated from the sum of the effects of its active ingredients.

It was hypothesised that:

1. Both carboxin and thiram will cause dose- and time-dependent cytotoxicity in the cell lines used
2. Both carboxin and thiram will cause apoptosis and cell cycle arrest
3. The toxicity of the ingredients in Vitavax 200FF will be synergistic and will be due to interaction between its active ingredients, carboxin and thiram.

4.2 Materials and methods

4.2.1 Preparation cell treatments

Stock solutions of carboxin, thiram, carboxin and thiram combined or Vitavax 200FF were prepared by dissolving the chemicals in DMSO. Cell treatments were prepared by diluting the stock solutions in culture medium or the solvent control containing 0.1% DMSO in culture medium. All cell treatments contained the same concentration of the solvent (0.1% DMSO).

4.2.2 Cytotoxic effects of soil extracts in human cells

HepG2 (20000 cells) ,HaCaT (10000 cells), or MRC-5 (10000 cells) cells were seeded into 96-well flat bottom plates and allowed to adhere for 24h at 37°C in a humidified atmosphere. For JAr cells, 20000 cells were seeded into 96-well flat bottom plates and allowed to adhere for 2h at 37°C in a humidified atmosphere. After the adherence time, the culture medium was removed and replaced with 200µl cell treatments and incubated for 1h, 4h, or 24h at 37°C in a humidified atmosphere. Cells were rinsed twice using PBS and cell viability was determined using the MTT (section 2.11.1) or crystal violet assay (section 2.11.2).

For WIL2NS cells, 6-well plates were seeded at 500000 cells/well. Cells were then exposed to 1ml of treatment in culture medium for 4h or 24h at 37°C in a humidified atmosphere. Cells were then rinsed twice using PBS and resuspended in 5ml fresh culture medium. 100µl of cell suspension was then transferred to 96-well round bottom plates and cell viability determined using the MTT assay (section 2.11.1) or trypan blue exclusion assay (section 2.11.3).

4.2.3 Analysis of cell cycle and apoptosis

Analysis of cell cycle and apoptosis in HepG2, HaCaT or WIL2NS cells was performed as according to section 2.12 and section 2.13.

4.2.4 Statistical Analysis

Linear regression was used to analyse the correlation between cell number and absorbance for standard curves of both MTT and crystal violet assays. To determine significant differences between media only and solvent controls, independent t-tests were conducted on cells per well values relative to the respective controls.

Statistical analyses were performed using SPSS software, version 18. Statistical differences in % cell viability between the solvent control and treatments in cytotoxicity assays were analysed by conducting one way univariate analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Results were considered to be statistically significant when $p \leq 0.05$. Two-way ANOVA using General Linear Model, Univariate analysis was used to determine non-additive effects. This method compares the expected additive response of two treatments with the actual response with results considered to be non-additive when $p \leq 0.05$. GraphPad Prism (version 5.01) was used in which log-transformed concentration values and their effect were fitted to a four parameter logistic equation and IC_{50} values were calculated from the line of best fit.

4.3 Results

4.3.1 Effects of the solvent control on cell viability

In this study, the stock solutions were prepared by dissolving the compounds in DMSO. Treatments were prepared by diluting the stock solution in culture medium or solvent control (containing 0.1% DMSO in culture medium) so that all doses tested contained 0.1% DMSO. Therefore, a solvent control was included to determine any effects of 0.1% DMSO on cell viability. All cell lines and exposure times showed no significant differences in cell viability between the medium and solvent controls (Figures 1, 2, 3 & 4), indicating that the effects observed in this study were solely attributable to the effects of the compounds tested.

4.3.2 Effects of Vitavax 200FF and its active ingredients on cell viability

Vitavax 200FF contains the active ingredients carboxin and thiram in equal concentrations (200g/L each). The effects of each active ingredient on cell viability were determined by treating cells with either carboxin or thiram individually.

Exposure to 50mg/L carboxin for 24h produced a significant decrease ($p \leq 0.05$) in cell viability to 49.7 ± 3.6% compared to the solvent control in the WIL2NS cell line using the Trypan Blue assay (Figure 4-3). The MTT assay also showed a slight decrease in cell viability to 78.3 ± 3.2% at the same dose and exposure time of carboxin in WIL2NS cells but did not reach significance. Exposure of HepG2 and HaCaT cells to 50mg/L carboxin for 24h also showed a slight reduction in cell viability in the MTT and Crystal Violet assays but was not statistically significant ($p > 0.05$) (Figure 4-3)

Calculation of IC_{50} values for carboxin revealed an IC_{50} of 49.7 mg/L for WIL2NS cells after 24h exposure in the Trypan Blue assay (Table 4-1). The effects of carboxin on

cell viability for all other cell lines, exposure times and assays did not reach 50% of the solvent control and therefore the IC_{50} could not be determined.

Thiram produced dose and time dependent decreases in cell viability in all cell lines as shown in Figure 4-4. After 24h exposure, significant decreases in cell viability were observed in both cytotoxicity assays used for HepG2, HaCaT, JAr, MRC-5 and WIL2NS cells at doses equal or greater than 5, 0.1, 0.5, 0.5 and 0.05 mg/L respectively (Figure 4-4)

Interestingly, the MTT assay detected greater thiram-induced reductions in cell viability compared to either Crystal Violet or Trypan Blue assays for all cell lines tested (Figure 4-4). The differences in cell viability results between the assays were more noticeable after shorter exposure times of 1h or 4h (Figure 4-4) resulting in greater differences in IC_{50} values between assays after 1h and 4h exposure compared to 24h exposure (Table 4-2).

WIL2NS cells were the most sensitive to thiram-induced cytotoxicity, showing the greatest reductions in cell viability at all exposure times (Figure 4-4). Consequently, WIL2NS cells also had the lowest IC_{50} values after 4 and 24h exposure to thiram with respective values of 0.11 and 0.03mg/L in the MTT assay and 7.82 and 0.03 mg/L in the Trypan Blue assay (Table 4-2)

The HepG2 cell line was the least sensitive to thiram-induced cytotoxicity at doses greater than 0.5mg/L after 24h exposure, followed by the JAr cell line (Figure 4-4). The sensitivities of the cell lines to thiram in the MTT assay, as determined from their calculated IC_{50} values (Table 4-2) was in the following order; WIL2NS cells > JAr cells > HaCaT cells > MRC-5 cells > HepG2 cells.

In order to determine the combined cytotoxic effects of the active ingredients of Vitavax 200FF in the absence of other ingredients in the formulated product, cells were exposed to carboxin and thiram in combination and in equal concentrations. Dose responses for carboxin and thiram combined are shown in (Figure 4-5) with calculated IC_{50} values shown in Table 4-3. All cell lines showed similar sensitivity to carboxin and thiram combined (Figure 4-5 and Table 4-3) as to thiram alone (Figure 4-4 and Table 4-2).

Cells were also exposed to Vitavax 200FF in order to determine the cytotoxic effect of the formulated product as a whole. Dose responses for Vitavax 200FF are shown in Figure 4-6 with calculated IC_{50} values shown in Table 4-4. All cell lines showed similar sensitivity to Vitavax 200FF (Figure 4-6 and Table 4-4) as to carboxin and thiram combined (Figure 4-5 and Table 4-3) or thiram (Figure 4-4 and Table 4-2).

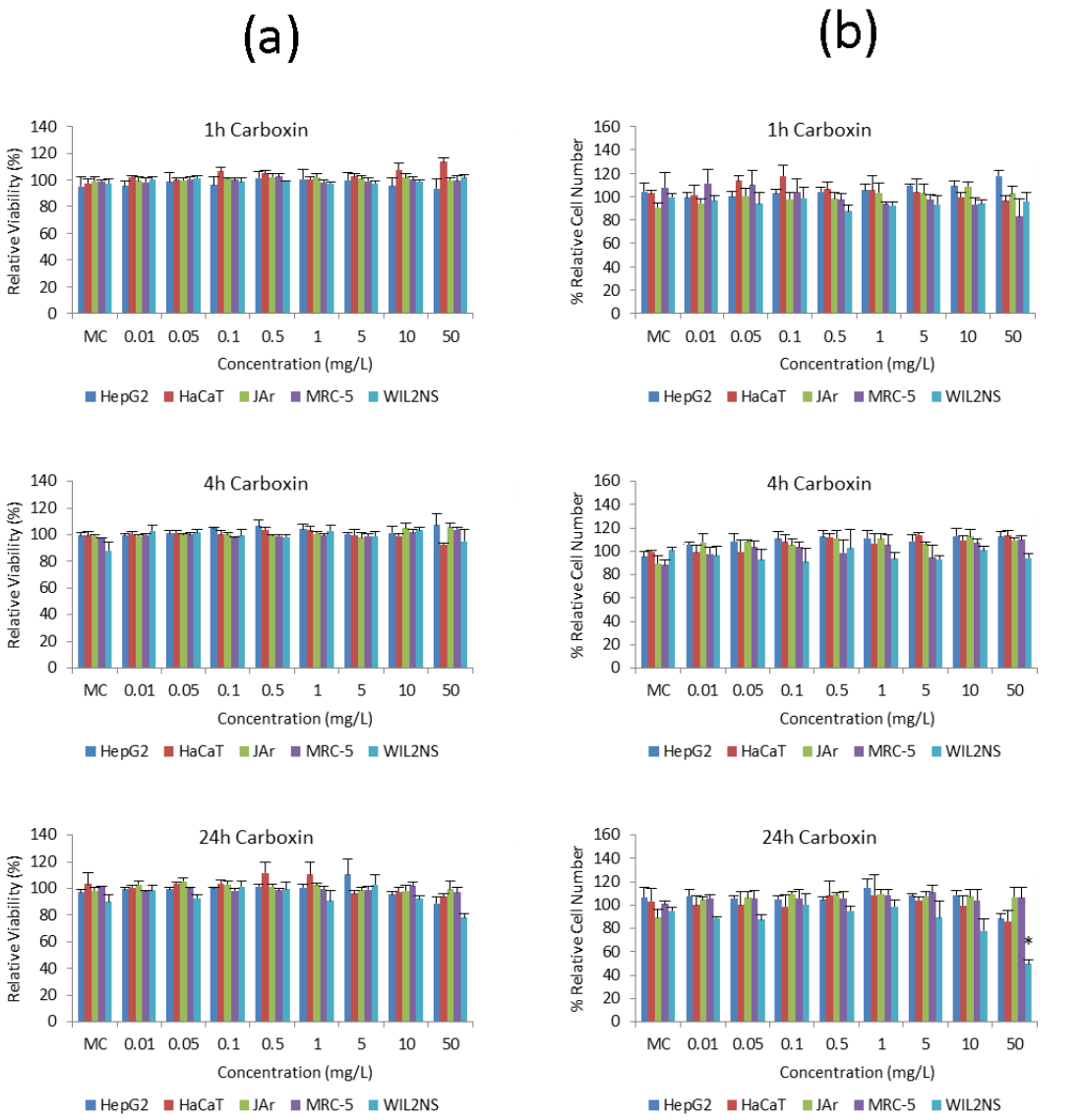


Figure 4-3. Effects of carboxin on cell viability after 1h, 4h or 24h exposure in five different human cell lines.

Cell viability was determined using the (a) MTT assay or (b) Crystal Violet Assay for adherent cells (HepG2, HaCaT, JAr and MRC-5) and Trypan Blue Assay for suspension cells (WIL2NS). MC = medium only control. For the MTT assay, results are expressed as % cell survival relative to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for HepG2, HaCaT, MRC-5 and WIL2NS; n=6 for JAr). For the Crystal Violet assay and Trypan blue assay, results are expressed as % relative cell number compared to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for all cell lines). * denotes a significant difference ($p \leq 0.05$) from the solvent control.

Table 4-1. IC_{50} values for carboxin.

Cells were exposed to 0 – 50 mg/L carboxin for 1, 4 or 24h and IC_{50} values were calculated from cell viability results determined from the MTT, Crystal Violet (CV) or Trypan Blue (TB) assays. Values in brackets () indicate 95% confidence intervals; ND = not determined

Assay	Cell line	IC_{50} (mg/L)		
		1h	4h	24h
MTT	HepG2	ND	ND	ND
	HaCaT	ND	ND	ND
	MRC-5	ND	ND	ND
	JAr	ND	ND	ND
	WIL2NS	ND	ND	ND
CV	HepG2	ND	ND	ND
	HaCaT	ND	ND	ND
	MRC-5	ND	ND	ND
	JAr	ND	ND	ND
TB	WIL2NS	ND	ND	49.58 (24.15 to 101.8)

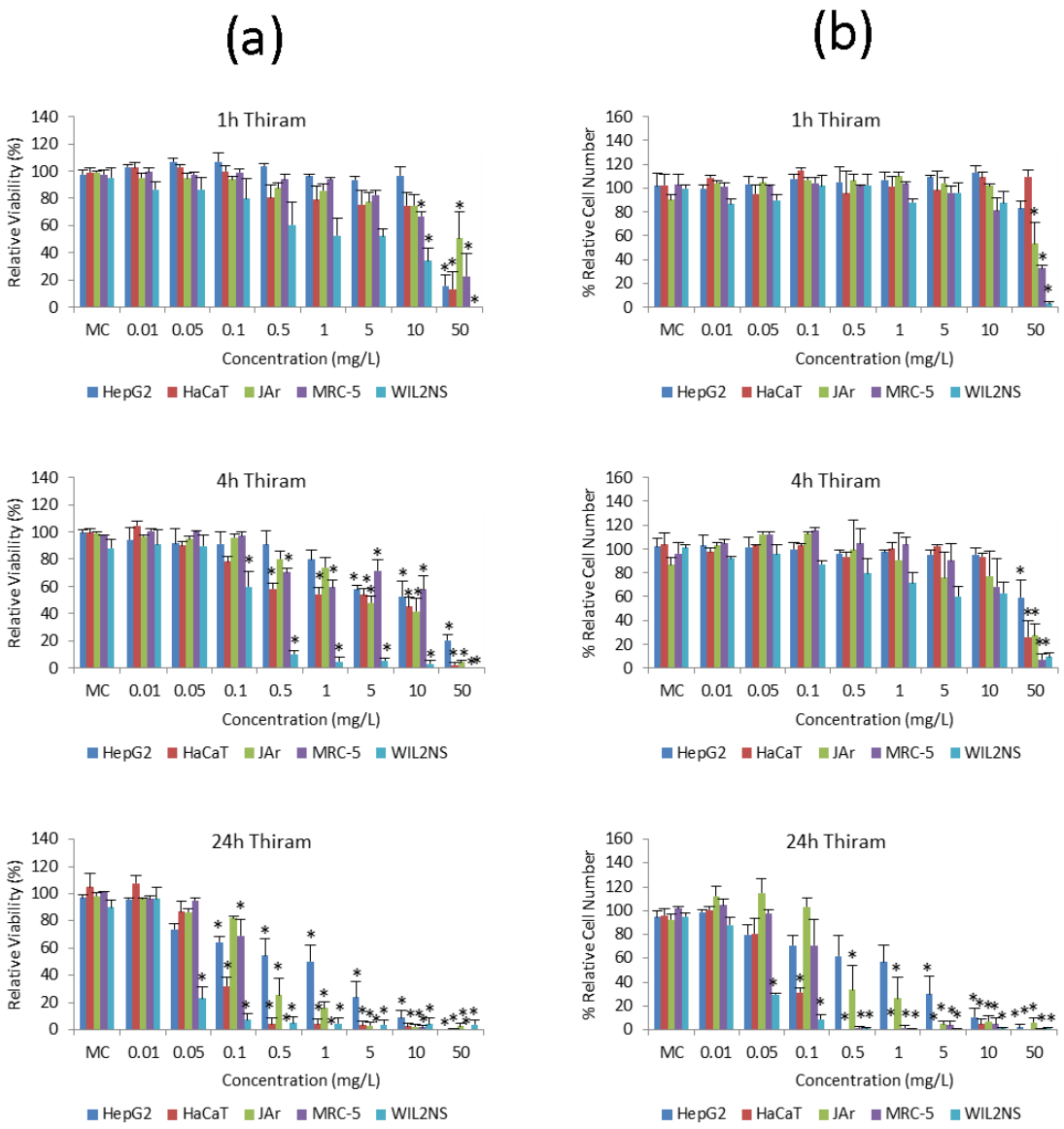


Figure 4-4 Effects of thiram on cell viability after 1h, 4h or 24h exposure in five different human cell lines

Cell viability was determined using the (a) MTT assay or (b) Crystal Violet Assay for adherent cells (HepG2, HaCaT, JAr and MRC-5) and Trypan Blue Assay for suspension cells (WIL2NS). MC = medium only control. For the MTT assay, results are expressed as % cell survival relative to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for HepG2, HaCaT, MRC-5 and WIL2NS; n=6 for JAr). For the Crystal Violet assay and Trypan blue assay, results are expressed as % relative cell number compared to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for all cell lines). * denotes a significant difference ($p \leq 0.05$) from the solvent control.

Table 4-2. IC_{50} values for thiram.

Cells were exposed to 0 – 50 mg/L thiram for 1, 4 or 24h and IC_{50} values were calculated from cell viability results determined from the MTT, Crystal Violet (CV) or Trypan Blue (TB) assays. Values in brackets () indicate 95% confidence intervals; ND = not determined.

Assay	Cell line	IC_{50} (mg/L)		
		1h	4h	24h
MTT	HepG2	32.43 (25.27 to 41.62)	8.9 (5.14 to 15.39)	0.39 (0.23 to 0.67)
	HaCaT	15.46 (8.47 to 28.23)	2.40 (1.31 to 4.38)	0.09 (0.08 to 0.10)
	MRC-5	18.04 (13.01 to 25.03)	6.34 (3.13 to 12.83)	0.13 (0.10 to 0.16)
	JAr	ND	4.26 (3.05 to 5.95)	0.05 (0.03 to 0.08)
	WIL2NS	1.73 (0.75 to 4.01)	0.11 (0.08 to 0.17)	0.03 (0.02 to 0.04)
CV	HepG2	ND	ND	0.91 (0.45 to 1.84)
	HaCaT	ND	32.27 (24.48 to 42.54)	0.08 (0.07 to 0.09)
	MRC-5	21.25 (15.64 to 28.87)	14.4 (8.53 to 24.32)	0.13 (0.07 to 0.21)
	JAr	ND	22.13 (8.87 to 55.23)	0.43 (0.26 to 0.69)
TB	WIL2NS	18.03 (10.28 to 31.63)	7.82 (3.93 to 15.58)	0.03 (0.03 to 0.04)

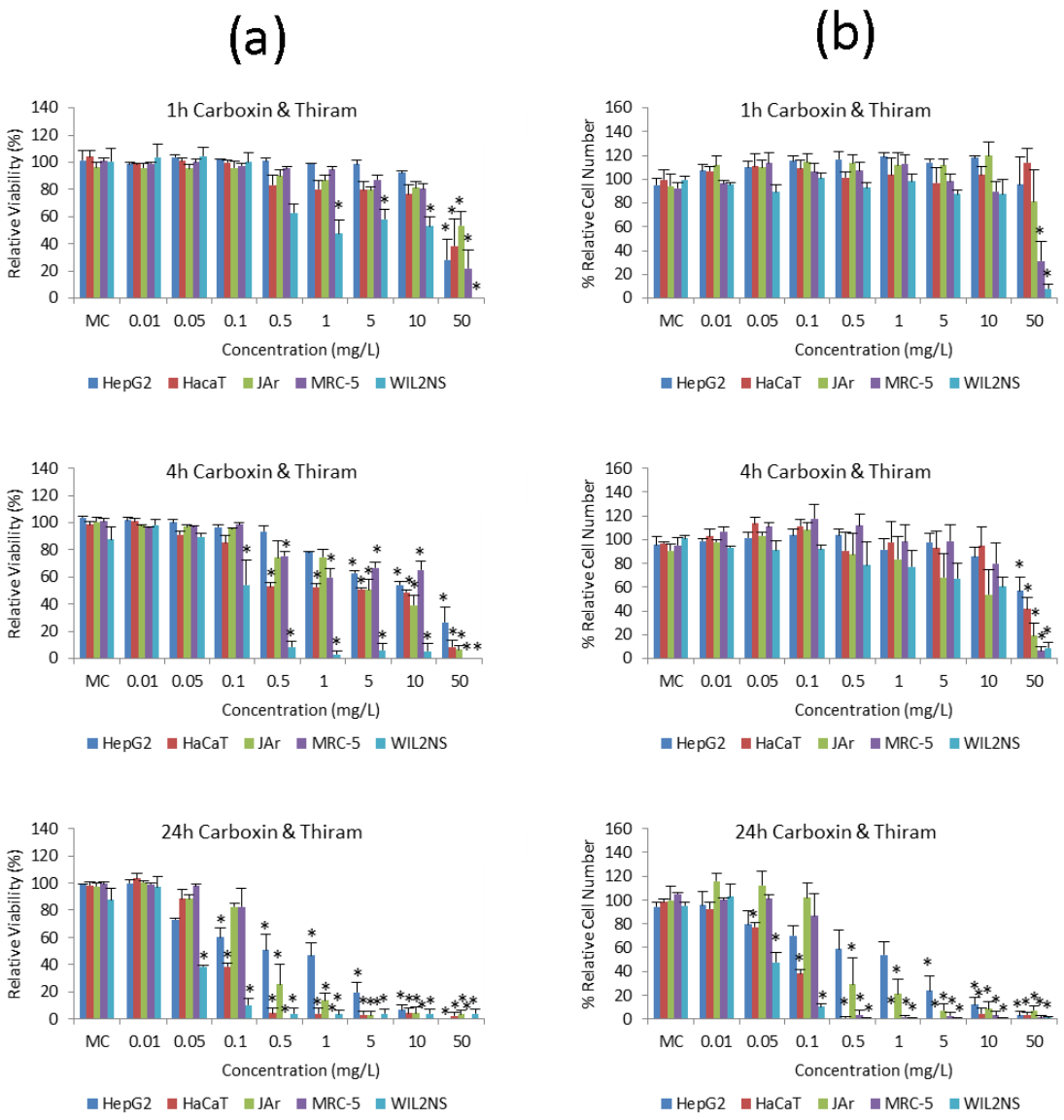


Figure 4-5 Effects of carboxin and thiram combined on cell viability after 1h, 4h or 24h exposure in five different human cell lines

Cell viability was determined using the (a) MTT assay or (b) Crystal Violet Assay for adherent cells (HepG2, HaCaT, JAr and MRC-5) and Trypan Blue Assay for suspension cells (WIL2NS). MC = medium only control. For the MTT assay, results are expressed as % cell survival relative to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for HepG2, HaCaT, MRC-5 and WIL2NS; n=6 for JAr). For the Crystal Violet assay and Trypan blue assay, results are expressed as % relative cell number compared to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for all cell lines). * denotes a significant difference ($p \leq 0.05$) from the solvent control.

Table 4-3. IC_{50} values for carboxin and thiram combined.

Cells were exposed to 0 – 50 mg/L carboxin and thiram in combination for 1, 4 or 24h and IC_{50} values were calculated from cell viability results determined from the MTT, Crystal Violet (CV) or Trypan Blue (TB) assays. Values in brackets () indicate 95% confidence intervals; ND = not determined

Assay	Cell line	IC_{50} (mg/L)		
		1h	4h	24h
MTT	HepG2	32.43 (25.27 to 41.62)	11.38 (8.21 to 15.79)	0.39 (0.23 to 0.67)
	HaCaT	34.88 (13.89 to 87.57)	2.54 (1.43 to 4.51)	0.09 (0.08 to 0.09)
	MRC-5	22.55 (17.37 to 29.26)	7.09 (3.61 to 13.92)	0.15 (0.08 to 0.26)
	JAr	ND	4.24 (2.84 to 6.34)	0.06 (0.03 to 0.09)
	WIL2NS	3.59 (1.61 to 8.02)	0.11 (0.09 to 0.15)	0.03 (0.02 to 0.05)
CV	HepG2	ND	ND	0.74 (0.38 to 1.46)
	HaCaT	ND	41.55 (24.30 to 71.07)	0.08 (0.07 to 0.09)
	MRC-5	21.05 (12.94 to 34.26)	17.25 (9.103 to 32.68)	0.18 (0.11 to 0.29)
	JAr	ND	10.93 (4.83 to 24.76)	0.38 (0.23 to 0.66)
TB	WIL2NS	20.03 (14.15 to 28.37)	9.07 (4.12 to 19.97)	0.05 (0.04 to 0.05)

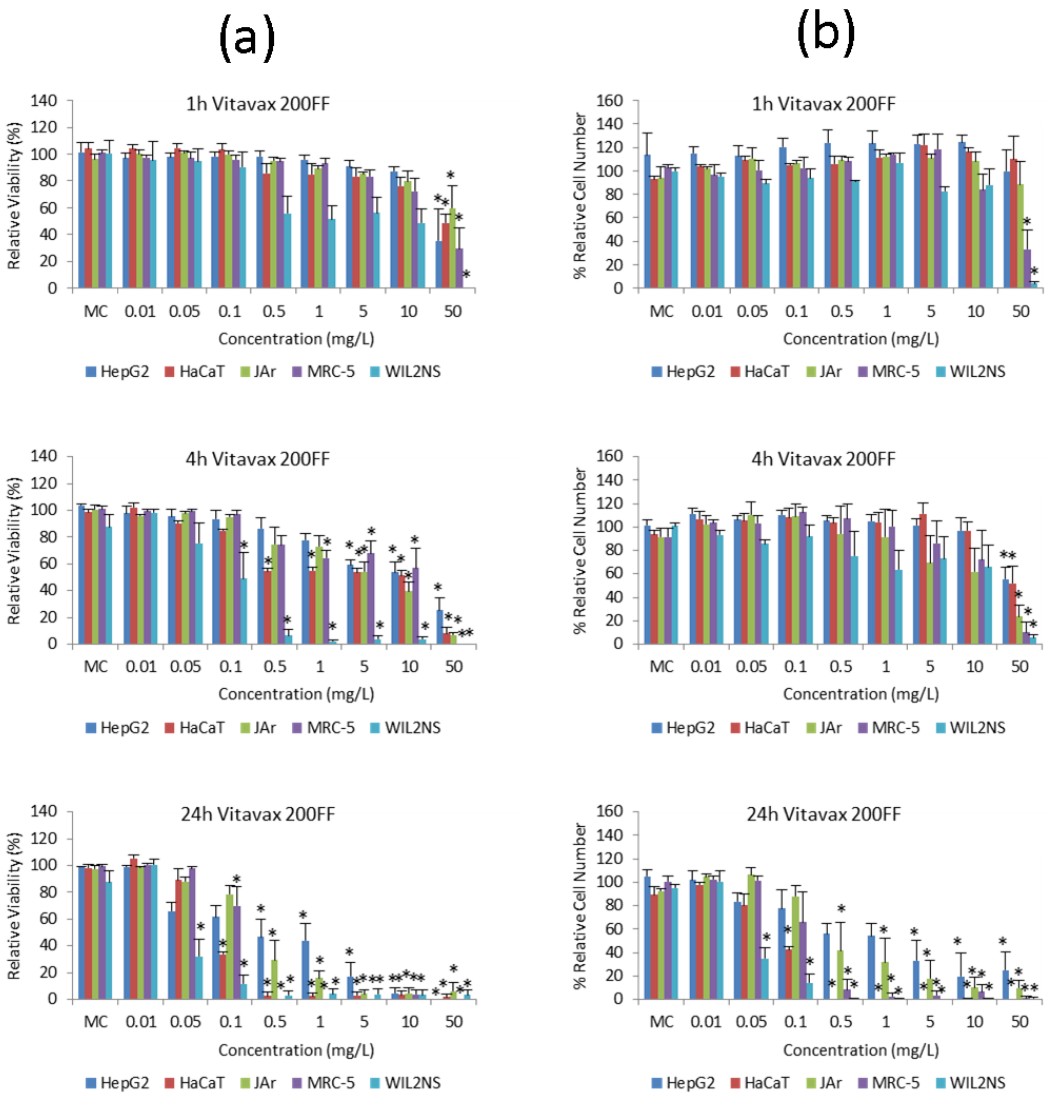


Figure 4-6 Effects of Vitavax 200FF on cell viability after 1h, 4h or 24h exposure in five different human cell lines

Cell viability was determined using the (a) MTT assay or (b) Crystal Violet Assay for adherent cells (HepG2, HaCaT, JAr and MRC-5) and Trypan Blue Assay for suspension cells (WIL2NS). MC = medium only control. Concentration shown is indicative of the equivalent concentrations of carboxin and thiram in the Vitavax200FF treatment. 1mg/L = 0.0005% Vitavax200FF. For the MTT assay, results are expressed as % cell survival relative to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for HepG2, HaCaT, MRC-5 and WIL2NS; n=6 for JAr). For the Crystal Violet assay and Trypan blue assay, results are expressed as % relative cell number compared to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for all cell lines). * denotes a significant difference ($p \leq 0.05$) from the solvent control.

Table 4-4. IC_{50} values for Vitavax 200FF.

Cells were exposed to concentrations of Vitavax 200FF containing equivalent concentrations of carboxin and thiram at 0 – 50 mg/L for 1, 4 or 24h. IC_{50} values were calculated from cell viability results determined from the MTT, Crystal Violet (CV) or Trypan Blue (TB) assays. 1mg/L = 0.0005% Vitavax200FF; Values in brackets () indicate 95% confidence intervals; ND = not determined

Assay	Cell line	IC_{50} (mg/L)		
		1h	4h	24h
MTT	HepG2	33.23 (21.82 to 50.60)	10.01 (6.24 to 16.05)	0.33 (0.18 to 0.59)
	HaCaT	47.05 (23.34 to 116.1)	3.03 (1.68 to 5.48)	0.08 (0.08 to 0.09)
	MRC-5	22.93 (15.41 to 34.11)	6.61 (3.42 to 12.80)	0.12 (0.09 to 0.17)
	JAr	ND	4.49 (2.94 to 6.86)	0.04 (0.02 to 0.08)
	WIL2NS	2.66 (1.04 to 6.79)	0.10 (0.07 to 0.14)	0.03 (0.02 to 0.05)
CV	HepG2	ND	ND	1.26 (0.48 to 3.31)
	HaCaT	ND	ND	0.09 (0.08 to 0.10)
	MRC-5	10.28 (4.65 to 27.34)	16.08 (8.749 to 29.54)	0.10 (0.03 to 0.19)
	JAr	ND	14.58 (5.60 to 37.91)	0.50 (0.27 to 0.95)
TB	WIL2NS	18.6 (12.03 to 28.76)	8.79 (2.48 to 31.10)	0.04 (0.03 to 0.05)

4.3.3 Synergistic effects of mixtures

Univariate analysis of variance (ANOVA) was used to determine non-additive effects (e.g. synergistic or antagonistic effects) between the mixtures Vitavax 200FF or carboxin and thiram combined and the sum of the individual effects of carboxin and thiram.

Exposure to 50mg/L Vitavax200FF produced a non-additive effect in HaCaT cells after 1h exposure in the MTT assay (Figure 4-7). Exposure to 50mg/L Vitavax 200FF produced a non-additive effect in WIL2NS cells after 1h or 24h exposure in the Trypan Blue assay (Figure 4-8). Exposure to 0.1mg/L or 50mg/L carboxin and thiram in combination also produced a non-additive effect in WIL2NS cells using the Trypan Blue assay (Figure 4-8). For each non-additive effect found in HaCaT or WIL2NS cells, the cytotoxic effect of the mixture was less than the sum of the individual cytotoxic effects of carboxin and thiram. No non-additive effects were found between the mixtures and the sum of the individual effects of carboxin and thiram for HepG2, JAr or MRC-5 cells as shown in Figure 4-9, Figure 4-10 and Figure 4-11 respectfully.

Non-additive effects (e.g. synergistic or antagonistic effects) of Vitavax 200FF resulting from the inclusion of other ingredients in the product formulation was also examined using one-way analysis of variance (ANOVA) to determine significant differences between the cytotoxic effects of Vitavax 200FF compared to carboxin and thiram in combination. No significant differences between Vitavax 200FF and carboxin and thiram in combination were found in any cell line, assay, exposure time or dose tested (Figure 4-7 to Figure 4-11).

The cytotoxic effects of the mixtures (Vitavax 200FF or carboxin and thiram in combination) appeared to be very similar to that seen for thiram for all cell lines (Figure 4-7 to Figure 4-11). Therefore, univariate analysis of variance was used to determine if

the cytotoxic effects of the mixtures were the statistically the same as that seen for thiram. No significant differences were found between the cytotoxic effects of either Vitavax 200FF or carboxin and thiram in combination to the cytotoxic effects of thiram (Figure 4-7 to Figure 4-11).

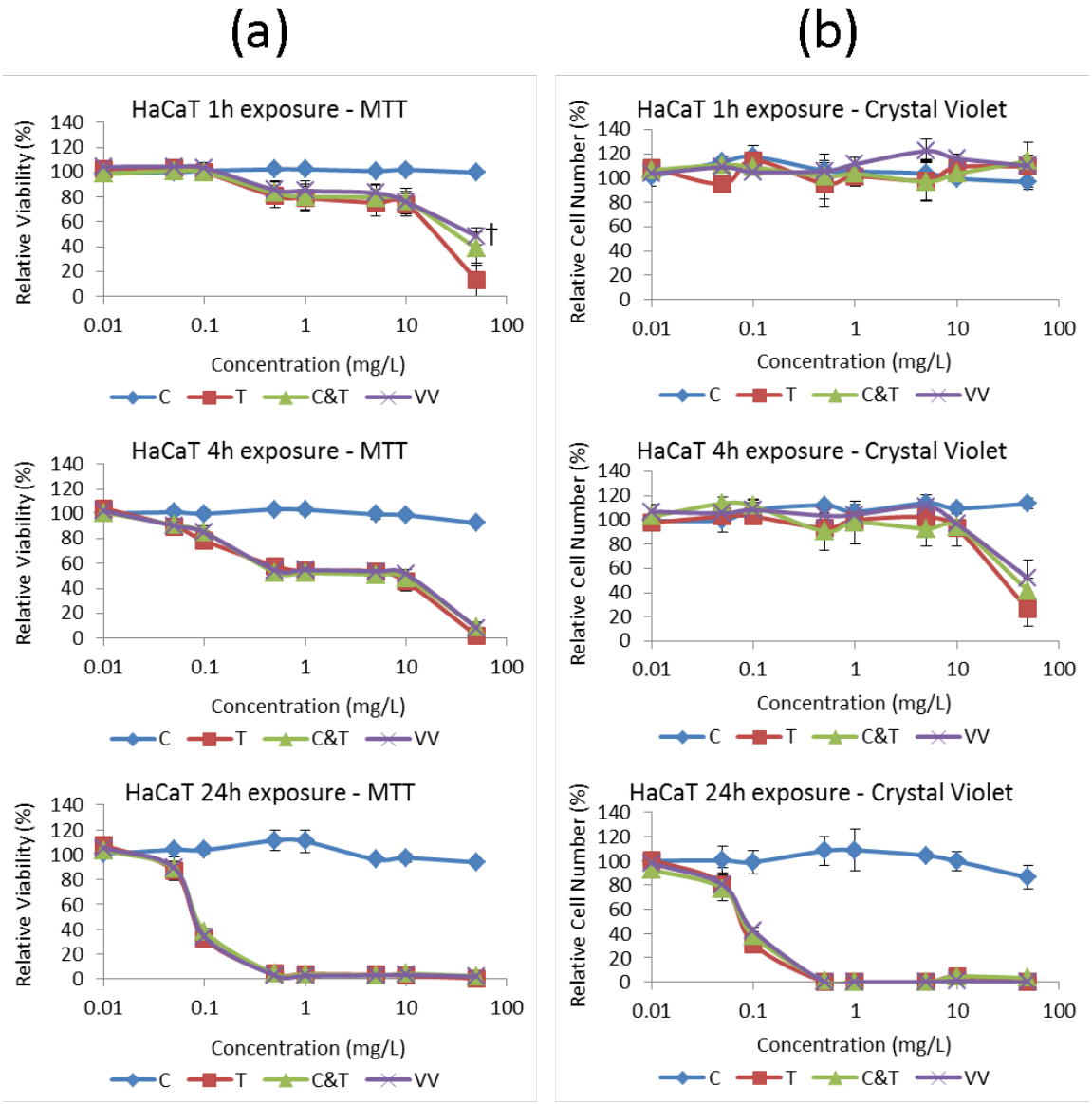


Figure 4-7. Non-additive effects of carboxin & thiram combined (C&T) or Vitavax 200FF (VV) in comparison to the individual effects of carboxin (C) or thiram (T) in HaCaT cells using the (a) MTT assay (MTT) or (b) Crystal Violet assay (CV).
Results are expressed as % cell survival (MTT) or % relative cell number (CV), relative to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for C or T, n=3 for C&T or VV). * indicates a non-additive response for C&T ($p \leq 0.05$); † indicates a non-additive response for VV ($p \leq 0.05$).

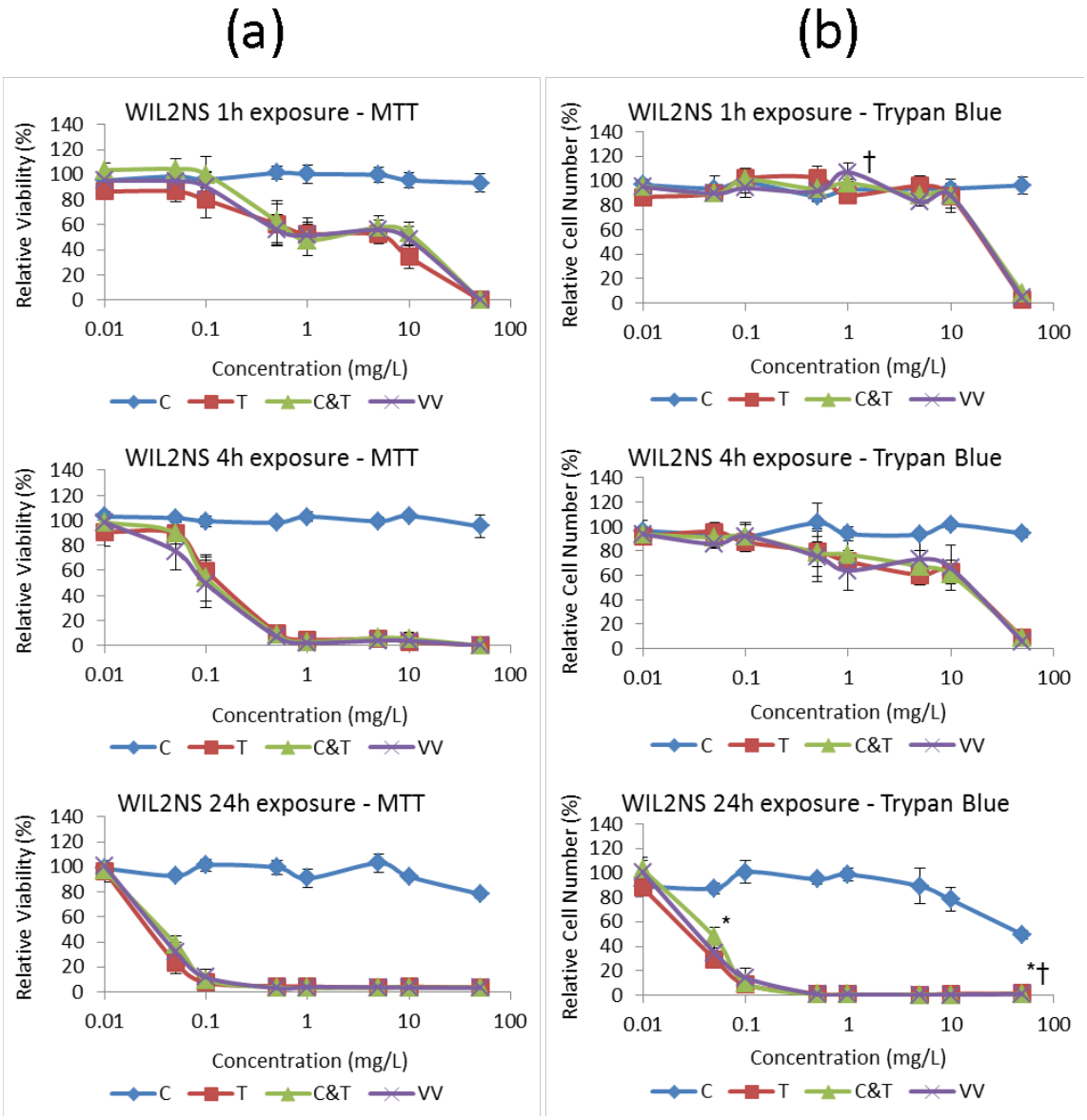


Figure 4-8. Non-additive effects of carboxin & thiram combined (C&T) or Vitavax 200ff (VV) in comparison to the individual effects of carboxin (C) or thiram (T) in WIL2NS cells using the (a) MTT assay (MTT) or (b) Trypan Blue Count (TB).

Results are expressed as % cell survival (MTT) or % relative cell number (CV), relative to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for C or T, n=3 for C&T or VV). * indicates a non-additive response for C&T ($p<0.05$); † indicates a non-additive response for VV ($p<0.05$).

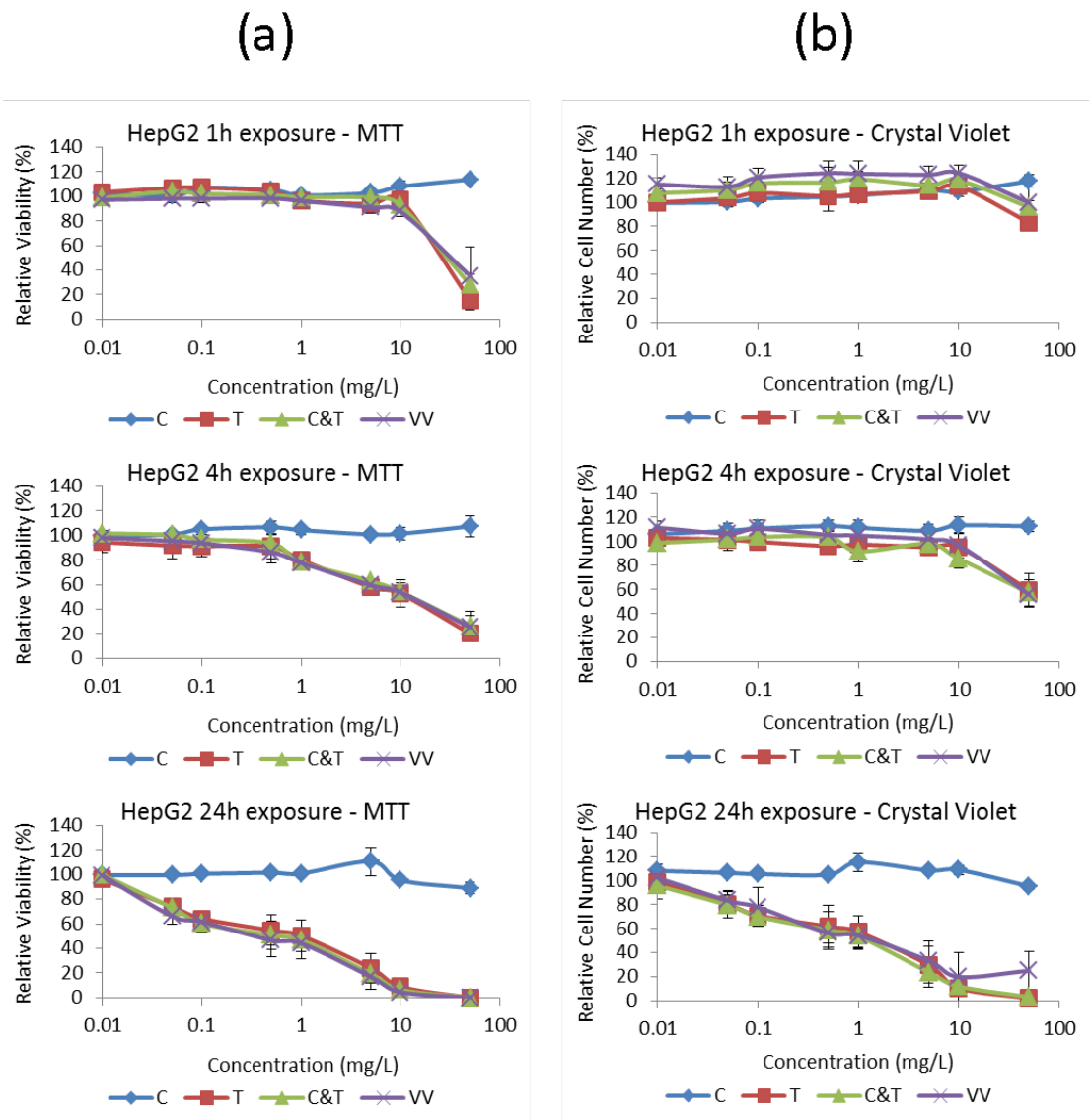


Figure 4-9. Non-additive effects of carboxin & thiram combined (C&T) or Vitavax 200FF (VV) in comparison to the individual effects of carboxin (C) or thiram (T) in HepG2 cells using the (a) MTT assay or (b) Crystal Violet assay.

Results are expressed as % cell survival (MTT) or % relative cell number (CV), relative to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for C or T, n=3 for C&T or VV). No non additive effects were found ($p>0.05$).

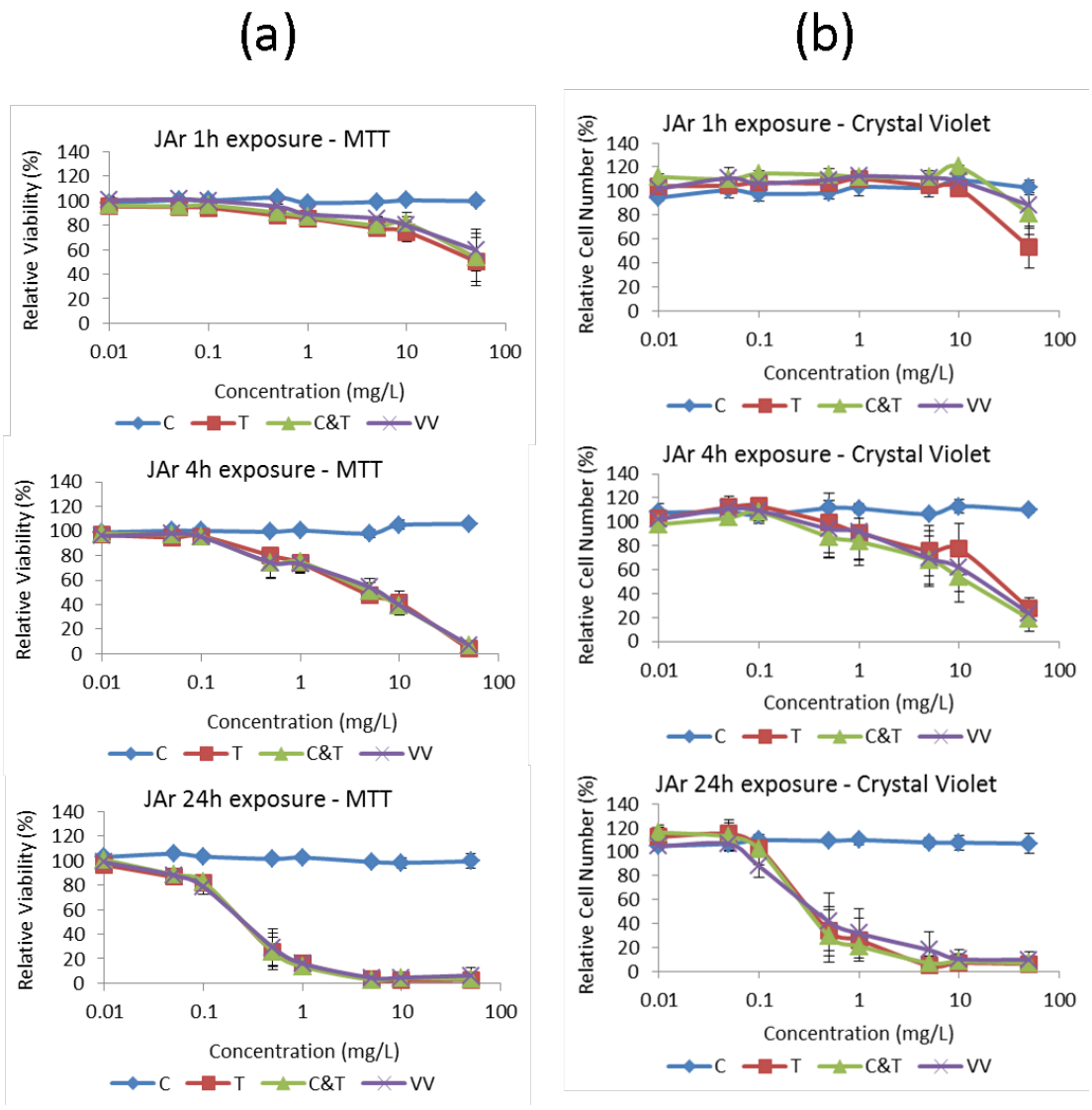


Figure 4-10. Non-additive effects of carboxin & thiram combined (C&T) or Vitavax 200FF (VV) in comparison to the individual effects of carboxin (C) or thiram (T) in JAr cells using the (a) MTT assay (MTT) or (b) Crystal Violet assay (CV).

Results are expressed as % cell survival (MTT) or % relative cell number (CV), relative to the solvent control and are presented as the mean ± S.E.M of separate experiments (For MTT, n=6 for C or T, n=3 for C&T or VV; for CV assay, n=4 for C or T, n=3 for C&T or VV. No non-additive effects were found ($p>0.05$).

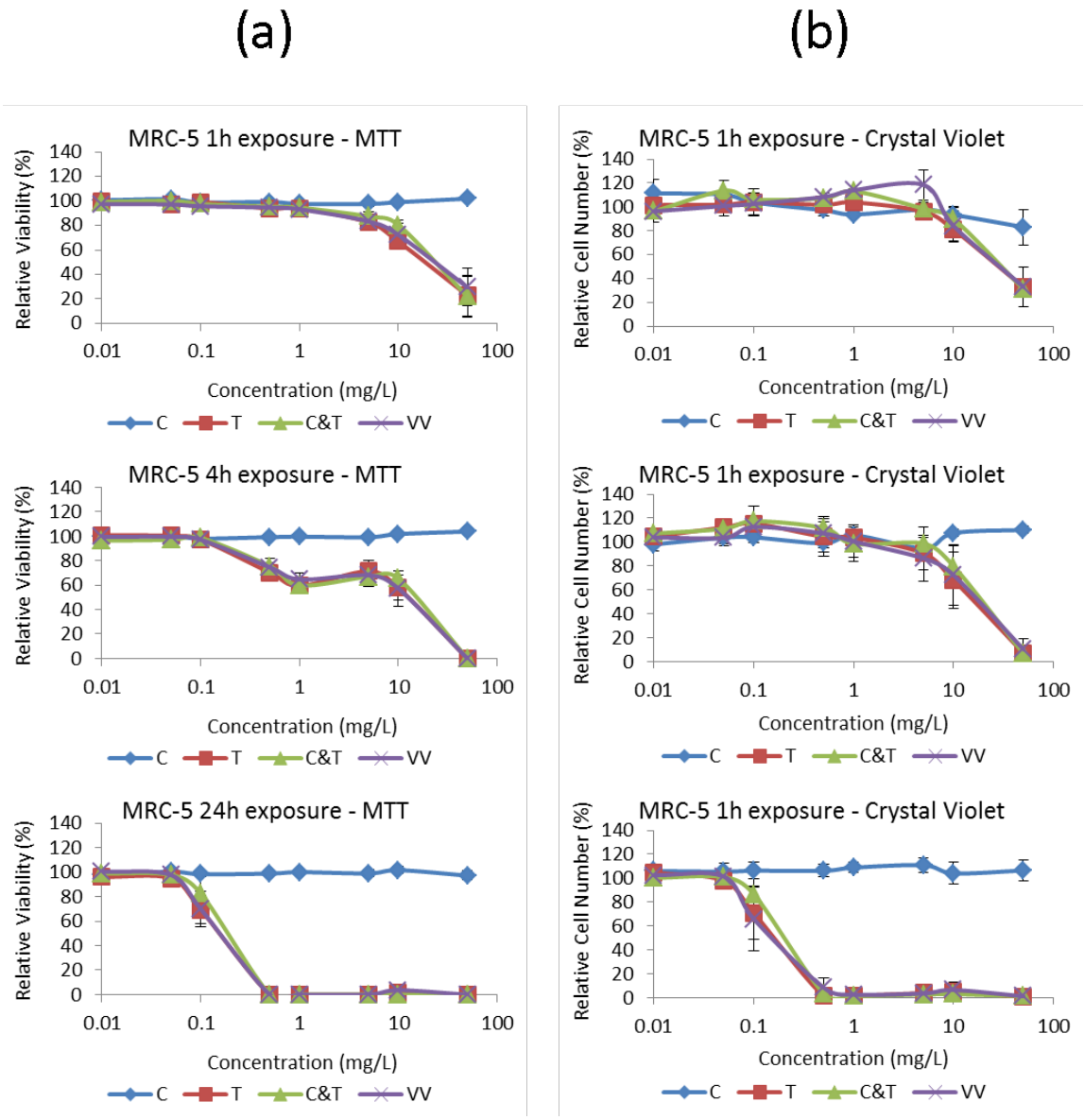


Figure 4-11. Non-additive effects of carboxin & thiram combined (C&T) or Vitavax 200FF (VV) in comparison to the individual effects of carboxin (C) or thiram (T) in MRC-5 cells using the (a) MTT assay (MTT) or (b) Crystal Violet assay (CV).

Results are expressed as % cell survival (MTT) or % relative cell number (CV), relative to the solvent control and are presented as the mean ± S.E.M of separate experiments (n=4 for C or T, n=3 for C&T or VV). No non-additive effects were found ($p>0.05$).

4.3.4 Effects on apoptosis and cell cycle

The cytotoxic effect of the concentrations of carboxin, thiram, carboxin and thiram combined or Vitavax 200FF selected for apoptosis and cell cycle studies was confirmed using the Trypan Blue assay (Figure 4-12). No significant differences in cell viability were found between the medium only control and the solvent control after 4h or 24h exposure (Figure 4-12).

Cell viability results for WIL2NS (Figure 4-12) were similar to that found previously using the Trypan Blue assay (Figure 4-3 to Figure 4-6). Cell viability results for HepG2 and HaCaT were similar to that observed for each treatment at the same dose and exposure time in the Crystal Violet assay (Figure 4-3 to Figure 4-6).

Exposure to the solvent control for 4h induced no significant differences in the populations of apoptotic or late apoptotic and necrotic cells compared to the medium only control for all cell lines (Figure 4-13A). Similarly, no differences in the population of cells in G_0/G_1, S, or G_2 phase were found between the solvent control and medium only control after 4h exposure in all cell lines (Figure 4-13B).

Results from single experimental replicates for HepG2 or HaCaT cells after 4h exposure showed that the treatments had little effect on apoptosis with differences compared to the solvent control of the population of cells undergoing early apoptosis or late apoptosis/necrosis no greater than ± 1.8% and ± 2.9% (Figure 4-13A). Similarly, the treatments induced minor differences in the population of cells in G_0/G_1, S or G_2 phases in comparison to the solvent control (Figure 4-13B). As significant effects on apoptosis or cell cycle compared to the solvent control were unlikely, additional replicates were not performed.

Exposure of WIL2NS to the treatments for 4h induced no significant changes to the population of early apoptotic or late apoptotic/necrotic cells (Figure 4-13A). It is noted that 4h exposure 0.1mg/L thiram, carboxin and thiram combined or Vitavax 200FF induced slight increases of 3.1-3.2% of late apoptotic/necrotic cells but were not significantly different from the solvent control (Figure 4-13A). At the same dose and exposure time, thiram, carboxin and thiram combined and Vitavax 200FF all induced a significant increase ($p \leq 0.05$) in the population of WIL2NS cells in S-phase (Figure 4-13B).

After 24h exposure, no significant differences in the populations of apoptotic or late apoptotic and necrotic cells between the solvent control and the medium only control were found for all cell lines (Figure 4-14A). Similarly, no significant differences in the population of cells in G_0/G_1, S or G_2 phase were found between the solvent control and medium only control after 24h exposure in all cell lines (Figure 4-14B).

24h exposure of HepG2 cells to the treatments induced no significant effects on apoptosis (Figure 4-14A) or cell cycle (Figure 4-14B). Exposure of HaCaT cells to thiram, carboxin and thiram combined or Vitavax 200FF for 24h induced a dose dependent increase in late apoptotic or necrotic cells and was significant at 5mg/L (Figure 4-14A). The highest dose of 5mg/L thiram, carboxin and thiram combined or Vitavax 200FF induced a significant ($p \leq 0.05$) decrease in cells in G_0/G_1 phase and was accompanied by a significant ($p \leq 0.05$) increase in cells in S-phase (Figure 4-14B).

24h exposure of WIL2NS cells to 50 mg/L carboxin induced a significant increase ($p \leq 0.05$) of apoptotic cells to 24.9 ± 4.9% of the population compared to the solvent control (9.3 ± 1.4%; Figure 4-14A). The increase of apoptotic cells was accompanied

with a significant increase ($p \leq 0.05$) of WIL2NS cells in S-phase and a significant decrease of cells in G_0/G_1 and G_2 phase (Figure 4-14B).

The effects of 24h exposure of WIL2NS cells to thiram, carboxin and thiram combined and Vitavax 200FF on apoptosis were similar with dose dependent increases of the population of late apoptotic or necrotic cells observed for each treatment (Figure 4-14A). The population of late apoptotic or necrotic cells was significantly higher compared to the solvent control at both 0.05 and 0.1mg/L thiram, carboxin and thiram combined or Vitavax 200FF (Figure 4-14A).

The effects of 24h exposure of WIL2NS cells to thiram, carboxin and thiram combined or Vitavax 200FF on cell cycle were also similar with dose dependent decreases of cells in G_0/G_1 phase and dose dependent increases of cells in S-phase (Figure 4-14B). The increase of cells in G_0/G_1 phase and decrease of cells in S-phase were significant for all thiram, carboxin and thiram combined or Vitavax 200FF at 0.1mg/L (Figure 4-14B).

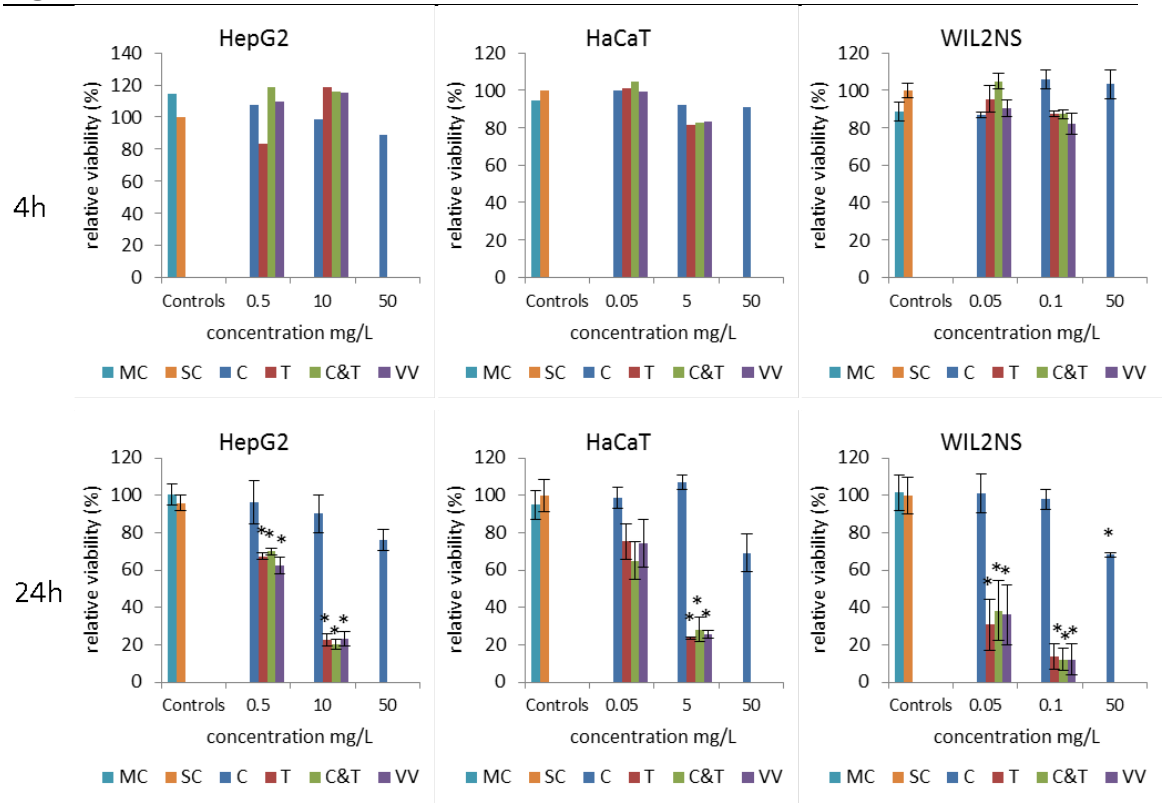


Figure 4-12. Effects of carboxin (C), thiram (T), carboxin and thiram combined (C&T), or the commercial product, Vitavax 200FF (VV) on HepG2, HaCaT or WIL2NS cell viability determined by the Trypan Blue assay.

Results for HaCaT and HepG2 cells after 4h exposure are shown for one experimental replicate (n=1). Results for HepG2 and HaCaT cells after 24h exposure and WIL2NS cells after 4 and 24h are expressed as the mean±SEM of three separate experiments (n=3). T, C&T and VV were not tested at 50mg/L. MC = medium only control; SC = solvent control. * indicates a significant difference ($p<0.05$) compared to the solvent control.

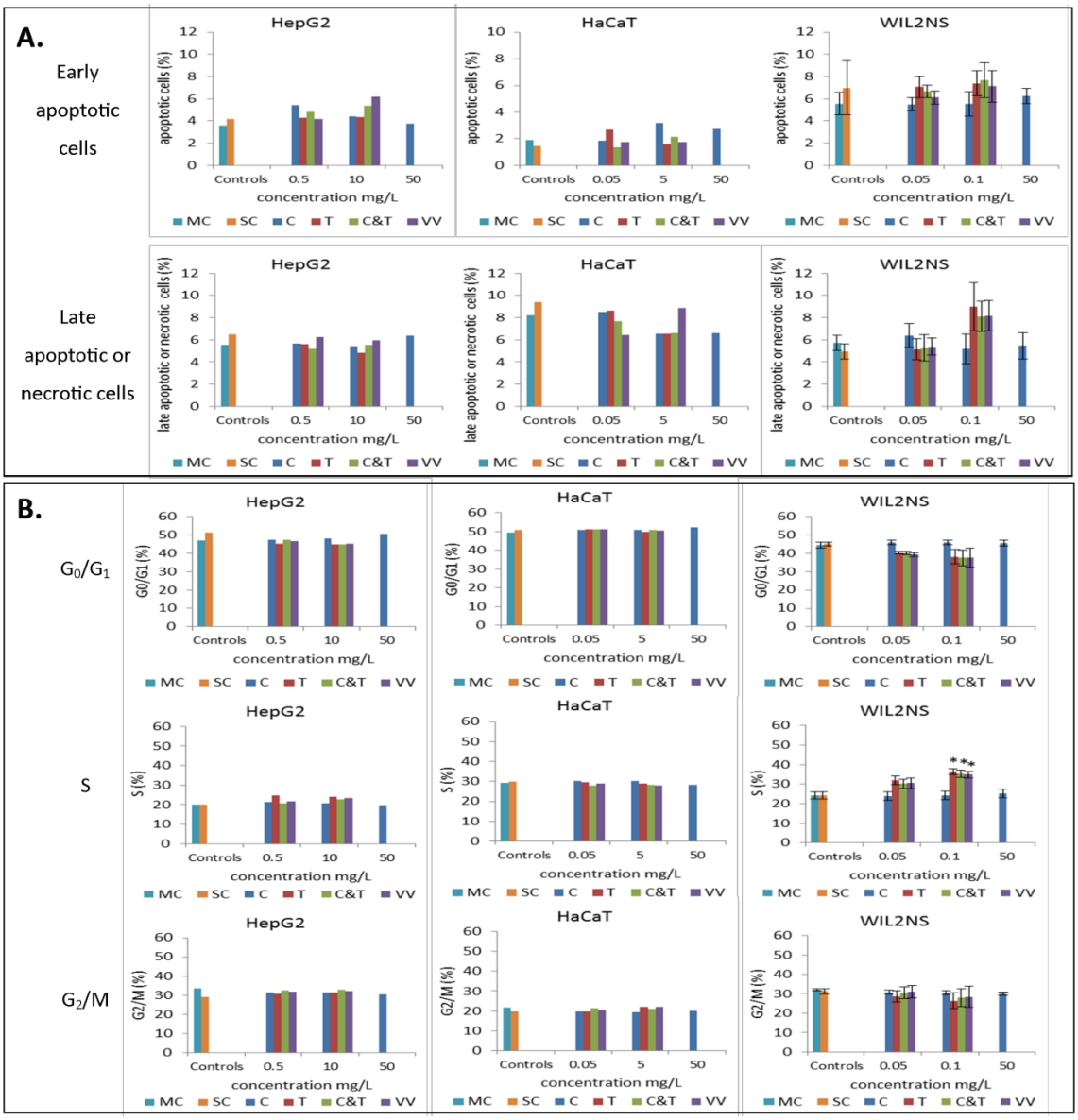


Figure 4-13. Effects of 4h exposure of carboxin and thiram individually, combined or in the commercial product Vitavax 200FF on apoptosis and cell cycle.

HepG2, HaCaT and WIL2NS cells were exposed to carboxin (C), thiram (T), carboxin and thiram combined (C&T) or Vitavax200FF for 4h and subsequently analysed for effects on apoptosis (A) or cell cycle (B). Effects on apoptosis (A) were determined by staining cells with PI and annexin V-FITC and analysed using flow cytometry. Results were obtained from 10000 events and expressed as a percentage of early apoptotic cells (annexin V-FITC positive) or late apoptotic / necrotic cells (PI positive and annexin V-FITC positive). Effects on cell cycle (B) were determined by staining cells with PI and analysing DNA content using flow cytometry. Results were obtained from 10000 events and are expressed as a percentage of cells in G0/G1 phase, S phase or G2/M phase. Results for WIL2NS cells are expressed as the mean±SEM of three separate experiments (n=3). Results for HaCaT and HepG2 cells are shown for one experimental replicate (n=1). T, C&T and VV were not tested at 50mg/L. MC = medium only control; SC = solvent control. * indicates a significant difference (p≤0.05) compared to the solvent control.

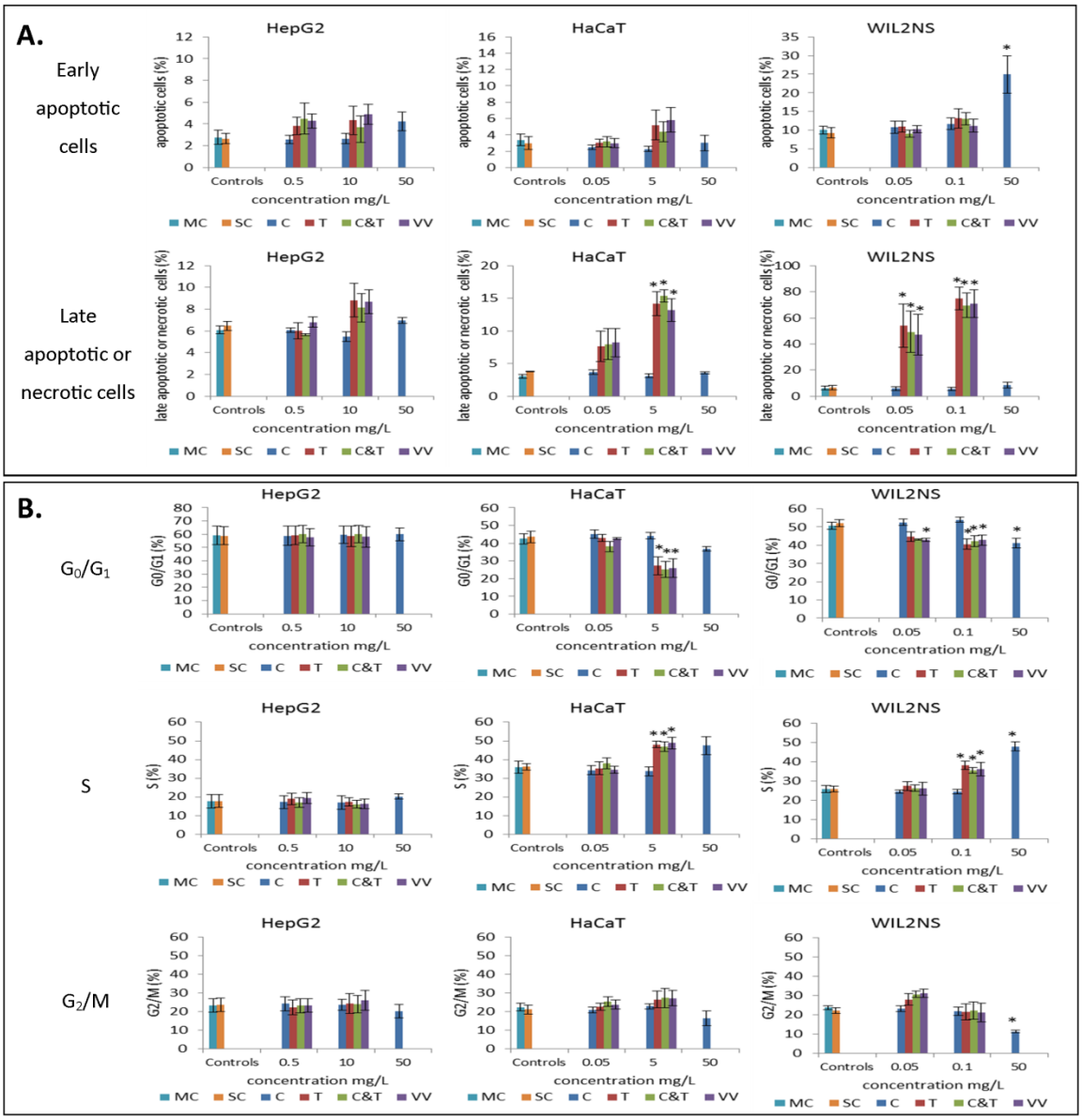


Figure 4-14. Effects of 24h exposure of carboxin and thiram individually, combined or in the commercial product Vitavax 200FF on apoptosis and cell cycle.

HepG2, HaCaT and WIL2NS cells were exposed to carboxin (C), thiram (T), carboxin and thiram combined (C&T) or Vitavax200FF for 24h and subsequently analysed for effects on apoptosis (A) or cell cycle (B). Effects on apoptosis (A) were determined by staining cells with PI and annexin V conjugated with FITC and analysed using flow cytometry. Results were obtained from 10000 events and expressed as a percentage of early apoptotic cells (annexin V-FITC positive) or late apoptotic / necrotic cells (PI positive and annexin V-FITC positive). Effects on cell cycle (B) were determined by staining cells with PI and analysing DNA content using flow cytometry. Results were obtained from 10000 events and are expressed as a percentage of cells in G_0/G_1 phase, S phase or G_2/M phase. Results for WIL2NS cells are expressed as the mean±SEM of three separate experiments (n=3). Results for HaCaT and HepG2 cells are shown for one experimental replicate (n=1). T, C&T and VV were not tested at 50mg/L. MC = medium only control; SC = solvent control. * indicates a significant difference ($p \leq 0.05$) compared to the solvent control.

4.4 Discussion

4.4.1 Effects of carboxin on human cell lines

Carboxin induced cell death was only observed in WIL2NS cells and was evident in both MTT and Trypan Blue assays showing a reduction in cell viability at the highest dose of 50mg/L for 24h. The slight reduction in cell viability seen in HepG2 and HaCaT cells, although not significant, may indicate a potential for carboxin-induced toxicity in human liver cells or keratinocytes at concentrations higher than 50mg/L or with exposure times longer than 24h. However, the absence of significant cell death in any of the doses in four out of the five cell lines used in this study would indicate low toxicity of the compound in human cell lines. The low toxicity of carboxin seen in this study is consistent with a previous study where the IC_{50} of carboxin after 6 days exposure was found to be greater than 47.06mg/L in the T-lymphoblastic leukemia cell line, CEM-SS using the XTT tetrazolium salt based assay (Bader *et al.*, 1991).

Carboxin induced apoptosis in WIL2NS cells after 24h exposure to 50mg/L. In addition, carboxin was also able to increase the population of WIL2NS cells in S-phase and decrease of cells in G0/G1 and G2 phase, indicating cell cycle arrest in S-phase. Therefore, this study shows the ability of carboxin to induce apoptosis in human cells after prolonged exposure. This is the first study to show the ability of carboxin to induce apoptosis and cell cycle arrest in human cells.

A previous study found that exposure to 3.85mg/L carboxin for 7 days was able to increase glutathione S-transferase (GST) activity in the liver of rainbow trout (Aydin *et al.*, 2012), indicating potential induction of oxidative stress via the generation of ROS. Therefore it is possible that carboxin induced the generation of ROS in WIL2NS cells to trigger subsequent apoptosis, however further studies are required for confirmation. WIL2NS cells are also glutathione-S-transferase M1 (GSTM1) null (Shield *et al.*, 2004a) and therefore the high sensitivity of WIL2NS cells to carboxin may be due to an

impaired ability to efficiently detoxify carboxin. The GSTM1 null genotype is common in many populations including Caucasian (60.9%) (Chen *et al.*, 1999), Turkish (48.1%) (Cora *et al.*, 2012), and Japanese (45.7%) (Hori *et al.*, 2009). Therefore, a high percentage of the population would have an increased risk if carboxin-induced effects were affected by GSTM1 status. GSTM1 expression or activity in HepG2, HaCaT, JAr and MRC-5 cells were not analysed in this study and therefore further studies are required to determine any definitive correlation between carboxin-induced cytotoxicity, apoptosis and cell cycle arrest and GSTM1 function.

Carboxin has also been shown to be an inhibitor of mammalian mitochondrial Complex II (Mowery *et al.*, 1976, Mowery *et al.*, 1977, Takahata *et al.*, 2016), a crucial enzyme in the tricarboxylic acid (TCA) cycle and electron transport chain (Cecchini, 2003). As an inhibitor of mitochondrial Complex II, 2h exposure to carboxin at 118mg/L was able to suppress ROS overproduction and decrease the magnitude of apoptotic cells in folate-deprived synoviocytes (Hsu *et al.*, 2016). This is in contrast to the carboxin-induced cytotoxicity, apoptosis and cell cycle arrest seen in WIL2NS cells in this study. The mechanism of carboxin-induced cell death remains to be fully understood and therefore further studies are required to gain a comprehensive understanding of the risk posed to the general population.

The maximum residue limit (MRL) of carboxin in cereal grains in Australia is 0.1mg/kg (Australian Pesticides and Veterinary Medicines Authority, 2016). Consumption of the amount of grain compliant with the MRL (>500kg) that could potentially cause the cytotoxic or apoptotic effects and cell cycle arrest seen in this study is improbable and therefore the current MRL of 0.1mg/kg is satisfactory. However, consumption of carboxin treated grain washed with water during a food shortage in a rural Malawi community was previously reported with a residual level of 57mg/kg carboxin (Schier

et al., 2012). In this case, the effects of carboxin seen in this study were more likely to occur and therefore posed a health risk to the community.

Exposure to carboxin can also occur during application of the fungicide to grain and subsequent handling of the treated grain (Grey *et al.*, 1983, White *et al.*, 2004). Exposure of seed treatment workers to carboxin was low (<0.5mg/h) provided that respirators and gloves were used (Grey *et al.*, 1983). Therefore, the likelihood of carboxin-induced effects seen in this study following acute exposure from application or handling treated grain is low and poses a low risk. However, exposure of seed treatment workers to low levels of carboxin may occur over extended periods during busy periods such as during harvest. In this case, future studies assessing the effects of long-term exposure to low levels of carboxin in human cells may be useful to gain a comprehensive view of any hazards to human health imposed by the chemical.

4.4.2 Effects of Thiram on human cell lines

Thiram is one of the listed active ingredients found in the commercial formulation Vitavax200FF. Exposure to thiram alone induced a reduction in cell viability using both end-points for cell viability in every cell line confirming that thiram does indeed induce cell death in the human cell lines used in this study. In addition, we demonstrated that the toxicity of thiram increased with dose and time in all cell lines. The level of toxicity of thiram to human cells observed in this study is consistent with other studies using human cell culture which reported 100% cell death in human skin fibroblasts after 6h exposure to 5mg/L thiram (Cereser *et al.*, 2001a) and 90% cell death in human lymphocytes exposed to 50mg/L thiram for 4h (Perocco *et al.*, 1989). Another study using human microvascular endothelial cells showed 60% and 30% cell death in the MTT and Trypan blue assay after 18h exposure to 5mg/L thiram (Kurpios-Piec *et al.*, 2015).

In this study, thiram showed greater toxicity in the MTT assay compared to the Crystal Violet or Trypan Blue Assay. Similar results were found in a study by Kurpios-Piec *et al.* (2015) where the MTT assay showed higher toxicity of thiram compared to the Trypan blue assay in human microvascular endothelial cells. This would indicate rapid onset of mitochondrial dysfunction before cell lysis. Mitochondrial dysfunction is a common feature in both apoptotic and necrotic cell death (Lemasters *et al.*, 1999). Mitochondrial dysfunction coupled with a rapid decrease in ATP inhibits apoptosis and instead promotes necrotic cell death (Eguchi *et al.*, 1997). Therefore the thiram induced necrosis seen in this study may be driven by rapid ATP depletion however further studies are required to confirm this.

Thiram has been shown to induce a decrease of intracellular glutathione levels in human skin fibroblasts (Cereser *et al.*, 2001b) and Chinese hamster fibroblasts (Grosicka *et al.*, 2005). In another study (York *et al.*, 1998), overexpression of glutathione S-transferase (GST) in *Sf*21 cells revealed an elevated ability to reduce MTT to formazan when supplemented with glutathione. Their experiments showed that an elevated reduction of MTT to formazan was only seen when both GST and GSH were present, suggesting the interdependency of GST in catalyzing the reduction of MTT by GSH. Therefore in our study the higher levels of cell death seen in the MTT assay compared to the Crystal Violet or Trypan Blue assay could also be partly attributed to a thiram-induced reduction of intracellular GSH and in turn decreased reduction of MTT to formazan.

Thiram-induced cell death in this study was necrotic and caused cell cycle arrest in S-phase. The finding that thiram induced death is necrotic is consistent with a study by Cereser *et al.* (2001a) where it was found that exposure of human skin fibroblasts to thiram at 0.3-5mg/L for 3-48h induced no nuclear characteristics of apoptosis (no

chromatin condensation, no nuclear fragmentation and no formation of membrane blebs) and no activation of caspase 3.

4.4.3 Effects of carboxin and thiram mixtures on human cell lines

A comparison of the effects of carboxin and thiram combined, the product formulation Vitavax200FF, and the sum of the effects of carboxin and thiram individually was performed to determine potential synergistic interactions between the two compounds used as active ingredients, or between active and non-active ingredients in the formulation.

The cytotoxic effects of the combination of the active ingredients carboxin and thiram were similar to that of the product formulation indicating the presence of other non-active ingredients had little or no effect on the toxicity of the product formulation. The effects of carboxin and thiram combined and Vitavax 200FF were predominantly due to the presence of thiram in the mixture. The effects of the mixtures were generally found to be additive and the few non-additive effects identified were only slightly antagonistic.

Therefore, estimation of the toxic effects of Vitavax 200FF based on the sum of the effects of its active ingredients was found to have a good correlation with actual toxicity of the formulated product. As the only non-additive effects found in this study were due to antagonism, estimation of the toxicity of the mixture based on the sum of the individual effects of carboxin and thiram was not likely to underestimate the risk posed to human health.

4.4.4 Concluding remarks

In conclusion, this study shows that carboxin induced cell death, apoptosis and cell cycle arrest in WIL2NS cells but had minimal effect on HepG2, HaCaT, JAr and MRC-5 cells. Thiram was more toxic than carboxin and induced cell cycle arrest and necrotic cell death. The cytotoxicity of Vitavax 200FF to cell lines was in the following order; WIL2NS cells > JAr cells > HaCaT cells > MRC-5 cells > HepG2 cells. The cytotoxicity was mainly due to the effects of thiram in the formulation. Estimation of the toxic effects of Vitavax 200FF based on the sum of the effects of its active ingredients was found to have a good correlation with actual toxicity of the formulated product and was unlikely to underestimate the risk to human health.

5 CHAPTER 5: Evaluation of the *in vitro* cytotoxicity of Vitavax 200FF and its active ingredients during degradation in soil

5.1 Introduction

Mixtures of compounds in the environment may show changes in their chemical profiles and toxicity over time depending on the different environmental factors they are subjected to. This is because chemicals can be modified and degraded by biotic and abiotic processes which may alter their toxicity (Paton *et al.*, 2006, Liang *et al.*, 2014b, Chin-Pampillo *et al.*, 2015) .

The toxicity of Vitavax 200FF and its active ingredients carboxin and thiram in human cells were investigated in Chapter 4. However, there is the potential for their toxic effects to be altered when present as a complex mixture in soil. This is because soils contain a diverse range of compounds including minerals and organic matter which may interact with compounds in Vitavax 200FF to alter their toxic effects. Soils have the potential to abiotically degrade chemicals to products with different toxicity (Svenson *et al.*, 1997, Liang *et al.*, 2014b, Guo *et al.*, 2015). In addition, soils contain a diverse range of microbes which may be able to biotically degrade Vitavax 200FF and its active ingredients to products with different toxicity (Marwood *et al.*, 1998, Phillips *et al.*, 2000). Furthermore, there is the potential for interaction between the degradation products and/or parent compounds of Vitavax 200FF themselves, or between the degradation products and other compounds within the soil.

Studies have been performed which investigate the toxicity of carboxin and its degradation products in various environmental matrices. The abiotic and biotic degradation of carboxin was previously examined in a study by Isidori *et al.* (2012). In

their study, both abiotic and biotic degradation of carboxin in soil resulted in a decrease in the toxicity of the soil to aquatic organisms over a period of 20 days. In a different study, DellaGreca *et al.* (2004) found that carboxin was photodegraded in water to its sulfoxide which exhibited a similar toxicity to the parent compound to aquatic organisms. It is important to note that these studies were perfomed using ecotoxicity models and may not reflect the toxicity of the soil to humans. Limitations of ecotoxicity models to predict the toxicity of chemicals to humans have been outlined in section 1.3.2.1. No current studies are avaliable which examine the effect of carboxin, and its degradation products in soil, to human cells.

The degradation of thiram in various environmental matrices has also been examined in previous studies. Gupta *et al.* (2012b) found that the major degradation product of thiram in soil was dimethyl dithiocarbamate due to hydrolysis of the parent compound. The study also identified other degradation products of thiram in soil, however it did not examine the toxicity of the soil extracts during degradation. Milenkovski *et al.* (2010) found that thiram was toxic to bacterial communities when present in soil, but toxicity analysis was only performed immediately after spiking and did not examine the change in the toxicity of the soil as it degraded. In a study by Sherif *et al.* (2011) the presence of thiram in soil caused an initial fluctuation of bacterial populations, which was alleviated after complete degradation of the compound. Dimethyldithiocarbamate and carbon disulfide, two major degradation products of thiram, were found to be less toxic in rats compared to their parent compound (Dalvi *et al.*, 2002). However, the rats were only treated with the pure compounds individually and therefore, the potential interaction of between degradation products from a soil matrix was not examined.

Similarly as for carboxin, it is important to note that these studies were perfomed using ecotoxicity and *in vivo* animal models and may not reflect the toxicity of the soil to humans. Limitations of ecotoxicity and *in vivo* models to predict the toxicity of

chemicals to humans have been outlined in section 1.3.2.1 and section 0. No current studies are avaliable which examine the effect of thiram as it degrades in soil to human cells.

The toxicity of Vitavax 200FF in a soil matrix has been examined previously (Alves *et al.*, 2013, Alves *et al.*, 2014). However these studies were performed using ecotoxicity models (earthworm and arthropod models) and may not reflect the toxicity of the soil to humans. Limitations of ecotoxicity models to predict the toxicity of chemicals to humans have been outlined in section 1.3.2.1. In addition, these studies only examined the toxicity of the soil immediately after spiking with Vitavax 200FF and did not examine the change in the toxicity of the soil as it degraded. There are currently no studies which investigate the degradation of the carboxin and thiram when present in combination as part of a commercial product and their toxicity to human cells.

The principal aims of this study were:

- To determine if carboxin and thiram can undergo abiotic and biotic degradation in soil
- To determine if degradation of carboxin and thiram in soil decreases its toxicity in human cells
- To determine if the product formulation Vitavax 200FF affects the degradation of carboxin and thiram in soil
- To determine if Vitavax 200FF can alter microbial populations in soil
- To determine the toxicity of the soil contaminated with Vitavax 200FF decreases as carboxin and thiram are degraded

5.2 Materials and Methods

5.2.1 Soil treatments

The concentration of the treatments selected in this chapter were based on the predicted environmental concentration of Vitavax 200FF in soil (Table 5-1) and the cytotoxic response of Vitavax 200FF in cells as determined in Chapter 4.

25 g of sterile or non-sterile air-dried soil was wetted to 60% water holding capacity by the addition of 3.07 ml sterile RO water. The soil was then spiked with carboxin, thiram carboxin and thiram in combination or Vitavax 200FF in acetone so that the final concentration of carboxin and thiram in the soil was as outlined in Table 5-2. The final concentration of acetone in all soil samples was constant (3.3%). A soil solvent control consisting of wetted soil spiked with 100 µl acetone and a soil only control was also included. Spiked soil samples were then mixed thoroughly using a spatula and incubated in the dark at 27°C for 0, 1, 3, 7, 14, 21 or 28 days. Soil samples were weighed every three days during the incubation period to enable maintenance of soil moisture. This involved replacing the weight of water that had evaporated from the sample using sterile RO water.

Table 5-1. Predicted environmental concentration (PEC) of Vitavax 200FF in soil following sowing of wheat seed coated with the product formulation.

The PEC of Vitavax 200FF in soil was estimated based on the manufacturer recommended volume of Vitavax 200FF required to treat wheat seed and recommended sowing guidelines. Calculations used for estimation of PEC are outlined in Appendix V.

Product	Active ingredients	PEC	
		% of Vitavax 200FF in soil	Equivalent amount of active ingredients in soil
Vitavax 200FF	200 g/L carboxin + 200 g/L thiram	0.00000375%	0.3 mg/kg each of carboxin and thiram

Table 5-2. Soil treatments.

C&T = carboxin and thiram combined; VV = Vitavax 200FF

Soil	Treatment	Equivalent concentration of carboxin (mg/kg)	Equivalent concentration of thiram (mg/kg)
Sterile	Soil Only Control	0	0
	Soil Solvent Control	0	0
	Carboxin	30	0
	Thiram	0	30
	C&T	30	30
	VV	30	30
Non-sterile	Untreated Control	0	0
	Soil Solvent Control	0	0
	0.0000375% VV	3	3
	0.000375% VV	30	30

5.2.2 Analysis of soil pH, microbial activity or populations

Soil samples were analysed immediately after treatment for the required number of days (section 5.2.1). Analysis of soil pH, microbial activity or populations were perfomed as described in section 2.3.1, section 2.14 and section 2.15.

5.2.3 Extraction and HPLC analysis of carboxin and thiram from soil

Extraction of carboxin and thiram from soil was performed using acetonitrile as described in section 2.5. For quantification, HPLC analysis of samples was performed as described in section 2.8.

5.2.4 Cytotoxic effects of soil extracts in human cells

The treatments were prepared by diluting the soil extracts (dissolved in methanol) using culture medium. The concentration of methanol in all treatments was 0.05%. A medium only control and a solvent control (0.05% methanol) was also included in each experiment.

HepG2 (20000 cells) and HaCaT (10000 cells) cells were seeded into 96-well flat bottom plates and allowed to adhere for 24h at 37°C in a humidified atmosphere. After the adherence time, the culture medium was removed and cells were exposed to 200µl of treatments for 4h or 24h at 37°C in a humidified atmosphere. Cells were rinsed twice using PBS and cell viability was determined using the MTT assay (section 2.11.1).

For WIL2NS cells, 6-well plates were seeded at 5×10^5 cells/well. Cells were then exposed to 1ml of treatment in culture medium for 4h or 24h at 37°C in a humidified atmosphere. Cells were then rinsed twice using PBS and resuspended in 5ml fresh culture medium. 100µl of cell suspension was then transferred to 96-well round bottom plates and cell viability determined using the MTT assay (section 2.11.1).

5.2.5 Statistical analysis

Linear regression was used to analyse the correlation between cell number and absorbance for standard curves of both MTT and crystal violet assays. To determine significant differences between media only and solvent controls, independent t-tests were conducted on cells per well values of the respective controls.

Statistical differences in % cell viability between the solvent control and treatments in cytotoxicity assays were analysed by conducting one way univariate analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Statistical differences in % cell viability, concentration of carboxin or thiram, pH, microbial activity or populations compared to day 0 were also analysed by conducting one way univariate analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Statistical differences between soil treatments compared to the untreated control following an equal number of days after spiking of soil were analysed by independent t-tests. Statistical analyses were performed using SPSS software, version 18. Results were considered to be statistically significant when $p \leq 0.05$.

To determine the correlation between thiram concentration and the cytotoxicity of the soil samples, soil doses were first expressed as their equivalent concentration of thiram. IC_{25} and IC_{50} values were determined using GraphPad Prism (version 5.01). IC_{25} and IC_{50} values in which the curve had an R^2 value of less than 0.65 were excluded from the results. Significant differences ($p \leq 0.05$) in the IC_{25} or IC_{50} values between thiram and the soil samples was determined in GraphPad Prism using the sum of all squares F test as was previously done in other studies (Dawson *et al.*, 2012, Ivanov *et al.*, 2014, Broekman *et al.*, 2015).

5.3 Results

5.3.1 Sterile soil

5.3.1.1 Soil pH

The rate at which carboxin and thiram are degraded in soil can be influenced by the pH of the soil (Chin *et al.*, 1970, Hustert *et al.*, 1999, Gupta *et al.*, 2012a). The active ingredients of Vitavax 200FF, carboxin and thiram or other undisclosed proprietary ingredients in the product formulation could potentially alter soil pH. Therefore, the pH of the sterile soil was tested at day 0 with and without spiking with the chemicals used in this study. The pH of the untreated soil was 5.80±0.04 (Figure 5-1). The addition of acetone to soil caused no significant changes to soil pH (Figure 5-1). Similarly, spiking sterile soil with with carboxin, thiram, carboxin and thiram combined or Vitavax 200FF caused no significant changes to soil pH (Figure 5-1).

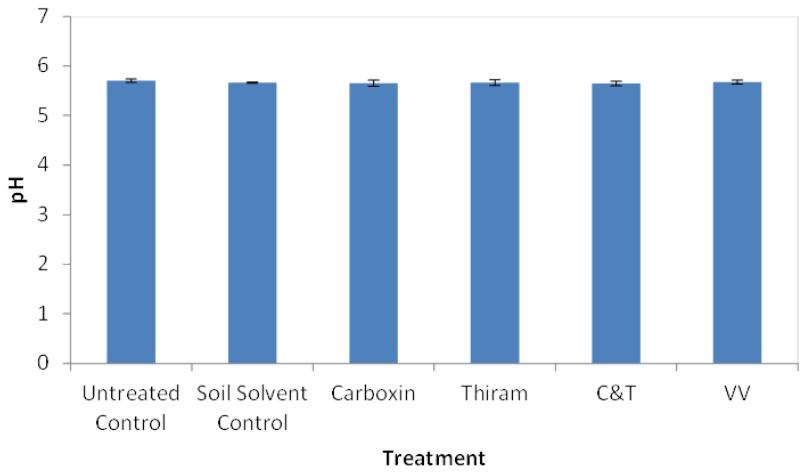


Figure 5-1. pH of sterile soil with and without spiking with treatments at day 0. Sterile soil was left untreated (untreated control), or spiked with acetone (soil solvent control), 30mg/kg carboxin or thiram individually, carboxin and thiram combined at 30mg/kg each (C&T), or Vitavax 200FF (VV) at a concentration equivalent to 30mg/kg each of carboxin and thiram. Results are expressed as the mean±SEM of three separate experiments. No significant differences compared to the untreated control were found ($p>0.05$).

5.3.1.2 Degradation of carboxin and thiram in sterile soil

To determine if the degradation of carboxin or thiram in sterile soil was affected by the presence of other ingredients, each compound was spiked in sterile soil at 30mg/kg individually, in combination (C&T), or in the product formulation Vitavax 200FF (VV) and monitored over a 28 day period.

The chromatograms for both the untreated control and soil solvent control showed little change throughout the 28 day period with all peaks having retention times of less than 5 minutes (Figure 5-2) and (Figure 5-3). In carboxin spiked soil, a peak with a retention time of 6.3 minutes was shown to increase over time as the peak for carboxin (retention time 15.9 minutes) decreased (Figure 5-4). Two additional peaks with retention times of 10.2 minutes and 10.8 minutes were also observed in carboxin spiked soil at days 21 and 28 (Figure 5-4).

In thiram spiked soil, two additional small peaks with retention times of 6.9 minutes and 9.0 minutes were observed at days 3, 7, 14, 21 and 28, together with thiram which had a retention time of 12.3 minutes (Figure 5-5). In soil spiked with both carboxin and thiram, a peak with a retention time of 6.3 minutes increased over time (Figure 5-6). A very small peak with a retention time of 9.0 minutes was also detected in some replicates at days 3, 7, 14, 21 and 28 for soil spiked with carboxin and thiram (Figure 5-6). For soil spiked with Vitavax 200FF, a peak with a retention time of 6.3 minutes increased over time as the peak for carboxin (retention time 15.9 minutes, Figure 5-7) decreased, similarly to that of carboxin or C&T spiked soil. Similarly to carboxin and thiram spiked soil, a very small peak with a retention time of 9.0 was also detected in some replicates at days 3, 7, 14, 21 and 28 for soil spiked with Vitavax 200FF (Figure 5-7).

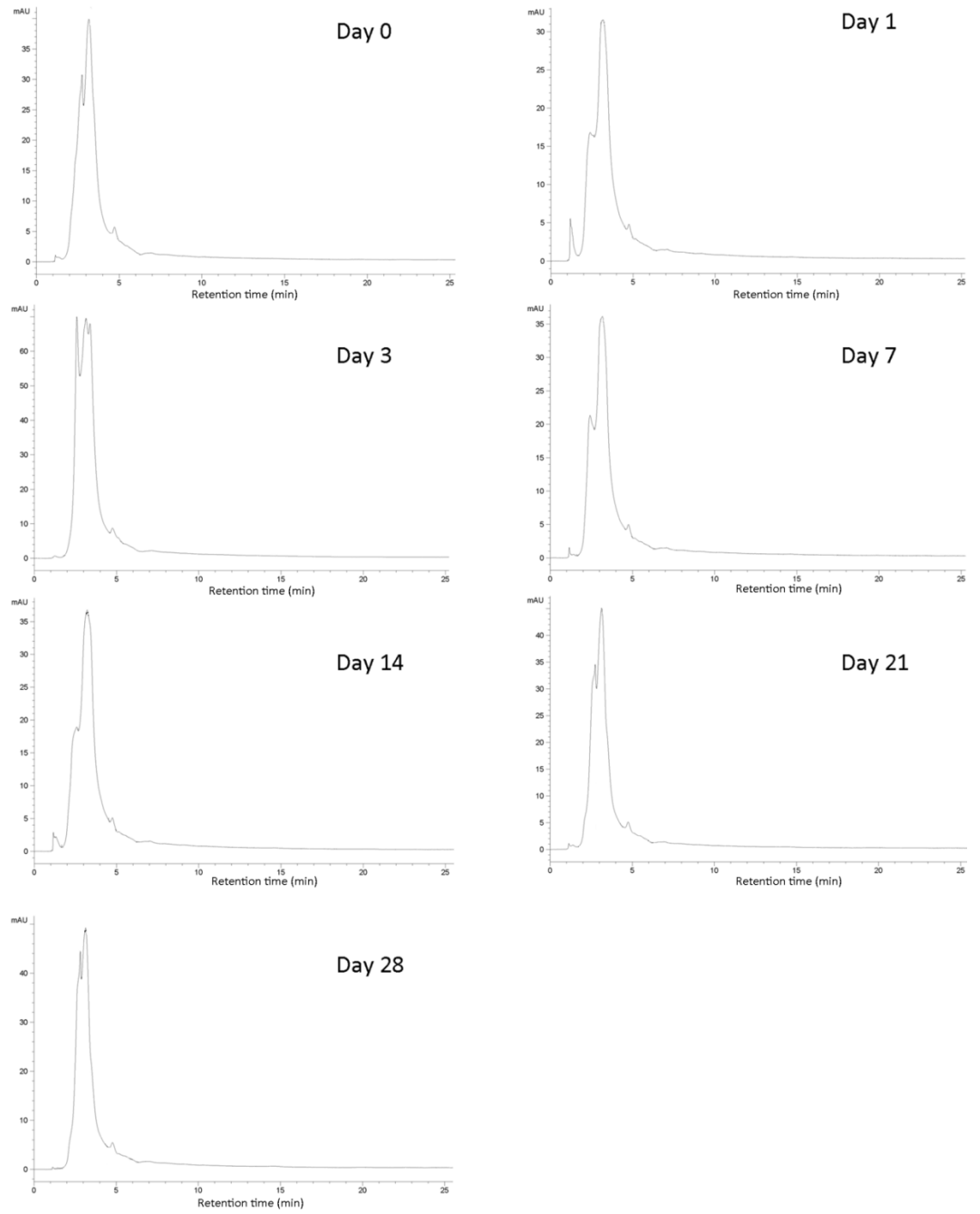


Figure 5-2. HPLC chromatograms of the Untreated Control over 28 days.

Sterile soil was left untreated and sampled over a 28 day period. Soil samples were extracted using acetonitrile followed by evaporation of the extract. The dried extract was dissolved in methanol and analysed using HPLC with detection at 254 nm.

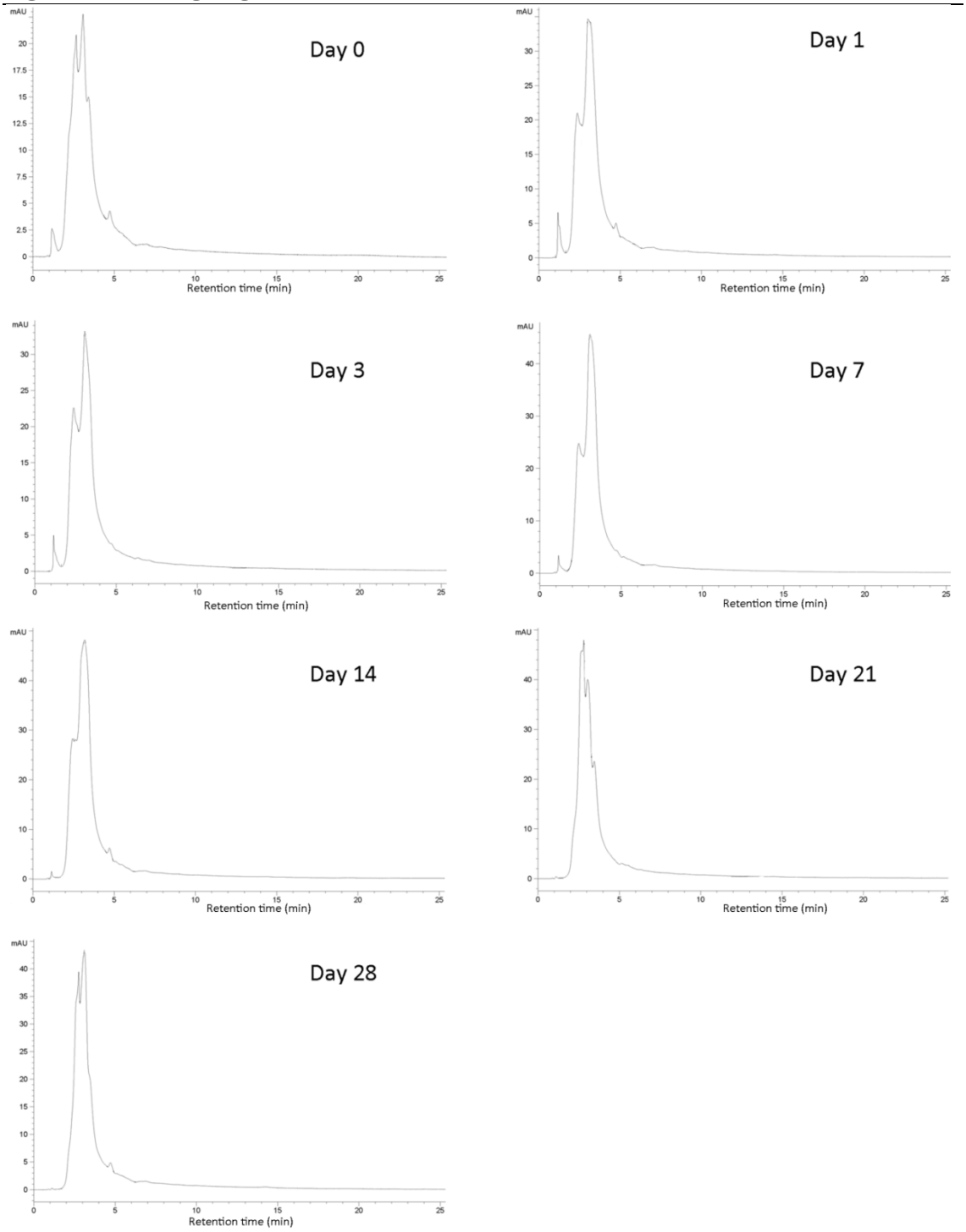


Figure 5-3. HPLC chromatograms of sterile soil spiked with acetone (Soil Solvent Control) over 28 days.

Sterile soil was spiked with acetone and sampled over a 28 day period. Soil samples were extracted using acetonitrile followed by evaporation of the extract. The dried extract was dissolved in methanol and analysed using HPLC with detection at 254 nm.

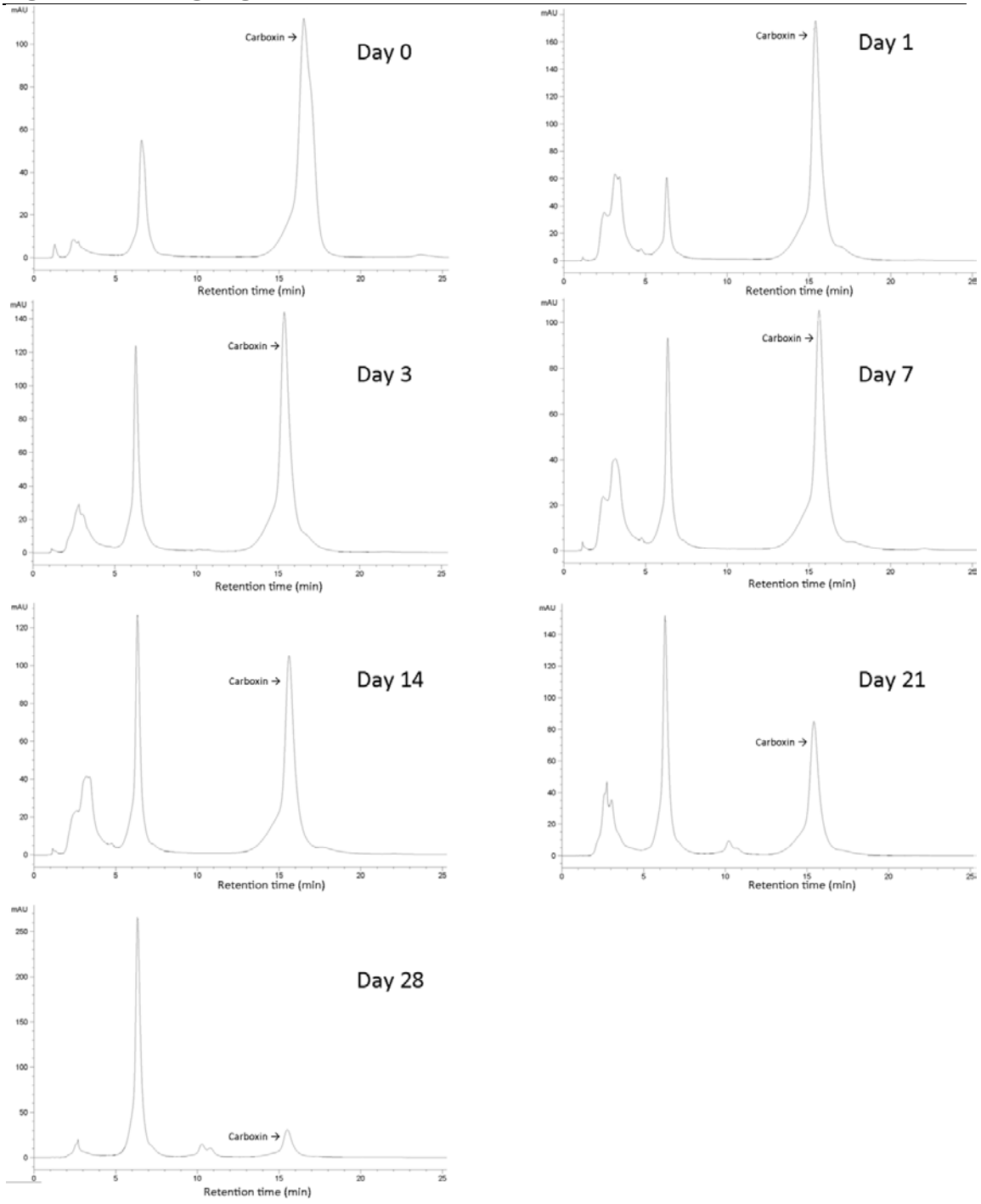


Figure 5-4. HPLC chromatograms of sterile soil spiked with carboxin over 28 days.

Sterile soil was spiked with carboxin at 30 mg/kg and sampled over a 28 day period. Soil samples were extracted using acetonitrile followed by evaporation of the extract. The dried extract was dissolved in methanol and analysed using HPLC with detection at 254 nm. The peak corresponding to carboxin is indicated on all chromatograms.

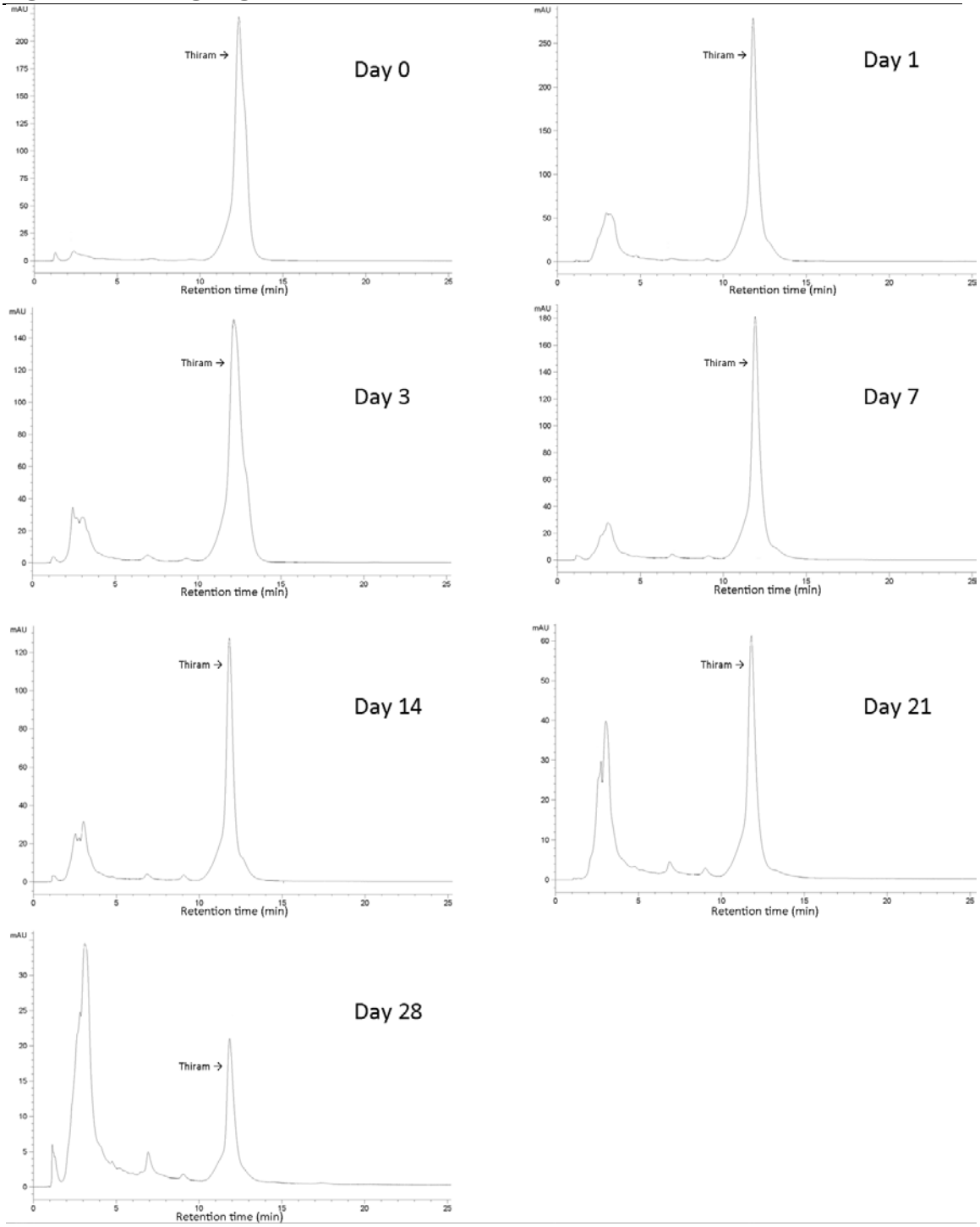


Figure 5-5. HPLC chromatograms of sterile soil spiked with thiram over 28 days. Sterile soil was spiked with thiram at 30 mg/kg and sampled over a 28 day period. Soil samples were extracted using acetonitrile followed by evaporation of the extract. The dried extract was dissolved in methanol and analysed using HPLC with detection at 254 nm. The peak corresponding to thiram is indicated on all chromatograms.

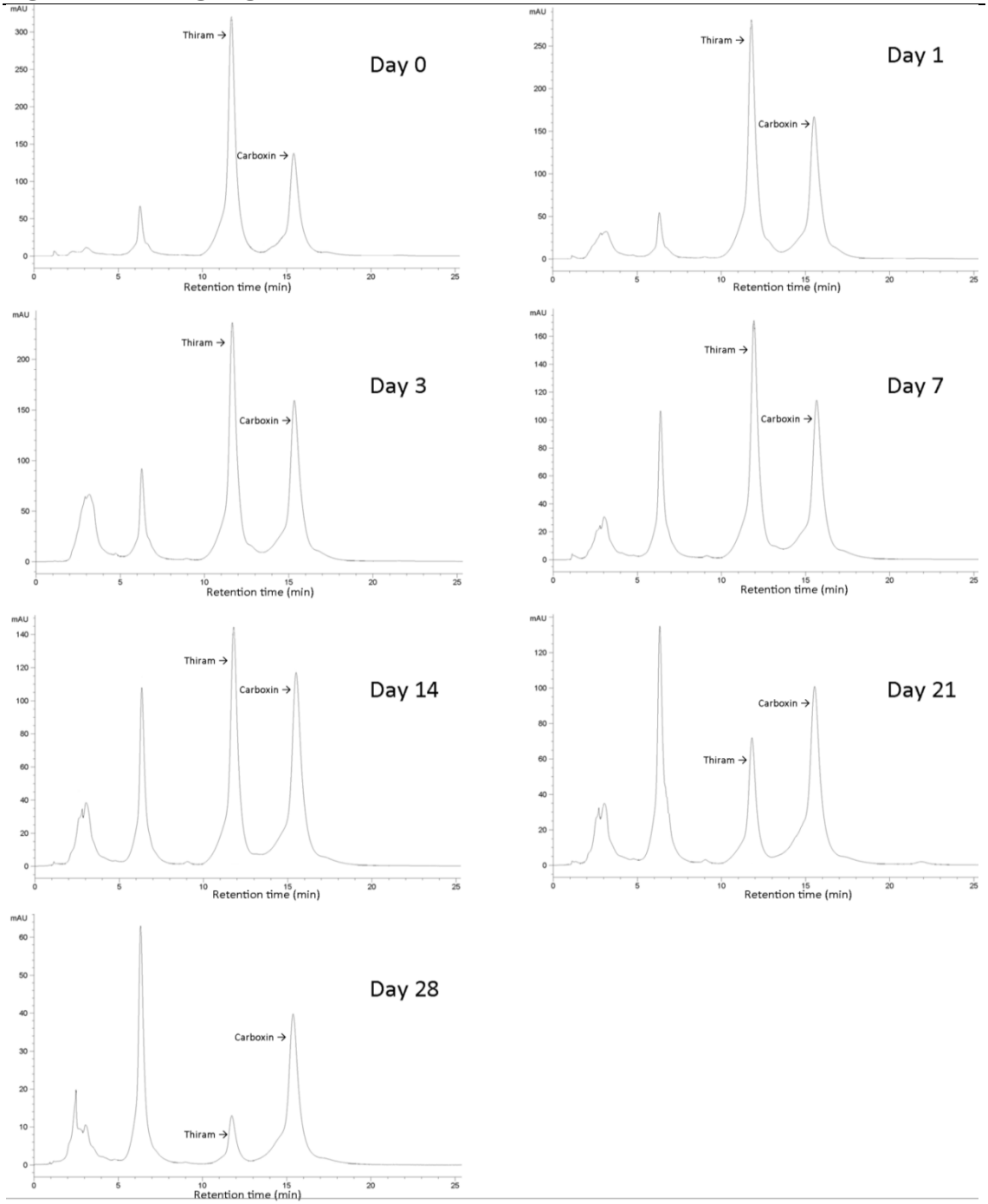


Figure 5-6. HPLC chromatograms of sterile soil spiked with carboxin and thiram over 28 days.

Sterile soil was spiked with carboxin and thiram at 30mg/kg each and sampled over a 28 day period. Soil samples were extracted using acetonitrile followed by evaporation of the extract. The dried extract was dissolved in methanol and analysed using HPLC with detection at 254 nm. The peaks corresponding to carboxin and thiram are indicated on all chromatograms.

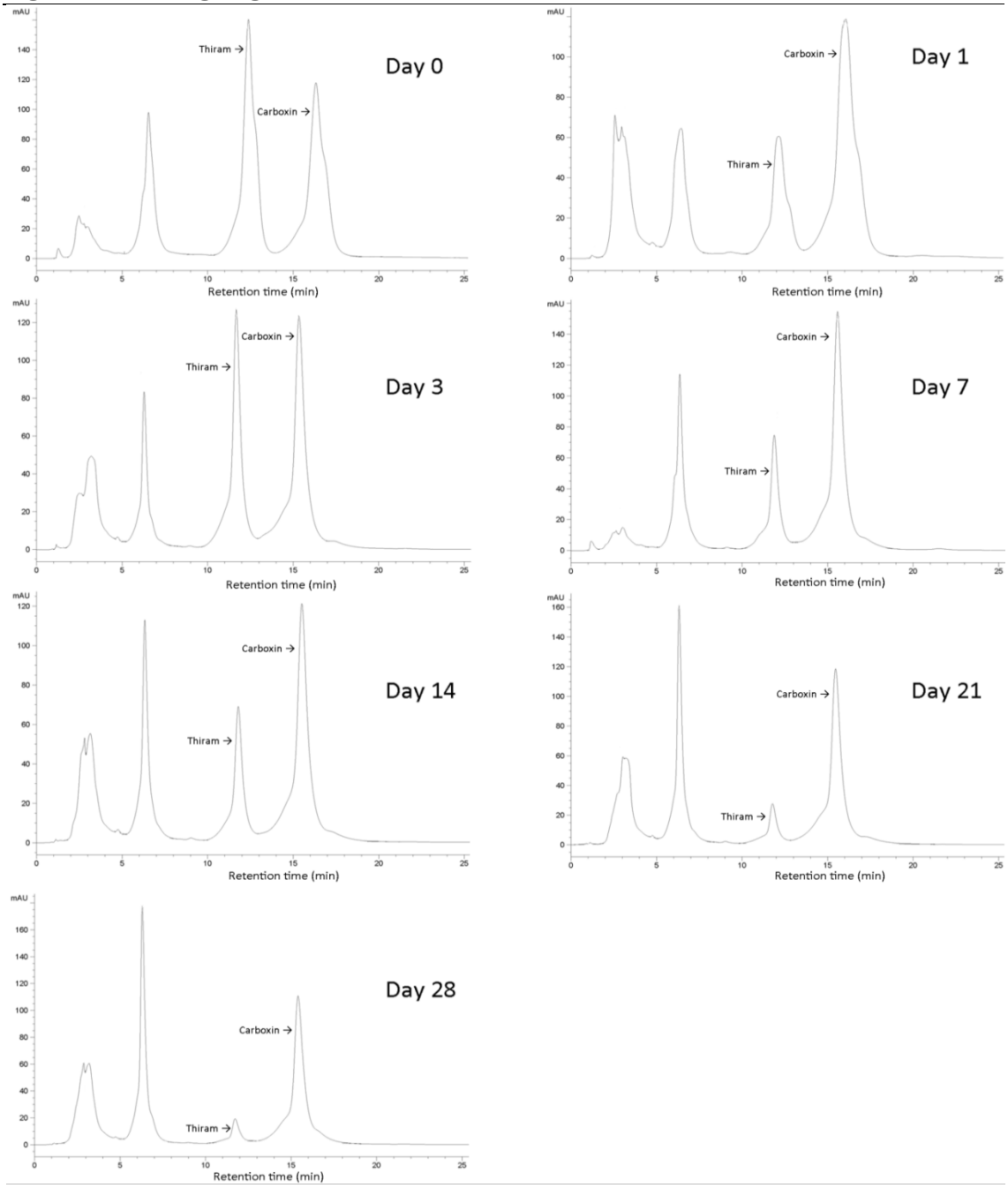


Figure 5-7. HPLC chromatograms of sterile soil spiked with Vitavax 200FF over 28 days.

Sterile soil was spiked with Vitavax 200FF equivalent to 30 mg/kg each of carboxin and thiram and sampled over a 28 day period. Soil samples were extracted using acetonitrile followed by evaporation of the extract. The dried extract was dissolved in methanol and analysed using HPLC with detection at 254 nm. The peaks corresponding to carboxin and thiram are indicated on all chromatograms.

The concentration of carboxin in all three spiked soils decreased over 28 days (Figure 5-8). The decrease in carboxin was significant compared to day 0 in soil spiked with carboxin individually at days 14, 21 and 28 (Figure 5-8). The decrease in carboxin observed in C&T and VV spiked soils did not reach statistical significance from day 0 within the 28 day period (Figure 5-8). The greatest degradation of carboxin occurred when soil was spiked with carboxin individually with 40.1±16.6% of carboxin remaining after 28 days of incubation compared to day 0 (Figure 5-8). Soil spiked with C&T showed slightly less degradation of carboxin over time compared to soil spiked with carboxin individually with a higher level of carboxin (60.2±26.5%) remaining in the C&T-treated soil after 28 days compared to day 0. Soil spiked with VV showed the least degradation of carboxin and showed the greatest amount of carboxin remaining in the soil (71.1±11.7%) after the 28 day period (Figure 5-8).

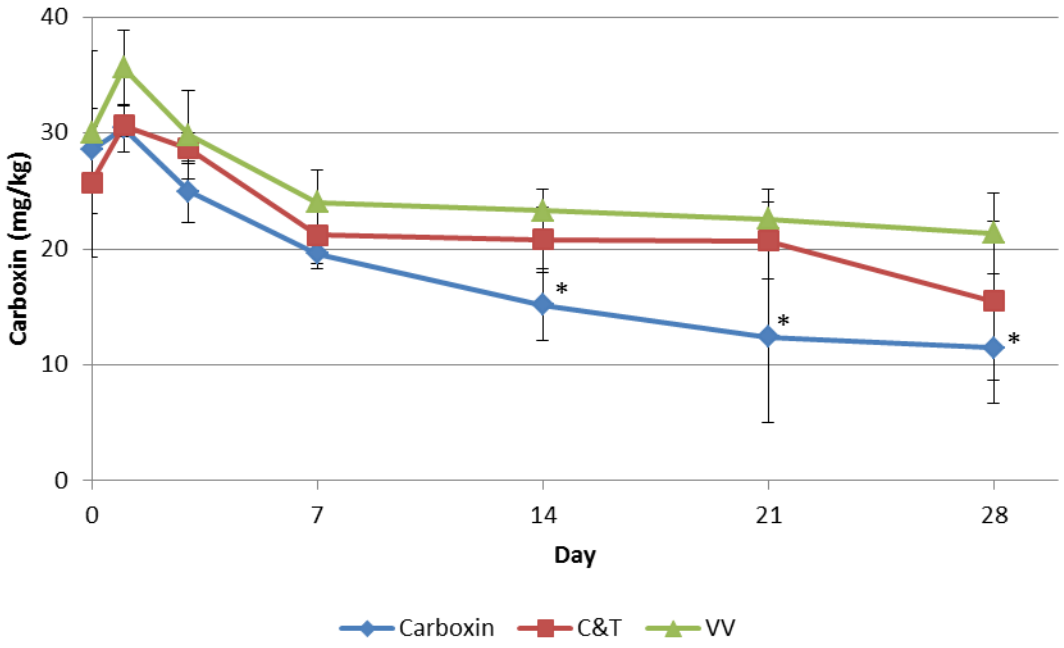


Figure 5-8. Degradation of carboxin in sterile soil. Sterile soil was spiked with 30 mg/kg carboxin individually, in combination with thiram (C&T) or in the product formulation Vitavax200FF (VV).
The concentration of carboxin remaining in the soil at each time point was determined using HPLC. Results are shown as the mean±SD of three separate experiments. Significant differences from Day 0 are denoted as *

Thiram also showed a decrease over time which reached statistical significance compared to day 0 in all three spiked soils during the 28 day degradation period (Figure 5-9). The amount of thiram remaining in thiram-only spiked soil and C&T spiked soil was similar at each time point (Figure 5-9). VV spiked soil showed a rapid initial decrease of thiram in soil after 1 day to 42.9±2.3% of the spiked amount. The amount of thiram remaining in thiram only, C&T and VV spiked soil after 28 days was 5.1±4.1%, 16.0±15.1% and 6.5±3.4% respectively (Figure 5-9).

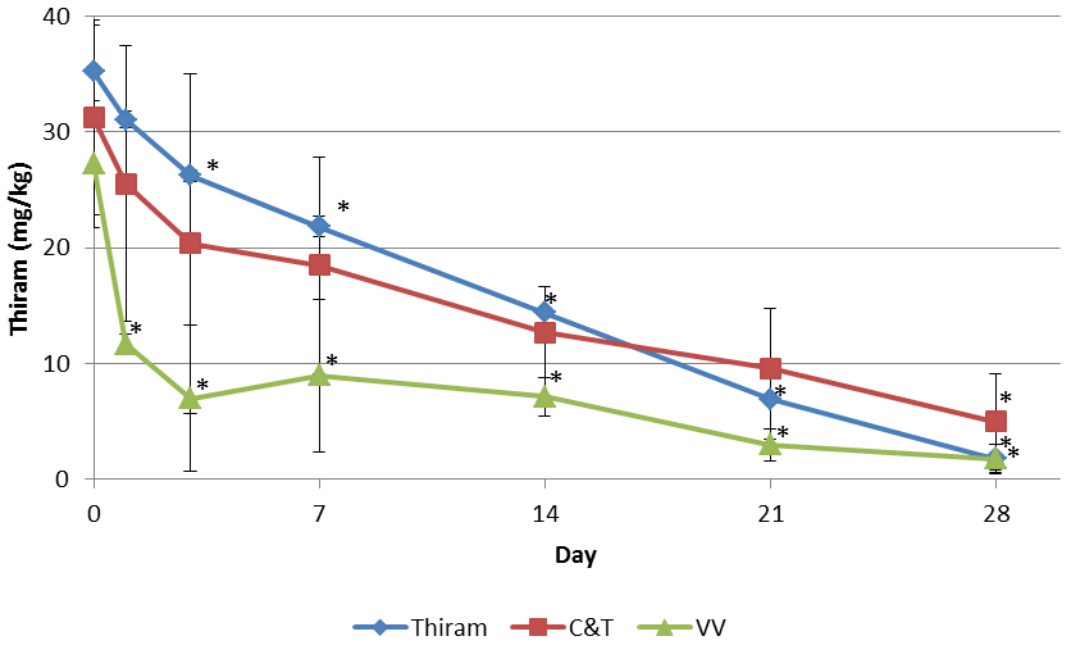


Figure 5-9. Degradation of thiram in sterile soil.

Sterile soil was spiked with 30mg/kg thiram individually, in combination with carboxin (C&T) or in the product formulation Vitavax200FF (VV). The concentration of thiram remaining in the soil at each time point was determined using HPLC. Results are shown as the mean±SD of three separate experiments. Significant differences from Day 0 are denoted as *

5.3.1.3 Effects of control soils on cell viability

Untreated sterile soil (Untreated Control) was monitored throughout the 28 day degradation period to determine any changes to its effects on cell viability (Figure 5-10). For all cell lines, no significant differences compared to the solvent control were found at any of the doses or exposure times tested (Figure 5-10).

The chemicals used in this study were dissolved in acetone prior to spiking sterile soil. Therefore, sterile soil was spiked with acetone only (Soil Solvent Control) and monitored over the 28 day degradation period to determine if the use of acetone during spiking of the soil affected cell viability (Figure 5-11). For all cell lines, no significant differences compared to the solvent control were found at any of the doses or exposure times tested (Figure 5-11).

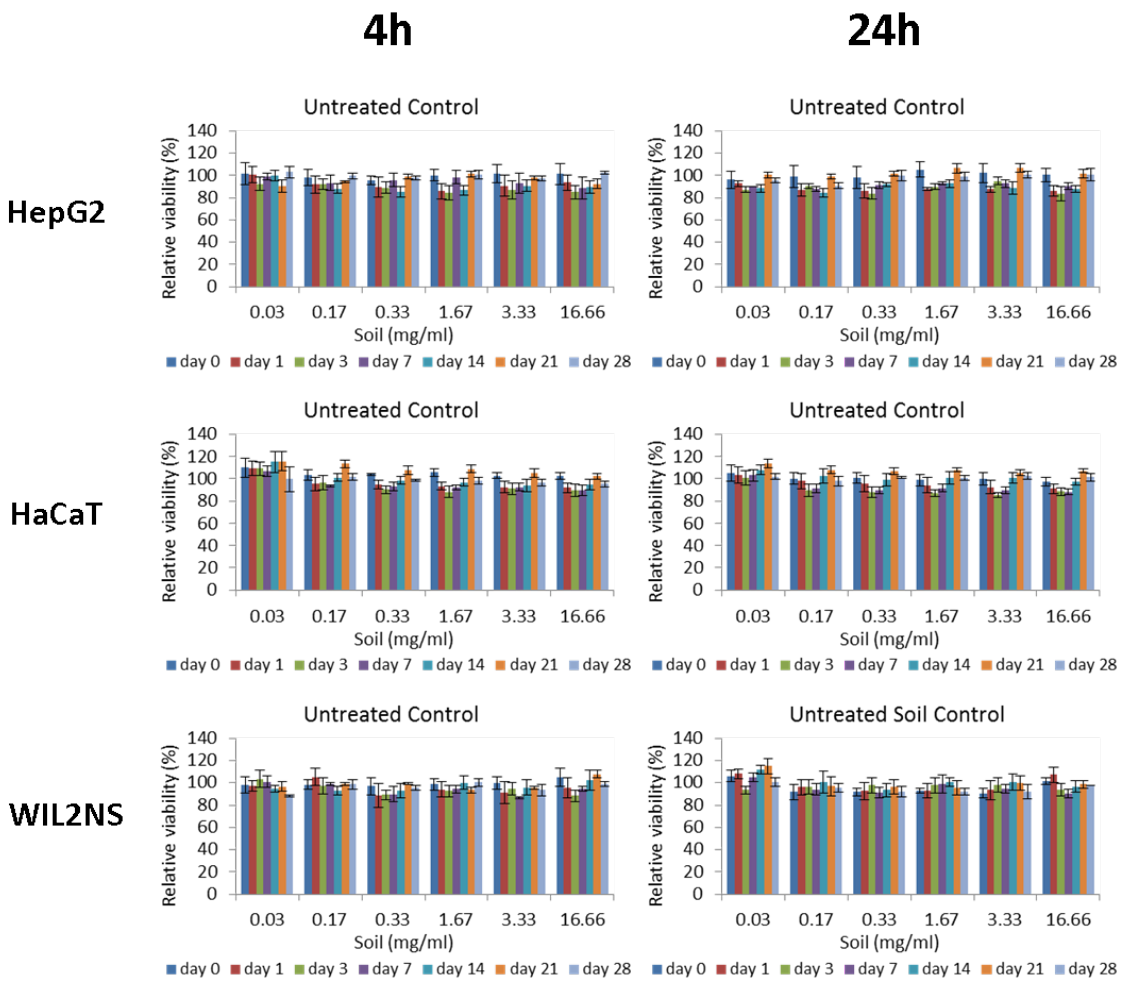


Figure 5-10. Effects of 4h or 24h exposure to untreated sterile soil (Untreated Control) on cell viability.

Sterile soil was left untreated and sampled over a 28 day period. Cells were exposed to the soil extracts for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). No significant differences compared to the solvent control were found ($p>0.05$).

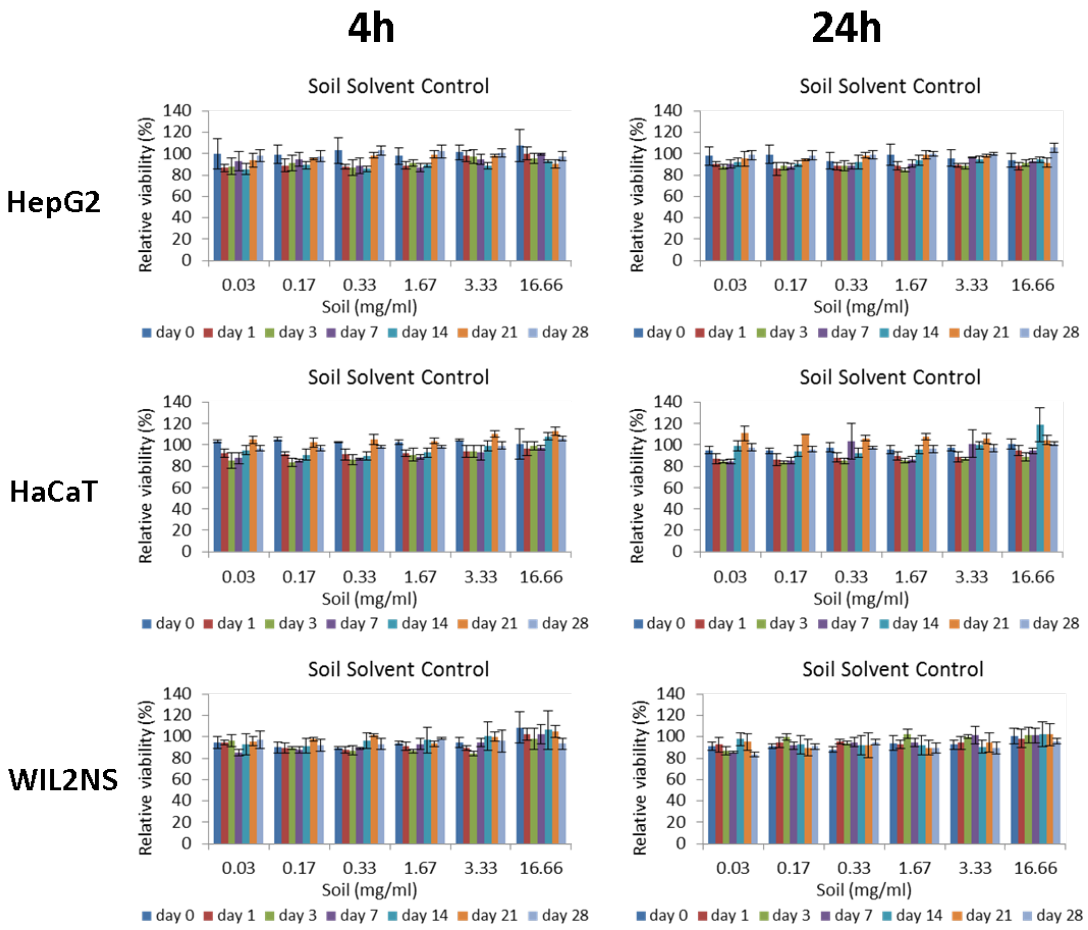


Figure 5-11. Effects of 4h or 24h exposure to sterile soil spiked with acetone (Soil Solvent Control) on cell viability.

Sterile soil was spiked with acetone and sampled over a 28 day period. Cells were exposed to the soil extracts for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). No significant differences compared to the solvent control were found ($p>0.05$).

5.3.1.4 Effects of degradation of Vitavax 200FF or its active ingredients in sterile soil on cell viability

In each cell line tested, carboxin spiked soil had no significant effect (defined as $p>0.05$) on cell viability compared to the solvent control at all doses and exposure times throughout the 28 day degradation period (Figure 5-12).

For thiram spiked soil, the decrease in cell viability induced by the soil extracts were both time- and dose-dependent in all cell lines (Figure 5-13). All cell lines showed a significant decrease ($p\leq0.05$) in cell viability after 24h exposure to 16.66 mg/ml soil from days 0-21 (Figure 5-13). After the 28 day degradation period, 24h exposure to 16.66 mg/ml thiram spiked soil induced no significant effect ($p>0.05$) on cell viability in HepG2 and HaCaT cell lines. In comparison, exposure to day 28 thiram spiked soil at the same dose and exposure period showed significantly lower cell viability ($p\leq0.05$) in WIL2NS cells of 55.17±11.9%, compared to the solvent control (Figure 5-13).

Sterile soil spiked with carboxin and thiram combined also induced time- and dose-dependent decreases in cell viability (Figure 5-14). Similarly to that seen for thiram spiked soil, all cell lines showed a significant decrease ($p\leq0.05$) in cell viability after 24h exposure to 16.66 mg/ml soil from days 0-21 (Figure 5-14).

For sterile soil spiked with Vitavax 200FF, exposure of cells to the soil also induced time- and dose-dependent decreases in cell viability (Figure 5-15). All cell lines showed a significant decrease ($p\leq0.05$) in cell viability after 24h exposure to 16.66 mg/ml soil from days 0-14 (Figure 5-15). Exposure to 16.66 mg/ml day 21 soil induced a significant decrease ($p\leq0.05$) of cell viability in HepG2 and WIL2NS cell lines to 72.98±5.3% and 39.53±5.5% respectively, whilst no significant effect was observed in HaCaT cells (Figure 5-15).

It is noted that 24h exposure to 16.66 mg/ml of sterile soil spiked with thiram, carboxin and thiram combined or Vitavax 200FF after 28 days resulted in a decrease in cell viability in all cell lines (Figure 5-13, Figure 5-14 and Figure 5-15). This coincided with residual levels of carboxin and thiram still remaining in the soil after the 28 day period (Figure 5-8 and Figure 5-9).

The changes in the cytotoxicity of the soil samples throughout the degradation period are shown in Figure 5-16. Carboxin spiked soil induced no effects on cell viability in all cell lines at day 0 and remained the same throughout the 28 day degradation period (Figure 5-16). Thiram, carboxin and thiram combined and Vitavax 200FF spiked soils all showed a gradual increase in cell viability over the 28 day degradation period in all cell lines (Figure 5-16). At day 28, both thiram and Vitavax 200FF spiked soils showed a significant increase ($p \leq 0.05$) in cell viability compared to day 0 after 24h exposure in cell lines (Figure 5-16). After 24h exposure, a significant increase ($p \leq 0.05$) in cell viability at day 28 compared to day 0 for carboxin and thiram combined soil was only observed in HaCaT cells (Figure 5-16). Although 24h exposure of HepG2 and WIL2NS cells to day 28 carboxin and thiram combined soil saw increased cell viability compared to day 0, the increase was not statistically significant ($p > 0.05$, Figure 5-16).

Soil spiked with thiram or carboxin and thiram combined showed similar changes in cytotoxicity over the 28 day degradation period after both 4h and 24h exposure in all cell lines (Figure 5-16). Soil spiked with carboxin and thiram combined generally showed slightly greater cytotoxicity compared to thiram spiked soil over the 28 day period (Figure 5-16). Exposure to day 28 carboxin and thiram combined soil for 24h induced a greater reduction of cell viability in all cell lines compared to day 28 thiram soil (Figure 5-16).

For all cell lines, Vitavax 200FF soil showed an initial sharp increase in cell viability from day 0 to day 3 (Figure 5-16). Vitavax 200FF soil was also less cytotoxic compared to thiram or carboxin and thiram combined soil at days 3-21 for all cell lines, with the differences being more prominent in HaCaT and WIL2NS cell lines (Figure 5-16).

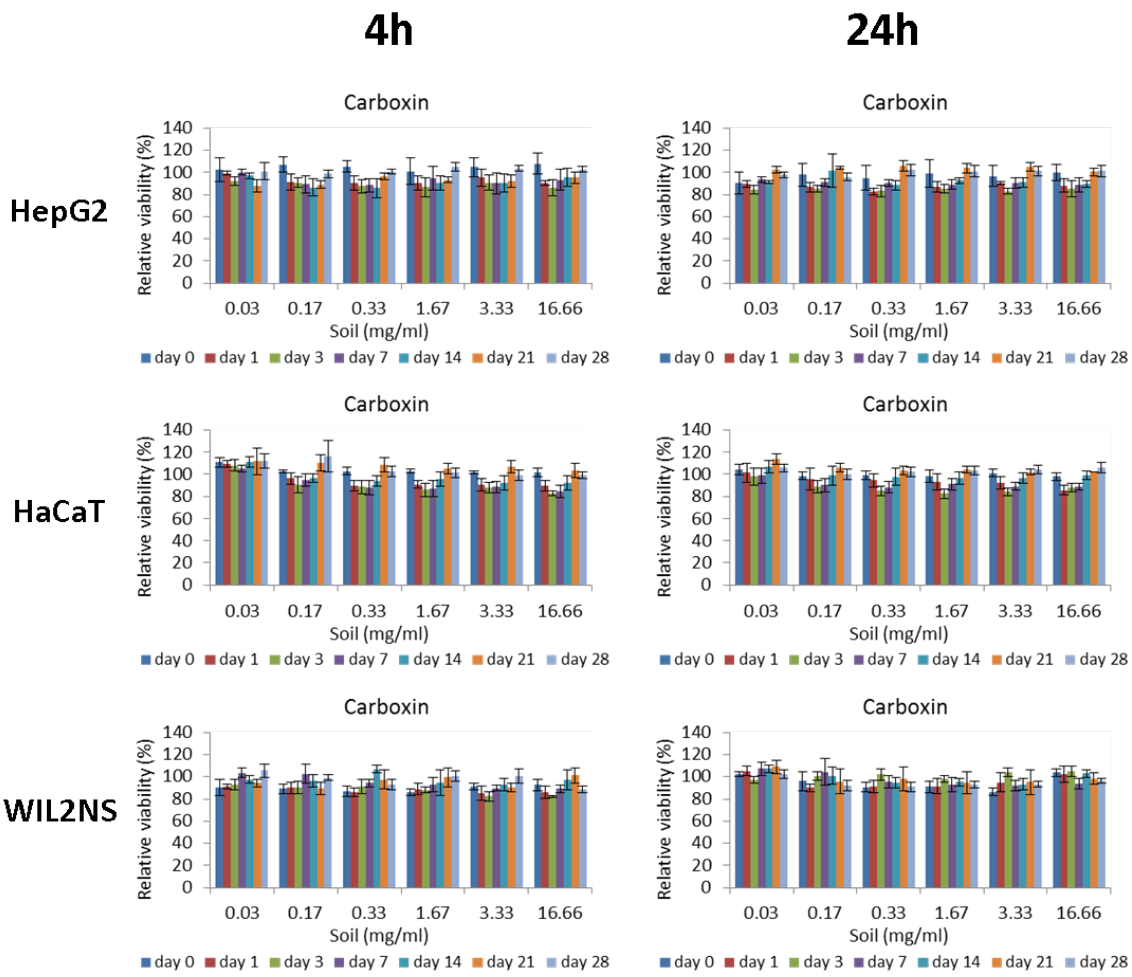


Figure 5-12. Effects of 4h or 24h exposure to sterile soil spiked with carboxin on cell viability.

Sterile soil was spiked with carboxin and sampled over a 28 day degradation period. Cells were exposed to the soil extracts for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). No significant differences compared to the solvent control were found ($p>0.05$).

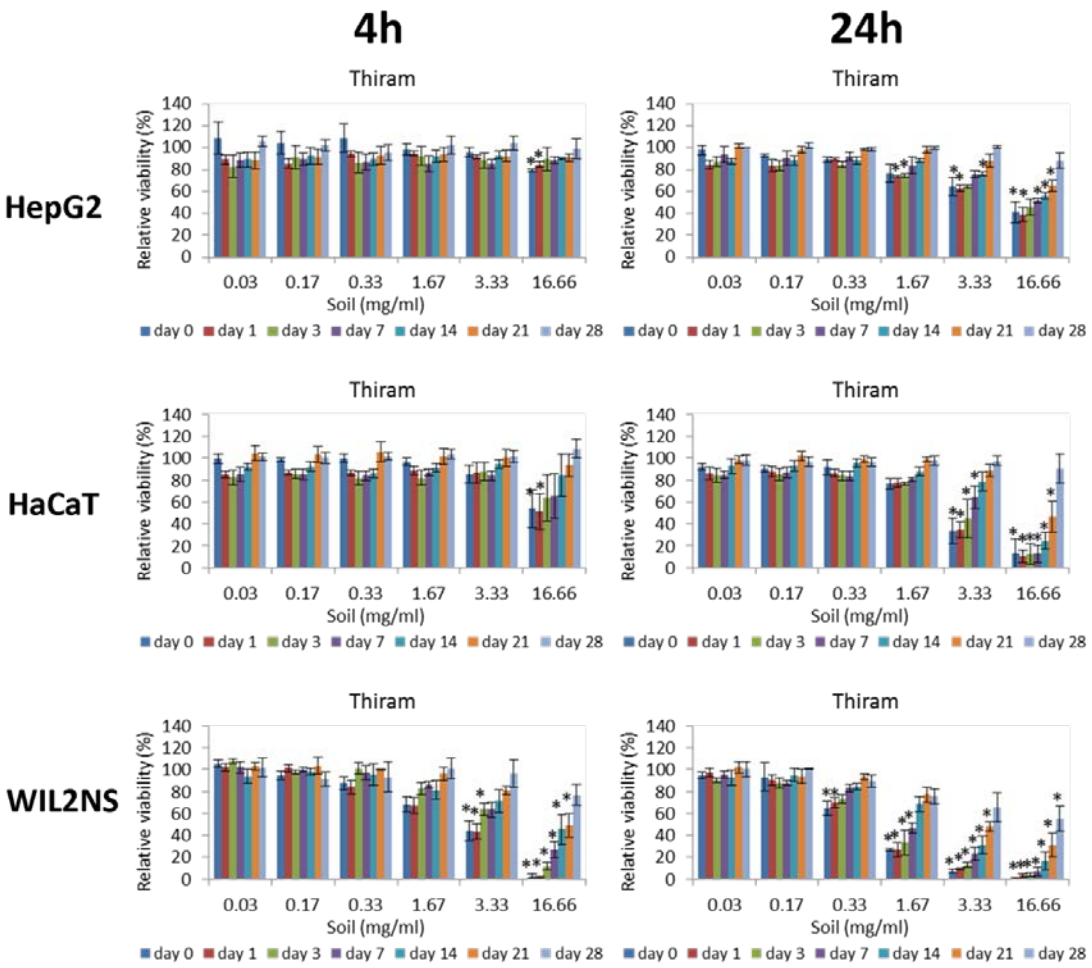


Figure 5-13. Effects of 4h or 24h exposure to sterile soil spiked with thiram on cell viability.

Sterile soil was spiked with thiram and sampled over a 28 day degradation period. Cells were exposed to the soil extracts for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). Significant differences ($p \leq 0.05$) compared to the solvent control are denoted as *.

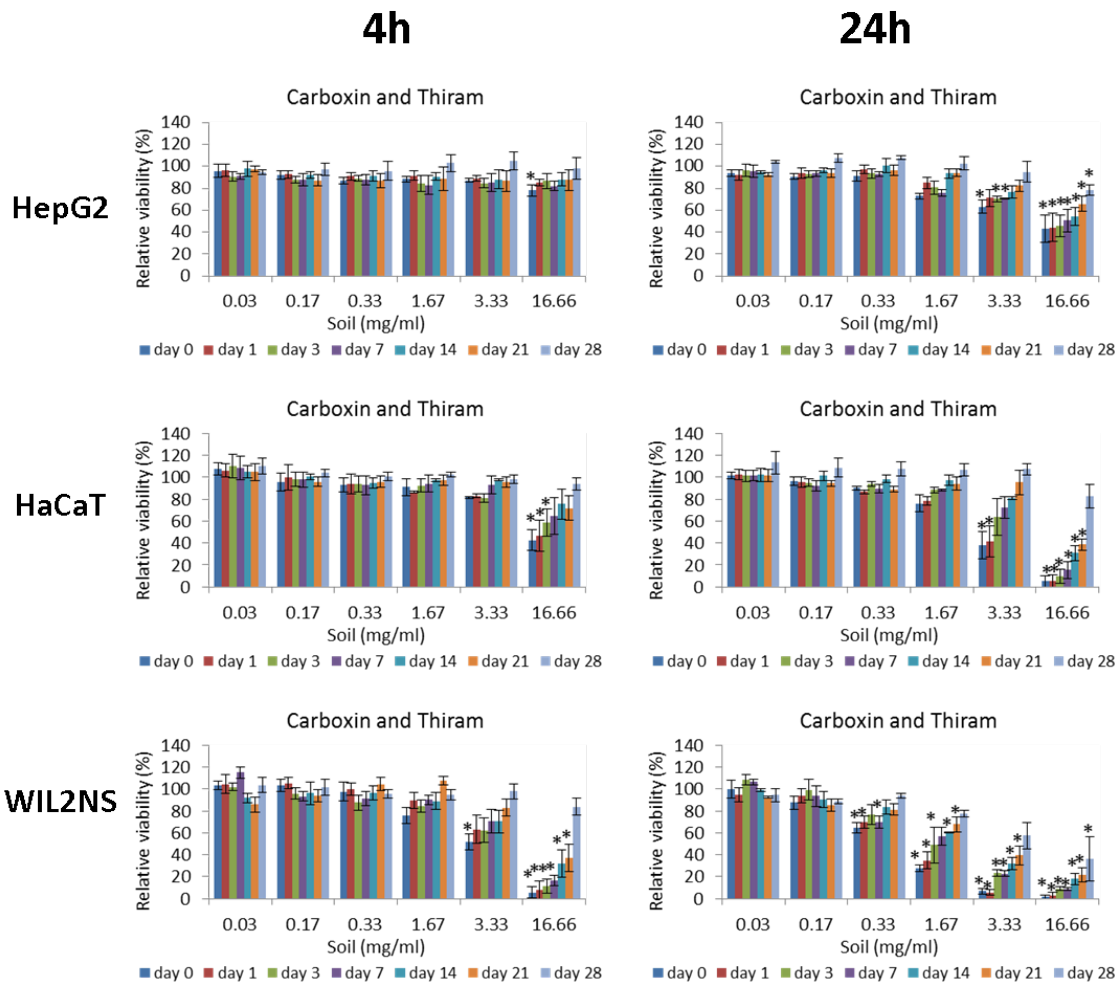


Figure 5-14. Effects of 4h or 24h exposure to sterile soil spiked with carboxin and thiram combined on cell viability.

Sterile soil was spiked with carboxin and thiram combined and sampled over a 28 day degradation period. Cells were exposed to the soil extracts for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). Significant differences ($p \leq 0.05$) compared to the solvent control are denoted as *.

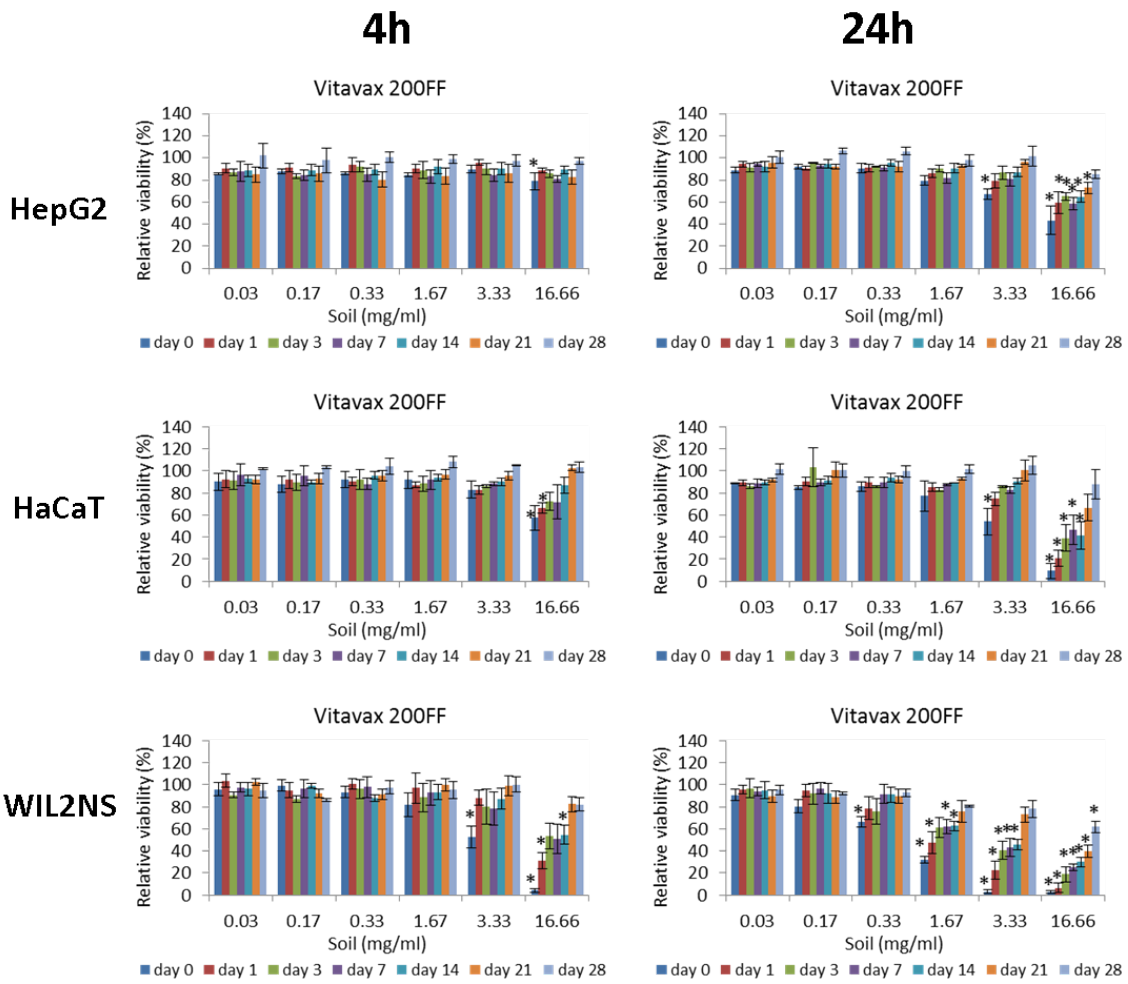


Figure 5-15. Effects of 4h or 24h exposure to sterile soil spiked with Vitavax 200FF on cell viability.

Sterile soil was spiked with Vitavax 200FF combined and sampled over a 28 day degradation period. Cells were exposed to the soil extracts for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). Significant differences ($p \leq 0.05$) compared to the solvent control are denoted as *.

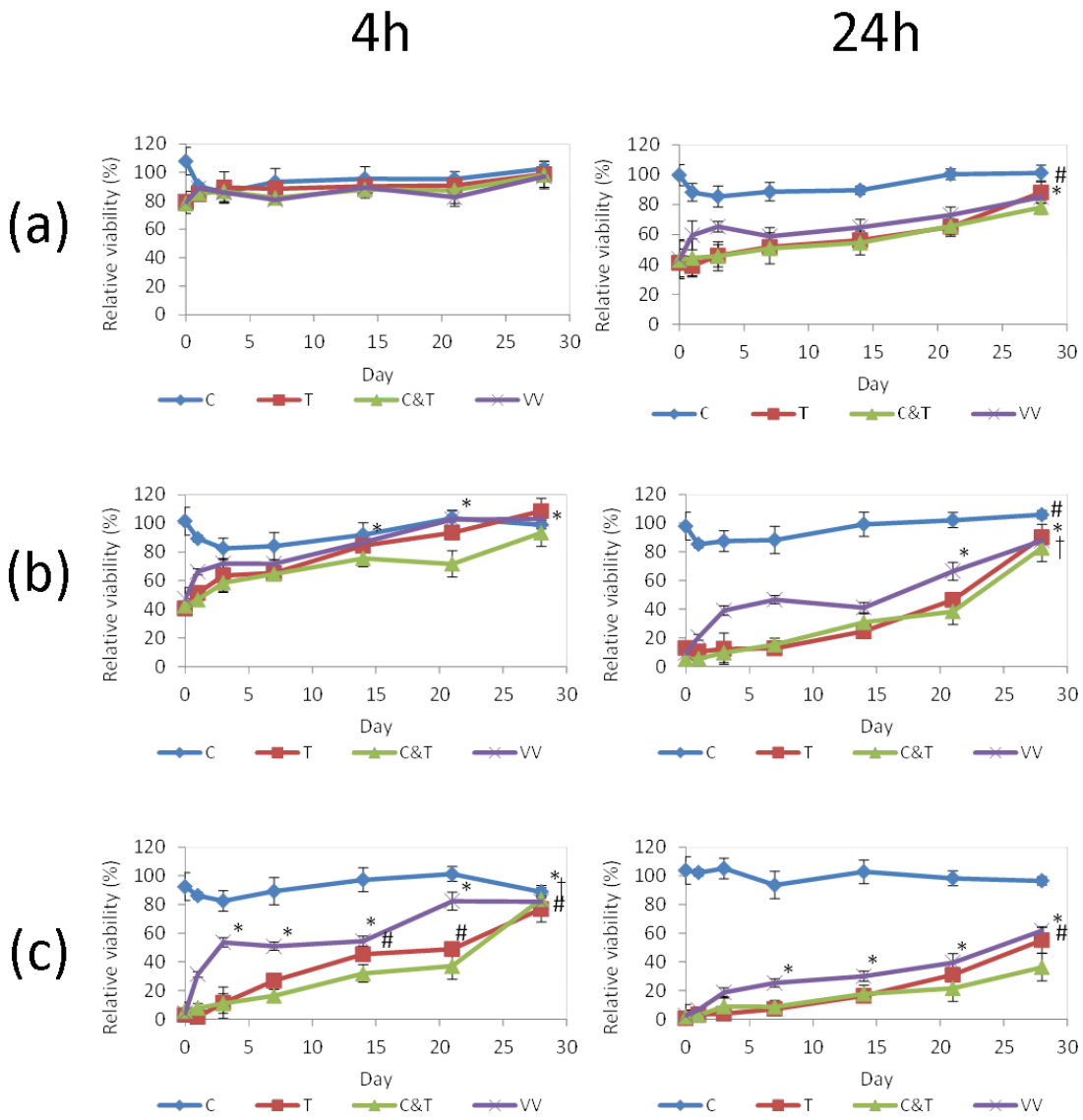


Figure 5-16. Changes in the toxicity of sterile soil spiked with various chemicals over 28 days to (a) HepG2, (b) HaCaT or (c) WIL2NS cells.

C = carboxin; T = thiram; C&T = carboxin and thiram combined; VV = Vitavax 200FF. Cells were exposed to soil extracts at 16.66 mg/ml for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). Significant differences ($p \leq 0.05$) compared to Day 0 are denoted as # for thiram, † for carboxin and thiram combined (C&T), or * for Vitavax 200FF (VV).

5.3.2 Non-sterile soil

5.3.2.1 Degradation of Vitavax 200FF in non-sterile soil

Non-sterile soil was spiked with a low dose of 0.0000375% Vitavax 200FF, which would represent a small spill of seed treated with Vitavax 200FF onto soil, contamination of the surrounding soil during seed treatment and subsequent transferring of the treated seed to containers in an open field, or cleaning of seed treatment equipment contaminated with Vitavax 200FF. Non-sterile soil was also spiked with a higher dose of 0.000375% Vitavax 200FF to represent a larger spill of seed treated with Vitavax 200FF onto soil, or spillage of the liquid product itself directly onto soil.

Both the untreated control (Figure 5-17) and soil solvent control (Figure 5-18) produced similar chromatograms throughout the 28 day period with all detectable peaks having retention times of less than 5 minutes. For soil spiked with the lower dose of 0.0000375% Vitavax 200FF, a peak with a retention time of 6.3 minutes was detected at day 0, with the size of the peak remaining similar throughout the degradation period (Figure 5-19). The same peak with a retention time of 6.3 minutes was also detected in soil spiked with the higher dose of 0.000375% Vitavax 200FF throughout the entire degradation period (days 0-28, Figure 5-20).

A small peak with a retention time of 5.1 minutes was detected in soil spiked with 0.0000375% Vitavax 200FF at days 1-28 (Figure 5-19). Both peaks with retention times of 6.3 and 5.1 minutes were also detected in soil spiked with the higher dose of 0.000375% Vitavax 200FF at days 1-28 (Figure 5-20).

Two additional peaks with retention times between 9.5-11.0 minutes were detected at day 1 and day 3 for soil spiked with 0.0000375% Vitavax 200FF and disappeared after day 7 (Figure 5-19). The two peaks with retention times of 9.5-11 minutes were also

present in soil spiked at the higher dose of 0.000375% Vitavax 200FF at days 1-21 and disappeared at day 28 (Figure 5-20).

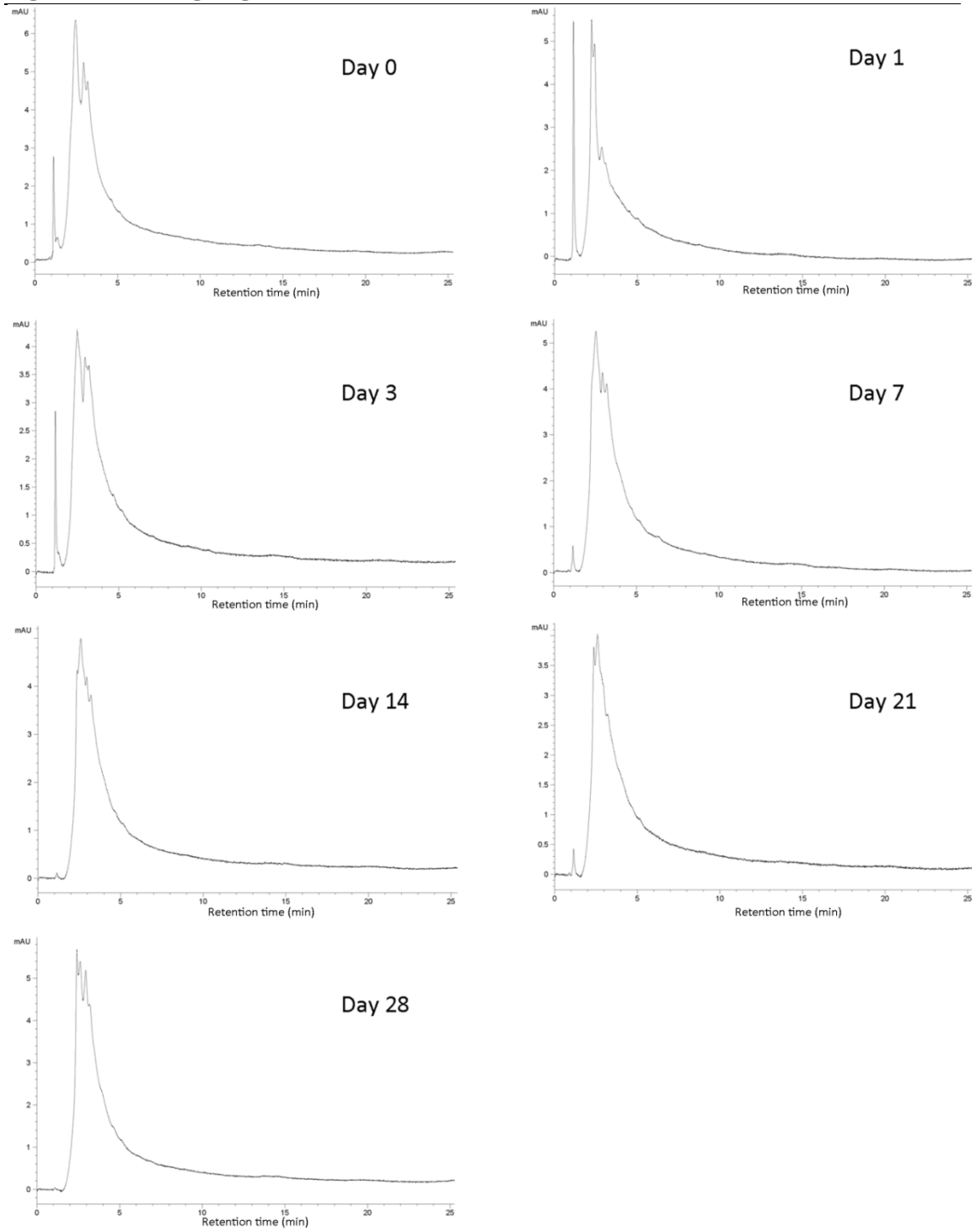


Figure 5-17. HPLC chromatograms of non-sterile soil over 28 days.

Non-sterile soil was left untreated (Untreated Control) and sampled over a 28 day period. Soil samples were extracted using acetonitrile followed by evaporation of the extract. The dried extract was dissolved in methanol and analysed using HPLC.

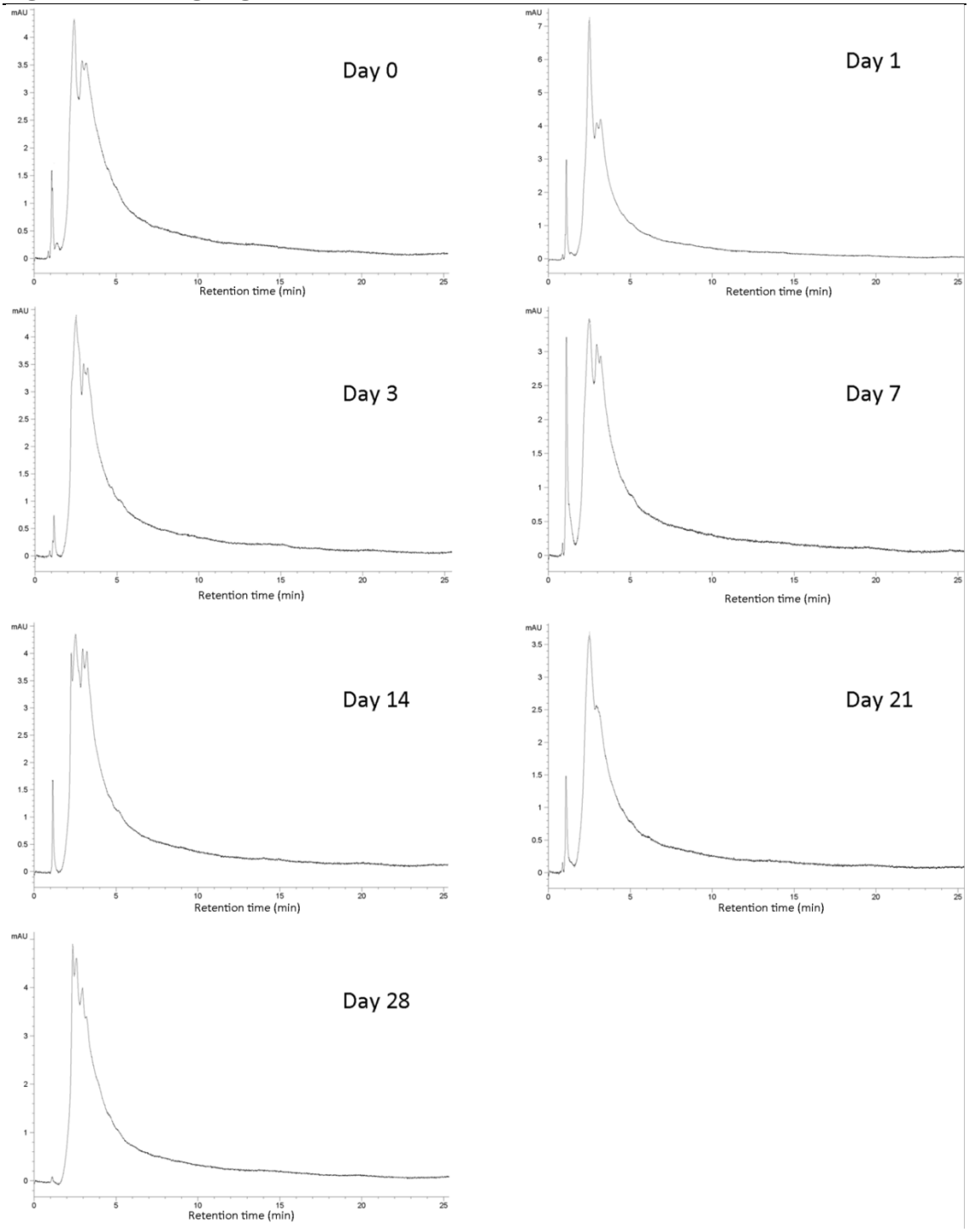


Figure 5-18. HPLC chromatograms of non-sterile soil spiked with acetone (Soil Solvent Control) over 28 days.

Non-sterile soil was spiked with acetone and sampled over a 28 day period. Soil samples were extracted using acetonitrile followed by evaporation of the extract. The dried extract was dissolved in methanol and analysed using HPLC.

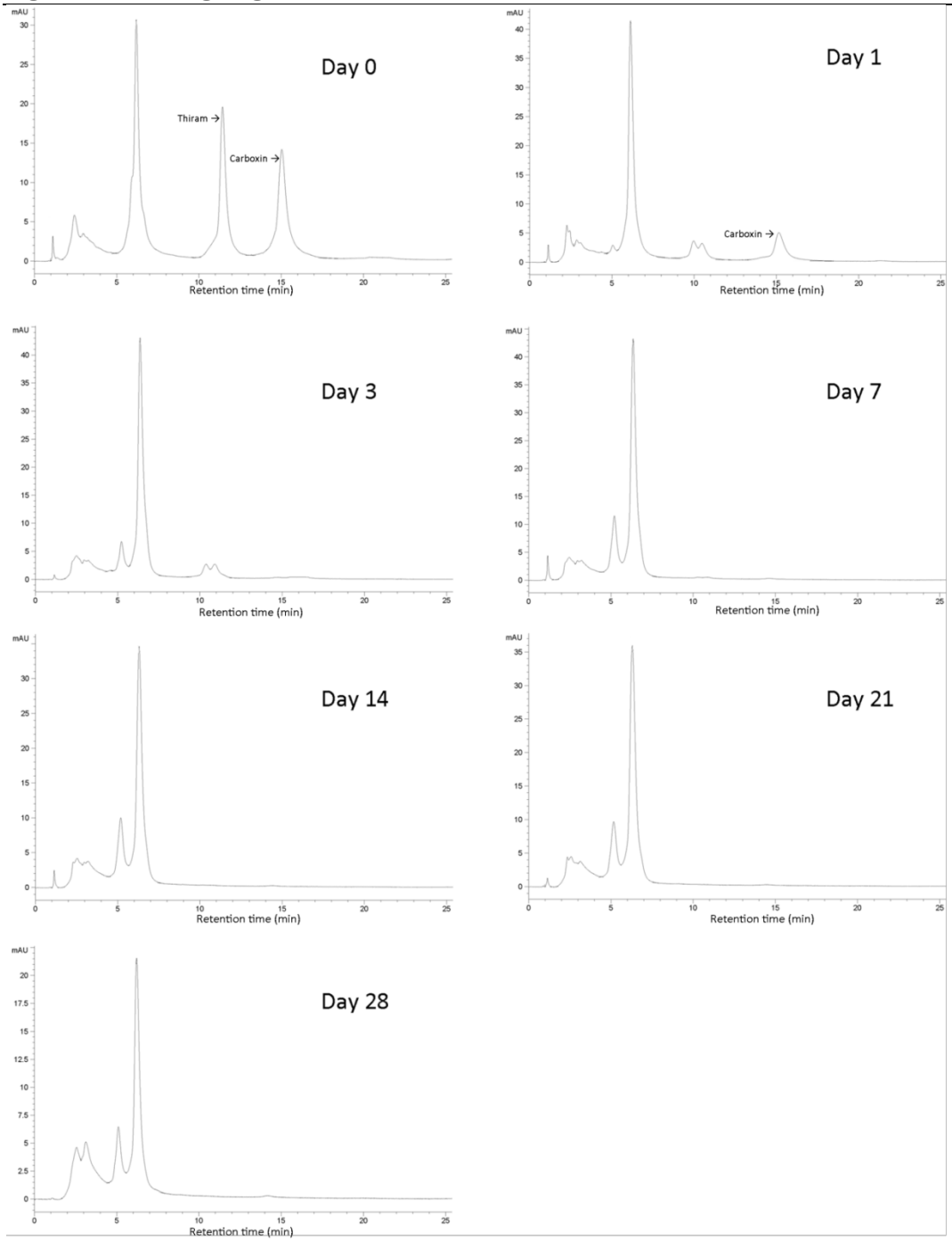


Figure 5-19. HPLC chromatograms of non-sterile soil spiked with 0.0000375% Vitavax 200FF (equivalent to 3mg/kg each of carboxin and thiram) over 28 days. Non-sterile soil was spiked 0.0000375% Vitavax 200FF and sampled over a 28 day period. Soil samples were extracted using acetonitrile followed by evaporation of the extract. The dried extract was dissolved in methanol and analysed using HPLC.The peaks corresponding to carboxin and thiram are indicated on the chromatograms.

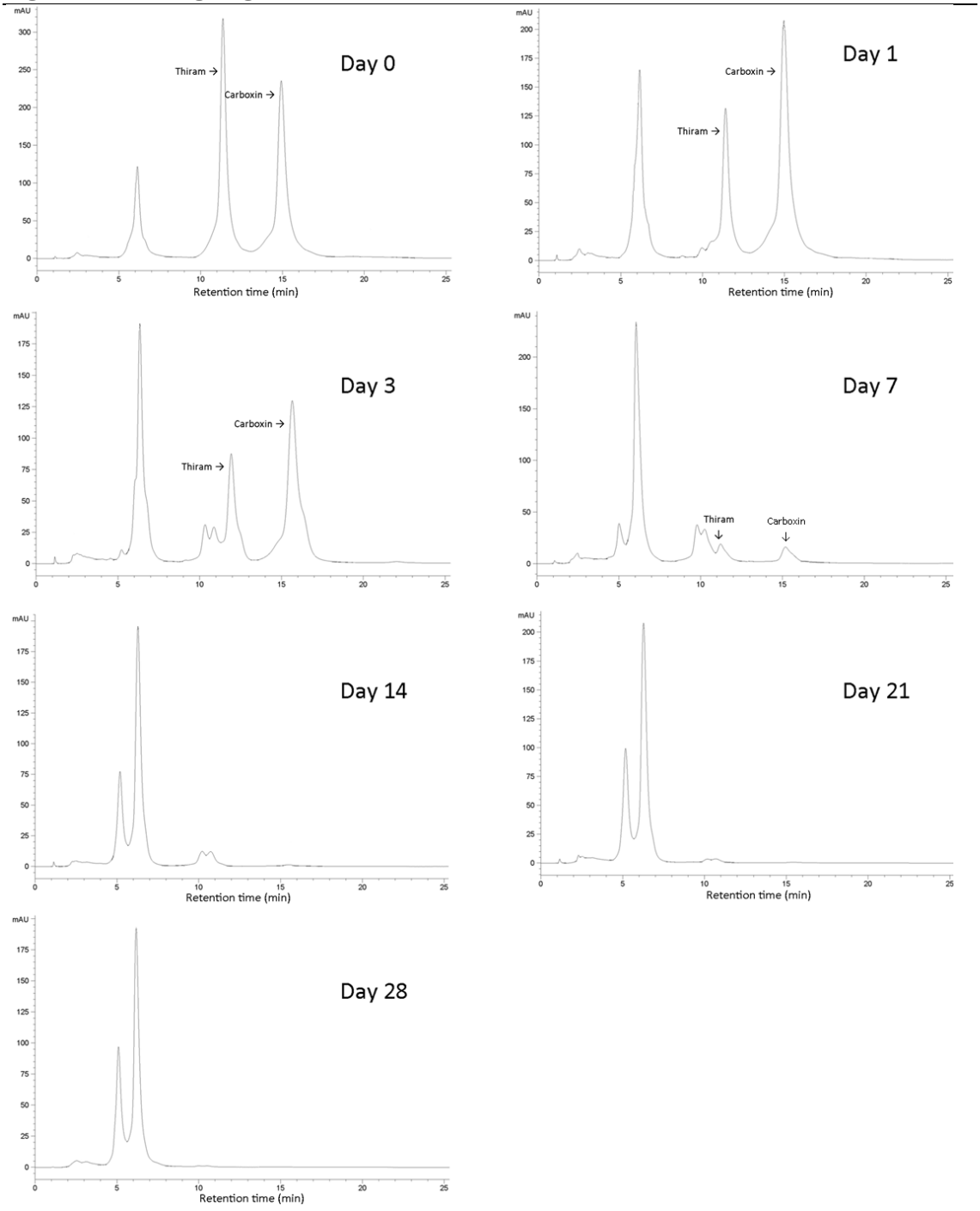


Figure 5-20. HPLC chromatograms of non-sterile soil spiked with 0.000375% Vitavax 200FF (equivalent to 30mg/kg each of carboxin and thiram) over 28 days. Non-sterile soil was spiked 0.000375% Vitavax 200FF and sampled over a 28 day period. Soil samples were extracted using acetonitrile followed by evaporation of the extract. The dried extract was dissolved in methanol and analysed using HPLC. The peaks corresponding to carboxin and thiram are indicated on the chromatograms.

Both soils spiked with Vitavax 200FF showed degradation of carboxin and thiram in the soil over time with thiram showing more rapid degradation compared to carboxin during the first 3 days (Figure 5-21). Degradation of carboxin and thiram in non-sterile soil spiked at the lower concentration of Vitavax 200FF (0.0000375%) occurred relatively quickly with no residual levels of either chemical detected after 3 days (Figure 5-21a).

Elimination of thiram from soil spiked with a higher concentration of Vitavax 200FF (0.000375%) took longer to achieve with no residual levels detected after 14 days (Figure 5-21b). A low level of carboxin was still detected in the soil at day 14 (0.6±1.0%), with no residual levels detected in the soil after day 21 (Figure 5-21b).

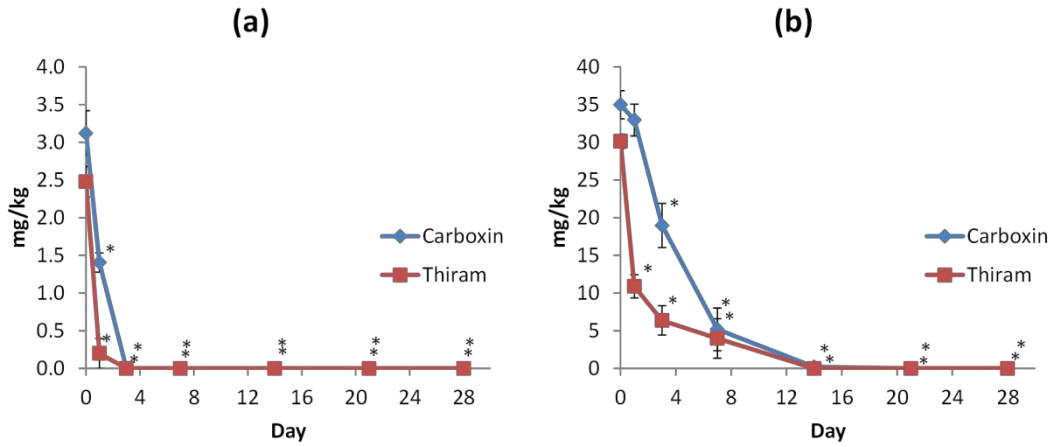


Figure 5-21. Degradation of carboxin and thiram in non-sterile soil spiked with (a) 0.0000375% Vitavax 200FF or (b) 0.000375% Vitavax200FF.

Non-sterile soil was spiked with Vitavax 200FF. Carboxin and thiram were extracted at the times shown for analysis by HPLC. Results are shown as the mean ± SEM of three separate experiments (n=3). Significant differences ($p \leq 0.05$) compared to day 0 are denoted as *.

5.3.2.2 Soil pH

The addition of chemicals to soil may change the pH of the soil and affect microbial growth (Rousk *et al.*, 2011, Fernández-Calviño *et al.*, 2016). In addition, metabolites produced by microbes may also change the pH of soil over time (Nazir *et al.*, 2010). As changes in soil pH have the potential to change the rate at which carboxin and thiram degrade (Gupta *et al.*, 2012a), the soil was monitored throughout the degradation period to determine the effects of the treatments on the pH of microbe containing soil (non-sterile soil).

No significant differences in soil pH ($p>0.05$) compared to day 0 of the same treatment were found (Figure 5-22). In addition, no significant differences ($p>0.05$) compared to the untreated control at the same day for each treatment were found (Figure 5-22).

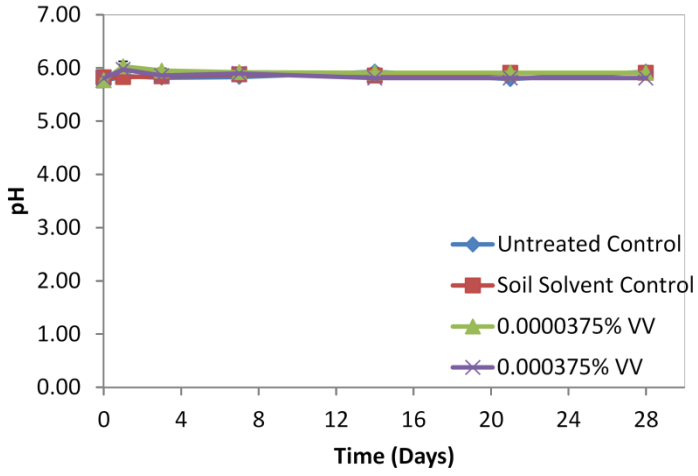


Figure 5-22. pH of non-sterile soil during incubation following spiking with Vitavax 200FF (VV).

Results are shown as the mean ± SEM of three separate experiments (n=3). No significant differences ($p>0.05$) compared to day 0 of the same treatment were found. For each treatment, no significant differences ($p>0.05$) compared to the untreated control at the same number of days following spiking were found.

5.3.2.3 Microbial Activity and Enumeration

Soils used in this study were monitored for changes in microbial activity that could potentially influence the cytotoxicity of the soil to HepG2, HaCaT and WIL2NS cells (Figure 5-23). For the untreated control, soil solvent control and soil spiked with the low dose of 0.0000375% Vitavax 200FF, no significant differences ($p>0.05$) in microbial activity compared to day 0 of the same treatment were found (Figure 5-23). In addition, the soil solvent control and soil spiked with the low dose of 0.0000375% Vitavax 200FF induced no significant differences ($p>0.05$) in microbial activity compared to the untreated control at the same day for each treatment (Figure 5-23).

In contrast, soil spiked with the high dose of 0.000375% Vitavax 200FF showed a significant reduction ($p\leq0.05$) of microbial activity, after 1 day to 242.9±26.9 µg/g compared to 346.5±13.3 µg/g at day 0 (Figure 5-23). Microbial activity remained similar to that seen after 1 day for the remainder of the degradation period (Figure 5-23). Microbial activity for soil spiked with the high dose of 0.000375% Vitavax 200FF after 1 day was also lower than that found in the untreated control after the same number of days following spiking, and reached significance ($p\leq0.05$) at days 3-28 (Figure 5-23).

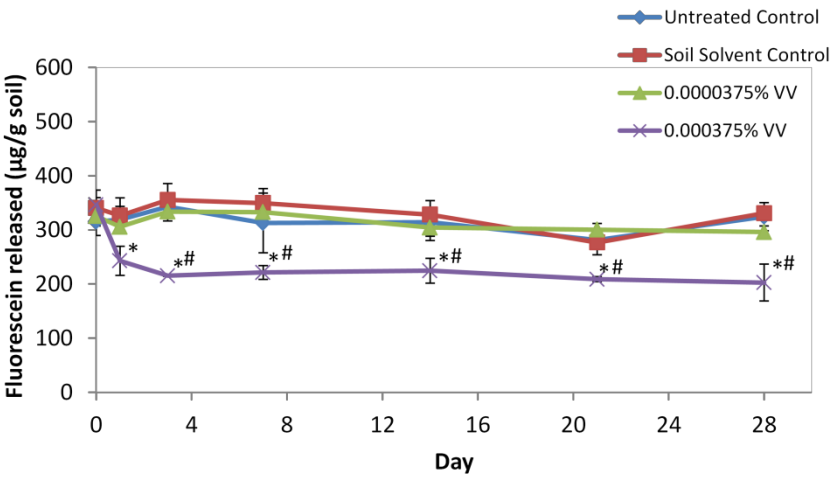


Figure 5-23. Total microbial activity of non-sterile soil as determined by the fluorescein diacetate (FDA) method following spiking with Vitavax 200FF (VV). Results are shown as the mean ± SEM of three separate experiments (n=3). Significant differences ($p \leq 0.05$) compared to day 0 of the same treatment are denoted as *. Significant differences ($p \leq 0.05$) compared to untreated soil at the same number of days following spiking are denoted as #.

Soils used in this study were also monitored for changes in the populations of bacteria, actinobacteria and fungi over the course of the degradation period (Figure 5-24). All soils showed a significant increase ($p \leq 0.05$) in the population of bacteria after days 1 and 3 compared to the population at day 0 (Figure 5-24). After 7 days, the population of total bacteria in the Untreated Control, Soil Solvent Control and 0.0000375% Vitavax 200FF spiked soil decreased back down to levels similar to that found at day 0 and remained constant for the remaining degradation period (Figure 5-24). In contrast, the population of total bacteria in soil spiked with the higher dose of 0.000375% Vitavax 200FF increased further after 7 days to $2.06x10^{-7} \pm 1.45x10^{-6}$ cfu/g before showing a decreasing trend after 14 days to reach a final population of $6.48x10^{-6} \pm 1.02x10^{-6}$ cfu/g after 28 days (Figure 5-24). The population of bacteria in 0.000375% Vitavax 200FF soil was higher than that found for the untreated control at days 3-28 (Figure 5-24).

The change in the population of actinobacteria for each soil treatment showed a similar trend to that seen for total bacteria (Figure 5-24). Untreated control, soil solvent control and 0.000375% Vitavax 200FF showed a slight increase of actinobacteria in the soil after 1 day, before decreasing back down to levels similar to that found at day 0 at days 3-28 (Figure 5-24). A significant increase ($p \leq 0.05$) in the population of actinobacteria in soil spiked with the higher dose of 0.000375% Vitavax 200FF ($3.08x10^{-7} \pm 1.55x10^{-6}$ cfu/g) compared to day 0 ($1.26x10^{-7} \pm 2.65x10^{-6}$ cfu/g) was observed after 3 days, which was followed by a gradual decrease over time to $2.06x10^{-7} \pm 2.37x10^{-6}$ cfu/g after 28 days (Figure 5-24). The population of actinobacteria in 0.000375% Vitavax 200FF soil was higher than that found for the untreated control at days 3-28 (Figure 5-24).

The population of fungi in each soil treatment showed no significant change ($p > 0.05$) over time compared to day 0 (Figure 5-24). For soil solvent control, 0.0000375%

Vitavax 200FF and 0.000375% Vitavax 200FF soils, no significant differences ($p>0.05$) were found compared to untreated control at the same number of days following spiking (Figure 5-24), however it is noted that 0.000375% Vitavax 200FF soil had slightly lower levels of fungi compared to the other soil at days 3-28 (Figure 5-24).

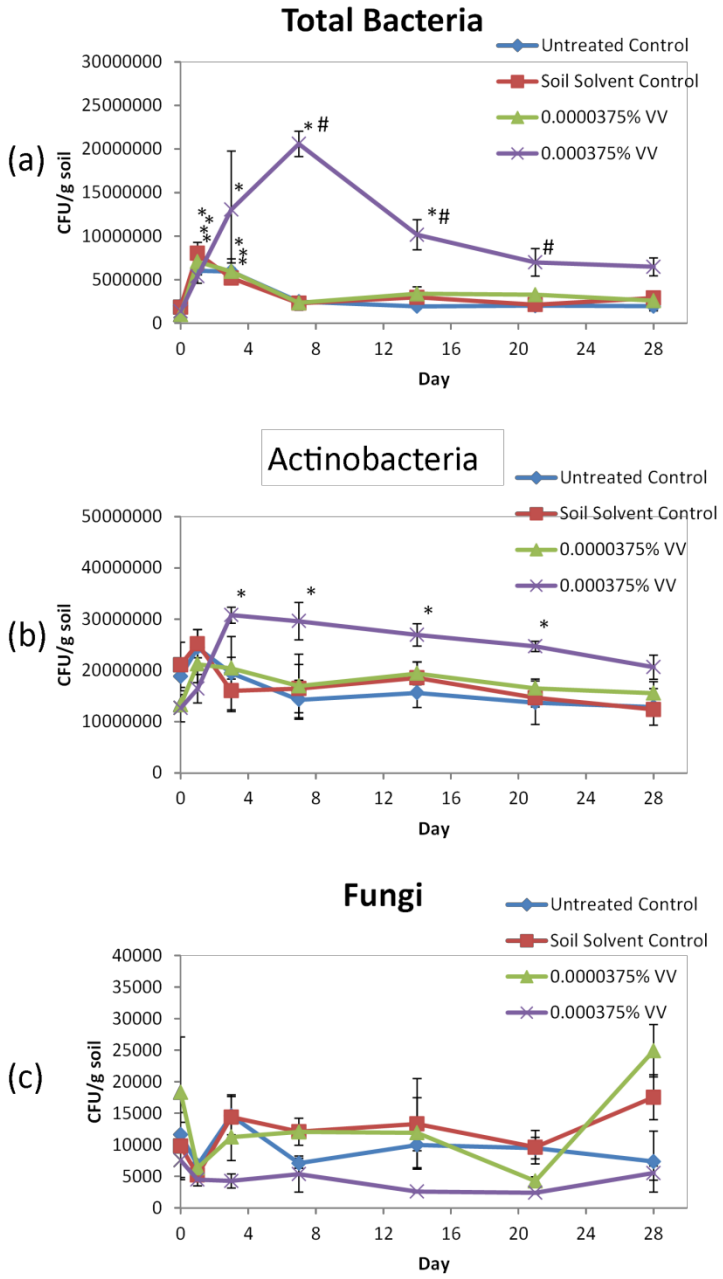


Figure 5-24. Enumeration of microorganisms in non-sterile soil following spiking with Vitavax 200FF (VV).

Populations of (a) total bacteria, (b) actinobacteria, or (c) fungi in soil were enumerated in non-sterile soil following spiking with Vitavax 200FF. Results are shown as the mean ± SEM of three separate experiments (n=3). Significant differences ($p \leq 0.05$) compared to day 0 of the same treatment are denoted as *. Significant differences ($p \leq 0.05$) compared to Untreated Control at the same number of days following spiking are denoted as #.

5.3.2.4 Effects of non-sterile soil controls on cell viability

Untreated non-sterile soil (Untreated Control) was monitored throughout the 28 day degradation period to determine any changes to its effects on cell viability (Figure 5-25). For all cell lines, no significant differences compared to the solvent control were found at any of the doses or exposure times tested (Figure 5-25).

The chemicals used in this study were dissolved in acetone prior to spiking sterile soil. Therefore, non-sterile soil was spiked with acetone only (Soil Solvent Control) and monitored over the 28 day degradation period to determine if the use of acetone during spiking of the soil affected cell viability (Figure 5-26). For all cell lines, no significant differences compared to the solvent control were found at any of the doses or exposure times tested (Figure 5-26).

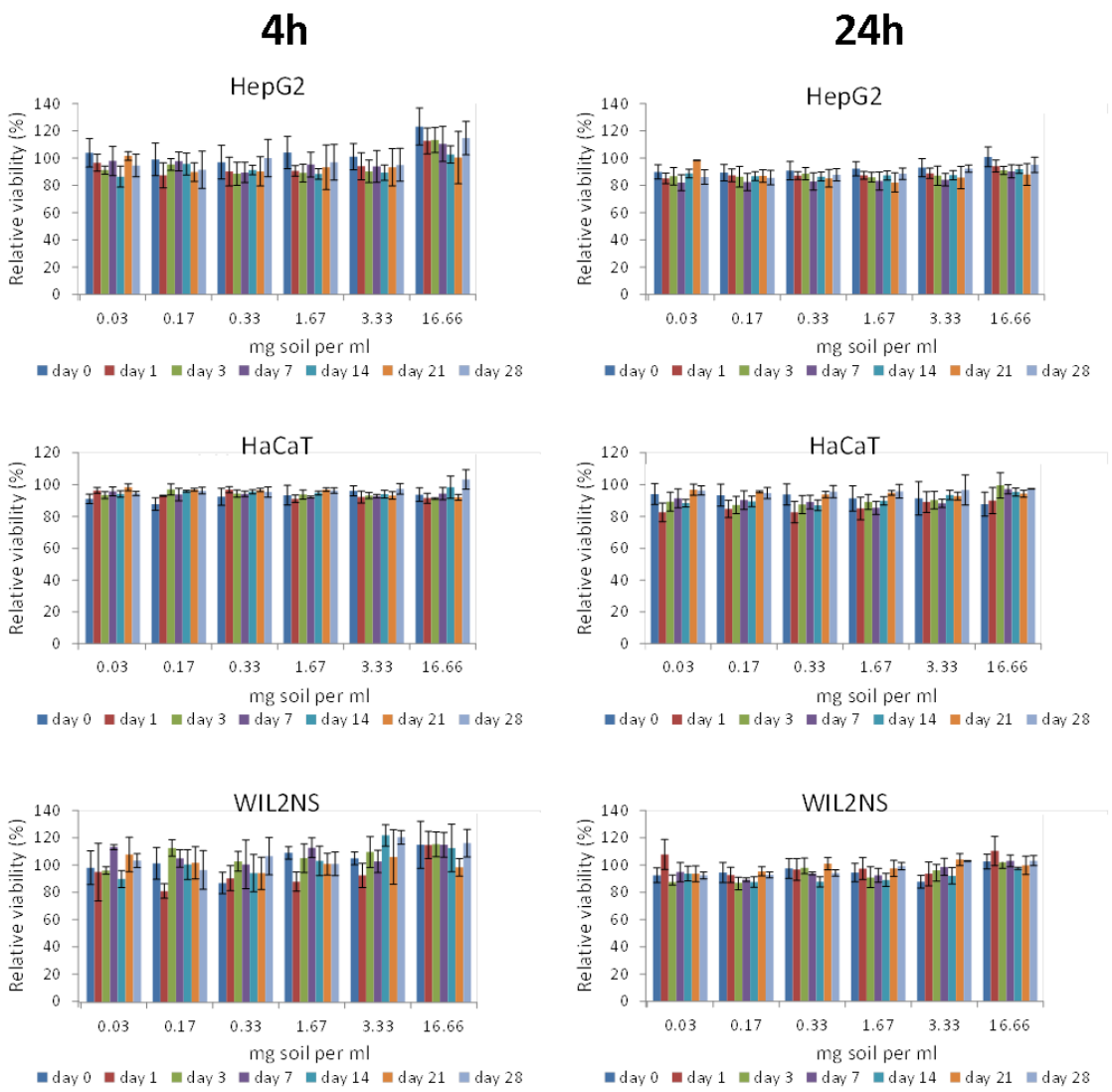


Figure 5-25 Effects of untreated non-sterile soil on cell viability of different cell lines after 4h or 24h incubation.

Non-sterile soil was left untreated and sampled over a 28 day period. Cells were exposed to the soil extracts for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). No significant differences ($p>0.05$) compared to the solvent control were observed in all cell lines and exposure times.

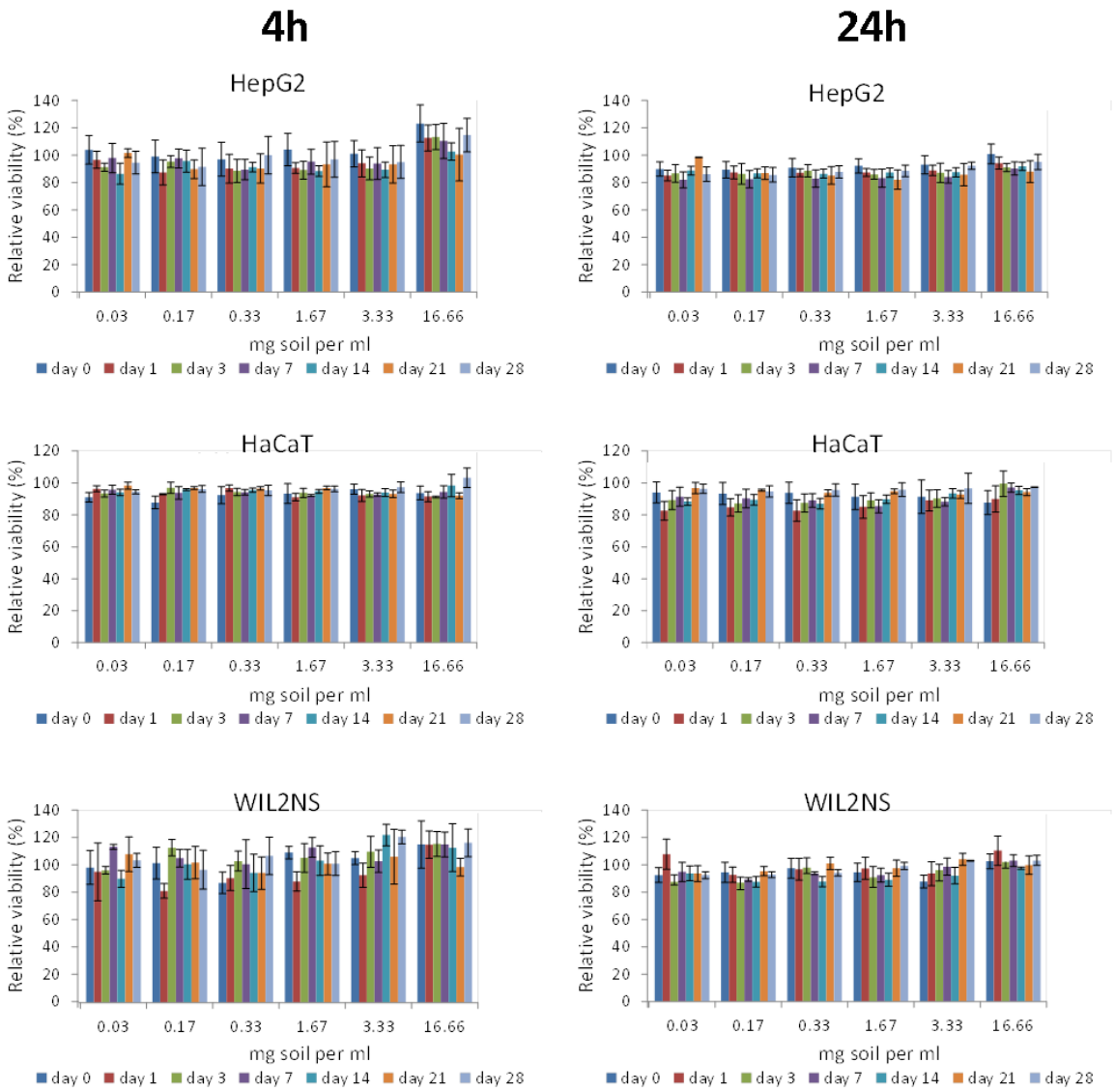


Figure 5-26 Effects of soil solvent control on cell viability of different cell lines after 4h or 24h incubation.

Non-sterile soil was spiked with acetone (soil solvent control) and sampled over a 28 day period. Cells were exposed to the soil extracts for 4h or 24h and cell viability was determined using the MTT assay Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). No significant differences ($p>0.05$) compared to the solvent control (0 mg soil per ml) were observed in all cell lines and exposure times.

5.3.2.5 Effects of degradation of Vitavax 200FF in non-sterile soil on cell viability

The cytotoxic effects non-sterile soil treated with a low dose of 0.0000375% Vitavax 200FF were determined in HepG2, HaCaT and WIL2NS cells (Figure 5-27). 4h or 24h exposure to the soil induced no significant changes to HepG2 or HaCaT cell viability at any of the doses tested throughout the 28 day degradation period (Figure 5-27). A significant decrease ($p \leq 0.05$) in cell viability was observed in WIL2NS cell after 24h exposure to 16.66 mg/ml of 0.0000375% Vitavax treated soil at day 0 (Figure 5-27).

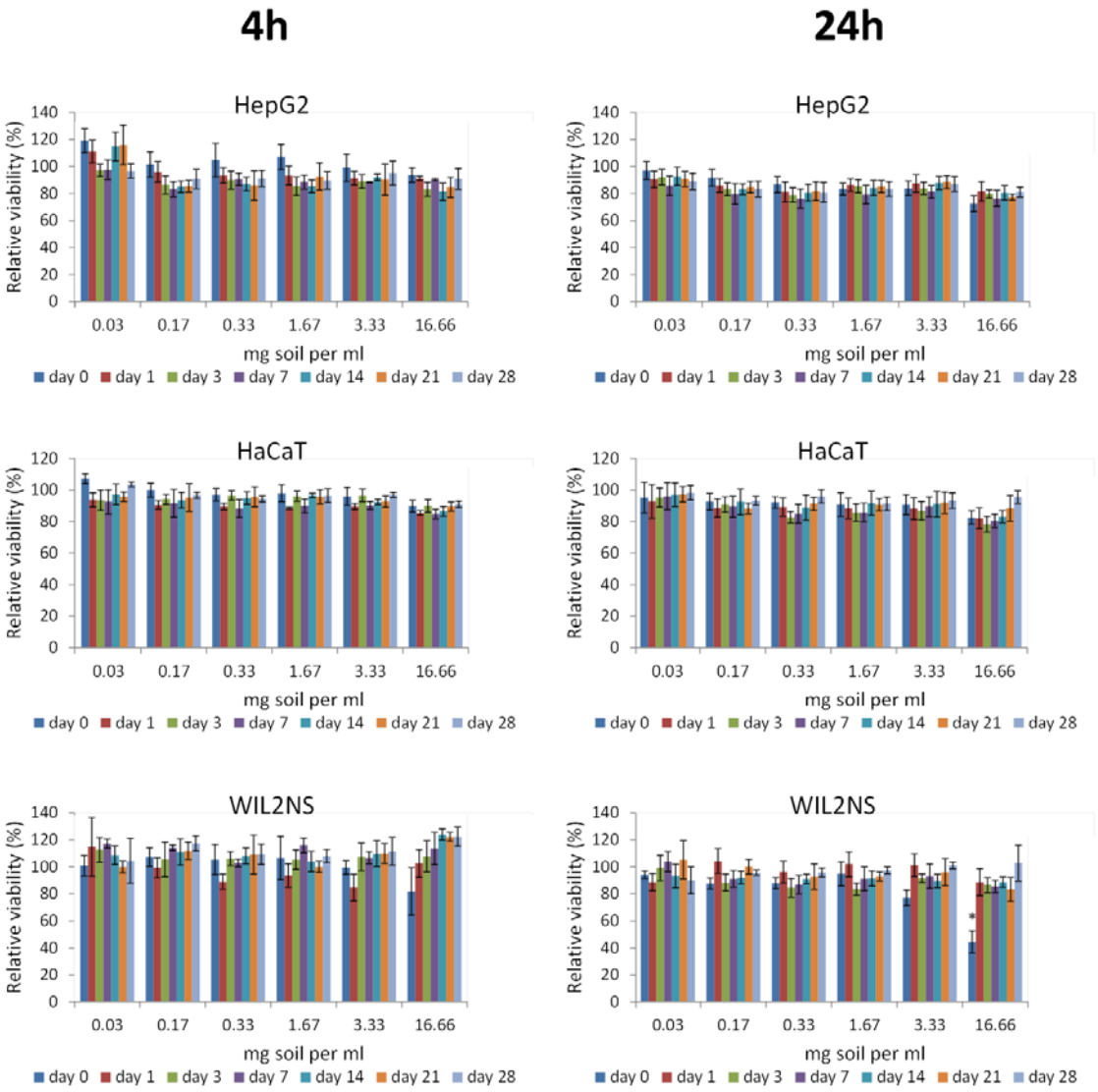


Figure 5-27 Effects of 0.0000375% Vitavax 200FF in non-sterile soil on cell viability of different cell lines after 4h or 24h incubation.

Non-sterile soil was spiked with Vitavax 200FF at 0.0000375% and sampled over a 28 day period. Cells were exposed to the soil extracts for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). No significant differences compared to the solvent control (0 mg soil per ml) were observed in all cell lines and exposure times. Significant differences ($p \leq 0.05$) compared to the solvent control (0 mg soil per ml) are shown as *

Non-sterile soil spiked with the higher dose of 0.000375% Vitavax 200FF induced time- and dose- dependent decreases in cell viability in all three cell lines tested (Figure 5-28). 24h exposure to 16.66 mg/ml of soil at days 0-14 induced significant ($p \leq 0.05$) decreases in cell viability in WIL2NS and HaCaT cells lines (Figure 5-28). Interestingly, a significant decrease in cell viability was still observed in HaCaT and WIL2NS cells after 24h exposure to the soil at day 14 (Figure 5-28), which contained only 0.36±0.21 mg/kg carboxin and no traces of thiram in the soil (Figure 5-20). A significant decrease in HepG2 cell viability was only observed after 24h exposure to day 0 soil at the highest dose of 16.66 mg/ml (Figure 5-28).

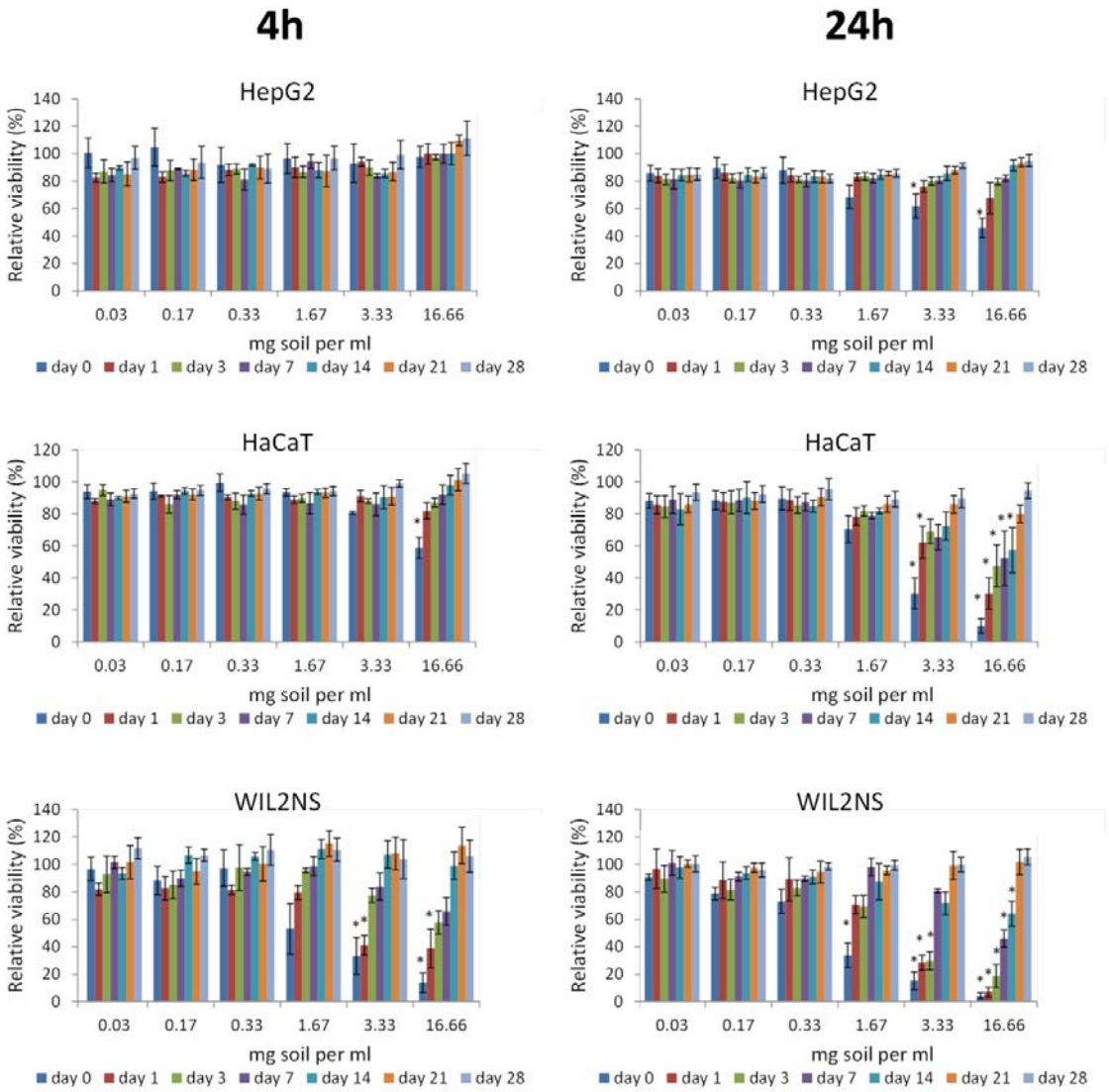


Figure 5-28 Effects of 0.000375% Vitavax 200FF in non-sterile soil on cell viability of different cell lines after 4h or 24h incubation.

Non-sterile soil was spiked with Vitavax 200FF at 0.0000375% and sampled over a 28 day period. Cells were exposed to the soil extracts for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). No significant differences compared to the solvent control (0 mg soil per ml) were observed in all cell lines and exposure times. Significant differences ($p \leq 0.05$) compared to the solvent control (0 mg soil per ml) are shown as *

The changes in the cytotoxicity of non-sterile spiked with Vitavax 200FF at either 0.0000375% or 0.000375% throughout the degradation period are shown in Figure 5-29. Soil spiked with the low dose of 0.0000375% Vitavax 200FF induced no significant changes in cell viability in HepG2 or HaCaT cells compared to Day 0 throughout the degradation period (Figure 5-29a and Figure 5-29b). In WIL2NS cells, allowing non-sterile soil spiked with 0.0000375% Vitavax 200FF to degrade for 1-28 days resulted in a significant increase in cell viability after 24h exposure compared to day 0 (Figure 5-29c).

Soil spiked with the higher dose of 0.000375% Vitavax 200FF generally showed an increase in cell viability in all cell lines the longer the soil was allowed to degrade (Figure 5-29). For HepG2 cells, the increase in cell viability was significantly higher compared to day 0 after 24h exposure to the soil that had been degraded for 7-28 days (Figure 5-29a). For HaCaT cells, the increase in cell viability was significantly higher when compared to day 0 after 4h exposure to soil that had been degraded for 3-28 days, or after 24h exposure to soil that had been degraded for 14-28 days (Figure 5-29b).

For WIL2NS cells the increase in cell viability was significantly higher when compared to day 0 after 4h exposure to soil that had been degraded for 14-28 days, or after 24h exposure to soil that had been degraded for 7-28 days (Figure 5-29c)

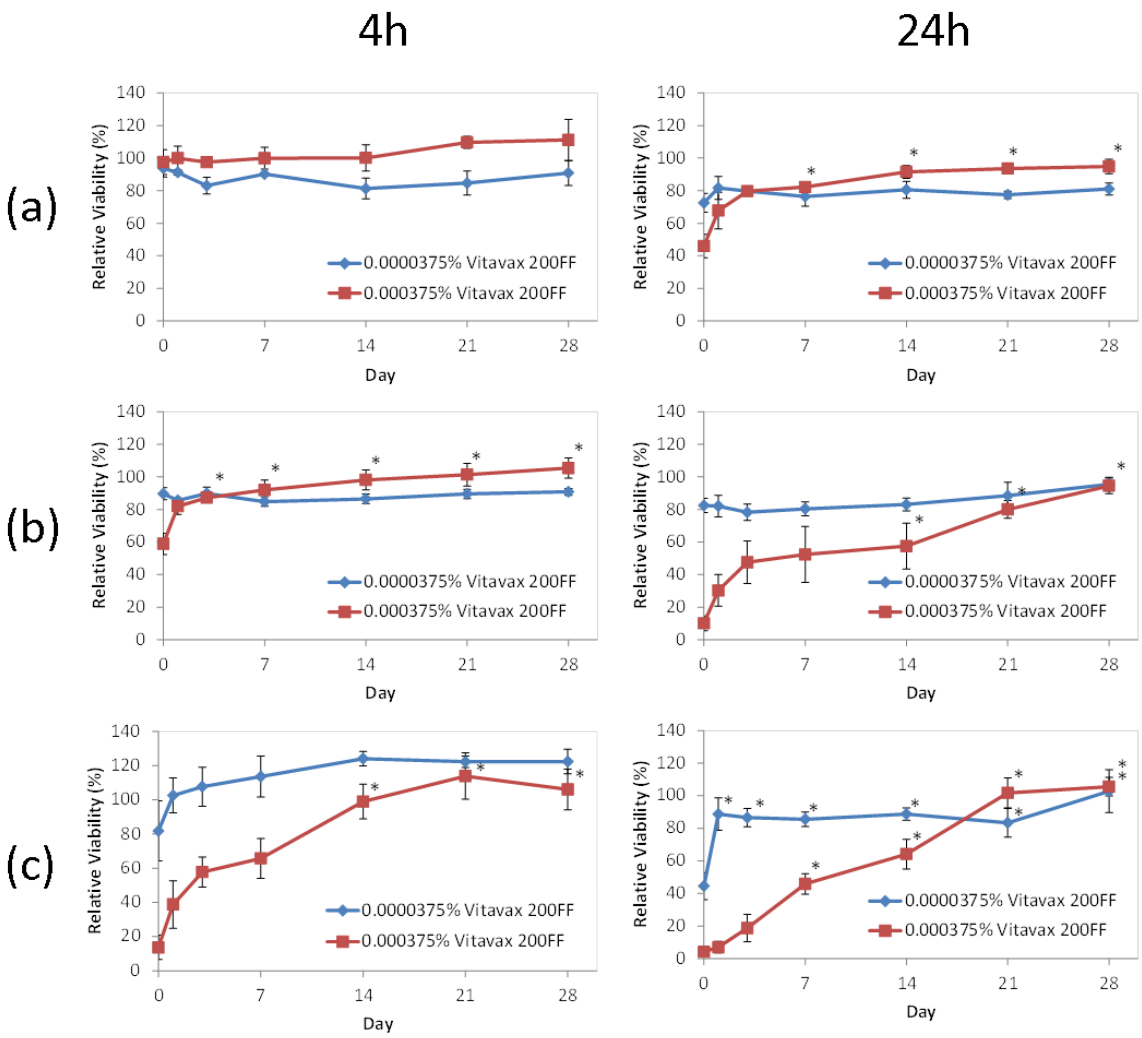


Figure 5-29 Changes in the toxicity of non-sterile soil spiked with Vitavax 200FF over 28 days to (a) HepG2, (b) HaCaT or (c) WIL2NS cells.

Cells were exposed to soil extracts at 16.66 mg/ml for 4h or 24h and cell viability was determined using the MTT assay. Results are expressed as % relative viability compared to the solvent control and are shown as the mean ± SEM of three separate experiments (n=3). Significant differences compared to Day 0 are denoted as *.

5.3.3 Correlation between thiram concentration and cytotoxicity

All soil doses from Figure 5-13, Figure 5-14, Figure 5-15, Figure 5-19 and Figure 5-20 were converted to equivalent thiram concentration to determine IC_{25} and IC_{50} values (Table 5-3, Table 5-4 and Table 5-5). The IC_{25} or IC_{50} values of each soil sample were then compared to the IC_{25} or IC_{50} values obtained for thiram when applied directly to cells as a single chemical using the extra sum of squares F test. This enabled the determination any correlation between thiram concentration and the cytotoxicity of the soil (Table 5-3, Table 5-4 and Table 5-5).

In HepG2 cells, no significant differences ($p>0.05$) were found between the IC_{25} and IC_{50} values of soil samples when compared to the IC_{25} and IC_{50} values of thiram as a single chemical (Table 5-3). In HaCaT cells, 24h exposure to sterile soil spiked with Vitavax 200FF after 3 days of degradation resulted in a significantly lower ($p\leq0.05$) IC_{25} (0.28 mg/L) compared to the IC_{25} of thiram as a single chemical (0.53 mg/L, Table 5-4). All other soil treatments in HaCaT cells induced no significant differences in IC_{25} and IC_{50} values when compared to the IC_{25} and IC_{50} values of thiram as a single chemical (Table 5-4).

In WIL2NS cells, 24h exposure to sterile soil spiked with carboxin and thiram combined after 7 days of degradation, sterile soil spiked with Vitavax 200FF after 3 days of degradation, or non-sterile soil spiked with 0.000375% Vitavax 200FF after 3 or 7 days of degradation resulted in significantly lower ($p\leq0.05$) IC_{25} values (0.006, 0.005, 0.004 and 0.007 mg/L) compared to the IC_{25} of thiram as a single chemical (0.014 mg/L, Table 5-5). All other soil treatments in WIL2NS cells induced no significant differences in IC_{25} and IC_{50} values when compared to the IC_{25} and IC_{50} values of thiram as a single chemical (Table 5-5).

Table 5-3. IC_{25} and IC_{50} values of thiram as a single chemical or in different soil treatments in HepG2 cells.

ND=could not be determined. No significant differences in IC_{25} or IC_{50} values were found between the soil treatments when compared to thiram as a single chemical ($p>0.05$ by extra sum of squares F test).

HepG2					
Treatment	Degradation period (days)	4h		24h	
		IC_{25} (mg/L)	IC_{50} (mg/L)	IC_{25} (mg/L)	IC_{50} (mg/L)
Thiram	N/A	1.7 (0.82 to 3.43)	8.9 (5.14 to 15.39)	0.068 (0.031 to 0.15)	0.390 (0.23 to 0.67)
Sterile soil - Thiram	0	ND	ND	0.033 (0.014 to 0.078)	0.290 (0.17 to 0.49)
	1	ND	ND	0.029 (0.016 to 0.053)	0.260 (0.16 to 0.42)
	3	ND	ND	0.025 (0.014 to 0.044)	0.270 (0.16 to 0.43)
	7	ND	ND	0.068 (0.040 to 0.12)	0.510 (0.29 to 0.91)
	14	ND	ND	0.052 (0.033 to 0.082)	0.420 (0.23 to 0.75)
	21	ND	ND	0.072 (0.051 to 0.10)	0.640 (0.29 to 1.4)
	28	ND	ND	ND	ND
Sterile soil - Carboxin and Thiram	0	ND	ND	0.044 (0.024 to 0.082)	0.320 (0.20 to 0.53)
	1	ND	ND	0.076 (0.042 to 0.14)	0.470 (0.27 to 0.81)
	3	ND	ND	0.051 (0.030 to 0.084)	0.340 (0.20 to 0.58)
	7	ND	ND	0.043 (0.026 to 0.071)	0.330 (0.19 to 0.56)
	14	ND	ND	0.068 (0.041 to 0.11)	0.480 (0.25 to 0.90)
	21	ND	ND	0.081 (0.047 to 0.14)	0.650 (0.31 to 1.4)
	28	ND	ND	ND	ND
Sterile soil - Vitavax 200FF	0	ND	ND	0.050 (0.027 to 0.094)	0.360 (0.21 to 0.60)
	1	ND	ND	0.053 (0.028 to 0.10)	0.420 (0.22 to 0.78)
	3	ND	ND	0.060 (0.039 to 0.090)	ND
	7	ND	ND	0.038 (0.022 to 0.065)	ND
	14	ND	ND	0.062 (0.040 to 0.096)	ND
	21	ND	ND	ND	ND
	28	ND	ND	ND	ND
Non-sterile soil - 0.0000375% Vitavax 200FF	0	ND	ND	ND	ND
	1	ND	ND	ND	ND
	3	ND	ND	ND	ND
	7	ND	ND	ND	ND
	14	ND	ND	ND	ND
	21	ND	ND	ND	ND
	28	ND	ND	ND	ND
Non-sterile soil - 0.000375% Vitavax 200FF	0	ND	ND	0.029 (0.011 to 0.074)	0.270 (0.17 to 0.44)
	1	ND	ND	ND	ND
	3	ND	ND	ND	ND
	7	ND	ND	ND	ND
	14	ND	ND	ND	ND
	21	ND	ND	ND	ND
	28	ND	ND	ND	ND

Table 5-4. IC_{25} and IC_{50} values of thiram as a single chemical or in different soil treatments in HaCaT cells.

ND=could not be determined. * Indicates a significant difference in IC25 or IC50 values when compared to thiram as a single chemical (p≤0.05 by extra sum of squares F test).

HaCaT					
Treatment	Degradation period (days)	4h		24h	
		IC_{25} (mg/L)	IC_{50} (mg/L)	IC_{25} (mg/L)	IC_{50} (mg/L)
Thiram	-	0.25 (0.11 to 0.59)	2.4 (1.3 to 4.4)	0.053 (0.045 to 0.064)	0.089 (0.077 to 0.10)
Sterile soil - Thiram	0	0.23 (0.10 to 0.49)	0.69 (0.32 to 1.5)	0.031 (0.015 to 0.065)	0.087 (0.052 to 0.15)
	1	0.17 (0.0037 to 7.3)	1.10 (0.17 to 6.4)	0.033 (0.019 to 0.057)	0.084 (0.058 to 0.12)
	3	ND	ND	0.035 (0.017 to 0.069)	0.089 (0.054 to 0.15)
	7	ND	ND	0.038 (0.022 to 0.067)	0.1 (0.065 to 0.16)
	14	ND	ND	0.046 (0.032 to 0.067)	0.11 (0.077 to 0.15)
	21	ND	ND	0.05 (0.029 to 0.085)	0.1 (0.072 to 0.15)
	28	ND	ND	ND	ND
Sterile soil - Carboxin and Thiram	0	0.21 (0.11 to 0.43)	0.69 (0.29 to 1.6)	0.05 (0.035 to 0.069)	0.085 (0.069 to 0.11)
	1	0.14 (0.065 to 0.32)	0.51 (0.20 to 1.3)	0.041 (0.028 to 0.061)	0.075 (0.058 to 0.098)
	3	ND	ND	0.051 (0.036 to 0.073)	0.095 (0.067 to 0.14)
	7	ND	ND	0.051 (0.037 to 0.069)	0.1 (0.078 to 0.14)
	14	ND	ND	0.058 (0.043 to 0.078)	0.11 (0.086 to 0.14)
	21	ND	ND	0.07 (0.040 to 0.12)	0.12 (0.085 to 0.16)
	28	ND	ND	ND	ND)
Sterile soil - Vitavax 200FF	0	0.14 (0.058 to 0.35)	0.95 (0.20 to 4.45)	0.035 (0.018 to 0.068)	0.099 (0.060 to 0.16)
	1	ND	ND	0.036 (0.024 to 0.056)	0.081 (0.056 to 0.12)
	3	ND	ND	*0.028 (0.013 to 0.061)	0.082 (0.041 to 0.16)
	7	ND	ND	0.036 (0.019 to 0.068)	0.14 (0.072 to 0.28)
	14	ND	ND	0.041 (0.025 to 0.068)	0.094 (0.064 to 0.14)
	21	ND	ND	0.04 (0.022 to 0.074)	0.072 (0.027 to 0.19)
	28	ND	ND	ND	ND
Non-sterile soil - 0.0000375% Vitavax 200FF	0	ND	ND	ND	ND
	1	ND	ND	ND	ND
	3	ND	ND	ND	ND
	7	ND	ND	ND	ND
	14	ND	ND	ND	ND
	21	ND	ND	ND	ND
	28	ND	ND	ND	ND
Non-sterile soil - 0.000375% Vitavax 200FF	0	0.19 (0.13 to 0.30)	0.78 (0.42 to 1.4)	0.029 (0.016 to 0.052)	0.07 (0.048 to 0.10)
	1	ND	ND	0.017 (0.0086 to 0.032)	0.069 (0.040 to 0.12)
	3	ND	ND	ND	ND
	7	ND	ND	ND	ND
	14	ND	ND	ND	ND
	21	ND	ND	ND	ND
	28	ND	ND	ND	ND

Table 5-5. IC_{25} and IC_{50} values of thiram as a single chemical or in different soil treatments in WIL2NS cells.

ND=could not be determined. * Indicates a significant difference in IC25 or IC50 values when compared to thiram as a single chemical (p≤0.05 by extra sum of squares F test).

WIL2NS					
		4h		24h	
Treatment	Degradation period (days)	IC_{25} (mg/L)	IC_{50} (mg/L)	IC_{25} (mg/L)	IC_{50} (mg/L)
Thiram	-	0.052 (0.034 to 0.077)	0.11 (0.079 to 0.17)	0.014 (0.009 to 0.023)	0.026 (0.018 to 0.037)
Sterile soil - Thiram	0	0.023 (0.012 to 0.043)	0.071 (0.045 to 0.11)	0.008 (0.005 to 0.014)	0.017 (0.012 to 0.023)
	1	0.037 (0.025 to 0.055)	0.082 (0.057 to 0.12)	0.009 (0.007 to 0.012)	0.021 (0.017 to 0.026)
	3	0.062 (0.049 to 0.078)	0.13 (0.089 to 0.20)	0.008 (0.006 to 0.012)	0.021 (0.016 to 0.028)
	7	0.053 (0.037 to 0.075)	0.14 (0.090 to 0.21)	0.010 (0.008 to 0.014)	0.029 (0.023 to 0.035)
	14	0.041 (0.018 to 0.093)	0.15 (0.095 to 0.23)	0.012 (0.006 to 0.021)	0.034 (0.023 to 0.051)
	21	0.04 (0.024 to 0.067)	0.11 (0.066 to 0.17)	0.009 (0.005 to 0.017)	0.034 (0.021 to 0.056)
	28			ND	ND
Sterile soil - Carboxin and Thiram	0	0.055 (0.040 to 0.076)	0.11 (0.077 to 0.16)	0.010 (0.007 to 0.014)	0.019 (0.015 to 0.025)
	1	0.066 (0.048 to 0.092)	0.13 (0.089 to 0.20)	0.009 (0.006 to 0.013)	0.02 (0.015 to 0.028)
	3	0.045 (0.031 to 0.066)	0.098 (0.066 to 0.14)	0.011 (0.006 to 0.022)	0.029 (0.018 to 0.045)
	7	0.055 (0.036 to 0.082)	0.12 (0.077 to 0.18)	0.009 (0.005 to 0.016)	0.027 (0.018 to 0.040)
	14	0.038 (0.020 to 0.073)	0.1 (0.066 to 0.16)	0.008 (0.005 to 0.013)	0.026 (0.018 to 0.037)
	21	0.059 (0.032 to 0.11)	0.12 (0.077 to 0.19)	*0.006 (0.003 to 0.012)	0.026 (0.017 to 0.042)
	28			ND	ND
Sterile soil - Vitavax 200FF	0	0.054 (0.039 to 0.074)	0.1 (0.070 to 0.15)	0.009 (0.006 to 0.014)	0.021 (0.015 to 0.028)
	1	0.066 (0.038 to 0.12)	0.13 (0.083 to 0.20)	0.008 (0.005 to 0.013)	0.017 (0.013 to 0.023)
	3	ND	ND	*0.005 (0.001 to 0.009)	0.019 (0.011 to 0.033)
	7	ND	ND	0.008 (0.004 to 0.014)	0.028 (0.019 to 0.042)
	14	ND	ND	0.008 (0.004 to 0.014)	0.026 (0.017 to 0.040)
	21	ND	ND	0.007 (0.004 to 0.014)	0.03 (0.015 to 0.059)
	28	ND	ND	0.009 (0.005 to 0.016)	ND
Non-sterile soil - 0.0000375% Vitavax 200FF	0	ND	ND	0.01 (0.006 to 0.020)	0.033 (0.018 to 0.061)
	1	ND	ND	ND	ND
	3	ND	ND	ND	ND
	7	ND	ND	ND	ND
	14	ND	ND	ND	ND
	21	ND	ND	ND	ND
	28	ND	ND	ND	ND
Non-sterile soil - 0.000375% Vitavax 200FF	0	0.026 (0.011 to 0.064)	0.07 (0.039 to 0.13)	0.008 (0.005 to 0.013)	0.023 (0.017 to 0.032)
	1	ND	ND	0.013 (0.007 to 0.025)	0.025 (0.017 to 0.036)
	3	ND	ND	*0.004 (0.002 to 0.008)	0.014 (0.008 to 0.025)
	7	ND	ND	*0.007 (0.004 to 0.012)	0.027 (0.018 to 0.040)
	14	ND	ND	ND	ND
	21	ND	ND	ND	ND
	28	ND	ND	ND	ND

5.4 Discussion

5.4.1 Abiotic degradation of carboxin and thiram individually, in combination, or in the product formulation Vitavax 200FF

In this study, abiotic degradation of Vitavax 200FF and its active ingredients, carboxin and thiram, in sterile soil was monitored over a 28 day period in the absence of light. The presence of thiram in the soil appeared to stabilize carboxin and inhibit its degradation in comparison to sterile soil spiked with carboxin alone. Degradation of carboxin in sterile soil was slowest when spiked as the product formulation Vitavax 200FF. Degradation of carboxin has been shown to be affected by soil pH (Chin *et al.*, 1970). However, the addition of carboxin and thiram or Vitavax 200FF to sterile soil did not affect soil pH and therefore was not a contributing factor to the difference in carboxin degradation seen in the different sterile soil treatments.

The decreased degradation rate of carboxin may indicate that thiram or its degradation products interacted with carboxin to slow its degradation in soil. Alternatively, the decreased degradation rate of carboxin in sterile soil spiked with Vitavax 200FF may also indicate further stabilization of carboxin by one or a combination of the undisclosed proprietary ingredients in the product formulation.

The addition of stabilizers to product formulations is common practice in the chemical industry (Cox et al. 2006; Surgan et al. 2010). Therefore it is highly likely that Vitavax 200FF contains other chemicals intended to inhibit degradation of carboxin or thiram as part of the product formulation. However, as the other ingredients in Vitavax 200FF are not disclosed on the label the presence of stabilizers in the formulation cannot be confirmed. Nevertheless, this study shows that the persistence of carboxin in soil is enhanced when present as the product formulation Vitavax 200FF. Increased persistence of carboxin in soil would enable a longer period of protection of seeds from

pathogenic fungi once they were sown in the soil and would therefore be beneficial for improving seedling health. The impact of the increased persistence of carboxin in soil on its toxicity in human cells is discussed later in this chapter.

Interestingly, thiram showed an accelerated rate of degradation in sterile soil when present as part of the product formulation Vitavax 200FF. Similarly to carboxin, the degradation of thiram has been shown to be affected by soil pH (Sharma *et al.*, 2003, Haque *et al.*, 2005, Gupta *et al.*, 2012a). No differences in soil pH were seen between the treatments in sterile soil in this study, and were therefore not a contributing factor.

The presence of carboxin in sterile soil did not appear to affect thiram degradation, indicating that the rapid degradation of thiram was due to other undisclosed ingredients in the product formulation. This study would indicate that Vitavax 200FF would reduce the period of protection from pathogenic fungi afforded by thiram to seeds in soil. The impact of the decreased persistence of thiram in soil on its toxicity in human cells is discussed later in this Chapter.

By using sterile soil in this study we have shown that both carboxin and thiram can be degraded by abiotic catalytic processes in soil. Previous studies have shown that degradation of carboxin or thiram in water does not occur in the absence of light (DellaGreca *et al.*, 2004, Filipe *et al.*, 2013). This indicates that hydrolysis of carboxin or thiram in a water only matrix requires light to catalyze the reaction. The soil samples in the present study were incubated in the dark. Therefore the abiotic degradation of carboxin and thiram was unlikely to be caused by the water in the soil alone. Possible causes of degradation of carboxin and thiram in this study include heat or interactions with other compounds or elements in the soil. The soil was kept at a constant temperature of 27°C during the degradation period and therefore could have caused some thermal degradation of carboxin and thiram.

The degradation of carboxin in sterile soil seen in this study is consistent with a study which found that abiotic degradation of carboxin in soil could occur in the absence of light and was reported to be due to its hydrolysis or oxidation to sulfoxide or enol respectively (Isidori *et al.*, 2012). For thiram, previous studies have looked at abiotic degradation of the chemical in water in the absence of light (Gupta *et al.*, 2012a). However, this is the first study to show that abiotic degradation of thiram in soil can occur in the absence of light.

The HPLC method used in this study only allowed for identification and quantification of the parent compounds, carboxin and thiram. Identification and quantification of the unknown degradation products would have required the use of an additional method such as nuclear magnetic resonance (NMR) as described in previous studies (Hustert *et al.*, 1999, DellaGreca *et al.*, 2004, Isidori *et al.*, 2012). The aim of our study was to compare the toxicity of soil in relation to the concentration of carboxin and thiram in the soil. Therefore, the HPLC method used in this study was deemed suitable for this purpose. Future studies which also identify and quantify the degradation products of carboxin and thiram generated in the soil would be useful to determine which abiotic catalytic processes are involved.

5.4.2 Degradation of Vitavax 200FF in non-sterile soil and its effects on soil microorganisms

Degradation of both carboxin and thiram in non-sterile soil occurred more rapidly compared to sterile soil. The pH of both sterile and non-sterile soils were found to be similar and were not a contributing factor to the differences in degradation of carboxin and thiram in the soil.

The lowest dose of Vitavax 200FF used in non-sterile soil in this study (0.0000375%) is ten times higher than the estimated environmental concentration due to sowing seeds coated with the product (Table 5-1) and would represent a small spill of treated seed. As the lowest dose of Vitavax 200FF in non-sterile soil had no effect on microbial activity or populations, the practice of sowing treated seed into soil or a small spill of treated seed is unlikely to have any detrimental effect on soil health.

The higher dose of Vitavax 200FF used in non-sterile soil in this study (0.000375%) is two orders of magnitude higher than the estimated environmental concentration for sowing seeds coated with the product (Table 5-1) and would better represent a spill of the undiluted product formulation. In this study, we show that contamination of soil with high levels of Vitavax 200FF can alter the population of microorganisms in the soil.

Differences in the degradation of carboxin and thiram in sterile and non-sterile soil was likely due to the participation of microbes. The increased degradation of carboxin and thiram seen in this study, coupled with the increase in the number of total bacteria and actinobacteria in non-sterile soil treated with the highest dose of Vitavax 200FF (0.000375%) could indicate utilization of carboxin and thiram by soil microorganisms. Alternatively, degradation of carboxin and thiram may be due to the microbial by-products promoting abiotic degradation of the compounds.

Total bacteria and actinobacteria populations were highest after 7 days and 3 days respectively which correlated with carboxin and thiram still being present in the soil. Therefore, it would appear that some bacteria and actinobacteria in particular were able to utilize the carboxin and thiram present in the soil to enhance their growth. Various soil microbes have been shown to be able to utilize a range of different environmental pollutants as carbon and nitrogen sources (Topp, 2001, Snellinx *et al.*, 2002, Bellinaso *et al.*, 2003, Radwan *et al.*, 2007, Singh *et al.*, 2011, Silambarasan *et*

al., 2012). Therefore, microbes capable of mineralizing carboxin and thiram to utilize the carbon and nitrogen from the chemicals may have been present in the soil used in the current study.

The gradual decrease in total bacteria and actinobacteria after 14 days correlated with a disappearance of carboxin and thiram from the soil. This could indicate that the inhibition of growth was due to the carboxin and thiram no longer being present in the soil, and therefore the microbes no longer being able to use the chemicals as a nutrient source.

A previous study found that exposure of soil to thiram at 22.8 mg/kg or 45.7 mg/kg resulted in an initial increase of the population of actinobacteria in the soil compared to the untreated control after 4 weeks, followed by a decrease of the population after 8 weeks (Sherif *et al.*, 2011). In the study, the decrease of the number of actinobacteria after 8 weeks correlated with the disappearance of thiram from the soil (Sherif *et al.*, 2011) and is similar to that seen in this study.

It is noted that although the highest dose of Vitavax 200FF (0.000375%) in non-sterile soil induced significant increases in the populations of bacteria and actinobacteria in soil, total microbial activity was significantly lower. In this study, total microbial activity was measured by the hydrolysis of fluorescein diacetate by microbes in the soil. Fluorescein diacetate is hydrolyzed by a wide range of soil microorganisms including bacteria and fungi (Schnürer *et al.*, 1982, Stubberfield *et al.*, 1990), as well as algae (Akram *et al.*, 2015) and protozoa (Schnürer *et al.*, 1985). Therefore, the reduction in total microbial activity was likely due to the toxicity of Vitavax 200FF to soil microorganisms that did not grow well on the agar media used for enumeration in this study.

The labelled purpose of Vitavax 200FF is to coat cereal seeds in order to protect seeds from pathogenic fungi (Arysta LifeScience Australia, 2016). However, we show in this study that the product was able to increase the population of bacteria including actinobacteria and potentially decrease the population of other non-target soil microorganisms. Both carboxin and thiram have been shown to be toxic to soil bacteria at 100mg/L and 4.4mg/L respectively (Milenkovski *et al.*, 2010). Another study found that that thiram was toxic to soil protozoa at 0.5mg/L (Dive *et al.*, 1984). It is possible that the increase in bacteria, including actinomycetes, seen in this study was partly due to Vitavax 200FF killing off other microorganisms that compete with the surviving bacteria for nutrients however, further study is required to confirm this hypothesis.

It is noted that the decrease in total microbial activity was associated with no significant change in fungal populations in soil treated with Vitavax 200FF and may indicate a higher toxicity to non-target soil microorganisms compared to the target pathogenic fungi. Studies have shown a correlation between soil microbe populations and plant productivity. This may be due to the presence of particular microbes in soil enabling increased nutrient uptake and growth (Sabir *et al.*, 2012, Zafar *et al.*, 2012, de Souza *et al.*, 2013, Liu *et al.*, 2013a), increased resistance to pathogens (Dutta *et al.*, 2008, Kumar *et al.*, 2012, Senthilraja *et al.*, 2013), or increased resistance to biotic and abiotic stress (Choudhary, 2012, Praveen Kumar *et al.*, 2014, Kakar *et al.*, 2016). In this study we demonstrated that Vitavax200FF was able to alter the soil microbial population. Therefore, Vitavax200FF has the potential to alter plant productivity.

The current study only investigated the effect of Vitavax 200FF on common groups of soil microbes and did not identify specific species within each group. As a result, further studies are required to identify the exact species that are affected by Vitavax 200FF in soil. This would also allow an investigation of whether Vitavax200FF has the

potential to cause adverse effects or enhance the growth of soil microbes that are known to be beneficial to plant productivity.

5.4.3 Soil toxicity during degradation of Vitavax 200FF and its active ingredients

The degradation products of chemicals in soil generated by abiotic and biotic processes can be very different and thus have differing toxic effects in cells. In addition, mixtures of parent compounds and their metabolites can interact synergistically to increase toxicity (Pesce *et al.*, 2010).

A soil-only control was included in both sterile and non-sterile soil experiments to determine if any cytotoxicity could be attributed to chemical or microbial changes in the soil itself. Similarly, a soil solvent control was included in both sterile and non-sterile soil experiments to determine if the acetone used to dissolve Vitavax 200FF and its active ingredients could cause changes to the cytotoxicity of the soil over the 28 day degradation period. None of the controls caused a change in cytotoxicity indicating that any cell death induced by the treated soils was due to the effects of Vitavax 200FF, its active ingredients, and/or its metabolites.

Abiotic degradation of carboxin had no effect on cell viability, indicating that its abiotic degradation products had similar or lower toxicity to the parent compound. Carboxin sulfoxide and enol are the major abiotic degradation products of carboxin in soil in the absence of light (Isidori *et al.*, 2012). Therefore our results are in agreement with previous studies which found that carboxin sulfoxide and enol had similar or lower toxicity compared to carboxin (DellaGreca *et al.*, 2004, Isidori *et al.*, 2012).
The toxicity of sterile soil spiked with thiram decreased from over 28 days. This would indicate that the abiotic degradation products of thiram were less toxic than the parent compounds. This is further supported by the finding that the toxicity of the soil showed

a direct correlation to the concentration of thiram throughout the degradation period in all cell lines. Our results are in agreement with a previous study which found that thiram caused hepatic damage in rat liver whilst two of its common metabolites in soil, dimethyldithiocarbamate and carbon disulfide showed no toxic effects (Dalvi *et al.*, 2002).

The toxicity of sterile soil spiked with carboxin and thiram in combination, or Vitavax 200FF also decreased over 28 days and would indicate the generation of abiotic degradation products with less toxicity compared to the parent compounds. The toxicity of the soil generally correlated with the concentration of thiram for all cell lines except for soil spiked with carboxin and thiram after 21 days or soil spiked with Vitavax 200FF after 3 days. In both cases, more than half of the initial amount of thiram had been degraded and may indicate potential interaction between the abiotic degradation products of thiram and other compounds in the soil. The disparity between toxicity and thiram concentration may also be due to to the accumulation of abiotic degradation products with some toxic potential. Indeed, many chemical degradation products often have greater persistence in the environment compared to their parent compounds (Boxall *et al.*, 2004). Therefore, although the degradation products of carboxin, thiram and Vitavax 200FF are likely to be low, their accumulation in soil over time may contribute to the toxicity of the soil, which was found to be cytotoxic in human cells. Further experiments which identify and quantify the abiotic degradation products of carboxin, thiram and Vitavax 200FF are required for confirmation.

The toxicity of non-sterile soil spiked with the low dose of 0.0000375% Vitavax 200FF at day 0 in WIL2NS cells was found to correlate with the concentration of thiram in the soil, indicating no or toxicologically insignificant interactions between compounds in the mixture. After one day of degradation, the toxicity of the soil to WIL2NS cells disappeared and was likely to be due to the rapid degradation of thiram in the soil. As

a consequence, the toxicity of soil from a field that had been recently sown with seed coated with Vitavax 200FF, or from a small spill with no history of previous contamination is likely to be accurately estimated from the chemical analysis of thiram.

The toxicity of non-sterile soil spiked with 0.00375% Vitavax 200FF decreased at a faster rate compared to the Vitavax 200FF in sterile soil in all cell lines tested. This was due to increased degradation of the parent compounds carboxin and thiram in non-sterile soil by microbes or their metabolites compared to sterile soil. Therefore, this study shows that the presence of microbes in soil can reduce the length of time that soil contaminated with Vitavax 200FF remains toxic to human cells.

Similarly to that seen for the low dose of Vitavax 200FF in non-sterile soil, the higher dose of 0.000375% Vitavax 200FF at day 0 was found to correlate with the concentration of thiram in all cell lines. This would indicate that accurate estimation of the toxicity of soil with no history of previous contamination can be achieved using chemical analysis if performed immediately after contamination with a large spill of Vitavax 200FF.

Estimation of the toxicity of soil contaminated with a high amount of Vitavax 200FF after subsequent degradation of the chemicals however, may not be accurate. This was demonstrated in this study where significant cell death was observed in HaCaT and WIL2NS cell lines after exposure to day 14 of non-sterile soil spiked with 0.00375% Vitavax 200FF, even though chemical analysis found no traces of thiram in the soil. The toxicity of day 3 and day 7 soil in WIL2NS cells was also greater than that observed for thiram alone and would also highlight the inadequacy of chemical analysis in toxicity estimation during degradation of Vitavax 200FF in soil.

The persistent toxicity of non-sterile soil spiked with 0.00375% Vitavax 200FF after degradation of its parent compounds may indicate an accumation of degradation products with toxic effects to human cells. Although the presence of microbes enabled faster degradation of the parent compounds to negligible levels, it could also have caused an accumulation of their degradation products to a level that was able to cause toxic effects.

It was also noted that high levels of Vitavax 200FF was able to alter the microbial population in soil. More specifically, total bacteria and actinomycete populations in particular increased prior to day 14. Metabolites or by-products of these microbes have the potential to be toxic to human cells. Consequently, their increased growth may also lead to an increased concentration of microbial by-products with cytotoxic effects, or that are able to interact with other compounds in the soil to induce a cytotoxic effect.

Nevertheless, non-sterile soil spiked with 0.00375% Vitavax 200FF was not toxic to any of the cell lines after day 21 and would indicate removal of the cytotoxic compounds present in day 14 soil. Although the possibility of interaction between compounds in day 21 soil cannot be ruled out, it can be concluded that any interaction between compounds in the soil after day 21 had negligible effects on cell viability.

For all soil samples tested, WIL2NS cell were the most sensitive to the cytotoxic effects of the soil followed by HaCaT cells and finally HepG2 cells. This is likely related to their xenobiotic metabolism capacities. Both HepG2 and HaCaT cells are known to express a number of phase II enzymes including glutathione-S-transferases (Knasmuller *et al.*, 1998, Hewitt *et al.*, 2004, Götz *et al.*, 2012b). In contrast, WIL2NS cells have been found to be deficient in glutathione-S-transferase M1 (Shield *et al.*, 2004b). Therefore, the increased sensitivity of WIL2NS cells to the soil may be due to a impaired metabolism of toxic compounds in the soil.

5.4.4 Concluding remarks

In this chapter we show that high concentrations of Vitavax 200FF can alter the population of soil microorganisms in soil to potentially impact soil health. We also demonstrated that degradation of the active ingredients of Vitavax 200FF, carboxin and thiram could occur via abiotic catalytic processes as well as via biotic transformation.

The toxicity of soil to human cells could be predicted from chemical analysis following initial contamination with Vitavax 200FF. Degradation products generated from both abiotic and biotic processes were likely to be less toxic than their parent compounds. However, it was found that the soil could remain toxic to human cells for a longer period of time than predicted from chemical testing. This residual toxicity is likely due to degradation products with some toxicity or interaction between compounds in the soil that are only detected using bioassays.

6 CHAPTER 6: *In vitro* cytotoxicity monitoring of bioremediated soil

6.1 Introduction

As discussed in section 1.1, the complexity of environmental mixtures can vary greatly between sources and therefore the toxicity of the mixture may be difficult to estimate by chemical analysis (see section 1.3.1). Chapter 5 found that the presence of microbes could affect the length of time that soil contaminated with chemical mixtures remained toxic to human cells. Therefore, this chapter investigated the toxicity of contaminated soil where exogenous microbes were deliberately added with the intention of decreasing the toxicity of the soil.

Petroleum based products are used extensively in the modern world. Due to their frequent transportation and storage, contamination of soil with petroleum hydrocarbons is unavoidable. Total petroleum hydrocarbons (TPH) are complex mixtures with toxic and carcinogenic potential (Agency for Toxic Substances and Disease Registry, 1999). Therefore many methods such as bioremediation have been developed that aim to reduce the hazard posed by the contaminated soil.

Bioremediation is considered to be an environmentally friendly and cost effective process which utilises microorganisms to degrade contaminating petroleum hydrocarbons in soil (Boopathy, 2000). Two common methods of bioremediation include natural attenuation (NA) or the combined treatment of bioaugmentation and biostimulation (BABS). BABS involves the addition of exogenous microorganisms and nutrients to the soil to enhance degradation of contaminants by microorganisms (Tyagi *et al.*, 2011) whereas NA refers to the unassisted degradation of contaminants by endogenous microbial populations in the soil (Bento *et al.*, 2005)

The end point of bioremediation is usually evaluated by chemical analysis to determine when the residual concentration of total petroleum hydrocarbons in the soil has decreased to levels deemed to be safe by regulatory authorities (Vidali, 2001). However, studies have shown a limited correlation between the results of chemical analysis and toxicity testing (Marwood *et al.*, 1998, Vasseur *et al.*, 2008, Mao *et al.*, 2009). Therefore, it is thought that chemical analysis alone may overlook other unknown hazardous compounds present in the soil (Plaza *et al.*, 2005).

For chemical analysis, reliable quantification requires the use of an internal standard containing known concentrations of a compound with a similar physico-chemical profile to the target compound (Shannon *et al.*, 1993). This poses a problem as soils in the environment vary greatly in their chemical makeup due to differences in nutrients, heavy metals, microbial activity, or soil type which may contribute to the toxicity of the soil. Microbial communities in particular can vary greatly in diversity and abundance between soils. Bundy *et al* (2002) demonstrated that contamination of different soil types with the same hydrocarbon mixture resulted in increased divergence of microbial communities indicating the potential for differences in metabolites or by-products formed with diverse or unknown toxicities. Therefore, bioremediation involving the use of microbes may also produce a multitude of metabolites or by-products with diverse or unknown toxicities.

Petroleum hydrocarbons are also complex mixtures and can vary in toxicity depending on the exact chemical composition (Vrabie *et al.*, 2009). Reliable identification and quantification of a contaminating compound using chemical analysis can only be performed if an appropriate standard is used. However, due to the chemical complexity of contaminated soils, a high number of expensive standards would be needed. Additionally, it is highly likely that internal standards would not be available for all of

the compounds that are present in the soil. Consequently, the identification and quantification of all compounds present in a soil sample via chemical analysis to determine potential hazards may not be practicable due to the complexity of each soil sample and the high number of compounds present. Furthermore, potential synergistic or additive effects between compounds within the soil sample means extrapolation by examining the toxicity of individual compounds may not accurately estimate the toxicity of the mixture (Donnelly *et al.*, 2004, Wei *et al.*, 2012). Therefore, alternative methods are required to evaluate the efficiency of bioremediation to reduce the toxicity of the contaminated soil in humans.

Bioassays have emerged as promising tools for detecting toxicity in soil samples by enabling a cost effective and simple approach that can overcome the limitations of chemical analysis (Maila *et al.*, 2005). While there is support for the incorporation of bioassays as part of the risk assessment (Molina-Barahona *et al.*, 2005, Plaza *et al.*, 2005, Torokne *et al.*, 2010, Raimondo *et al.*, 2015), current bioassays for soil toxicity are primarily based on ecotoxicity studies using plant and earthworm models or on bacterial models. Differences between the biological activities of these models and human cells mean that the results may not accurately predict the potential risks to human health.

The human liver cell line HepG2 has shown potential to detect toxicity of complex mixtures and is widely used in toxicity and carcinogenicity studies (Knasmuller *et al.*, 2004). The human derived cells are metabolically competent (Knasmuller *et al.*, 1998) and are sensitive to the toxicity of petroleum hydrocarbons and their metabolites (Diamond *et al.*, 1980, Valentin-Severin *et al.*, 2003, Rudzok *et al.*, 2009). They are therefore an ideal model system to use to assess the human toxicity of complex environmental samples such as contaminated and bioremediated soil.

Petroleum hydrocarbons have the potential to cause direct toxicity to mammalian cells (Bekki *et al.*, 2009, Vrabie *et al.*, 2009) but can also be metabolised within human cells to toxic metabolites (Leadon *et al.*, 1988). On the other hand, metabolism and degradation of TPH compounds by microbes used in bioremediation may also result in compounds with direct toxicity to human cells.

Differences in xenobiotic metabolising enzymes and activities between species (Graham *et al.*, 2008) may result in different metabolites or varied levels of metabolites produced. Therefore, metabolites produced by microorganisms such as bacteria and fungi utilised in the bioremediation process may be different from those produced by human liver cells and hence have different toxicities in humans.

The use of HepG2 cells in this study is designed to enable monitoring of the toxicity of parent compounds and metabolites formed in HepG2 cells as well as the toxicity of the breakdown products formed during bioremediation (Figure 6-1).

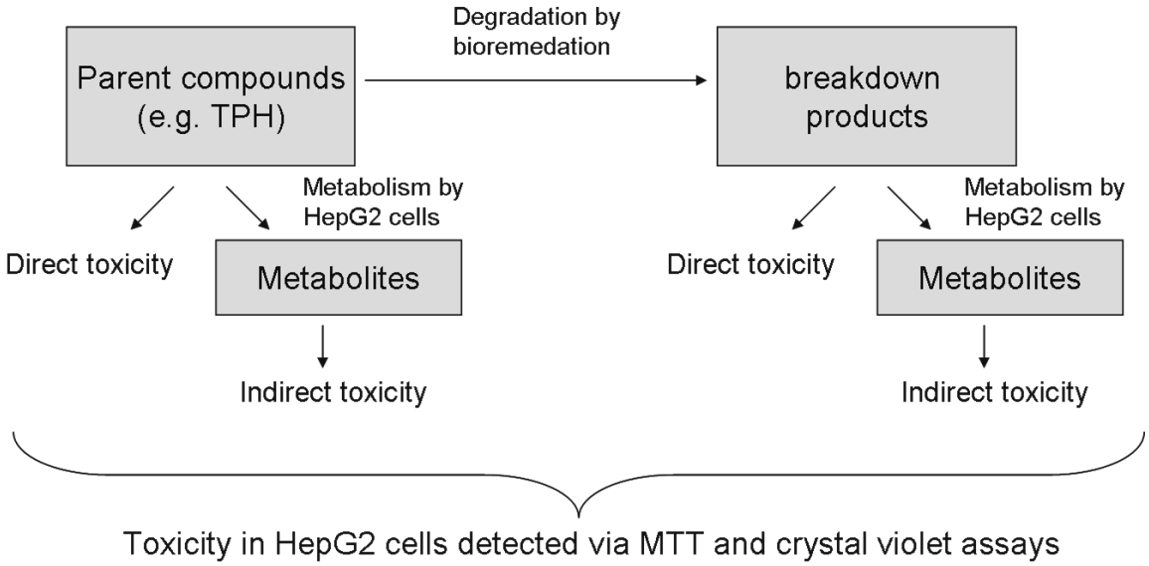


Figure 6-1. Schematic of possible toxic compounds present during bioremediation of petroleum hydrocarbon contaminated soil that can affect viability of HepG2 cells

It is hypothesised that toxicity directly caused by parent TPH compounds in the soil or indirectly via their metabolism in HepG2 cells to toxic metabolites (Figure 6-1) should decrease over time as TPH levels decrease. Toxicity directly caused by breakdown products of bioremediation with a long half-life in the soil or indirectly via their subsequent metabolism in HepG2 cells would be likely to increase over time as the breakdown products accumulate in the soil. On the other hand, toxicity caused by breakdown products with short half lives in soil would likely cause a transient increase in toxicity.

The use of HepG2 cells to monitor bioremediation therefore has the potential to detect both the changes in toxicity to humans over time, as well as help identify the possible causes of toxicity. This would provide a better evaluation of the risk posed by human exposure to the contaminated soil compared to chemical analysis alone or ecotoxicity testing.

Therefore the aims of this study were to:

- Compare the effectiveness of two different bioremediation methods in degrading petroleum hydrocarbons in contaminated soil
- Monitor the cytotoxicity in HepG2 cells of the soil before, during and after bioremediation by either NA or BABS
- Evaluate the effectiveness of using HepG2 human cells to predict toxicity of contaminated and bioremediated soil.

6.2 Materials and methods

6.2.1 Soil collection, extraction and HPLC analysis to determine TPH content

Soil was obtained from Flinders Bioremediation as described in section 2.2.2. Extraction of TPH from soil was performed as described in section 2.7. Determination of TPH content in soil extracts was performed using gas chromatography as described in section 2.9.

6.2.2 Cell treatments

In order to examine the effects of the extracted TPH on HepG2 cells, the stock 9g soil per ml extract was diluted in culture medium to a concentration of 10 mg soil per ml. The remaining concentrations (0.625, 1.25, 2.5, 5 mg soil per ml) were prepared by diluting the 10 mg soil per ml solution in the solvent control (0.01% heptane, 0.1% DMSO in media). A medium only control, solvent control (0.01% heptane, 0.1% DMSO in media) and a positive control, 40 µM 5-FU was also included in the assay. Oil sludge treatments containing equivalent concentrations of TPH were prepared by diluting the oil sludge extract in culture medium to obtain a 200 µg/ml TPH working solution. The remaining oil sludge treatments were prepared by diluting the 200 µg/ml TPH working solution in the solvent control.

20,000 HepG2 cells were seeded in wells of 96-well flat-bottom plates and incubated for 24 hours to allow cells to attach. The medium was removed and the cells were exposed to the cell treatments in four or six replicate wells (for MTT or crystal violet assays respectively) for 24, 48 or 72h. The treatment medium was then removed and the cells were washed twice with 100µl PBS followed. The number of surviving cells was determined using the MTT assay (section 2.11.1) or the crystal violet assay (section 2.11.1)

6.2.3 Statistical analysis

Linear regression was used to analyse the correlation between cell number and absorbance for standard curves of both MTT and crystal violet assays. Examples of MTT and crystal violet standard curves are shown in Appendix II. To determine significant differences between media only and solvent controls, independent t-tests were conducted on cells per well values of the respective controls. Statistical differences in % cell viability between the solvent control and treatments, exposure time, length of bioremediation, bioremediation method or cytotoxicity assay were analyzed by conducting two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. All statistical analyses were performed using SPSS software, version 18. Results were considered to be statistically significant when $p \leq 0.05$.

6.3 Results

6.3.1 TPH content of soil extracts

A blank extraction was conducted in order to determine any potential contamination from solvents or insufficient washing of glassware. GC analysis of the blank extraction was below the level of detection. At day 0, TPH concentrations were 20000 mg/kg for NA and 19000 mg/kg for BABS (Figure 6-2). Both bioremediation strategies induced a reduction in the concentration of total petroleum hydrocarbons in soil over time. The rate of TPH degradation was highest during the first four weeks of bioremediation with NA and BABS resulting in a 48.5% and 48.4% reduction to 10,300 and 9,800 mg/kg of TPH respectively in the contaminated soil after week 4 compared to week 0 (Figure 6-2). In addition, by week 4 of bioremediation TPH levels in the BABS soil decreased to 9,800 mg/kg and therefore was below the safe landfill disposal limit of 10,000 mg kg (Environmental Protection Authority, 2010). TPH levels in NA soil fell below the safe landfill disposal limit after 8 weeks of bioremediation to 9,400 mg/kg. The amount of TPH degradation observed decreased after 4 weeks with NA and BABS showing TPH decreases of only 1900 mg/kg and 2300 mg/kg respectively after week 12 compared to week 4 (Figure 6-2).

No noticeable difference in the rate of degradation was observed between NA and BABS over time with both bioremediation strategies showing a similar degradation curve (Figure 6-2). Comparison of chromatogram profiles also indicated that degradation of individual petroleum hydrocarbons were similar between the two bioremediation strategies during bioremediation (Figure 6-3). Degradation over time was more prominent for petroleum hydrocarbons that had retention times (RT) between 6-13 min, with little change observed for petroleum hydrocarbons with retention times greater than 13 min (Figure 6-3).

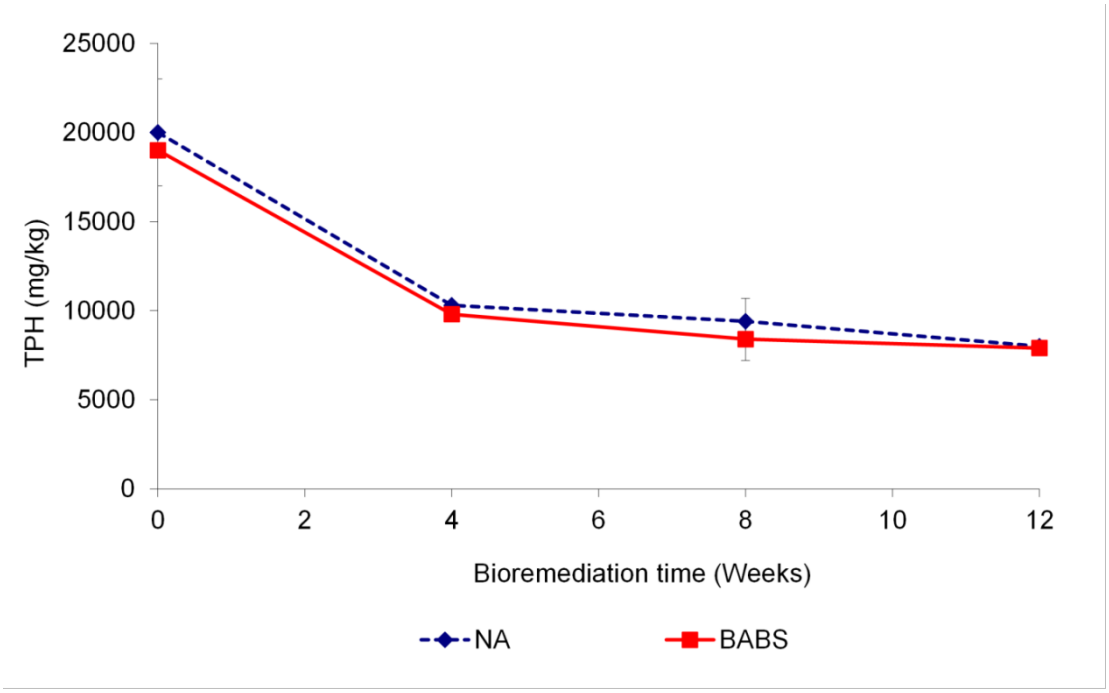


Figure 6-2. Degradation of total petroleum hydrocarbons (TPH) in bioremediated soil as determined by gas chromatography (GC).

GC analysis of the blank extraction without soil was below the level of detection. NA – natural attenuation, BABS – bioaugmentation and biostimulation. Error bars indicate the standard deviation of the sample analysed in duplicate (n = 2).

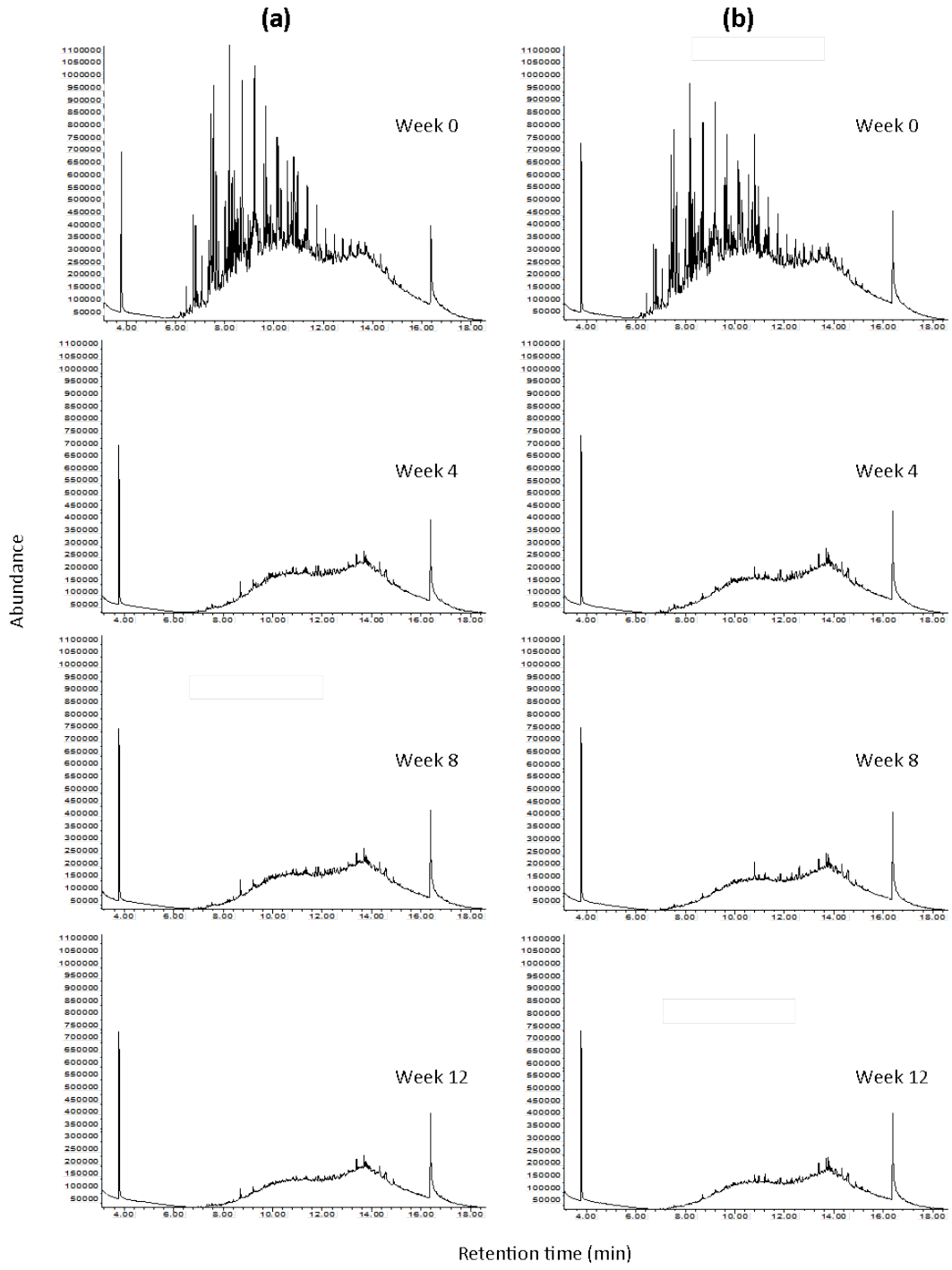


Figure 6-3. GC chromatograms showing TPH content present in oil-contaminated soil bioremediated by (a) natural attenuation or (b) bioaugmentation and biostimulation at time zero and after weeks 4, 8 and 12.

6.3.2 Cytotoxicity of soil extracts to HepG2 cells

The solvent control did not significantly affect cell viability ($p>0.05$) compared to the medium only control at any of the exposure times tested in both MTT and crystal violet assays (Figure 6-4). The extraction procedure used required evaporation of the extract which could potentially concentrate any contaminants within the solvent and affect cell viability. Thus the blank extraction without soil served as a negative control and was analysed to confirm that evaporation of the solvent did not affect cell viability. The blank extraction did not affect cell viability at any of the doses or exposure times tested in both the MTT and crystal violet cytotoxicity assays (Figure 6-5)

5-FU was used as a positive control to confirm the sensitivity of the MTT and crystal violet assays to detect cell death (Longley *et al.*, 2003). 5-FU induced significant cell death compared to the solvent control ($p<0.05$) after 24h, 48h and 72 exposure in both MTT and crystal violet assays (Figure 6-5)

The dose dependency of NA and BABS soil was examined by exposing HepG2 cells to increasing concentrations of soil extracts (Figure 6-5). Dose dependent decreases in cell survival were observed for all soil extracts at all exposure times and in both cytotoxicity assays. All soil extracts produced a significant decrease in cell viability ($p<0.05$) in HepG2 cells after 72h exposure at 10 mg/ml (Figure 6-5).

Time-dependent decreases in cell viability were also observed with an increased exposure time. This aligned with decreased cell viability for each dose of soil extract (Figure 6-5). Results reached significance ($p<0.05$) at 10mg/ml with lower cell viability observed after 72h exposure when compared to 24h for all soil extracts

The length of bioremediation had no effect on the cytotoxicity of the soil extracts with no significant differences in cytotoxicity ($p>0.05$) detected at any bioremediation time

compared to week 0 for both NA and BABS soil (Figure 6-6). BABS soil extracts produced a greater reduction in cell viability than NA soil extracts when compared at the same bioremediation time (Figure 6-6). Results were significant ($p<0.05$) at week 12 for all exposure times in the MTT assay, and at week 0 after 24h and 48h exposure in the crystal violet assay.

NA and BABS soil extracts generally produced a greater reduction in cell viability compared to an equivalent concentration of TPH from sludge oil (Figure 6-6). In the MTT assay, significantly lower cell viability was observed for BABS and NA in weeks 4, 8 and 12 after 48 or 72h exposure compared to an equivalent concentration of TPH from sludge oil. Week 0 of NA or BABS soil extract also showed lower cell viabilities compared to their equivalent concentration of TPH from sludge oil in the MTT assay, but only reached significance after 72h ($p=0.049$ and $p=0.044$ respectively; Figure 6-6).

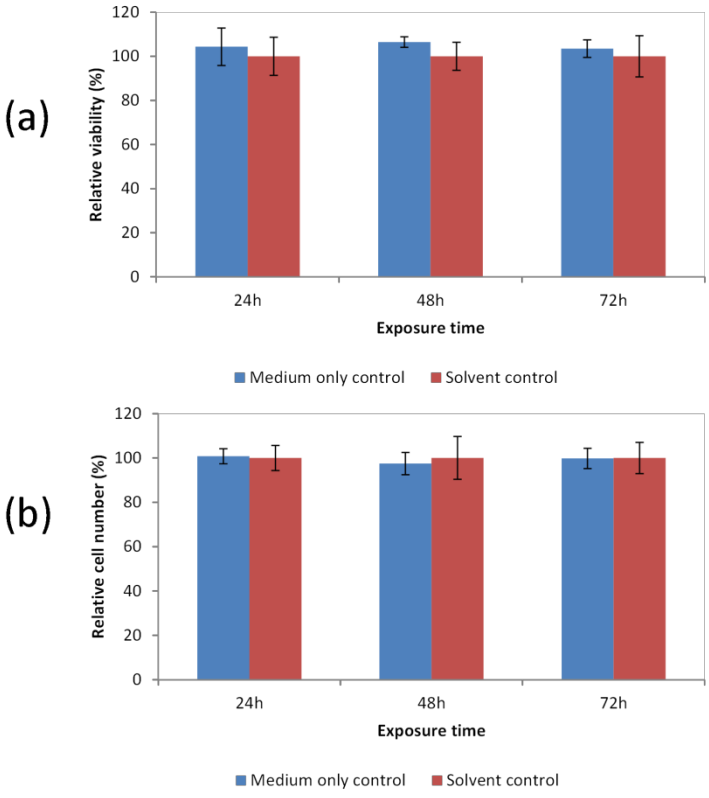


Figure 6-4. Effect of the solvent control on cell viability.

Cells were exposed to the medium only control or the solvent control for 24, 48 or 72h and cell viability determined using the (a) MTT assay or (b) crystal violet assay. Results are shown as % relative viability (MTT) or % relative cell number (crystal violet) compared to the solvent control (100%) and are presented as the mean ±SEM of four separate experiments (n=4). No significant differences were found between the medium only and solvent controls ($p>0.05$).

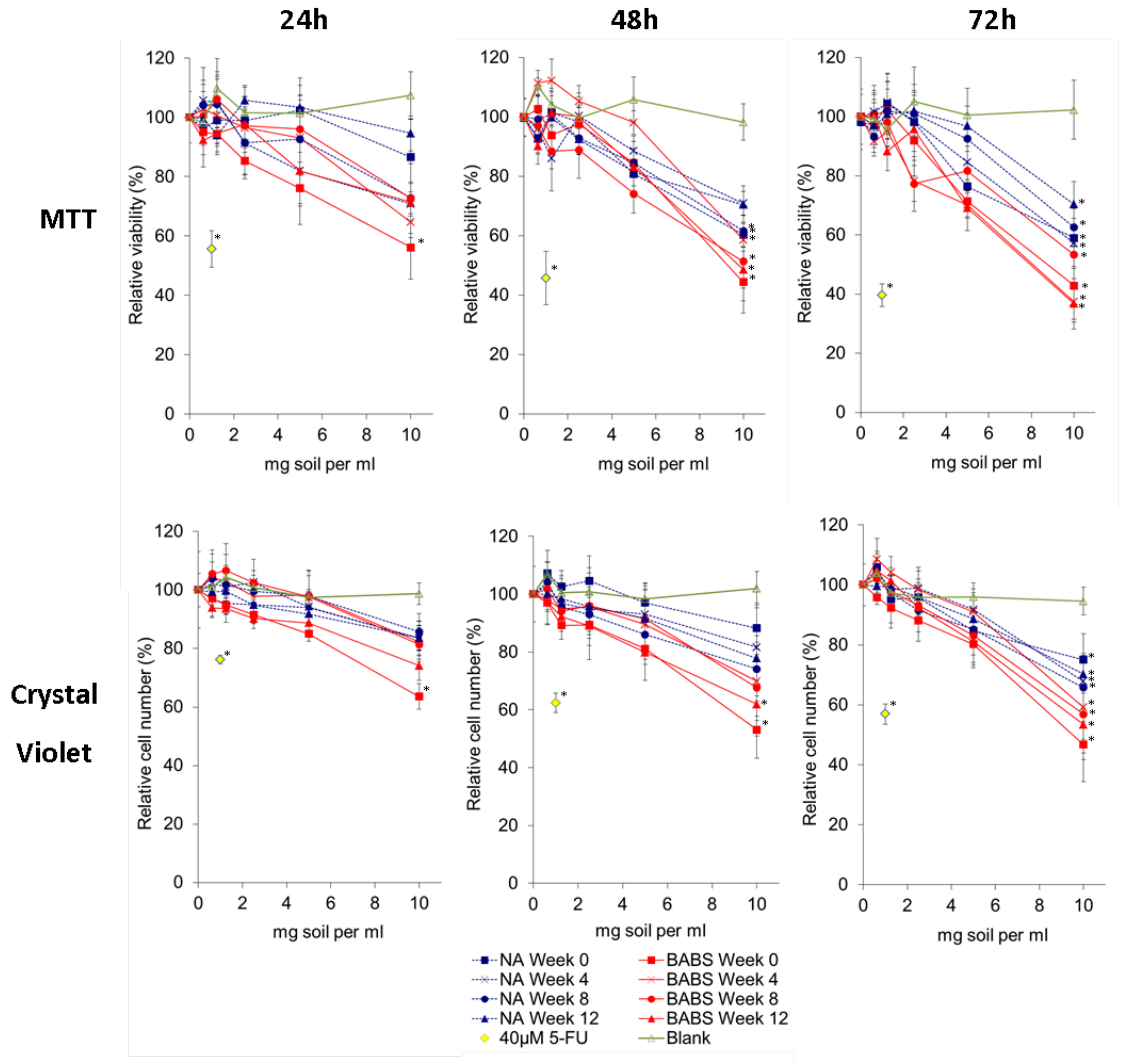


Figure 6-5 Dose dependency of extracts of bioremediated soil in HepG2 cells exposed for 24, 48 or 72h using the MTT or Crystal Violet assay.

Petroleum hydrocarbon contaminated soil was bioremediated using natural attenuation (NA) or bioaugmentation and biostimulation (BABS). The blank extract was prepared without soil. 40µM 5-FU was used as the positive control. Results are shown as % relative viability (MTT) or % relative cell number (crystal violet) compared to the solvent control (100%) and are presented as the mean ±SEM of four separate experiments (n=4). * Significant difference ($p \leq 0.05$) compared to the solvent control.

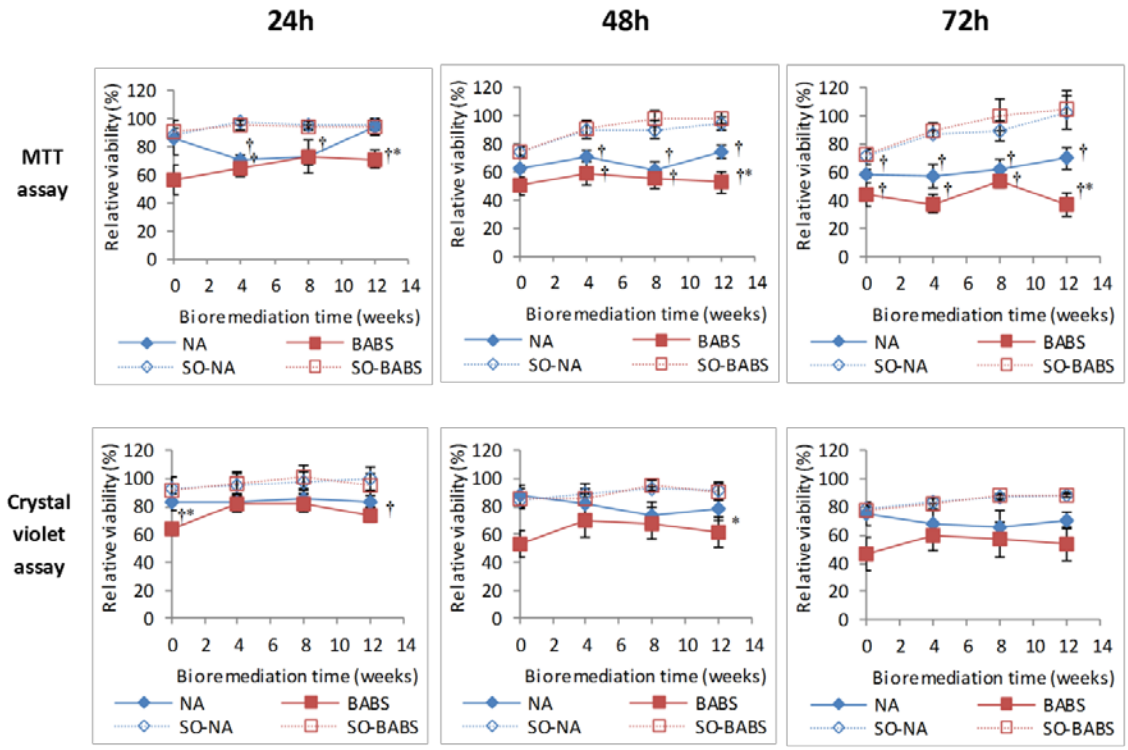


Figure 6-6. Time dependency of bioremediation in HepG2 cells exposed with 10mg/ml of soil extracts for 24, 48 or 72h using the MTT or Crystal Violet assay

NA = natural attenuation; BABS = bioaugmentation and biostimulation; SO-NA = sludge oil containing an equivalent concentration of TPH to NA at each sampling time; SO-BABS = sludge oil containing an equivalent concentration of TPH to BABS at each sampling time. Results are shown as % relative viability (MTT) or % relative cell number (crystal violet) compared to the solvent control and are presented as the mean ±SEM of at least three separate experiments. * Significant difference ($p \leq 0.05$) compared to NA at the same bioremediation time. † Significant difference ($p \leq 0.05$) compared to an equivalent concentration of TPH from sludge oil.

6.4 Discussion

6.4.1 Effectiveness of NA or BABS to degrade TPH levels in soil

In this study, two different bioremediation strategies, NA and BABS were employed to degrade contaminating hydrocarbons in soil. Both methods resulted in similar rates of TPH degradation throughout the duration of bioremediation. BABS treatment, involving the addition of nutrients and microbes to contaminated soil and periodical monitoring is more costly than NA treatment, which involves periodical monitoring alone. The addition of nutrients and exogenous fungal populations to BABS soil did not increase the rate of TPH degradation compared to NA soil in this study. It is noted that the soil used in this study came from a biopile that was previously contaminated with TPH and successfully bioremediated to below 10,000 mg/kg. Previous studies on petroleum hydrocarbon contaminated soil have shown a population shift of microbial communities towards hydrocarbon degrading microorganisms during bioremediation (Vinas *et al.*, 2005, Margesin *et al.*, 2007, Grace Liu *et al.*, 2011). Therefore, successful bioremediation of the initial biopile suggests the probable presence of a dominant population of hydrocarbon degrading microorganisms. It is likely that there was already an optimal amount of hydrocarbon degrading microorganisms present in the initial biopile and amending the soil with BABS did not increase TPH degradation. Therefore, NA treatment would be selected as the most suitable method of bioremediation for the soil used in this study.

After 12 weeks both bioremediation methods resulted in a decrease of TPH levels in the soil to below 10,000 mg/kg and could therefore be disposed of in landfill as low level waste soil (Environmental Protection Authority, 2010). As the degradation of TPH by microorganisms in the soil is likely to continue after 12 weeks, a longer bioremediation period would likely result in further decreases of TPH levels in the soil over time. However as the rate of TPH degradation decreased after 4 weeks, the

amount of time required for TPH levels to drop below 1,000 mg/kg and thus be suitable for disposal in residential areas (Environmental Protection Authority, 2010) is undetermined.

In addition, degradation of TPH was more effective for compounds with RT ≤ 13 min in both bioremediation methods indicating greater efficiency in degradation of more volatile and/or more polar compounds.

6.4.2 Toxicity of soil before, during and after bioremediation

TPH levels decreased over time in both NA and BABS soil and would suggest a reduction in toxicity if relying on chemical analysis alone for risk assessment. Gas chromatogram profiles also indicated the reduction of many individual compounds within the mixture over time. However, a reduction in toxicity over time was not seen in the cytotoxicity assays when using the human HepG2 cell line. Instead, both NA and BABS soils showed no significant change in toxicity from Day 0 at any point during bioremediation. This may indicate that toxic intermediary metabolites were formed which have similar toxicity to their parent compounds. It is also possible that the toxicity observed was due to the less volatile and/or more non-polar compounds with RT > 13 min, which showed little degradation during bioremediation.

The lower cell viability observed for NA and BABS soil compared to an equivalent concentration of TPH from sludge oil would indicate some toxicity originating from the soil itself. This may indicate the presence of other compounds in the soil extract that were not detected using chemical analysis, but could have contributed to its toxicity. In any case, we show here that different toxicities can be observed from samples containing the same concentration of TPH. Our results support other studies which have shown that the toxicity of the soil could not be predicted solely from measuring the concentration of TPH (Hubálek *et al.*, 2007, Mao *et al.*, 2009, Steliga *et al.*, 2012)

Previous studies have also shown bioremediation of some petroleum hydrocarbon contaminated soils may increase its toxicity (Marwood *et al.*, 1998, Phillips *et al.*, 2000). Thus, previous bioremediation of the soil may have produced toxic intermediary metabolites that were not detected using chemical analysis. Unfortunately, soil samples taken prior to the previous bioremediation were not available, hence it was not possible to determine if the toxicity observed was due to prior bioremediation, or the soil itself.

The oil extraction procedure used in this study is solvent based and is likely to extract other compounds in the soil including nutrients and other contaminants in addition to TPH. The use of solvents in the extraction procedure may also disrupt the cell membranes of some microorganisms in the soil, causing the release of cellular contents. Water soluble compounds in the soil are removed during subsequent washing steps with water however other compounds insoluble in water including cellular material such as lipids and some proteins may be retained in the final extract and thus affect the overall toxicity of the soil extract.

As the GC analysis method used in this study only examined compounds with retention times between the internal standards n-decane (C_{10}) and n-tetracontane (C_{40}), the presence of other compounds in the extract with retention times outside that of the internal standards cannot be ruled out. In addition, non-volatile or thermally labile compounds present in the soil cannot be detected using this method. Therefore, it is possible that toxic compounds were present in the soil samples but were not detected using the GC analysis used in this study.

6.4.3 Effect of the bioremediation method on the toxicity of soil

The addition of nutrients, surfactants or microorganisms to a soil sample may reduce the toxicity of the soil by increasing the degradation rate of toxic compounds. On the other hand, metabolism of the multitude of compounds present in a soil sample by different organisms may result in the formation of different metabolites or by-products with varying toxicity and persistence in the environment. Therefore, there is the potential for varying toxicity of the same soil when using different bioremediation methods.

In this study, BABS soil was slightly more toxic than NA soil at time 0 and indicates that the nutrients and fungi added to the initial soil sample probably increased its toxic potential. As the addition of nutrients and exogenous fungal populations to BABS soil also did not increase the rate of TPH degradation, it would appear that BABS treatment had no beneficial effects on reducing the hazard posed by the soil compared to NA. This highlights the need for careful initial assessment of the bioremediation method so that any unnecessary treatments that increase the hazard posed by the soil may be prevented.

6.4.4 The effectiveness of using HepG2 cells in the MTT and crystal violet cytotoxicity assays to evaluate the toxicity of soils

The HepG2 cells used in this study mimic human liver cells. Therefore, any toxicity observed in the cells following exposure to the soil would also indicate the potential for toxicity in humans. The HepG2 cells were sensitive to the soil extracts used in this study in both the MTT and crystal violet cytotoxicity assays. The MTT assay enables the determination of metabolically active cells (Mosmann, 1983) while the crystal violet assay stains the nuclei of viable cells (Gillies *et al.*, 1986b). The use of two cytotoxicity assays which measure different end points is necessary to enable confirmation of cell death and reduce the probability of other factors affecting results such as altered

metabolism or altered levels of nuclear material. Both cytotoxicity assays used in this study produced similar dose-responses after exposure of HepG2 cells to the soil extracts, indicating that the reduction in cell viability was indeed due to cell death.

It is noted that only metabolically active HepG2 cells were used in this study which enables the detection of the total toxicity of the soil. Therefore, it is unclear whether the toxicity observed in the study is due to toxic compounds produced from HepG2 metabolism, toxic compounds produced from bioremediation or a combination of both. Future studies using non-metabolically competent cells such as the hepatocellular HepaRG cell line (Aninat *et al.*, 2006, Al-Attrache *et al.*, 2016), which only detect the presence of parent compounds or products of bioremediation with direct toxicity (Figure 6-1) may assist in elucidating mechanisms of toxicity. Determination of the mechanisms of toxicity would be useful to determine if the toxic effects of the soil are likely to be localised to tissues with high metabolic capacity such as the liver, or if there is the potential for damage to other cell types.

6.4.5 Conclusions

In this study we show that the reduction of TPH levels detected via chemical analysis over time did not correlate with a reduction of cytotoxicity over time. We also showed that treating TPH contaminated soil via the addition of nutrients and microorganisms could potentially be more harmful than allowing the contaminants to degrade in the soil unassisted. Thus, we reinforce the view that assessment using chemical analysis alone is insufficient to determine potential hazards to human health.

Screening of the soil samples using the HepG2 cell line allowed the toxicity of the soil to be detected and thus indicated a potential hazard to human health. Therefore,

inclusion of bioassays using human derived cells in addition to chemical analysis in current risk assessment protocols would enable a more comprehensive evaluation of the toxicity of contaminated and bioremediated soil to humans.

7 CHAPTER 7: General discussion and future directions

7.1 General discussion

The nature of this thesis was that it was divided into a series of subprojects and therefore each Chapter has a detailed discussion. As a consequence, this final discussion chapter only looks at the overarching themes that have arisen from the research and explores future directions.

The overall aim of this study was to use human cell lines to monitor the cytotoxicity of various environmental mixtures and their degradation in soil. This was achieved by using a range of human cell lines including HepG2, HaCaT, MRC-5, JAr, and WIL2NS to monitor the cytotoxicity of environmental mixtures of varying levels of complexity.

Current guidelines for assessing the human health hazard of environmental mixtures such as agricultural chemicals and bioremediated soil allow for predictions based solely on chemical analysis (Australian Pesticides and Veterinary Medicines Authority, 2009a, Australian Pesticides and Veterinary Medicines Authority, 2009b, Larsson *et al.*, 2013). In contrast, toxicity analysis of the mixture using *in vitro* bioassays may provide a more comprehensive assessment of the risk posed by the mixture to human health. Therefore, another main aim of this thesis was to determine if *in vitro* cytotoxicity monitoring of environmental mixtures using human cell lines was more sensitive for detecting changes in toxicity compared to chemical analysis.

The addition of soil to a chemical mixture increases its complexity. This is because soil contains a multitude of compounds in whose exact composition can vary between sources (Mattsson *et al.*, 2009, Larsson *et al.*, 2013, Liang *et al.*, 2014b). Therefore, there is the potential for chemical interactions to occur between compounds in the chemical mixture and soil, and hence affect its toxicity to human cells. In this study, the toxicity of the chemical mixtures, Vitavax 200FF and petroleum hydrocarbons from

crude oil, were examined in the presence or absence of a soil matrix. The toxicity of the chemical mixtures in soil immediately after spiking to humans cells was equivalent to the effects of the chemical mixture in the absence of soil. However both soils showed some disparities between *in vitro* cytotoxicity and chemical analysis as the mixtures degraded in the soil. In particular, it was demonstrated that soil contaminated with Vitavax 200FF (Chapter 5) or TPH (Chapter 6) could remain toxic to human cells even when chemical analysis indicated a reduction of the contaminants to non-toxic levels. This could indicate the presence of toxic compounds or interactions between compounds within the soil that would otherwise be overlooked when only relying on chemical analysis for hazard assessment.

Another factor to consider is the role of degradation products. Studies have also shown that many chemical degradation products can persist in the environment for a longer period of time compared to their parent compound (Boxall *et al.*, 2004). In this case, chemical analysis may not detect the presence of the degradation product if it is not selected as a compound of interest for analysis. Even if all the degradation products in a mixture were identified, toxicological data for all the products may not be available to enable accurate estimation of the toxic effects of the mixture to humans. This has implications for assessing the risk posed by soil with a history of previous contamination, particularly if the degradation products are able to induce an adverse effect to humans. In this study, *in vitro* cytotoxicity tests detected toxic effects in degraded soil that was not detected using chemical analysis (Chapters 5 and 6). Therefore, this project has demonstrated that *in vitro* cytotoxicity monitoring using human cell lines may be more valuable than chemical analysis in determining the hazard posed by soils with a history of previous contamination.

The impact of microbes must also be taken into account when assessing the potential hazard of contaminated soil to human health. In this thesis, it was demonstrated that

the presence of microbes in soil can influence the toxicity of soil contaminated with chemical mixtures. Chapter 5 demonstrated that the presence of microbes in soil was able to increase the degradation rate of carboxin and thiram in soil. However, the soil remained toxic to human cells despite the levels of carboxin and thiram in soil being below the level of detection of chemical analysis. Meanwhile, it was demonstrated in Chapter 6 that the addition of exogenous microbes to soil could increase the toxicity of soil, without affecting the degradation of rate of TPH. Therefore, microbes present in the soil may be contributing to the toxicity of the soil by generating metabolites with toxic effects to human cells. Indeed, the ability of microbes to generate metabolites with toxic effects to human cells has been demonstrated in previous studies (Malpuech-Brugere *et al.*, 2001, Caldwell *et al.*, 2009, Khanal *et al.*, 2012).

Alternatively, microbes in soil may generate metabolites which in turn may cause degradation of other compounds present in the soil via chemical interaction, resulting in products with toxic effects to human cells. It is acknowledged that the methods used in this thesis were unable to distinguish if the observed toxicity was due to the direct effect of microbial metabolites, or if microbial metabolites caused subsequent degradation of soil compounds to products with toxic effects in human cells. However, the use of human cell based bioassays in this thesis was advantageous in that they were able to detect the toxicity of the soil without needing to know the microbial or chemical content of the soil.

The results from this study adds to the body of evidence which highlights the limitations of solely relying on chemical analysis to assess the hazard posed by complex environmental mixtures to human health (Alexander *et al.*, 2002, Dévier *et al.*, 2011, Hu *et al.*, 2012, Mesnage *et al.*, 2014). The results presented in this thesis demonstrated that the use of *in vitro* cytotoxicity monitoring using human cells was able to detect toxicity in soil that was not a direct consequence of the compounds

selected for chemical analysis. It was identified that other factors such as degradation products, compounds within soil, or interaction between compounds may contribute to the toxicity of the soil and may not be detected using chemical analysis.

It is acknowledged that the exclusive use of *in vitro* bioassays for assessing the hazard of chemical mixtures to human health would have some limitations in specific situations. This is because *in vitro* bioassays are unable to identify the compound(s) responsible for the effect (Brack, 2003). This would be problematic in a situation where identification of the compounds responsible for the effect is required to investigate their originating source. One such example would be the investigation of contaminated soil, where the chemical composition and distribution of the contaminant(s) in the soil is required to identify the polluting source (Chang *et al.*, 2009, Lu *et al.*, 2010, Jiang *et al.*, 2011, National Environment Protection, 2013). Identification of contaminants in soil would also provide valuable information to aid identification of the mechanism(s) by which the contaminated soil exerts its toxic effects.

The limitation of *in vitro* bioassays to identify the compound(s) responsible for the toxic effect can be overcome through the use of chemical analysis. Therefore, a combined approach which includes both chemical and *in vitro* cytotoxicity testing of chemical mixtures would provide a more comprehensive evaluation of the toxicity posed by complex environmental mixtures.

7.2 Future directions

A number of limitations of the current study were identified. These limitations could be addressed through additional studies and are outlined in the following subsections 7.2.1 – 7. 2.5.

7.2.1 Mechanism of cell death

Results in Chapter 4 indicated that carboxin induced cell cycle arrest and apoptotic cell death in WIL2NS cells and could indicate activation of the p53 pathway. In cells, the p53 pathway is activated in response to cellular stress such as DNA damage to trigger cell cycle arrest and apoptosis (Oren, 1999). Therefore, activation of the p53 pathway may indicate mutagenic potential through induction of DNA damage. Considering the widespread use of carboxin as an agricultural fungicide, future studies which investigate whether carboxin induces DNA damage and subsequent activation of the p53 pathway would be warranted.

Results from Chapter 4 indicated that thiram induced necrotic cell death in human cell lines. Recent studies have found that cells can die through a programmed form of necrosis, which is regulated by key mediators including RIPK (Cho *et al.*, 2010, Moquin *et al.*, 2013, Newton *et al.*, 2014, Newton, 2015). Inhibition of programmed necrosis has been found to reduce disease severity in mouse models (Newton, 2015). Therefore, elucidation of whether thiram induces programmed necrosis may indicate a potential avenue for reducing the toxic effects of thiram in human cells.

Many diseases including cancer and neurodegenerative diseases are linked to dysregulation of cell cycle and cell death (Hetz *et al.*, 2005, Zhivotovsky *et al.*, 2010). A more comprehensive study on the mechanism of cell death induced by carboxin and thiram may provide insight on whether repeated exposure to the chemicals has the potential to lead to a diseased phenotype.

7.2.2 Bioavailability

The methods used to extract chemicals from soil for subsequent toxicity assessment in this thesis employed the use of organic solvents such as acetonitrile, acetone and heptane in order to investigate the absolute toxicity of the soil (Chapters 5 and 6). However, studies have shown that only a portion of the total chemicals present in soil are bioavailable (Rodriguez *et al.*, 2003). Therefore, the extraction methods used in this thesis may overestimate the hazard posed by the soil.

In previous studies, different concentrations of calcium chloride solutions (0.01M – 0.1M) were used to extract the bioavailable fraction of various pesticides and fungicides from soil (Barriuso *et al.*, 2004, Regitano *et al.*, 2006, Sopeña *et al.*, 2013, Wang *et al.*, 2015, Ren *et al.*, 2016). Therefore, future studies which examine the toxicity caused by the calcium chloride extract of soil contaminated with Vitavax 200FF or its active ingredients may provide toxicity data that is more relevant to its bioavailability.

Also, a study by Andersson *et al.* (2009) used n-butanol to extract the bioavailable portion of polycyclic aromatic hydrocarbons from soil. It is therefore probable that future studies which examine the toxicity caused by the n-butanol extract of soil contaminated with TPH may provide toxicity data that is more relevant to its bioavailability.

7.2.3 Elucidation of degradation products

As previously described in section 7.1, a complete chemical profile of compounds in contaminated soil is required in order to identify possible sources of contamination. Different chemicals may produce distinct degradation products as a result of their biotic or abiotic degradation (Hustert *et al.*, 1999, Sharma *et al.*, 2003, Sherif *et al.*, 2011, Abramović *et al.*, 2013). Therefore, characterisation of the degradation products generated by Vitavax 200FF and its active ingredients carboxin and thiram may enable the identification of soils that have a history of contamination by the parent compounds. Previous metabolomic studies have used MS and NMR spectroscopy to detect the photodegradation products of carboxin or thiram in water or soil (Hustert *et al.*, 1999, Gupta *et al.*, 2012b). Therefore, MS and NMR spectroscopy would be ideal methods to use to characterise the degradation products generated by Vitavax 200FF in soil.

7.2.4 Characterisation of the metabolic capacity of cell lines used in this thesis

Chapters 4 and 5 identified different sensitivities of the HepG2, HaCaT, JAr, MRC-5 and WIL2NS cell lines to Vitavax 200FF or its active ingredients. Data from the current literature suggested that the differences in sensitivity could be due to the different metabolic capacities of each cell line (Knasmuller *et al.*, 1998, Shield *et al.*, 2001, Götz *et al.*, 2012b). However, the expression of phase I and II enzymes in HepG2 cells has been shown to differ between sources (Hewitt *et al.*, 2004). Therefore, it is acknowledged that the metabolic activities of all the cell lines used in this thesis should be confirmed. This could be achieved by determining the activities of major phase I and II enzymes in each cell line using methods previously described by Hewitt *et al.* (2004) , Götz *et al.* (2012a) and Götz *et al.* (2012b).

7.2.5 Identification of specific microbes that degrade carboxin and thiram

Identification of specific microbial species which degrade carboxin and thiram may be useful for bioremediation of sites contaminated with high concentrations of the two chemicals. Experimental results from Chapter 5 of this thesis suggested that the non-sterile soil contained microbes with the potential to degrade carboxin and thiram, and thus reduce the length of time the soil remained toxic to human cells. In particular, the population of actinomycetes increased in response to spiking soil with the higher dose of Vitavax 200FF. The increase in the population of actinomycetes also correlated with a decrease in the concentration of carboxin and thiram in soil. Consequently, this study identified actinomycetes as good candidates for future studies which examine their carboxin or thiram degrading potential and suitability for bioremediation. A common technique used to identify and monitor microbial populations in complex samples such as soil is 16S rDNA gene sequencing (Elsayed *et al.*, 2015, Feld *et al.*, 2015). Therefore future studies which use 16S rDNA gene sequencing to monitor the change in the microbial community in soil contaminated with Vitavax 200FF would allow a more comprehensive search for microbes with carboxin or thiram degrading potential.

7.3 Summary

Humans are typically exposed to a multitude of chemical mixtures in the environment with varying impacts on human health. This work focused on the use of human cell lines to monitor the cytotoxicity of various environmental mixtures as they degraded in soil.

It was found that soil contaminated with the agricultural product, Vitavax 200FF, could remain toxic to human cells for a longer period of time than predicted from chemical testing. It was also found that bioremediation of soil contaminated with petroleum hydrocarbons did not correlate with a reduction of cytotoxicity.

The results of this thesis reinforced the view that assessment using chemical analysis alone is insufficient to determine potential hazards to human health. It was found that a combined approach which includes both chemical and *in vitro* cytotoxicity testing of chemical mixtures using human cells provided a more comprehensive evaluation of the toxicity posed by complex environmental mixtures. Future metabolomics studies which identify the degradation products of Vitavax 200FF or petroleum hydrocarbons during their degradation in soil, would allow for potential identification of the individual compounds or groups of compounds which are responsible for the residual cytotoxic effect of the soil to human cells.

APPENDIX I: Extraction of carboxin and thiram from soil using hexane:acetone (1:1)

Introduction

Accurate quantification of carboxin and thiram in soil requires that the extraction method has a high recovery of the compounds from soil. An extraction method was proposed by Gopal *et al.* (2006) which enabled simultaneous extraction of carboxin and thiram from product formulations and wheat seed.

Therefore the aim of this experiment was to assess the suitability of the method proposed by Gopal *et al.* (2006) to extract carboxin and thiram from soil.

Methods

Extraction of carboxin and thiram from soil - Hexane:acetone method

25g of soil spiked with carboxin and thiram (refer to section 3.2.1) was added to 150 ml hexane:acetone (1:1) and sonicated in a chilled sonicating water bath for 15 min. The extract was passed through a column of neutral alumina (5g). The column was further eluted using 50 ml hexane:acetone (1:1) and the total collected extract was evaporated using a rotary evaporator. A 33.3 g/ml extract was prepared by dissolving the dried extract in 750 µl methanol. The extracts were centrifuged at 10000 g for 10 min to remove remaining debris and the supernatant was used for HPLC analysis.

HPLC analysis of soil extracts

HPLC analysis of the soil extracts was performed as described in section 2.8.

Results

Recovery of carboxin from soil was less than 60% for all spiked concentrations (Figure A1). Recovery of thiram from soil was less than 25% for all spiked concentrations (Figure A1).

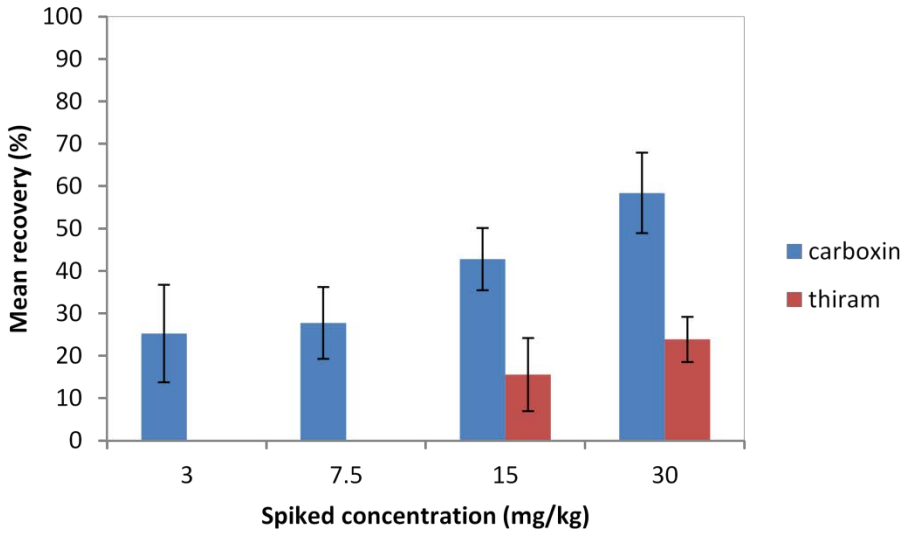


Figure A1. Recovery of carboxin and thiram from soil extracted using hexane:acetone (1:1)
Results are presented as a percentage of the spiked concentration and is presented as the mean±relative standard deviation (RSD) of three separate experiments (n=3)

Discussion

Extraction of carboxin and thiram using hexane:acetone (1:1) yielded low recovery of the compounds from soil. Therefore the method was deemed to be unsuitable for accurate quantification of carboxin and thiram from soil.

The method proposed by Gopal *et al.* (2006) was performed using product formulations and wheat seed. However this thesis uses soil samples. Therefore the low recovery found in the current experiment is likely due to compounds within the soil matrix interfering with extraction of carboxin and thiram from the soil.

APPENDIX II: Examples of standard curves generated in the MTT and crystal violet assays

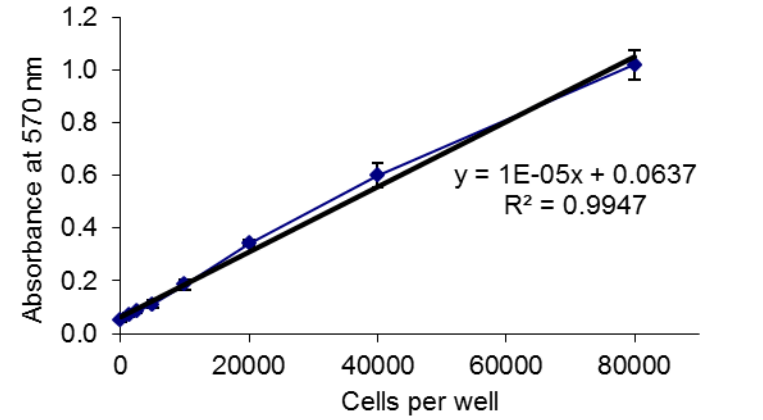


Figure A2. Example MTT assay standard curve

HepG2 cells were seeded at 0-80,000 cells per well. After a 24h adherence time, cells were incubated with 2.5 mg/ml MTT for 4h followed by 18h incubation with 20% SDS in 20mM HCl. The absorbance was measured at 570nm with reference absorbance 630nm. Results are shown as the mean ± SD of four replicate wells.

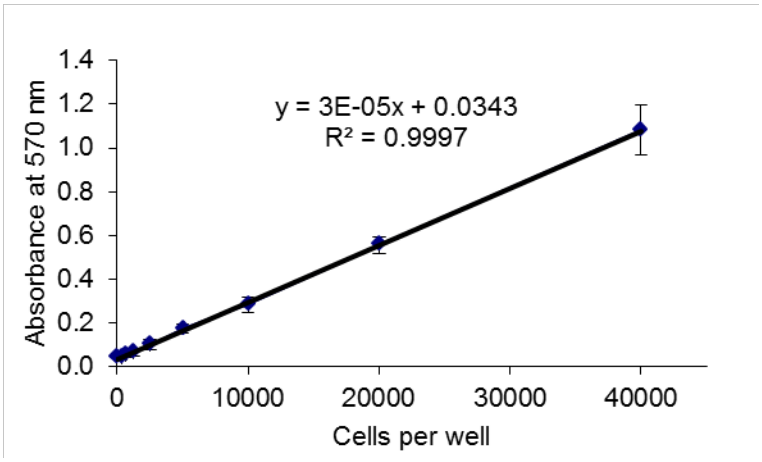


Figure A3. Example crystal violet assay standard curve

HaCaT cells were seeded at 0-40,000 cells per well. After a 24h adherence time, cells were stained using crystal violet for 10 min followed by destaining with 33% acetic acid for 10 min. The absorbance was measured at 570nm. Results are shown as the mean ± SD of six replicate wells.

APPENDIX III. Examples of flow cytometry histograms used for analysis of cell cycle

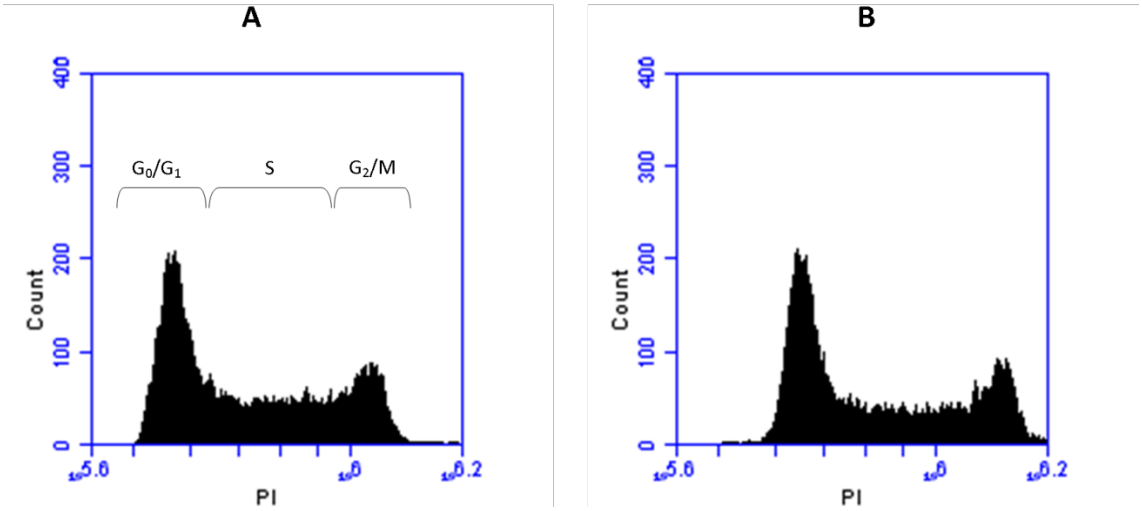


Figure A4. Examples of flow cytometry histograms used for analysis of cell cycle.

HaCaT cells were treated with (A) solvent control or (B) 5 mg/L thiram for 24h. Cells were then harvested and stained with PI and cellular DNA content analysed using flow cytometry (section 2.12). Cell cycle distribution of cells in G_0/G_1, S or G_2/M phases is indicated for the solvent control.

APPENDIX IV. Examples of flow cytometry histograms used for analysis of apoptosis

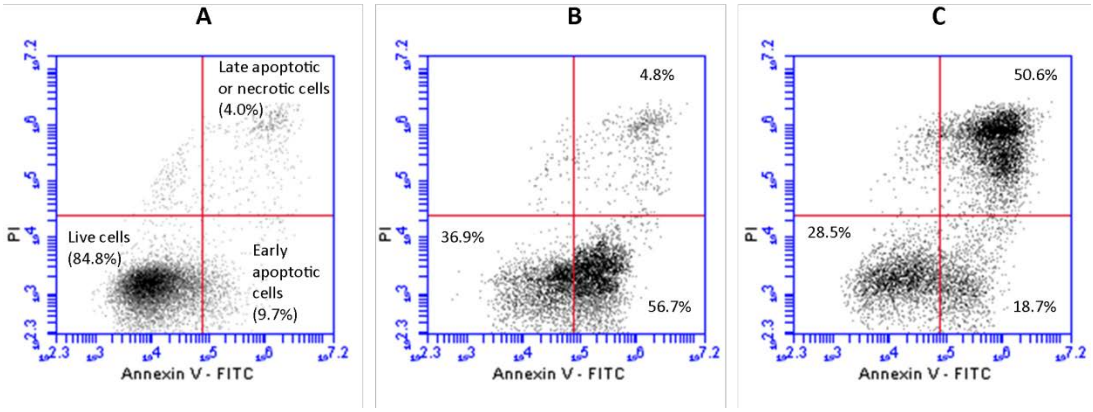


Figure A5. Examples of flow cytometry histograms used for analysis of apoptosis.

WIL2NS cells were treated with (A) solvent control, (B) 50 mg/L carboxin or (C) 0.1 mg/L thiram for 24h. Cells were then harvested and stained using Annexin V-FITC and PI (section 2.13). Live cells are negative for both Annexin V-FITC and PI (lower left quadrant); early apoptotic cells are positive for Annexin V-FITC but negative for P (lower right quadrant); late apoptotic or necrotic cells are positive for both Annexin V-FITC and PI (upper right quadrant). The percentage of cells in each quadrant is indicated on each histogram.

APPENDIX V: Calculations for determining the predicted environmental concentration (PEC) of Vitavax 200FF

Calculations for determining the PEC of Vitavax 200FF in soil were based the assumptions shown in Table A1.

Table A1. Assumptions used for determining the PEC of Vitavax 200FF.
Sources: (Hazelton *et al.*, 2007, Department of Environment and Primary Industries, 2012)

Vitavax 200FF active ingredients	200g/L each of carboxin and thiram
Recommended application rate	500ml per 100kg of wheat seed (equivalent to 1 mg each of carboxin and thiram per g seed)
Germination rate	90%
Recommended wheat crop density	200 plants/m^2
Seed weight per 100 seeds	4.5g
Sowing depth	25mm
Soil density	1.3 g/cm^3 (loamy soil)

Appendix V

The method for calculating the PEC of Vitavax 200FF in soil is outlined in parts A-D below:

Part A. Weight of wheat seed required to achieve 200 plants per m^2:

$$= seed\ weight\ per\ 100\ seeds\ x\ 200\ plants\ x\ (\frac{1}{germination\ rate\ x\ 100})$$

$$= 10\ g$$

Part B. Weight of soil in the top 25mm of a $1m^2$ plot:

$$= 1m^2\ x\ 25mm\ x\ soil\ density$$

$$= 32500\ g$$

Part C. Amount of carboxin and thiram present in the weight of wheat seed required to achieve 200 plants per m^2 (Part A):

$$= 10g\ x\ 1mg/g$$

$$= 10mg\ each\ of\ carboxin\ and\ thiram$$

Part D. Concentration of carboxin and thiram in soil after sowing:

$$= 10mg\ in\ 32500g\ soil$$

$$= 0.3\ mg/kg$$

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