E

January, August & Final Reports

REPORTS

Part 1 - Summary Details

Please use your TAB key to complete part 1 & 2.

CRDC Project Number: **CSE77C**

January Report:	☐	Due 29-Jan-01
August Report:	☐	Due 03-Aug-01
Final Report:	☒	Due within 3 months of project completion

Project Title: Bioremediation Enzymes for Endosulfan

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Research Program: Insect Management

Part 2 – Contact Details

Administrator: Mr Mark Hardwick, Finance Manager
Organisation: CSIRO Entomology
Postal Address: GPO Box 1700, Canberra ACT 2601
Ph: 02 6246 4008 **Fx:** 02 6246 4095 **E-mail:** mark.hardwick@ento.csiro.au

Principal Researcher: Dr John Oakeshott
Organisation: CSIRO Entomology
Postal Address: GPO Box 1700, Canberra ACT 2601
Ph: 02 6246 4157 **Fx:** 02 6246 4173 **E-mail:** john.oakeshott@ento.csiro.au

Supervisor: Dr John Oakeshott
Organisation: CSIRO Entomology
Postal Address: GPO Box 1700, Canberra ACT 2601
Ph: 02 6246 4157 **Fx:** 02 6246 4173 **E-mail:** john.oakeshott@ento.csiro.au

Researcher 2 Dr Ivan Kennedy
Organisation: University of Sydney
Postal Address: Department of Agricultural Chemistry and Soil Science, University of Sydney, NSW 2006
Ph: 02 9351 3546 **Fx:** 02 9351 5108 **E-mail:** i.kennedy@acss.usyd.edu.au

Signature of Research Provider Representative: _____

Part 3 – Final Report Format

The points below are to be used as a guideline when completing your final report.

1. Outline the background to the project.

The chlorinated cyclic sulfite diester, endosulfan (Thiodan®, bicyclo-[2.2.1]-2-heptene-5,6-bisoxymethylene sulfite), is a broad-spectrum insecticide that has been used extensively for over 30 years on a variety of crops. Endosulfan is often classified as a cyclodiene and has the same primary action and target site as other cyclodienes (Casida, 1993). However, it has significantly different chemical and physical properties from other cyclodiene insecticides that affect both its environmental and biological fates. In particular, endosulfan has a relatively reactive cyclic sulfite diester group (Van Woerden, 1963) and, as a consequence, its environmental persistence is lower than that of other cyclodienes, albeit still higher than many other insecticides. Since the deregistration in many countries of most cyclodiene insecticides, the ongoing availability of endosulfan has become important as an alternative option in resistance management strategies of pest species. Additionally, compared to many other available insecticides, it has low toxicity to many species of beneficial insects, mites and spiders (Goebel *et al.*, 1982). However, endosulfan is extremely toxic to fish and aquatic invertebrates, and it has been implicated increasingly in mammalian gonadal toxicity (Singh and Pandey, 1990; Sinha *et al.*, 1995; Sinha *et al.*, 1997; Turner *et al.*, 1997), genotoxicity (Chaudhuri *et al.*, 1999) and neurotoxicity (Paul and Balasubramaniam, 1997). These environmental and health concerns have led to an interest in post-application detoxification of the insecticide.

Enzymatic detoxification of pesticides is receiving serious attention as an alternative to existing methods of toxic waste remediation, such as incineration and landfill. In particular, enzymatic insecticide bioremediation has been the focus of extensive study following the isolation of a phosphotriesterase capable of detoxifying a range of organophosphate compounds from several bacterial species (for review see Dave *et al.*, 1993 and references within). Indeed, the OpdA OP hydrolysing enzyme of Horne *et al.* (submitted for publication) has recently been shown to be effective in the decontamination of irrigation run-off water from a cotton field (M. Selleck, personal communication). An essential initial step in the investigation of an enzymatic method for endosulfan detoxification is the definitive identification of a biological source of endosulfan degrading activity.

Microorganisms have increasingly been investigated as sources of xenobiotic-degrading enzymes. Numerous studies have described the degradation of endosulfan in microbial cultures. However, the susceptibility of endosulfan to alkali hydrolysis at pH $\geq$7.0 makes it difficult to estimate the contribution of enzymes to this degradation as distinct from from chemical hydrolysis, since growth of the microbes often increases the alkalinity of the growth medium.

The aim of the research supported by this grant was to isolate an endosulfan-degrading gene as a potential source of an enzymatic bioremediating agent to treat endosulfan contaminated waste water. Using endosulfan as the only available sulfur source, we enriched soil inocula for microorganisms capable of releasing the sulfur from endosulfan, thereby providing a source of sulfur for growth. Since removal of the sulfur moiety dramatically decreases the vertebrate toxicity of endosulfan (Dorough *et al.*, 1978; Goebel *et al.*, 1982), this results in concurrent detoxification of the insecticide. From the soil culture with endosulfan-degrading activity we isolated a pure bacterium that demonstrated

activity. We isolated a gene encoding an endosulfan-degrading protein from the bacterium using novel molecular biological techniques.

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Dorough, H. W., K. Huhtanen, T. C. Marshall, and H. E. Bryant. 1978. Fate of endosulfan in rats and toxicological considerations of apolar metabolites. Pest. Biochem. Physiol. **8**:241-252.
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Singh, S. K., and R. S. Pandey. 1990. Effect of subchronic endosulfan on plasma gonadotropins, testosterone, testicular testosterone and enzymes of androgen biosynthesis. Ind. J. Expt. Biol. **28**:953-956.
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Sinha, N., R. Narayan, and D. K. Saxena. 1997. Effect of endosulfan on testis of growing rats. Bull. Environ. Contam. Toxicol. **58**:79-86.
Turner, K. O., M. Syvanen, and S. Meizel. 1997. The human acrosome reaction is highly sensitive to inhibition by cyclodiene insecticides. J. Androl. **18**:571-575.
Van Woerden, H. F. 1963. Organic Sulfites. Chem. Rev. **63**:557-571.

2. List the project objectives and the extent to which these have been achieved.

Objective 1.1: Compare individual strains of Anabaena (and possibly other microorganisms) for endosulfan degrading ability (University of Sydney).

Initial studies demonstrated that the endosulfan degradation reported in *Anabaena* was a result of increases in the pH of the growth medium (University of Sydney).

Enrichment cultures were initiated to isolate soil microorganisms capable of degrading endosulfan. From these enrichment cultures a bacterium was isolated that could degrade endosulfan. An assay system was developed to ensure degradative activity was enzymatic rather than chemical (CSIRO Entomology).

Objective 1.2: Determine biochemical pathways for endosulfan degradation in Anabaena strains from 1.1. (University of Sydney).

Not determined for *Anabaena* for reasons stated above (University of Sydney). As a result, attention was directed to white rot fungi *(Phanerocheate chrysosporium)* instead.

Objective 1.3: Establish molecular protocols and adapt Kennedy's assay system for use in the expression cloning of Anabaena genes and directed evolution of prototype hydrolase and Anabaena genes with endosulfan degrading potential (CSIRO Entomology and University of Sydney).

Not determined for *Anabaena* for reasons stated above (University of Sydney).

Prototype hydrolases were tested for their ability to hydrolyse or bind endosulfan. These included naturally occuring and synthetic mutants of esterase E3 from the sheep blowfly, various *Drosophila* esterases, dienelactone hydrolase, an *Archaebacterial* thermostable esterase and a sulfatase. None of these showed measurable binding or activity (CSIRO Entomology).

Given the results discussed above showing that the endosulfan hydrolytic activity of *Anabaena* strain was chemical rather than biochemical in nature, the major project objectives were revised in the Annual Report Survey of 6 August 1999.

Revised Objective 1.4: Characterise crude-enzyme kinetics of bacterial enzyme(s) responsible for endosulfan / endosulfan sulfate degradation in strains from Objective 1.1, and continue the isolation of endosulfan / endosulfan sulfate degrading soil microorganisms should the first round of hydrolytic enzymes prove not to be useful for endosulfan bioremediation (CSIRO Entomology).

Biochemical pathway analysis of endosulfan degradation by the soil bacterium isolated in 1.1 above revealed that endosulfan was hydrolysed to endosulfan monoaldehyde (ES-M) a product predicted to be non-toxic (CSIRO Entomology).

Revised Objective 1.5: Clone individual bacterial genes encoding enzymes with endosulfan degrading activity from the most promising strains from Objective 1.1 and 1.4 (CSIRO Entomology).

This objective was achieved (see 4 and 5 below; CSIRO Entomology).

Revised Objective 1.6: Characterise the endosulfan hydrolysing activities of white rot fungal strains, and if any of the activities are biological, determine the biochemical pathway(s) involved in the hydrolysis (University of Sydney).

This objective was achieved (see 4 and 5 below; University of Sydney).

Revised Objective 1.7: Develop an immunoassay for the detection of endosulfan diol (University of Sydney).

This objective was re-defined as low priority and therefore not met (see 4 below; University of Sydney).

Revised Objective 3.1: Construct a genomic cosmid or plasmid library of the bacterial strain with endosulfan hydrolytic activity in appropriate shuttle vectors and using appropriate host strains, screen for endosulfan hydrolytic activity and, if successful, optimise expression of the enzyme (CSIRO Entomology).

This objective was achieved (see 4 and 5 below; CSIRO Entomology).

Revised Objective 3.2: *Survey gene databases for genes similar to that encoding ESD, and analyse homologous genes using molecular techniques (CSIRO Entomology).*

This objective was achieved (see 4 and 5 below; CSIRO Entomology).

Revised Objective 3.3: *Characterise the endosulfan degradative pathways identified in the three white rot fungal strains from Objective 1.6, focussing on the activity of cell-free extracts and the purification of protein(s) with enzymatic activity (University of Sydney).*

This objective was achieved in part in that activity was obtained in hyphal homogenates, but insufficient amounts of material meant that the protein(s) responsible for the activity could not be purified (see 4 and 5 below; University of Sydney).

3. How has your research addressed the Corporations three outputs: Sustainability, profitability and international competitiveness, and/or people and community?

The goal of cleaning up endosulfan residues is important for all three of the Corporation's outputs: 1) because it improves the quality of waste waters, 2) because it lessens non-target residue problems, which in turn helps retain registration of cheap and efficacious insecticides and 3) because it should lead to overall reduction in residues in the environment of local communities.

4. Detail the methodology and justify the methodology used.

A) Isolation of an endosulfan-degrading bacterium

Soil. As soil contaminated with xenobiotics has frequently been used as a source of pesticide-degrading organisms, we investigated endosulfan contaminated soil as a source of endosulfan-degrading organisms. The soil used in this study was collected from a cotton field near Narrabri, New South Wales, at the end of the growing season. The field had generally received several applications of endosulfan in the summer months for at least the previous five years. The soil was fertile grey clay at pH 7.5. Top soil was collected from the first 15cm, air dried and stored at 4°C for up to one month prior to enrichment.

Chemicals. Technical grade endosulfan (99% pure) for bacterial growth was a gift from Hoechst Schering AgrEvo Pty Ltd. Technical-grade endosulfan (used commercially) is a mixture of two diastereoisomers: *alpha*-endosulfan and *beta*-endosulfan in a ratio of 7:3, respectively. With the exception of endosulfan diacetate, insecticide and metabolite standards (at least 99% pure) were purchased from Chem. Services Inc. (PA, USA). Endosulfan diacetate was synthesised by peracetylation of endosulfan diol with acetic anhydride in dry pyridine at 80°C for 1 hour and purified by silica chromatography. The *O*-benzyl oxime of endosulfan monoaldehyde was prepared by the reaction of the putative

aldehyde (recovered by TLC on alumina) with a fivefold excess of benzylhydroxylamine hydrochloride (Alltech, NSW, Australia) in dry pyridine at room temperature for 8 hours. All other chemicals used were at least of reagent grade.

Thin layer chromatography (TLC). A TLC method was developed to measure rates of endosulfan-degradation and to measure formation of endosulfan metabolites. Cultures were extracted with equal volumes of ethyl acetate. The organic phase was passed through a 6 cm $MgSO_4$ column in a Pasteur pipette stoppered with glass wool to remove any residual water, gently evaporated under a dry nitrogen stream, dissolved in acetone and then applied to neutral aluminium oxide F_{254} TLC plates (Alltech, NSW, Australia). The plates were developed in either 4:1 petroleum ether-acetone or 3:1 chloroform-ethyl acetate. R*f* values for endosulfan and its metabolites in these solvent systems are given in Table 1. The aqueous phase was reduced to dryness by rotary evaporation and the resultant residue extracted with dichloromethane (DCM) to recover any hydrophilic metabolites. The DCM-soluble products were spotted onto TLC plates as above and developed in methanol. Chlorine containing constituents were visualised by spraying plates with silver nitrate-saturated methanol then exposing them to UV light. The lower limit of detection of this method for endosulfan and metabolites containing the hexachlorinated ring structure was 0.1 µg (data not shown). As detection is based on formation of silver chloride, dechlorinated metabolites will have a detection limit relative to the level of dechlorination.

Isolation of soil bacteria. Endosulfan is a poor biological energy source as it contains only six potential reducing electrons; indeed, previous attempts to enrich for endosulfan-degrading microorganisms using the insecticide as a carbon source have all been unsuccessful (Martens, 1972; Guerin, 1999). However, endosulfan has a relatively reactive cyclic sulfite diester group (Van Woerden, 1963). In this study microorganisms were therefore selected for their ability to release the sulfite group from endosulfan and use this as a source of sulfur for growth. The enrichment strategy also addressed two potential problems in selecting microorganisms for their ability to degrade endosulfan: endosulfan is virtually insoluble in water and spontaneously hydrolyses at alkaline pH.

A study into the distribution of endosulfan in sterile microbial broth showed that it concentrated at the glass/medium interface. Therefore we included Tween 80 (a mix of oleic, linoleic, palmitic and stearic acids) in the enrichment broth to emulsify the endosulfan (Guerin and Kennedy, 1992), thereby increasing the amount of insecticide in contact with the soil bacteria. This detergent has previously been used to solubilize pyrethroids during the isolation of microorganisms capable of metabolising permethrin (Maloney *et al.,* 1988).

Endosulfan is susceptible to alkaline hydrolysis (Martens, 1976), with approximately ten-fold increases in hydrolysis occurring with each increase in pH unit. Many previous studies have been unable to differentiate between chemical and biological hydrolysis of endosulfan because microbial growth has led to increases in the alkalinity of the culture medium (Martens, 1976; Miles and Moy, 1979). To minimise non-biological hydrolysis the enrichment medium was buffered to pH 6.6 and cultures were monitored constantly to ensure that growth did not decrease hydrogen ion concentrations. We observed detectable levels of endodiol (>0.1ppm) in sterile media inoculated with 50µM endosulfan at pH 7.2 after four days, and ensured that the pH of the medium for biodegradation studies of endosulfan remained below pH 7.0.

Soil (approx. 15 g) was first enriched for endosulfan-degrading organisms by the addition of 2 mg technical-grade endosulfan in 100 µl acetone to remoistened soil, followed by incubation in the dark at room temperature for 1 month. Further enrichment was then achieved by initiating shake flask enrichment cultures from these samples using endosulfan as the only added source of sulfur. The enrichment medium (pH 6.6-6.8) consisted of 20 mg technical grade endosulfan (99% pure), 0.05% Tween 80, 2.0 g KH_2PO_4, 7.5 g K_2HPO_4, 1.0 g NH_4Cl, 0.5 g NaCl, 1.0 g glucose, 0.1 g $MgCl_2$, 0.86 mg *p*-amino benzoic acid, 0.86 mg nicotinic acid and 10 ml of a trace element solution per litre. The stock trace element solution contained 20 mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 50 mg H_3BO_3, 30 mg $ZnCl_2$, 3 mg $CoCl_2.6H_2O$, 10 mg $(CH_3COO)_2Cu.H_2O$, and 20 mg $FeCl_2.6H_2O$ per litre. According to impurity data provided by Sigma Chemical Co.(NSW, Australia) the maximum limit of sulfite/sulfate contamination in the enrichment medium was less than 0.4×10^{-3} g l^{-1}. Approximately one gram of endosulfan enriched soil was inoculated into 50 ml enrichment media and cultured in a 400ml Erlenmeyer flask on a rotary shaker (200 rpm) at 28°C for up to 14 days. Substrate levels were measured using thin layer chromatography (TLC) and when approximately 50% of the endosulfan had degraded relative to sterile controls, 5 ml of the culture was transferred into 50 ml fresh enrichment medium. Ten different soil samples were enriched for endosulfan-degrading activity. An endosulfan-degrading culture was obtained from only one of these samples. After approximately six transfers into enrichment media cultures were transferred into sulfur-free media (see below) for further enrichment.

A 'sulfur-free' medium was also designed because contaminating sulfur in the enrichment medium could promote culture growth, resulting in increases in optical density at 595 nm (OD_{595}) of the culture from 0.05 to 0.3. A second soil culture was initiated for the sole purpose of preparing medium free of contaminating sulfur. Sulfur-free medium was prepared by growing the second soil culture overnight in enrichment medium without endosulfan then removing cells by centrifugation and filtering the supernatant through a 0.22 µm pore filter. After inoculation of this medium with either the endosulfan-degrading culture or *Escherichia coli* strain TG1 no growth was observed until the addition of a source of sulfur. After the addition of either 50 µM sodium sulfite or magnesium sulfate both the endosulfan-degrading culture and *Escherichia coli* strain TG1 culture were able to grow to at least an OD_{595} of 0.8. The sterility of the sulfur-free medium was confirmed by the absence of growth when aliquots were incubated on rich media agar plates.

Using this method of enrichment we were able to isolate a Mycobacterial species, *Mycobacterium* strain ESD, that was capable of degrading endosulfan to form the novel product, endosulfan monoaldehyde (Sutherland *et al.*, 2000; Sutherland *et al.* 2001).

Gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) analysis. GC and GC/MS were used to characterise endosulfan metabolites. As endosulfan and its chlorine-containing metabolites are strongly electronegative, previous studies have employed electron-capture GC (GC-ECD) for detection of these compounds. As we demonstrate in the present study, flame ionisation detection (FID) can replace this if preliminary steps are used to recover the metabolites selectively. In addition, FID enables the use of DCM for clean and efficient solvent extraction.

Cultures (15 ml) were extracted with DCM (10 ml) and the organic phase dried with $MgSO_4$, as described above. The solution of endosulfan and its lipophilic metabolites was diluted with hexane to yield a 20% hexane-DCM solution, which was applied to a 5 cm silica column (DCC silica gel, 63-200, Aldrich) within a Pasteur pipette. The column was flushed

with a further 3 ml of 20% hexane-DCM. Control experiments demonstrated that endosulfan hydroxyether and endodiol were the only metabolites retained by the silica under these conditions. Endosulfan diacetate (50 µg) was added as an internal standard to the combined eluate and washings, which were then concentrated to 25 µl under a gentle stream of nitrogen before storage at -20°C and subsequent GC analysis using FID.

The more polar metabolites, endosulfan hydroxyether and endodiol, were subsequently eluted from the silica column with 10% methanol-DCM (8 ml). Endosulfate (40 µg) was added as the internal standard and the recovered solution was evaporated to near dryness under a gentle stream of nitrogen. The residue was taken up in DCM (10 µl) and bis(trimethylsilyl) trifluoroacetamide (BSTFA, 25 µl) was added with initial vortex mixing to silylate the metabolites (6 hours, room temperature) before storage at -20°C and GC analysis.

The addition of the internal standards to the fractions enabled both qualitative assessment of the metabolites from their relative retention times by GC and also quantitative evaluation of the metabolic pathways. Losses from volatilisation and extraction efficiencies ranged from 15-40% (depending on length of time incubated, media composition and compound) and were calculated by comparison with stocks of known concentration. GC was performed using a Varian model 3300 with a cool on-column injector, a flame ionisation detector and a computer with data acquisition and processing software. The capillary column was 5% phenyl methylsilicone (SE54, Alltech Econocap, 30 m x 0.32 mm ID, 0.25 µm film thickness) with a helium flow rate of 2 ml min^{-1}. The column was preceded by a retention gap of deactivated silica (2 m) to preserve the integrity of the column. A typical temperature program for analysis of the endosulfan metabolites comprised an initial period after injection of 2 min at 40°C, temperature gradient of 20°C min^{-1} to 200°C for 10 min, followed by a temperature gradient of 10°C min^{-1} to 300°C.

The identities of the known metabolites in the fractions were confirmed by GC/MS using a VG Trio 2000 mass spectrometer interfaced to a Hewlett Packard 5890 gas chromatograph (cool on-column injector), with VG MassLynx software for control and data acquisition. The GC column was 5% phenyl methyl silicone (SE54, Alltech Econocap, 30 m x 0.32 mm ID, 0.5 µm film thickness) with a helium flow rate of 1 ml min^{-1}. Ionisation modes used for mass spectrometry of the metabolites were either electron ionisation (EI, 70 eV) or positive-ion chemical ionisation (PCI, ammonia reagent gas, source pressure 60 Pa). The molecular and fragment ions were generally represented by peak distributions over several masses because their respective chlorine compositions included the additional natural isotope ^{37}Cl.

B) Cloning of the endosulfan-degrading gene from* Mycobacterium *strain ESD

Initial attempts at cloning the endosulfan-degrading gene using *E. coli*-based traditional cloning methods were unsuccessful. This was later attributed to an inability of the *E. coli* translational mechanisms to recognise the endogenous promotor preceeding the endosulfan-degrading gene. Therefore a novel cloning strategy was designed to screen for activity in a related *Mycobacterium* strain, *Mycobacterium smegmatis*.

Isolation of genomic DNA. Genomic DNA was extracted from *Mycobacterium* strain ESD using a method adapted from Anderberg *et al.*, 1995. Cells were grown until turbid in LB supplemented with 0.05% Tween 80, then pelleted and resuspended in 1 ml TE plus 200 $\mu g.ml^{-1}$ proteinase K and 10 $mg.ml^{-1}$ lysozyme. After 1 hr incubation at 37°C cells were

pelleted and resuspended in 750 µl 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl. Glass beads (450-600 microns, Sigma, St Louise, MO) were added and the cells and beads vigorously vortexed for ten minutes. The beads were settled by a brief centrifugal pulse, then the supernatant was removed and extracted with phenol/chloroform twice. The DNA was then precipitated with ethanol, resuspended in TE and stored at –20 °C.

Construction of cosmid library. A *Mycobacterium* strain ESD genomic library was constructed in the *Mycobacterium/E. coli* cosmid shuttle vector, pYUB415 as follows. Genomic DNA of *Mycobacterium* strain ESD was partially digested with Sau3A restriction endonuclease and then separated by gel electorphoresis in a 1% low melting point agarose gel. An agarose gel slice containing DNA fragments of 30 to 45 kb was melted and DNA was in-gel ligated to BamH1 digested, calf intestinal alkaline phosphatase treated, pYUB415. After ligation, GELase Agarose Gel-Digesting enzyme preparation (Epicenter Technologies, Madison, WI) was added according to manufactures instructions. The ligated DNA was packaged into lambda using MaxPlax Packaging Extracts and used to infect freshly prepared *E. coli* strain EP1305 cells according to manufactures instructions (Epicenter Technologies, Madison, WI). *E. coli* containing pYUB415 were grown in the presence of 100 $\mu g.ml^{-1}$ ampicillan and *M. smegmatis* in 100 $\mu g.ml^{-1}$ hygromycin.

Screening of cosmid clones and subsequent sub-clones. Cosmid DNA was isolated from 2 ml cultures of individual library clones using alkaline lysis (Sambrook *et al.*, 1989), purified using the QIAquick system (Qiagen, Victoria, Australia), then electroporated into freshly prepared *M. smegmatis* (prepared according to Jacobs *et al.*, 1991). Concentrations of 5-500 pg DNA in 1µl distilled H_20 were incubated with 50µl prepared cells for 1 min on ice then transferred to a cuvette with a 0.2 cm gap (Bio-Rad, NSW, Australia). This was exposed to one electrical pulse at 2500 V, 25 µF with resistance set at 1000 ohms. Electroporated cells were incubated for 3 hrs in LBT then plated onto LBagar containing 100 $\mu g.ml^{-1}$ hygromycin and incubated at 28 °C for four days. Colonies from each electroporation were then combined, washed with sulfur-free media and used to inoculate SFM containing 100 $\mu g.ml^{-1}$ hygromycin and 50 µM *beta*-endosulfan. After 7 days at 28 °C and 180 $rev.min^{-1}$ the culture was analysed for endosulfan degradation activity by TLC.

Martens, R. 1972. Der Abbau von Endosulfan durch Mikroorganismen des Bodens. Schriftener. Vers. Wasser-boden-Lufthyg., Berlin-Dahlem, Heft 37, 167 pp.

Martens, R. 1976. Degradation of [8,9-^{14}C] endosulfan by soil microorganisms. Appl. Environ. Microbiol. **6**:853-858.

Miles, J. R. W., and P. Moy. 1979. Degradation of endosulfan and its metabolites by a mixed culture of soil microorganisms. Bull. Environ. Contam. Toxicol. **23**:13-19.

Sambrook, J.E.F. Fritsch and T. Maniatis. 1989. Molecular cloning – A laboratory Manual. 2nd Ed. Cold Spring Harbour Laboratory Press, USA.

Van Woerden, H. F. 1963. Organic Sulfites. Chem. Rev. **63**:557-571.

C) Characterisation of the endosulfan hydrolysing activities of white rot fungal strains

As a result of endosulfan degradation in *Anabaena* being shown to be largely chemical degradation as a result of elevation of the pH of the medium, *Phanaerochaete chrysosporium*, the white rot fungus, which has been shown to degrade and mineralize a wide variety of industrial and agricultural environmental pollutants (Joshi and Gold, 1993) was investigated instead. For some time, the white rot fungi basidiomycete *Phanerocheate chrysosporium*

has been used as an agent for liquid effluent and soil bioremediation. The extended biodegradation properties of the fungus towards many environmentally persistent chemicals (Higson, 19991; Shah *et al*, 1992; Steven, 1990) have been credited to its lignin degrading system (LDS), which includes mainly extracellular lignin peroxidases (LiPs) and manganese dependant peroxidases. The enzymes are produced under substrate-limiting growth conditions, and they are not induced by pollutants.

The metabolic pathway utilized by *P. chrysosporium* for the biodegradation of endosulfan has been worked out to a certain extant (Kullman and Matsumura, 1996).

Chemicals. All pesticides and metabolites were purchased from Riedel-de-Haën (technical-grade, 99% pure). The pesticides and metabolites studied include: α-endosulfan, β-Endosulfan, endosulfan sulfate, endosulfan diol, endosulfan ether, endosulfan hydroxy ether, endosulfan lactone. The solvents; acetone and hexane used in the extraction and analysis, were purchased from Mallinckrodt. All solvents used were of the highest analytical grade (nanopure) and were employed without further purification.

Culture conditions for growth of fungus. Cultures of white rot fungi were maintained on agar slants containing 1% glucose, 1% malt, 0.2% yeast extract, 0.1% aspargine, 0.2% KH_2PO_4, 0.1% $MgSO_4.7H_2O$ and 1mg of thiamine per liter and stored at 4^0C. The growth conditions used for white rot fungi were adopted from literature (Kirk et al., 1975, 1978; Ulmer et al., 1983). The fungus was maintained on 2% malt agar slants. Aqueous suspensions of fungal conidia were used for inoculation of the liquid growth medium. The basal growth medium described (Kirk et al., 1978) was used in the experiments. The basal medium was supplemented with 56mM D-Glucose and 0.6mM each of NH_4NO_3 and L-aspargine as nitrogen sources, as well as 20mM of 2,2-dimethylsuccinate, with pH adjusted to 4.5 by adding aqueous NaOH. The buffered glucose solution was autoclaved. Basal medium and nitrogen sources were filter sterilized (Whatman, 0.22μm filter).

Phanerocheate chrysosporium ATCC 24725 was grown from a conidial inoculum as stationary cultures in 90-mm-diameter polystyrene petri dishes and incubated in a moist atmosphere at 30^0C. Approximately 10 mL of sterile medium was pipetted onto the petri plates and spores were dislodged by means of a sterile glass spreader. Suspensions were removed from the surface of the plates by sterile pipette and filtered through sterile pads of glass wool to remove mycelial debris. The suspension was diluted using one-quarter strength of the basal medium and the absorbance was adjusted to 0.5 at 650nm, equivalent to 2.5 X 10^6 spores/mL (Kirk *et al.* 1978).

Each culture contained 3mL of either nitrogen deficient (2.4 mM N) or nitrogen rich (24 mM N) medium in 20 mL glass vials. The contents of the medium were used as described by Kirk *et al.* (1978), unless otherwise stated, with 1% glucose as the carbon source and asparagine and ammonium nitrate as nitrogen sources. Carbon-deficient medium contained 24 mM nitrogen and 0.1% glucose as a carbon source. The media were buffered with 10 mM sodium 2,2-dimethyl succinate (pH 4.5). Heat killed controls were prepared by autoclaving cultures which had been pregrown in 250 mL conical flasks or 3mL vials and incubated for the same period of time as indicated above. Hyphal homogenates were prepared by extensive grinding of 5 day cultures with glass homogenisers.

Degradation studies. After five days of preliminary incubation, each culture (3 mL) received 30 μL of α-endosulfan from a stock of 100ppm; 13.6 μL of β-endosulfan from a stock of

220ppm and 20 µL of endosulfan sulfate from a stock of 150ppm (all stock solutions were prepared in hexane) to achieve a final concentration of 1ppm. The controls were autoclaved prior to the addition of the pesticides. All the cultures were incubated at 30^{0}C for various intervals of time up to 5 days after the addition of the pesticide. Following the incubation, cultures were collected at various time intervals and stored at -20^{0}C until extracted.

Analyses. The spiked cultures, which were incubated and stored were acidified with two drops of concentrated HCl and homogenized with a Teflon homogenizer. The homogenized material was then extracted thrice with 3mL of acetone-hexane (20-80 [vol/vol]) and the material was vortexed thoroughly for 1min each and centrifuged at 2000X *g* for 3 minutes. The supernatants were then pooled together and re-extracted with hexane only by ratovapor at 40^{0}C. The samples so obtained were made upto 5mL and were quantified using GC-MS directly.

Quantitative analysis of endosulfan and its metabolites was conducted by gas chromatography performed on HP5890 series II (5971 series with mass selective detector) equipped with an electron capture detector (ECD) and an auto-sampler (HP 7673). Separation was done on a column, DB-4 Supelco capillary column (0.32mm [inside diameter] by 30m [length]: 0.25 µm film thickness). Nitrogen was used as both carrier gas and make-up gas. The oven temperature was programmed for an initial temperature of 55^0 C and raised to 250^0 C at 10^0 C/min. Injector and detector temperatures were 250^0 C and 280^0 C respectively. The resulting information was analysed by HP Chemstation software.

Aust, S.D. 1990. Degradation of environmental pollutants by *Phanerocheate chrysosporium*. Microb. Ecol. 20: 197-209.

Higson, F.K., 1991. Degradation of xenobiotics by white rot fungi. Rev. Environ. Cont. Toxicol. **122**: 111.52

Joshi, D. K. and M. H. Gold. 1993. Degradation of 2,4,5-trichlorophenol by the lignin-degrading basidiomycete *Phanerochaete chrysosporium* . Appl Environ Microbiol. **59**:1779-1785.

Kirk, T. K., Schultz, E., Conners, W.J., Lorenz, L. F. and Zeikus, J. G. 1978. Influence of culture parameters on lignin metabolism by *Phanaerochaete chrysosporium*. Arch Microbiol. **117**:277-285.

Kullman, S. W. and Matsumura, F. 1996. Metabolic pathways utilized by *Phanerochaete chrysosporium* for degradation of the cyclodiene pesticide endosulfan. Appl Environ Microbiol **62**:593-600

Shah, M. M., Bare, D. P., Chung, N. and Aust, S. D., 1992. Use of white rot fungi in the degradation of environmental chemicals. Toxicol. Lett., 64/65:493-501.

Steven, D. Aust., 1990. Degradation of Environmental Pollutants by *Phanerocheate chrysosporium*. Microb. Ecol. 20: 197-209.

Sutherland, T.D., Horne, I., Lacey, M.J., Harcourt, R.L., Russell, R.J. and Oakeshott, J.G. 2000. Enrichment of an endosulfan- degrading mixed bacterial culture. Appl Environ Microbiol **66**: 2822-2828.

Ulmer, D. C.; Leisola, M. S. A.; Schmidt, B. H.; Fiechter, A. Appl. Environ. Microbiol. 1983. 45, 1795-1801.

D) Antibody for endosulfan diol

Although attempts were made to prepare an antibody to endosulfan diol using a specially designed hapten for this purpose, these were not successful. With limited resources, it was not judged of sufficiently high priority to persist with this revised objective (1.7).

5. Detail results including the statistical analysis of results.

A) Isolation of endosulfan-degrading bacterium.

After approximately six rounds of successive sub-culturing in enrichment media followed by four rounds in sulfur-free media, TLC analysis and optical density measurements of the enrichment culture confirmed substantial disappearance of endosulfan with a simultaneous increase in bacterial mass. The culture was incapable of growth in sulfur-free medium without the addition of a sulfur source. Addition of either *alpha-*, *beta-* or technical grade endosulfan promoted growth to varying degrees. No significant growth was seen in the absence of endosulfan and endosulfate could not substitute for endosulfan as a utilisable source of sulfur.

A pure bacterial culture capable of degrading endosulfan was obtained from the mixed culture after six months successive subculture (approximately 30 rounds with 1% inoculum) followed by three rounds of subculturing using very dilute inocula (1% to 0.001% late-log phase culture). In each of the latter three rounds, the most dilute inoculum to subsequently exhibit growth also demonstrated endosulfan metabolism and was used as the starting culture for another round of dilution subculturing. Plating of the final culture onto sulfur-free agar media with endosulfan, or tryptic soy agar media, gave rise to slow growing translucent colonies that became easily visible after 3-4 days and reached 3 mm diameter after 6 days. Broth cultures of individual colonies degraded endosulfan by a hydrolytic pathway to produce the putative monoaldehyde product and endosulfan hydroxyether, and an oxidative pathway to produce endosulfate as described in Sutherland *et al.* (2000) for the parental mixed culture. This isolate was named strain ESD (Endosulfan Degrading).

Analysis of the 16S rDNA gene sequence of strain ESD revealed it to be within the genus *Mycobacterium*, most similar (98.3%) to *Mycobacterium* strain LB501T which was described in a study of bacteria degrading polycyclic hydrocarbons (GenBank accession number AJ245702). A distance neighbour joining tree was constructed based on the comparison of related 16S rDNA sequences available on GenBank, which showed strain ESD clustered with the fast growing Mycobacteria. This subgroup includes many other *Mycobacterium* species that have demonstrated xenobiotic-degrading activities.

B) Characterisation of endosulfan metabolites.

TLC and GC analysis indicated the disappearance of both diastereomers of endosulfan and the concomitant formation of endosulfan metabolites. The known metabolites of endosulfan are not diastereomeric. Three metabolites were identified as endosulfan hydroxyether, endosulfate and endodiol on the basis of co-migration with authentic standards on TLC plates developed in different solvent systems, coincident retention times on GC (Table 1), and structural confirmation by GC/MS (data not shown). A single additional metabolite, with mobility on TLC similar to that of endosulfate, was also detected. Mass spectral analysis (70 eV EI) of the compound after purification by TLC indicated a molecular ion of *m/z* 342 ($^{35}Cl_6$), isomeric with that of endosulfan ether. The fragmentation pattern was also similar to that obtained with endosulfan ether, except for the absence of a prominent fragment ion of *m/z* 69 derived from the pentacyclic ether moiety. An analogous ion of *m/z* 85 was observed in the 70 eV EI mass spectrum of endosulfan hydroxyether. Thus the molecular structure of the novel isomer does not include a pentacyclic ether ring.

The positive-ion chemical ionisation mass spectrum (PCI(NH_3)) of the novel metabolite displayed the molecular parent ions $[M+H]^+$ and $[M+NH_4]^+$ of *m/z* 341 and *m/z* 358, respectively, confirming the molecular mass ($^{35}Cl_6$) indicated previously in the EI mass spectrum (Figure 2). The preliminary evidence indicated that the molecular structure of the novel isomer was that of endosulfan monoaldehyde. The PCI mass spectrum of the metabolite also displayed fragment ions indicating consecutive losses of two molecules of HCl from $[M+H]^+$ ions. Since the most probable site for gas-phase proton attachment in the putative structure would be the carbonyl oxygen atom, the initial HCl loss may be rationalised as elimination of the reagent proton together with the vicinal bridgehead chlorine atom *via* a favoured six-centred transition structure.

Support for the structure of the novel metabolite is provided by the observation that it forms an *O*-benzyl oxime derivative. Although the expected molecular ion is absent in its 70eV EI mass spectrum, an $[M - CH_3]^+$ ion of *m/z* 430 is present, indicating a relative molecular mass M of 445 ($^{35}Cl_6$) for the derivative and substantiating a monoaldehyde structure for the metabolite.

C) Isolation and characterisation of the endosulfan-degrading gene.

<u>*Cloning of the gene.*</u> The host *Mycobacteria* (*M. smegmatis* strain mc^2) and *E. coli* (strain TG1) used in screening the *Mycobacterium* strain ESD cosmid library could both grow in the SFM supplemented with either 50 µM magnesium sulfate or sodium sulfite. Neither species had detectable endosulfan hydrolysing activity. Three hundred and seventy clones from the *Mycobacterium* strain ESD cosmid library were screened in *M. smegmatis,* from which only one demonstrated endosulfan-degrading activity (Cosmid 172). When this clone was electroporated into *E. coli* strain TG1 it did not grow in SFM containing endosulfan, nor have detectable endosulfan-degrading activity in MLB containing ampicillan and endosulfan.

DNA fragments generated by various restriction nuclease digests of cosmid 172 were cloned into pYUB415 and screened for endosulfan-degrading activity in *M. smegmatis.* Fragments with activity were further subcloned and screened in the same manner until the DNA fragment containing activity was reduced to a 3.0kb *Apa*I DNA fragment (pYUB415::*Apa*3; Figure 1).

Nucleotide sequence analysis and homology to other proteins. DNA sequence analysis of the 3.0 kb ApaI fragment revealed that there were two ORFs, which were transcribed in the same orientation. The restriction map of this fragment is shown in Figure 1. The deduced amino acid sequence of the two ORFs were compared with sequences of other proteins in the SwissProt and SpTrEMBL database. The first ORF (ORF1), encoding a protein of 448 amino acids, had significant homology to several other proteins (Figure 2). The highest identity (50%) was to *tds*A, a thermophilic flavomonoxygenase of *Paenibacillus* sp. A11-2 that catalyses the conversion of dibenzothiophene-5-5-dioxide to 2-(2'-hydroxyphenyl) benzene sulphinate (Ishii *et al.*, 2000). ORF1 also had significant homology (46% identity) to *dsz*A (formerly *sox*A), the *tds*A homolog in *Rhodococcus* strain IGTS8 (Denome *et al.*, 1994), and (38% identity) to component A of a nitrilotriacetate monooxygenase of *Chelatobacter heintzii* ATCC 29600 (Knobel *et al.*, 1996).

The second ORF (ORF2) had low homology (21% identity) to d*sz*C (formerly *sox*C) of *Rhodococcus* strain IGTS8 (Denome *et al.*, 1994). The *dsz*C gene is part of the same operon as *dsz*A and its product catalyses the conversion of dibenzothiophene to dibenzothiophene-5-5-dioxide to 2-(2'-hydroxyphenyl) benzene sulphinate

The endosulfan degrading activity of *Mycobacterium* strain ESD is completely inhibited in the presence in of low concentrations of sulfate. We also observed this repression when the endosulfan-degrading activity was expressed in *M. smegmatis* under the control of its native promotor. Analysis of pYUB415::*Apa*3 revealed that there was insufficient space upstream from ORF2 to contain a promotor (X nucleotides), whereas ORF1 was preceded by a region apparently containing extensive secondary structure identified because of difficulties obtaining DNA sequence across this region. This suggested thet ORF1 was most probably preceded by a promotor and was therefore the most likely candidate to be responsible for the endosulfan-degrading phenotype. ORF1 was amplified by PCR and cloned behind the mycobacterial heat shock promotor of pMV261. This construct (designated pMV261::*esd*) transferred the endosulfan-degrading phenotype to *M. smegmatis* without the repression by sulfate observed with pYUB415::*Apa*3 confirming that ORF1 encoded an endosulfan-degrading gene.

Denome, S.A., Oldfield, C., Nash, L.J. and Young, K.D. 1994. Characterisation of the desulfurization genes from *Rhodococcus* sp. Strain IGTSG. J. Bacteriol. 176:6707-6716.

Knobel, H-R., Egli, T. and van der Meer, J. R. 1996. Cloning and characterisation of the genes encoding nitrilotriacetate monooxygenase of Chelatobacter heintzii ATCC 29600. J. Bacteriol. 178:6123-6132.

Ishii Y, Ohshiro, T., Aoi, Y., Suzuki, M. and Izumi, Y. 2000. Identification of the gene encoding a NAD(P)H-flavin oxioreductase coupling with dibenzothiophene (DBT)-desulfurizing enzymes from the DBT-nondesulfurizing bacterium *Paenibacillus polymyxa* A-1. J. Biosci. Bioeng. 90:220-222.

D) Degradation of endosulfan by whole cultures of Phanerocheate chrysosporium ATCC 2472.

Although white rot fungi were capable of being cultured successfully using the methods given from spore cultures, it proved difficult to obtain quantitative data regarding specific

rates of endosulfan degradation. Because of the slow rate of growth, it is not possible to monitor increases in the biomass in cultures with reliability. The protein content of mycelia is also extremely small and accurate measures of increasing protein content cannot be obtained. This seems to have been a general experience, since previously published work also simply uses a standard protocol for growth of cultures from spores for comparative studies, without giving quantitative data of the amount of cell material such as protein content or specific activities. Under these circumstances, while it is possible to obtain clear-cut evidence of endosulfan breakdown in individual experiments, with appropriate controls, it is difficult to compare the results from one experiment to the next because of variation in the viability of spore cultures used for inoculation.

Endosulfan was confirmed to be degraded by *Phanerocheate chrysosporium* ATCC 24725. Degradation was determined by monitoring the disappearance of endosulfan in cultures up to 11 days, using GC-ECD detection of extracts. In general, nearly all of the degradation occurred during the first 5 days with the activity declining after the first day. Because the pH of the medium was maintained below 7, all of disappearance could be attributed to biodegradation rather than chemical hydrolysis possible at increasingly alkaline pH as observed with *Anabaena.*

Significant degradation occurred in all three media used, whether N-deficient, C-deficient or a rich medium with no significant differences between the rates of disappearance. The biological nature of the degradation was confirmed by heat-killed controls of fungus, which retained approximately 98% of the substrate indicating that there was very little substrate degraded chemically or volatilized.

Using β-endosulfan as the substrate the amount of degradation / disappearance was observed as follows: in N-deficient medium: 46%, in C-deficient medium: 4% and in full medium: 64%, indicating that the degradation of β-endosulfan was more in the full medium. Using endosulfan sulfate as the substrate irrespective of the medium, the amount of degradation was found to be relatively slow over a longer period, as indicated by the following percentages loss of the original concentration in the respective media: N-deficient: 13%, C-deficient: 13% and full medium: 12% .

Metabolism of endosulfan . GC analysis of the acetone-hexane extracts indicated the formation of the following metabolites: endosulfan diol (major metabolite), endosulfan ether, endosulfan hydroxyether, endosulfan lactone. The amounts and types of metabolites formed varied with each substrate.

In hyphal homogenates, where individual hyphae were no longer obvious by microscopy, the rates of degradation observed with alpha, beta endosulfan and with endosulfan sulfate as the substrates in full medium were at least as great or even greater than with intact hyphae, with the following breakdown over a 12 hours time period: α-endosulfan degradation 77% (0%); β-endosulfan degradation 98% (30%); and endosulfan sulfate 15% (13%). The amounts of substrates lost as volatilized or as chemical degradation is shown in the brackets for the respective substrate systems. Although a significant proportion of the enzymic breakdown appeared in the supernatant from centrifugation at 10,000xg for 10 min on some occasions, most of the activity appeared to remain associated with the fraction sedimenting at 10,000xg, probably broken membranes. Despite numerous attempts to solubilise the endosulfan-degrading activity using various agents such as the detergents Tween, sodium cholate or sodium deoxycholate, this could not be achieved. Apparently, the

activity requires factors that remain firmly associated with the membranous materials. The difficulty in obtaining enough material during the course of experiments to yield more than trace amounts of protein obviously exacerbated this problem.

6. Discuss the results, and include an analysis of research outcomes compared with objectives.

Chlorinated aromatic pesticides generally are mineralised very slowly, or not at all, in the environment. This recalcitrance has lead to the banning of the use of most of these compounds throughout the world. An exception to this is endosulfan, which differs from other chlorinated aromatic compounds by the presence of a relatively reactive sulfite moiety that results in a considerably shorter environmental half-life. However, endosulfan is highly toxic to fish (Goebel *et al.*, 1982) and aquatic contamination due to run-off from arable soils is of considerable concern. Additionally, while endosulfan itself does not bioaccumulate (Goebel *et al.*, 1982), the toxic metabolite endosulfate can be stored in animal fat (Beck *et al.*, 1966; Maier-Bode, 1968; Dorough *et al.*, 1978). As a result, contamination of pastures due to incorrect application practices can lead to unacceptably high endosulfate residues in locally grown production animals. These concerns have led to growing interest in the bioremediation of the insecticide in the environment.

We have exploited the reactive nature of the sulfur group to isolate a strain of *Mycobacterium* (strain ESD) able to use this compound as a nutrient source, concurrently degrading the insecticide. Previous studies attempting the isolation of endosulfan-degrading microbes have been unsuccessful. Our study differs from previous studies by the application of strong selective pressure on the culture to release the sulfur moiety from the insecticide, allowing us to enrich for the degradative activity.

Initial attempts at isolating a gene with endosulfan-degrading activity from *Mycobacterium* strain ESD using *E. coli* cosmid or plasmid vectors were unsuccessful. Therefore we designed a novel cloning strategy in which we cloned the genes into a *Mycobacterium-E.coli* shuttle vector and screened for activity directly in a related *Mycobacterium* strain (*M. smegmatis)* that does not degrade endosulfan. Using this approach we isolated a 3kb DNA fragment that was able to confer the sulfate concentration-dependent endosulfan-degrading phenotype on *M. smegmatis*. Within this fragment was an ORF encoding a putative 448 amino acid protein (predicted to be 49.8 kDa) that, when expressed under the regulation of a heat shock promotor in *M. smegmatis*, resulted in sulfate concentration-independent endosulfan-degradation and expression of an approximately 50 kDa protein.

Analysis of the sequence of the endosulfan-degrading gene revealed it to be most similar to *dsz*A, a gene in the *dsz* operon that is involved in biodesulfurization of fossil fuels. The *dsz* operon has received considerable attention because it acts to remove sulfur from the hetrocyclic compound dibenzothiophene, the major sulfur containing compound in fossil

fuels, without the destruction of C-C bonds. Selective removal of sulfur is predicted to reduce the release of toxic sulfur dioxides into the environment and therefore reduce the levels of acid rain and air pollution without comprimising the fuel value of the fuel. Biodesulfurization is being intensively investigated to complement, or as an alternative to, hydrodesulfurization, the costly, energy-intensive current method for the removal of sulfur from fuels. The significance of this similarity is that we can draw on the research into commercialisation of the *dsz* genes for the development of an enzymatic product for endosulfan bioremediation for the cotton industry.

Unfortunately, work on *Anabaena* and white rot fungi conducted at the University of Sydney on naturally occurring enzymes for degradation of endosulfan has not contributed substantially to the outcome of this project in terms of new enzymic material suitable for gene cloning and the preparation of remediation enzymes. Information on the characteristics of the catalysis of endosulfan hydrolysis by white rot fungi has been achieved, but this work would need further development before it could be of use for production of bioremediation enzymes. By contrast the work conducted by Dr Tara Sutherland using specifically selected bacteria has been remarkably successful.

Beck, E.W., J.C. Johnson, D.B. Woodham, D.B. Leuck, L.H. Dawsey, J.E. Robbins, and M.C. Bowman. 1966. Residues of endosulfan in meat and milk of cattle fed treated forages. J. Econ. Entomolo. 59:1444-1450.
Dorough, H. W., K. Huhtanen, T. C. Marshall, and H. E. Bryant. 1978. Fate of endosulfan in rats and toxicological considerations of apolar metabolites. Pest. Biochem. Physiol. **8**:241-252.
Goebel, H., S. Gorbach, W. Knauf, R. H. Rimpau, and H. Huttenbach. 1982. Properties, effects, residues and analytics of the insecticide endosulfan. Residue Reviews. **83**:40-41.
Maier-Bode, H. 1968. Properties, effect, residues, and analytics of the insecticide endosulfan. Residue Rev. 22:1-44.

7. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. Where possible include a statement of the costs and potential benefits to the Australian cotton industry and future research needs.

The research supported by this grant has isolated an enzyme that could potentially be used to detoxify endosulfan residues in waste water. Our licencee, Orica Australia, has the development of bioremediating agents to treat pesticides in cotton waste water as a top priority, specifically the development of a single product containing enzymes that degrade organophosphate (OP) insecticides, endosulfan and endosulfan sulfate. Currently CSIRO Entomology has isolated an enzyme that degrades OP's, and large scale field trials using this enzyme demonstrated a 90% loss of OP residues in irrigation run-off after 10 minutes treatment. A bioremediation product for cotton requires the characterisation of the enzyme isolated by the project described here and the isolation and characterisation of an enzyme to degrade endosulfan sulfate.

The work with white rot fungi indicates this organism has enzymic activity for hydrolysis of endosulfan to endosulfan diol and other related metabolites at

significant levels. However, this is unlikely to find application as products containing enzymes.

8. Describe the project technology (eg. commercially significant developments, patents applied for or granted licenses etc).

The *esd* gene described in this report is the first description of a gene encoding an endosulfan-degrading enzyme and will form the basis of a patent application. A patent position will include the gene sequence (as described in this report), characterisation of cofactor requirements, field trial results, and kinetic analysis.

The work on white rot fungi has not yielded a commercially significant outcome.

9. Provide a technical summary of any other information developed as part of the research project. Include discoveries in methodology, equipment design, etc.

Not applicable

10. Detail a plan for the activities or other steps that may be taken;

(a) to further develop or to exploit the project technology.

The Orica Australia development plan requires the characterisation of both the *esd* enzyme and an equivalent enzyme for endosulfan sulfate, involving requirement of co-factors for activity, methods of delivery, and field trials. At CSIRO Entomology we will establish cell free assay systems to investigate the co-factor requirements for the *esd* enzyme. This work will determine the method of delivery of the *esd* product. Based on the results of this work Orica Australia will develop a prototype commercial product to be used in field trials.

CSIRO Entomology is involved in an Australian cotton CRC/CRDC funded project to isolate an endosulfan-sulfate degrading enzyme. This project is on target to isolate an enzyme in 2002. The enzyme will then be cloned and characterised biochemically, especially in relation to possible co-factor requirements.

Insufficient progress has been made in the work with white rot fungi to project application of the endosulfan-degrading activity found. However, further development may occur as described above in section 7.

(b) for the future presentation and dissemination of the project outcomes.

After the establishment of a patent position results will be published in scientific journals – see below.

11. List the publications arising from the research project.

1. Sutherland, TD, I Horne, MJ Lacey, RL Harcourt, RJ Russell, and JG Oakeshott. 2000. Enrichment of an endosulfan-degrading mixed bacterial culture. **Appl. Environ. Microbiol.** 66:2822-2828.

2. Sutherland, TD, KM Weir, MJ Lacey, I Horne, RJ Russell, and JG Oakeshott. Enrichment of a bacterial culture capable of degrading endosulfate, the toxic metabolite of endosulfan. Submitted to J. Appl. Micro. (Passed Internal CSIRO review)

Refereed Scientific publications, passed internal review but submission delayed until the establishment of a patent position

3. Sutherland, TD, I Horne, RL Harcourt, RJ Russell, and JG Oakeshott. 2000. Isolation of an endosulfan-degrading Mycobacterium species. Prepared for Appl. Environ. Microbiol. (Passed internal CSIRO review).

4. Sutherland, TD, I Horne, RJ Russell, and JG Oakeshott. Cloning and molecular characterisation of a gene involved in degradation of endosulfan. In preparation for Appl. Environ. Microbiol.

In preparation

5. Rao Srinivas, N.A. Lee and I.R. Kennedy. Biotransformation studies of endosulfan by white rot fungi.

12. Are changes to the Intellectual Property register required?

Patent position pending.

Part 4 – Final Report Plain English Summary

Provide a half to one page Plain English Summary of your research that is not commercial in confidence, and that can be published on the World Wide Web.

Endosulfan is a broad-spectrum insecticide that has been used extensively for over 30 years on a variety of crops. Endosulfan is often classified as a cyclodiene and has the same primary action and target site as other cyclodienes. However, it has significantly different chemical and physical properties from other cyclodiene insecticides that affect both its environmental and biological fates. In particular, endosulfan has a relatively reactive cyclic sulfite diester group and, as a consequence, its environmental persistence is lower than other cyclodienes, albeit still higher than many other insecticides. Since the deregistration in many countries of most cyclodiene insecticides, the ongoing availability of endosulfan has become important as an alternative option in resistance management strategies of pest species. Additionally, compared to many other available insecticides, it has low toxicity to many species of beneficial insects, mites and spiders. However, endosulfan is extremely toxic to fish and aquatic invertebrates, and it has been implicated increasingly in mammalian toxicity. These environmental and health concerns have led to an interest in post-application detoxification of the insecticide. Many of the residue problems with endosulfan could be avoided if water at risk could be quickly decontaminated on farms. This is feasible using the catalytic properties of enzymes.

The aim of this research was the isolation of an enzyme system capable of single step detoxification of endosulfan. Enzymatic detoxification of pesticides is receiving serious attention as an alternative to existing methods such as incineration and landfill. In particular, enzymatic insecticide bioremediation is the focus of extensive study after the isolation of a phosphotriesterase capable of detoxifying a range of organophosphate compounds, which our licencee, Orica Australia, has recently shown to be effective in the decontamination of irrigation run-off water from a cotton field (M. Selleck, personal communication).

Using endosulfan as the only available sulfur source, we enriched soil inocula for microorganisms capable of releasing the sulfur from endosulfan, thereby providing a source of sulfur for growth and concurrently detoxifying the insecticide. From the soil culture with endosulfan-degrading activity we isolated a bacterium that demonstrated the degrading activity and from this bacterium a gene encoding an enzyme responsible for this activity. We are currently undertaking a biochemical characterisation of the enzyme before transfer to our commercial partner for production and field trials. Related work using white rot fungi has shown lesss potential for exploitation of endosulfan-biodegrading enzymic activity found in this organism.