The Microbial Degradation of the DDT Metabolite Dichlorobenzophenone (DBP)

A thesis

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Abstract

Soil samples which had been previously exposed to chlorinated aromatics were screened for ability to degrade dichlorobenzophenone (DBP). Of the samples tested soil from the Dow Elanco Agricultural Farm and soil from the AgResearch Winchmore Research Station showed apparent degradative capabilities. Degradation was not sustainable in these soil samples and a time scale study showed DBP was stable in soil over a 24 week period. Samples from Winchmore were further used to establish enrichment cultures capable of degrading DBP and its non-chlorinated analogue benzophenone (BP) through selection pressure. BP proved to be readily degraded but DBP degradation was only achieved after fungal suppressants were used. Degradation of DBP was enhanced with the addition of yeast extract and sodium salicylate to the enrichment cultures. Degradation of DBP was confirmed by capillary gas chromatography and the detection of the metabolite *p*-chlorophenyl acetic acid by gas chromatography-mass spectroscopy and thin layer chromatography. The enrichment cultures established on DBP also extensively degraded BP, *p*-chlorobenzophenone and *p*-chlorobenzoic acid. When inoculated back into soil, the enrichment cultures degradative capabilities were significantly reduced due to competition from other organisms, availability of alternative carbon sources and the bioavailability of DBP due to binding to soil particles.

Two organisms capable of degrading BP as sole carbon source were isolated and identified as a *Rhodococcus* spp. and a *Streptomyces* spp. BP degradative capabilities were not maintained by these organisms and lost when subculturing on nutrient media Three presumptive DBP degraders were isolated and presumptively identified as two separate *Streptomyces* spp. and a *Pseudomonas vesicularis.* Of the three none were able to degrade DBP as sole carbon source in liquid culture although the *P. vesicularis* was able to co-metabolise DBP with the addition of yeast extract and sodium salicylate. Preliminary genetical studies of the *P. vesicularis* isolated were carried out.

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List of Abbreviations

B16	Border 16 soil, AgResearch Winchmore Research Station
BH	benzhydrol
BP	benzophenone
CBP	*p*-chloro-benzophenone
DBH	*p,p*-dichlorobenzhydrol
DBP	*p,p*-dichlorobenzophenone
DDA	bis(*p*-chlorophenyl)acetic acid
DDD	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane
DDE	1,1-bis(*p*-chlorophenyl)-2-dichloroethylene
DDM	bis(*p*-chlorophenyl)methane
DDT	1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane
DMSO	dimethyl sulfoxide
DPM	diphenylmethane
GC	gas chromatography
GCMS	gas chromatography / mass spectrometry
PCP	pentachlorophenol
PCPA	*p*-chlorophenylacetic acid
PSM	phosphate salts medium
Sal	sodium salicylate
SDS	sodium dodecyl sulfate
2,4,5-T	2,4,5-trichlorophenoxy acetic acid
TEM	transmission electron microscopy
TLC	thin layer chromatography
TSA	tryptic soy agar
TSB	tryptic soy broth
YE	yeast extract

Section 1:

Introduction

1.1 Chlorinated pesticides

Chlorinated pesticides have played an important role in chemical crop protection as well as in the prevention of human diseases. They combine high insecticidal effectiveness against a broad spectrum of insects with generally low mammalian toxicity and long lasting protection due to their chemical stability. Ultimately these compounds proved to be not only persistent but lipophilic and the development of trace analysis showed residues were persisting in all sectors of our environment including plant, animal and human tissues. Increased public concern over the occurrence of these residues and the possible effects on human health due to long term exposure has resulted in their use being banned in most industrialised countries.

The chlorinated pesticides are considered xenobiotics; no naturally occurring structural analogues are known to exist in nature. Consequently the chlorinated pesticides have only been present in the environment for the last 50 years or so from when they first came into use around the 1940's and are considered by many to be non-degradable. It is therefore, not surprising that residues from these recalcitrant compounds still persist in the environment. Microorganisms may not have already possessed the metabolic systems capable of dealing with these compounds and during the short time that microbes have been exposed to these compounds, may not have evolved the systems required to metabolise them. The fact that residues are still persisting in the environment over 20 years after being banned continues to question the dogma of "microbial infallibility". Are there chemicals that can not be degraded by microorganisms?. Such a

question can not be answered categorically however, the fact that a compound remains stable in the environment over time is not sufficient to prove it is non-biodegradable. It may simply be the environmental parameters present that are limiting the biodegradability of these so called non-degradable compounds.

For these compounds to be degraded, favourable environmental conditions such as temperature, pH, redox potential, nutrient balance, substrate concentration, bioavailability due to insolubility, absorption, competing polymerisation, complexing and binding reaction need to be established. These factors can all act singly or in combination to render an otherwise biodegradable compound recalcitrant. The environmental parameters that act upon the chlorinated pesticides are also obviously coupled to the presence of the appropriate microorganisms and their possible preferential utilisation of alternative substrates. Some of the environmental and biological causes of recalcitrance as mentioned above can be overcome by bioremediation. The majority of successful bioremediation efforts for xenobiotics identify and correct the environmental restraints that render a xenobiotic pollutant recalcitrant (Bartha 1986a).

Molecular features of the chlorinated pesticides add to their recalcitrant nature. The degree and position of chlorination often determines whether or not a compound is degraded (Boyle 1989). The chlorine atom is a large, bulky atom that has been shown to cause steric interference with the degradative activity of ring-cleaving enzymes. Chlorine atoms are strongly electronegative and will form stable bonds to the carbon ring due to delocalisation of the pi electrons (Sayler *et al.*, 1984).

Several other problems arise with chlorinated pesticides that can limit the mineralisation of the compound in question. Rarely were they applied in a pure chemical state and structural analogues of the compound along with additives and contaminants can hinder breakdown. This can be seen with pentachlorophenol (PCP) where contaminants include dioxins and dibenzofurans which are some of the most toxic compounds known (Rochkind-Dubinsky 1987). Additionally, any metabolite produced could be more recalcitrant than the parent compound. For example 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) is broken down into the metabolite 1,1-bis(*p*-chlorophenyl)-2-dichloroethylene (DDE) which is considered more recalcitrant than DDT (Bumpus *et al.,* 1993). Additionally a metabolite may be more toxic. Chloro-catechols are produced from the breakdown of chlorobenzoic acids, meta-cleavage is the principal route for cleavage of catechol, however meta-cleavage of chloro-catechols produces highly reactive "suicide inhibitors" that inactivate the ring cleaving enzymes (Rochkind and Dubinsky 1987).

Chlorinated pesticides are rarely used as sole energy and carbon sources for individual bacteria and when metabolism occurs it is usually via the process of co-metabolism. Co-metabolism is the transformation of a substance by a microorganism utilising another substance. The co-metabolised substance is not incorporated into an organism's biomass and the organism does not derive energy from the transformation of that substance (Atlas 1988). It is then possible that this somewhat reduced form has the potential to be reduced further by other unrelated organisms. It appears that these types of synergistic reactions are most likely to lead to complete mineralisation of the

chlorinated pesticide to CO_2 and H_2O while converting the organochlorine to its mineral state.

Often when degradation of a particular xenobiotic is demonstrated it can be attributed to plasmid encoded mechanisms. Several of these plasmids have been described (Table 1.1).

Table 1.1. Plasmids associated with xenobiotic catabolism. *

Plasmid	Degradative compound
pAC21	*p*-Chlorobiphenyl
pBS3	Dicofol
pKF1	*p*-Chlorobiphenyl
pJP2	2,4-Dichlorophenoxyacetic acid (2,4-D)
pJP4	2,4-D, 3-chlorobenzoate
pWR1	3-Chlrobenzoate
pAC25	3-Chlorobenzoate
pAC27	Chlorobenzoate
pAC31	3,5-Dichlorobenzoate
TOL	Xylene, toluene
SAL	Salicylate

* Adapted from Karns *et al.*, 1984.

It has been suggested that plasmids provide the genetic plasticity through which adaptive processes work. Therefore it is highly likely that any organism capable of degrading a chlorinated pesticide, which has only been in the environment for a relatively short time span, has acquired the genetical capabilities via plasmid transfer.
Under natural conditions, many plasmids are transmitted to new hosts by a process known as bacterial conjugation (for review see Willets and Wilkens 1984). Conjugative transfer of plasmids, encoding for degradation of xenobiotic compounds, to alternative

hosts will provide the new host with the degradative capabilities encoded for by the plasmid. The ability of these plasmids to be transferred conjugatively will depend on their compatibility grouping, promiscuity and whether or not they are indeed transmissible in the environment (Datta 1979).

1.2 DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane)

Probably the best known example of a chlorinated pesticide is DDT . This compound has been used in vast quantities to control numerous insect pests. The discovery of DDT's undesirable effects with regards to recalcitrance and bioaccumulation was quickly followed by public outcry for a safer, alternative insecticide. The application of DDT has been banned in most industrialised countries since 1972. Despite the ban on DDT, several insecticides containing the DDT metabolite dicofol were still in use and therefore DDT was still being applied to soils as a contaminant (Graham 1984). DDT and its residues are still detected in New Zealand soils and agricultural products (Morton, 1990). Due to its strong lipophilic nature, DDT can accumulate in both microoganisms and invertebrates and in these forms it can be transferred through the food chain (Chacko and Lockwood 1967).

Farm management practices have been shown to influence the levels of DDT and its residues in New Zealand soil (Boul *et al.*, 1994). In irrigated soils, levels of DDT residues are significantly reduced. Boul *et al.*, postulated that this is due to increased microbial degradation of DDT to 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD) in anaerobic microsites which may be more prevalent in irrigated soils. Anaerobic conditions favouring the formation of DDD also lead to the formation of dichlorobenzophenone (DBP) (Wedemeyer 1967, Guenzi & Beard 1967, Pfaender & Alexander 1972).

1.2.1 Non-biological degradation of DDT

DDT is considered to be very stable in the soil environment (Alexander 1965, Edwards 1966), however some non-biological transformation has been reported. Castro (1964) showed that under sterile conditions, reduced iron porphyrin complexes are oxidised by DDT with DDD being formed. Miskus *et al.,* (1965) also reported reductive dechlorination of DDT to form DDD using an aqueous solution of reduced porphyrins under anaerobic conditions. Glass (1972) reported that the iron redox system in water saturated soil was capable of degrading DDT to DDD with the rate of DDD formation dependent on the rate of ferrous iron formation. More recently, Prakash *et al.,* (1994) have shown that reactive oxygen species can be used to degrade both lindane and DDT. They used photosensitisers capable of generating reactive oxygen species by an energy or electron transfer reaction. A maximum degradation of 89% for DDT was achieved using the photodynamic agent benzophenone with solar irradiation. Benzophenone, the non-chlorinated analogue of DBP, is itself a proposed degradation product of DDT and is capable of generating hydroxy-radicals under ultra-violet (UV) radiation (290-320 nm). Unfortunately the product or products of DDT degradation were not reported by Prakash *et al.*, (1994).

Despite these reports the extent of non-biological degradation of DDT appears to be minimal with the only reported metabolite being DDD and as stated by Crosby (1969), many of the *in vitro* studies of pesticide degradation may involve both biological and

purely chemical processes with the interface between the processes almost impossible to determine.

1.2.2 Biological degradation of DDT

It is only through the study of microbes that the degradation of DDT and its analogues have been demonstrated, albeit to a very limited extent. Reviews concerning DDT degradation in microbial systems have been published by Johnsen (1976), Esaac and Matsumura (1980) and Rochkind-Dubinsky *et al.,* (1987).

For many years DDE and bis(*p*-chlorophenyl)acetic acid (DDA) were considered to be the only primary degradation metabolites of DDT from biological systems. In 1963 DDD was shown to be formed from DDT by Finley and Pillmore and has since been demonstrated to be the major metabolite formed. Initial degradation of DDT typically occurs by way of three metabolic routes, all involving attack on the aliphatic portion of the compound as shown in Fig 1.2.1.

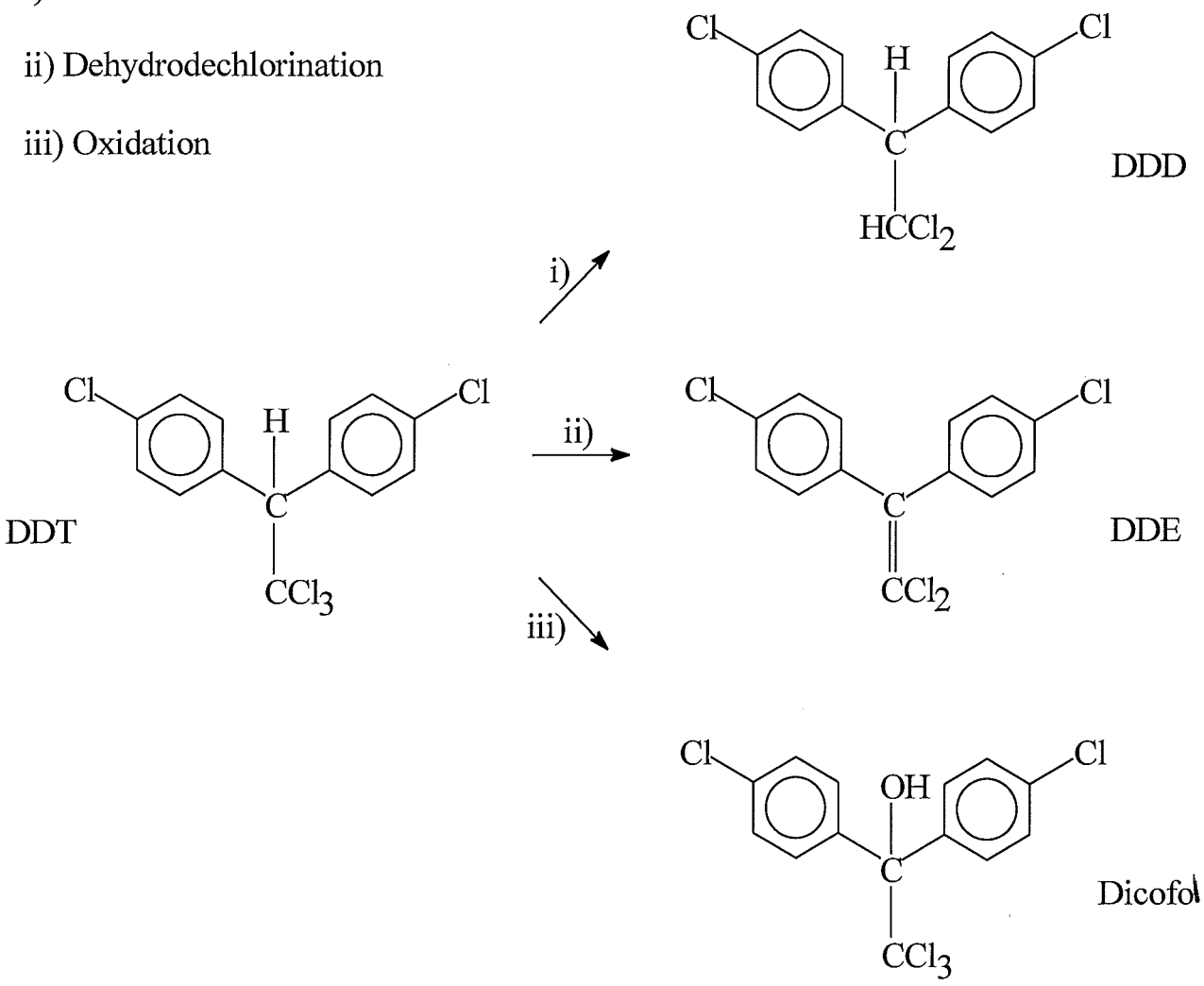
An exception to these three routes was reported by Nadeau *et al.,* (1994) where degradation of DDT by *Alcaligenes eutrophus* A5 was achieved by direct attack on one of the aromatic rings of DDT under aerobic conditions. Nadeau *et al.,* suggested that hydroxylation occurred at both the *ortho* and *meta* positions of the aromatic ring, which subsequently undergoes ring fission resulting in *p*-chlorobenzoic acid as the major stable metabolite formed.

Figure 1.2.1. Three major routes of DDT degradation.

i) Reductive dechlorination

ii) Dehydrodechlorination

iii) Oxidation



The formation of DDE which usually occurs under aerobic conditions has been termed a 'dead-end side reaction' as DDE apparently does not undergo further biological alteration (Johnsen 1976). However an exception to this was demonstrated by Bumpus et

al., (1993) when DDE was reportedly mineralised to CO_2 by the white rot fungus *Phanerochaete chrysosporium.* Small amounts of radiolabelled $^{14}CO_2$ were detected, in addition to the metabolites dicofol and dichlorobenzophenone (DBP).

Dicofol is not a commonly detected metabolite of DDT degradation. The production of dicofol requires the oxidation of DDT, which rarely occurs in microorganisms due principally to a lack of a defined mixed-function oxidase (Walker, 1991). A genetically engineered *Pseudomonas aeruginosa* BS827 containing the plasmid pBS3 (encoding naphthalene oxidation) (see Table 1.1), was found to significantly decrease dicofol levels in soil experiments (Golovleva *et al.*, 1988). One of the metabolites detected was DBP.

Wedemeyer (1966) showed that under anaerobic conditions DDD was clearly the major metabolite produced from the degradation of DDT by three facultatively anaerobic organisms, *Escherichia coli, Aerobacter aerogenes* and *Klebsiella pneumoniae*. *A. aerogenes* effected up to 80% conversion to DDD, although a 2-3% conversion to DDE always occurred. Further studies conducted by Wedemeyer on *K. pneumoniae* using metabolic inhibitors, detected several other metabolites and Wedemeyer proposed a metabolic pathway for the anaerobic transformation of DDT to DBP by way of the intermediary compounds DDD, 1-chloro-2,2-bis(*p*-chlorophenyl)ethylene (DDMU), 2,2-bis(*p*-chlorophenyl)ethylene (DDNU) and DDA (Fig 1.2.2).

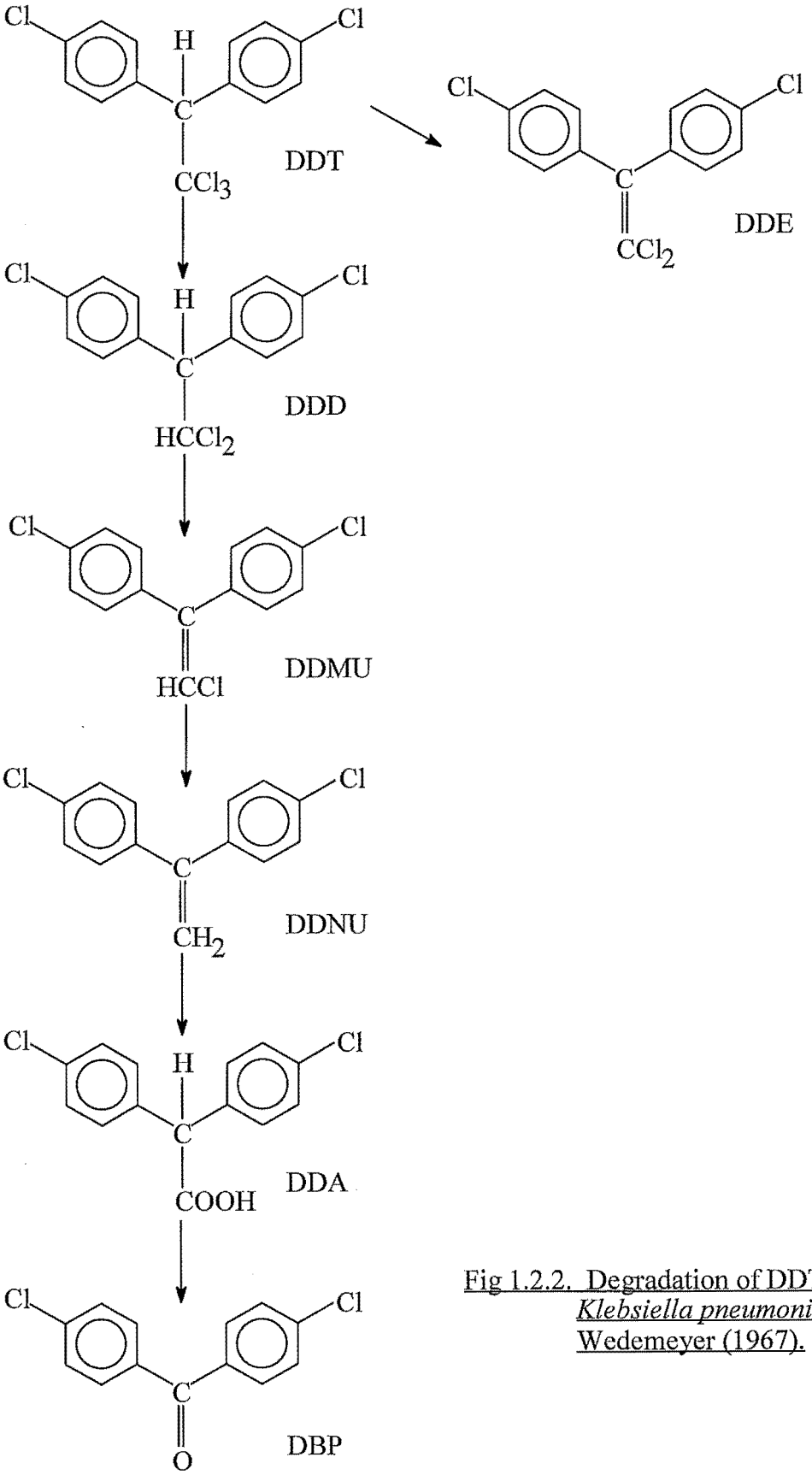


Fig 1.2.2. Degradation of DDT by *Klebsiella pneumoniae.* Wedemeyer (1967).

Guenzi and Beard (1967) reported large scale conversion of DDT to DDD using natural soil microflora under anaerobic conditions, recovering seven possible metabolites including DBP. DBP having one carbon removed from the aliphatic portion of the structure is considered the most degraded form. Pfaender and Alexander (1972) had similar results using a *Hydrogenomonas* sp. under anaerobic conditions, with DDD and DBP the main products formed. However they found that upon aeration a new metabolite,

p-chlorophenylacetic acid (PCPA), was formed. This compound has one of the aromatic rings cleaved and was at the time, the most degraded DDT metabolite detected. These results suggest that DDT can only be degraded to DBP under anaerobic conditions and for further degradation to occur aerobic conditions are required. Pfaender and Alexander also noted that DBP accumulated with cell-free systems, using only bacterial enzymes, as well as with microorganisms in model ecosystems. Further investigations into the fate of DBP in sewage were performed. DBP at a concentration of 40 ug/ml was added to raw sewage collected in autumn. Rapid disappearance was observed with total removal in four weeks. A repeat of the experiment using sewage collected in winter failed to show any reduction in DBP levels. This was possibly as a result of seasonal fluctuations in the sewage microflora.

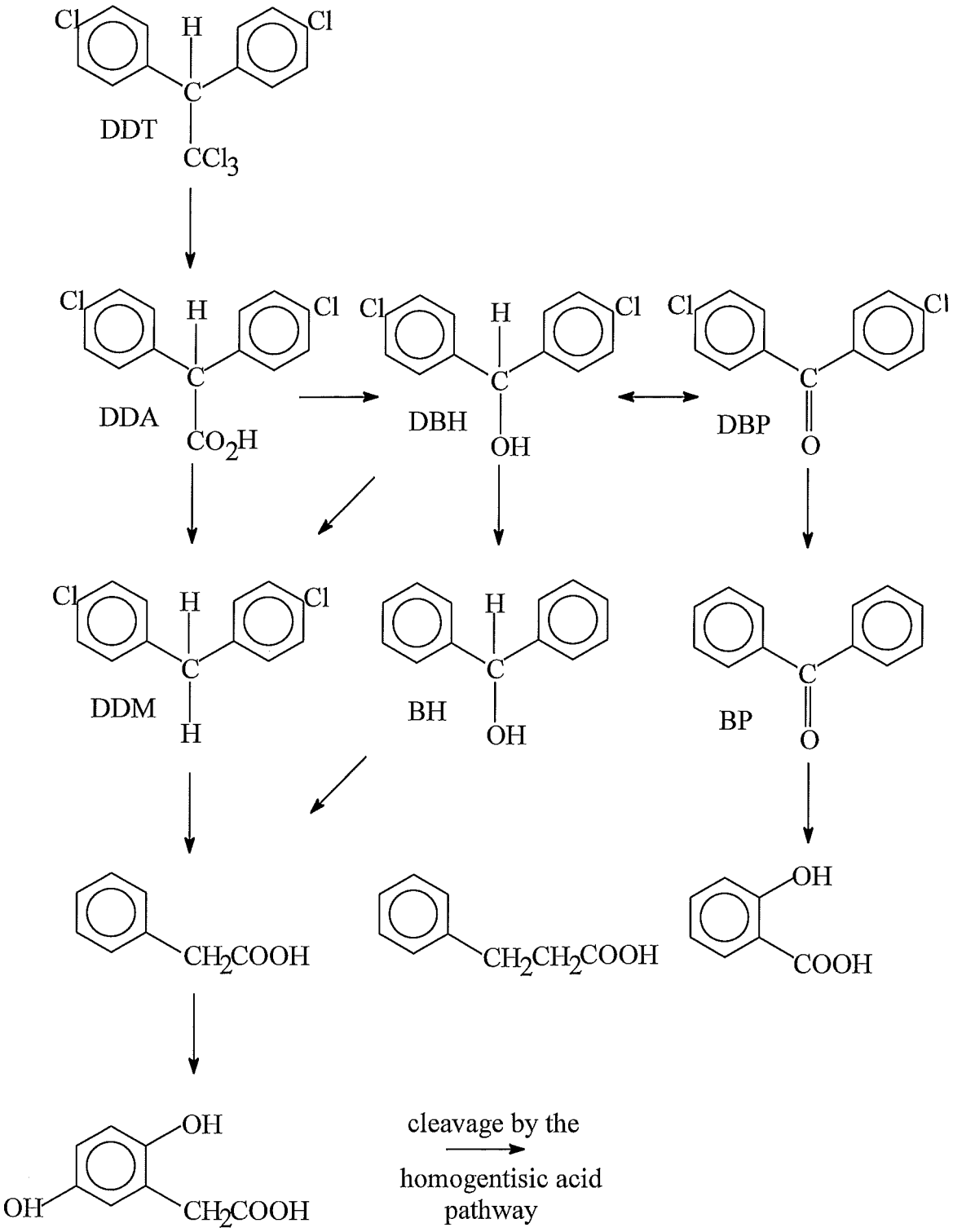
Pfaender and Alexander (1973) studied the effects of nutrient additions on the cometabolism of DDT in raw sewage. They confirmed that DDD, DDE and DBP were the major products formed from DDT breakdown, concluding that nutrients did not significantly alter overall DDT degradation. However, nutrient addition did alter the

concentrations of various metabolites formed; DDD formation was enhanced while DBP biosynthesis was reduced.

Juengst and Alexander (1976) isolated several microorganisms from sea water that were capable of converting between 5 and 10% of the DDT supplied, to water soluble products. One of these organisms, identified as *Mucor alternans,* generated several water soluble products that were partially identified and found to be different from known products of DDT metabolism. These products represented the most extensive degradation achieved by any single organism. However the metabolic products were not clearly identified and no degradation occurred using model marine ecosystems.

In 1981 Golovleva and Skryabin showed total degradation of DDT as a cometabolite with a single organism, *Pseudomonas aeruginosa* 640x. The degradation pathway is complex and a succession of different cosubstrates and aeration conditions are required for complete degradation of DDT to be achieved. *P. aeruginosa* 640x could not utilise DDT, DDD, DDE, DDA, *p,p*-dichlorobenzhydrol (DBH), DBP or bis(*p*-chlorophenyl)methane DDM as sole carbon source. Golovleva and Skryabin proposed a pathway for DDT degradation with three different routes for breakdown from DDA via DDM, DBH and DBP (Fig 1.2.3). Given the complexity of the reactions and conditions required, it is anticipated that under *in situ* conditions co-metabolic degradation of DDT will require initially anaerobic conditions to allow for dechlorination of the aliphatic fragment of the compound. It is then postulated that cleavage and mineralisation of the final products of anaerobic metabolism, such as DBP, can occur aerobically.

Fig 1.2.3. DDT degradation by *Pseudomonas aeruginosa* 640x.*



* Adapted from Golovleva and Skryabin (1981).

Several researchers concentrated their studies on the degradation of known DDT metabolites and their non-chlorinated analogues in an effort to determine the most probable route for complete DDT degradation. Focht and Alexander (1970a, 1970b, 1971) used a strain of *Hydrogenomonas* capable of growth on diphenylmethane (DPM) as sole carbon source to test the ability of this organism to degrade DDM, DBH and DBP and their mono-chlorinated and non-chlorinated analogues. They found that DBH and DDM were cometabolised by *Hydrogenomonas* while DBP was not transformed, although its mono and non-chlorinated analogues *p*-chlorobenzophenone (CBP) and benzophenone (BP) were cometabolised, in that partial oxidation of these compounds occurred without growth of the organism.

Subba-Rao and Alexander (1977a, 1977b) studied the degradation of DDM, DBH and DBP using *Pseudomonas putida.* This organism was capable of utilising DPM and benzhydrol (BH) as sole carbon source and produced similar results as found by Focht and Alexander (1970a, 1970b, 1971) with the transformation of DDM and DBH shown. In addition, Subba-Rao and Alexander also showed that DDM and DBH were converted to DBP. Other metabolites of DDM were identified as BP, BH and PCPA. BP, the non-chlorinated analogue of DBP, was formed from BH and not as a result of the dechlorination of DBP. No degradation of DBP was demonstrated.

Further work by Subba-Rao and Alexander (1985) using a range of different bacteria and fungi achieved conversion of DDT to DDD, DDE, DBH and DBP. Degradation of 35.2% of DBP, supplied to the fungus *Aspergillus niger,* was achieved in a single experiment but the same results could not be achieved after several repetitions of

the experiment. Products of DBP degradation were identified as CBP and a methylated chlorobenzophenone. This was the first report in which the products of DBP metabolism had been detected and identified but as the results were achieved in a single unrepeatable experiment, the validity of these results must be questioned.

1.3 Summary

As discussed previously DDT has three primary initial degradation routes which form the compounds DDD, DDE or Dicofol. DBP has been reported as a degradation product of all three compounds, but where it fits into the overall pathway of DDT degradation is unclear. It has been proposed that complete DDT degradation could occur via DBP by either dechlorination to produce BP or by direct ring cleavage. This pathway of degradation has yet to be elucidated. Proposed degradation pathways of DDT occurring via DBP include those proposed for the white rot fungus *Phanaerochaete chrysosporium* (Bumpus and Aust, 1987) and bacteria (Golovleva and Skryabin 1981, Wedemeyer 1966). Only Pfaender and Alexander (1972) and Subba-Rao and Alexander (1985) have demonstrated any degradation of DBP and in both cases the results could not be repeated. BP has yet to be identified as a breakdown product and CBP has been identified as a metabolite of DBP in only a single, unrepeatable experiment (Subba-Rao & Alexander 1985). It appears that extensive degradation of DDT may occur far more readily via the intermediates DBH and DDM as is proposed to occur by Golevleva and

Skyrabin (1981) (Fig 1.3). This is supported by the findings of Focht and Alexander (1970a, 1970b, 1971) and Subba-Rao and Alexander (1977) who demonstrated mineralisation of DBH and DDM while DBP remained untransformed.

DBP has been shown to be formed from DBH and DDM. These compounds are detected in much lower concentration than DBP suggesting that they are more readily degraded to compounds other than DBP. If complete degradation of DDT can occur via these two compounds, without the formation of DBP, overall rates of DDT and DDT metabolite degradation should be enhanced.

DBP does not show the levels of accumulation seen with DDE so either it is being degraded or alternatively the major pathway for DDT degradation does not involve DBP and the low levels produced are due to minor pathways. Golovleva and Skyrabin (1981) postulated an equilibrium-like conversion between DBP and DBH (Fig 1.2.3). DBP degradation may thus occur via DBH and BH, although this has yet to be demonstrated.

1.4 AIMS

Much research has been carried out on the initial degradation of DDT under anaerobic conditions and DBP has been clearly identified as being one of the main metabolites formed. It has been postulated the aerobic conditions are required for ring cleavage of DDT metabolites to occur. The aim of this research was to investigate the fate of DBP under aerobic conditions using soils with previous exposure to chlorinated compounds.

I aim to determine the ability of these soils to degrade DBP in their natural state and use them to develop a consortia of bacteria with enhanced degradative abilities against DBP and its non-chlorinated analogue BP through prolonged selective pressure in enrichment cultures. Degradation products of DBP are to be screened for in the hope of elucidating the pathway by which degradation of DBP occurs.

Bacteria exhibiting degradative capabilities against DBP or BP are to be isolated and studied for the mechanisms of degradation used

Section 2:

Materials and Methods

2.1 Soil Slurries

2.1.1 Biodegradation of dichlorobenzophenone in soil slurries

Aerobic enrichment cultures were established in Shuckla's (1986) phosphate salts medium (PSM) which contains solution A (per litre) Na_2PO_4-4.26g, KH_2PO_4-2.65g and NH_4NO_3-1g, solution B (per 100 ml) $MgSO_4.7H_2O$-2g, $MnSO_4.H_2O$-0.2g, $FeSO_4.7H_2O$-0.5g, $CaCl_2$-0.2g and $Na_2MoO_4.2H_2O$-0.01g. Solution B had the pH adjusted to 3 and was filter sterilised. 1 ml of solution B is added to 99 ml solution A. Sources of inocula were samples from sites known to be contaminated with chlorinated aromatics, these included sludge from the Tasman Pulp and Paper waste treatment pond (Kawerau), soil from the Dow Elanco Agricultural Farm (New Plymouth) known to be contaminated with 2,4,5-T (Bhamidimarri *et al.*, 1990), and soil from the AgResearch Winchmore Research Station (Mid-Canterbury) known to contain DDT (Boul *et al.*, 1994). The site at Winchmore is organised into borders for flood irrigation, separated by mounded soil levees. Soil samples were taken from three of these borders, borders 6, 9 and 16.

Cultures established from the soil samples were maintained by monthly subculture of 5 ml into 95 ml fresh PSM in 250 ml conical flasks enriched with 0.1 mM dichlorobenzophenone (DBP) (Aldrich 99% purity) dissolved in acetone (BDH). Flasks were incubated at 30°C in a rotary incubator at 200 revolutions per minute (rpm) to maintain aerobic conditions.

After one month incubation, each subculture was chemically analysed for DBP content by capillary gas chromatography (GC). With the addition of an internal standard, added immediately prior to extraction of flasks containing a test compound it is possible to quantitatively determine the amount of the test compound extracted. Comparing the peak area ratios generated by GC analysis of the test compound and the internal standard in control flasks (both constants), to the ratios generated in the experimental flasks where the internal standard is still a constant, the concentration of the test compound remaining can be calculated. Diphenylmethane (DPM) (Aldrich, 99+% purity) was chosen as the internal standard to use due to structural similarities with DBP and when the two compounds are analysed by GC they both have clearly distinct peak retention times from each other. DBP standards of varying concentrations were prepared and extracted with 0.2 mM DPM added as the internal standard to confirm the DBP concentration could be calculated against controls.

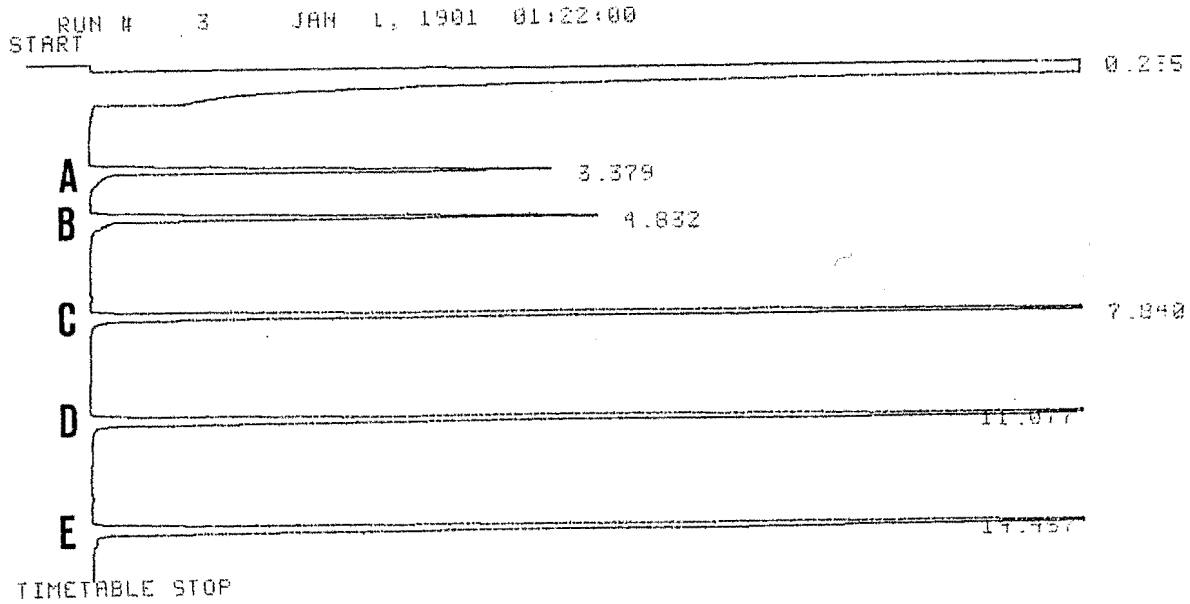
Prior to extraction the soil slurries were acidified to pH 1 with 1 ml concentrated HCL (cHCL) to lyse the cells and 0.2 mM DPM was added as an internal standard. The cultures were extracted three times with 20 ml diethyl ether (BDH) in a 500 ml separatory funnel and the pooled extracts dried over anhydrous Na_2SO_4 (BDH) and concentrated on a rotary evaporator at 70°C to approximately 1 ml. The concentrated extracts were analysed by GC on a Hewlett Packard 5890 series 2e gas chromatograph equipped with a flame ionisation detector using an HP1 fused silica column (5 m by 0.530 mm). The operating parameters were injection port at 270°C, detector at 300°C, column

temperature 120°C isothermal for 1 min, 5°C/min increase to 170°C: He as the carrier gas.

Standards were prepared of the following compounds, DBP (Aldrich, 99% purity), DPM and the proposed metabolites of DBP degradation benzophenone (BP) (Sigma, 99+%), *p*-chlorobenzophenone (CBP) (Aldrich, 99%), *p*-chlorophenylacetic acid and Cl-benzoic acid (Sigma) to determine retention times of peaks appearing during GC analysis to enable identification (Fig 2.1.1).

Figure 2.1.1. GC analysis of control compounds.

Peaks shown correspond to the compounds: A= *p*-chlorobenzoic acid, B= DPM, C= BP, D= CBP and E= DBP.



2.1.2 Time scale study of DBP degradation in soil

Field moist soil (20% moisture content) from the AgResearch Winchmore Research Station, border 16 (B16) was prepared by sieving through a 5 mm mesh to remove large pieces of soil, increasing the surface area and to remove plant material. 5 g of soil was added to 100 ml of PSM containing 0.2 mM DBP (dissolved in 250 µl acetone) in a 250 ml conical flask to form slurries. DBP dissolved in acetone was added to hot PSM to volatilise off the acetone minimising the contaminating carbon source supplied in addition to the DBP. Sterile controls were obtained by autoclaving (121°C) the soil samples three times in 24 hour intervals. Sterility of the controls was confirmed by plating out a dilution series of the autoclaved soil onto 0.1 strength tryptic soy broth solidified with 1.5% w/v agar (Difco) (0.1 TSA).

Soil slurries were extracted in triplicate along with a sterile control after 1, 2, 4, 6, 12 and 24 weeks incubation. Extraction and analysis of samples by GC is as described above and any disappearance of DBP over time and consequent build up of metabolites monitored.

2.1.3 Establishment of enrichment cultures

Enrichment cultures were established to develop consortia of bacteria with degradative ability against DBP and BP through prolonged exposure and selective pressure. Soil slurries using B16 soil were established with 0.2 mM DBP or 0.2 mM BP as sole added carbon source as described previously. Cultures were also established with the addition of 0.001% (w/v) yeast extract (YE) (Gibco BRL) as a nutrient supplement, or 0.001% YE with 0.05% (w/v) sodium salicylate (Sal) (BDH) as a possible co-metabolite. The cultures were incubated as described previously. 5 ml samples of each enrichment culture were subcultured monthly into 95 ml fresh PSM containing the same nutrient sources provided in the previous subculture. Six consortia were developed and assigned the names DBP only, DBP/YE, DBP/YE/Sal, BP only, BP/YE and BP/YE/Sal. These cultures were then used as a source of microorganisms and were also themselves extracted and analysed to determine if a consortium could develop that was capable of utilising either DBP or BP as either sole carbon source or as a co-metabolite. After the twelfth subculture 0.001% (w/v) cycloheximide (Sigma) was added to the flasks containing the DBP consortias (DBP only, DBP/YE and DBP/YE/Sal) to suppress fungal growth as bacterial isolation steps were hampered by an overgrowth of fungi. Subcultures with and without added cycloheximide were maintained with monthly subculturing and monitored for the removal of DBP/BP.

2.1.4 Screening for metabolites of DBP degradation

During GC analysis of the extracts from the enrichment cultures the appearance of peaks, not relating to the added DBP or DPM, was monitored in an attempt to detect the appearance of metabolites and comparisons made to the retention times of peaks generated by the addition of control compounds.

The concentrated extracts of the DBP/YE/Sal consortium subculture 13 were further analysed by gas chromatography / mass spectroscopy (GCMS). GCMS work was carried out by Bruce Clark (Chemistry Department, University of Canterbury) on a Kratos MS80RFA mass spectrometer directly coupled to a Carlo Erba gas chromatography fitted with a J&W DB-1 column.

When DPM was initially chosen as an internal standard to determine the amount of DBP removed in experimental flasks, the compound PCPA was not available. Upon obtaining a sample of PCPA it was shown to have identical peak retention time with DPM when analysed by GC. Hence GC analysis of extracts of experimental cultures would not have shown the appearance of PCPA. Thin layer chromatography (TLC) was able to separate DPM and PCPA, so consequent screening for PCPA as a possible metabolite of DBP degradation was carried out using TLC. The TLC plates used were silica gel plates (Whatman) 5 by 20 cm, fluorescent at 254 nm. Samples of concentrated extracts and control compounds were applied as thin bands to the plates and developed with hexane-diethyl ether-acetic acid (BDH) (100:10:5). After each plate had been developed, they were dried and examined under UV light (254 nm) and any fluorescent

bands marked. Any band appearing on the plates had its Rf. value compared to those of prepared standards. Any bands of further interest were re-extracted from the TLC plates and analysed by GC.

To further confirm degradation of DBP an attempt was made to detect the release of free chloride ions. Detection of chloride ion release was by the method of Krockel & Focht (1987). 0.5 ml of a 0.1 M silver nitrate solution in 5 M phosphoric acid was added to a 5 ml sample from the subcultures that had been centrifuged to remove any bacterial cells. The A_{525} was recorded with a spectrophotometer (Hitachi U-2000) and compared to a standard curve prepared by the measurement of a range of six standards 0 to 0.4 mM of sodium chloride.

2.1.5 Study of the microbial consortia in the enrichment cultures

The three DBP consortias obtained in 2.1.3 (DBP only, DBP/YE, and DBP/YE/Sal) were screened for their bacterial composition by selection and isolation of colonies obtained by spread plating onto plates prepared with 0.1 TSA in a dilution series. Isolates obtained in pure culture were presumptively identified using API 20NE bacterial identification strips (Bio Merieux) where appropriate along with supplementary tests including Gram stain, motility, oxidase and catalase as described in Gerhardt *et al.,*(1981). Comparisons were made between the bacterial composition of the three developed consortias.

2.1.6 The effect of B16 soil conditions on the degradative capabilities of the developed consortias.

Flasks of 100 ml PSM enriched 0.2 mM DBP were supplied with 5 g field moist soil (20% moisture content) from B16 (sterile or non-sterile soil, obtained by autoclaving 3 times in 24 hourly intervals). Some of these were then inoculated with the consortia developed with DBP as sole added carbon source (see 2.1.3) as a 5 ml sample of subculture 15 so that the following conditions were provided, sterile uninoculated soil, sterile inoculated soil, non-sterile uninoculated soil and non-sterile inoculated soil. The effects of DBP bioavailability due to binding to soil particles, effects of competition from existing B16 bacteria and the ability of the inoculated consortia to degrade DBP when alternative substrates were made available, were studied by comparing the amounts of DBP removed in the various flasks. Flasks were extracted after four weeks incubation and analysed for DBP content by GC as described previously.

2.1.7 Ability of consortia to degrade potential DBP metabolites

Flasks containing a 5 ml inocula of the DBP only consortia (subculture 15) and either 0.2 mM BP, 0.2 mM CBP or 0.4 mM *p*-chlorobenzoic acid as sole carbon sources or the addition of 0.001% YE or YE plus 0.05% Sal were prepared. The flasks were incubated as described previously but for two weeks only as it was postulated that the compounds tested would be degraded at a faster rate than DBP hence the incubation time

was reduced from the four weeks used for DBP to two weeks. Extraction and analysis of experimental flasks by GC is as described previously.

2.2 Isolation of Microorganisms

2.2.1 Isolation of Acetone degraders

As DBP was added to experimental flasks dissolved in acetone (see 2.1.1, 2.1.2, 2.1.3), the DBP enrichment cultures were screened to determine if acetone degraders were being selected for as well as DBP degraders. 100 ml PSM, supplied with 0.25 ml acetone as sole carbon source, was inoculated from enrichments cultures established on DBP dissolved in acetone. After the medium became turbid, indicating growth, microorganisms were isolated from these enrichments by spread plating onto 0.1 TSA plates. Representative colonies were inoculated into PSM amended with 0.1% acetone as sole carbon source to confirm their ability to metabolise acetone in pure culture. All cultures were incubated at 30°C and uninoculated control flasks were also prepared. Bacteria of interest were presumptively identified using API 20NE strips where appropriate with supplementary tests of Gram stain, spore stain, acid fastness, catalase, oxidase, motility, flagella, glucose utilisation and fluorescence on Kings B media as described in Gerhardt *et al.,* (1980) and visualisation by light microscopy and transmission electron microscopy (TEM) was used where appropriate.

2.2.2 Isolation of BP degraders

Presumptive BP degrading organisms were isolated from the BP only consortium (see 2.1.3) onto 0.1 TSA plates. Organisms isolated in pure culture were plated onto PSM agar plates amended with BP as sole carbon source. Growth on these plates was compared to isolates plated onto PSM agar with no added carbon source. Those isolates showing positive growth on BP amended plates were grown overnight in 0.1 strength tryptic soy broth (TSB), and the cells then centrifuged down and washed with sterile distilled water. The cells obtained were used to inoculate 250 ml conical flasks containing 100 ml PSM amended with 0.2 mM BP as sole added carbon source or BP plus 0.001% YE. All presumptive BP degraders isolated were also inoculated into flasks containing 0.2 mM DBP as sole carbon source as well as DBP plus 0.001% YE. All experimental flasks were incubated at 30°C for 4 weeks before being extracted and analysed using GC as described previously. Any bacteria with BP degradative capabilities confirmed by GC analysis were identified as described previously (see 2.2.1).

2.2.3 Isolation of DBP degraders

Presumptive DBP degrading bacteria were isolated directly from the DBP enrichment cultures (see 2.1.3) using a method similar to that of Bogardt *et al.*, (1992). Agar plates with a base of PSM solidified with 1.5% purified agar (Oxoid) were prepared.

They were then overlaid with 4 ml PSM agar amended with 4 mg of DBP dissolved in 0.2 ml dimethyl sulfoxide (DMSO) (BDH). The DBP appeared on the plates as a white precipitate. Plates were inoculated from all three DBP consortias (DBP only, DBP/YE and DBP/YE/Sal) by spread plating. Presumptive DBP degraders were detected by noting zones of clearing around colonies. Isolates that produced a halo of clearing on the DBP plates were isolated onto 0.1 TSA plates and DBP overlay plates. Isolated bacteria were also plated onto control plates which contained 0.2 ml DMSO only in the agar overlay. Any isolate that showed further halo production on DBP overlay plates and failed to show growth on the control plates were grown overnight in 0.1 TSB. Cells grown were centrifuged and cleaned as for the presumptive BP degraders. These cells were used to inoculate 250 ml conical flasks containing 100 ml PSM amended with 0.2 mM DBP as sole added carbon source or with the addition of 0.001% YE or YE plus 0.05% Sal. These flasks were incubated, extracted and analysed as for the BP degraders.

Additional flasks were prepared as for above but inoculated with halo producing colonies taken directly off DBP overlay plates as an agar plug.

2.2.4 Analysis of zones of clearing exhibited by presumptive DBP degraders on DBP overlay plates

To further confirm DBP was being degraded by the isolates obtained in 2.2.3 the halos of clearing around colonies on the DBP overlay plates were removed and extracted. A method was developed similar to that of Yang *et al.*, (1994). Agar plugs containing a

halo producing colony were cut out from the DBP overlay plates with a 5 mm core borer and mixed with 500 µl cHCl, 7.5 ml double distilled water, 7.5 ml analytical grade ethyl acetate (BDH) and 0.08 mM DPM dissolved in 50 µl acetone as the internal standard, in a 28 ml centrifuge tube. This was mixed and slowly heated in a water bath to 70°C and held at that temperature for 10 min. The tubes were once again mixed and centrifuged at 8000 rpm for 10 min. The supernatant was collected and analysed by GC as described previously. Controls were included using agar plugs removed from areas on the DBP overlay plates where no bacterial growth or associated clearing around colonies had occurred.

2.2.5 Substrate specificity of presumptive DBP degraders

Presumptive DBP degraders isolated in 2.2.3 were tested for any ability to degrade three possible DBP metabolites CBP, BP and *p*-chlorobenzoic acid in liquid culture. Experimental flasks were prepared with 100 ml PSM medium amended with either 0.2 mM CBP, 0.2 mM BP or 0.4 mM chlorobenzoic acid as sole added carbon sources or with the addition of 0.001% YE or YE plus 0.05% Sal. The flasks were inoculated by taking 12 halo producing colonies of each isolate directly of a DBP plate as an agar plug. Flasks were incubated for 4 weeks, extracted and analysed as described previously.

2.2.6 Plasmid study of the presumptive DBP degrader DBP1

Plasmid curing was carried out following the method of Aislabie et al., (1985). One presumptive DBP degrader (DBP1) was inoculated into 0.1 TSB at 30°C and after overnight growth had 0.05% w/v sodium dodecyl sulfate (SDS) (Bio-Rad) added. After 24 hours incubation a subculture was made into fresh 0.1 TSB containing 0.05% SDS. A total of 5 subcultures into SDS containing media were made with 24 hr intervals between each transfer. A small scale plasmid preparation of DBP1 in its original state was made to confirm that plasmid curing had been achieved following the method outlined in Sambrook *et al.,* (1989) and run on a agarose (0.2%) w/v, Tris-acetate (TAE) gel.

Section 3:

Results and Discussion

3.1 Soil Slurries

3.1.1 Biodegradation of DBP in soil slurries

Aerobic enrichment cultures were established in PSM with 0.01 mM DBP dissolved in acetone. DBP is poorly soluble in water which may limit its bioavailability to microorganisms capable of degrading the compound. To alleviate problems with insolubility, several methods can be used to increase the solubility of the compound of interest. In some cases this has allowed the isolation and study of organisms capable of degrading insoluble compounds. For example, surfactants have been used to isolate a bacteria able to degrade fluoranthene (Mueller *et al.,* 1990), isoquinoline (Aislabie *et al.,*1989), solvents used for carbazole (Ouchiyama *et al.,* 1993)and vapour phase used for biphenyl (Davidson *et al.,* 1994). Biphasic cultures have also been employed (Ascon-Cabrera & Le Beault 1993).

In these experiments, DBP was dissolved in acetone and added directly to the enrichment cultures. It was postulated that because of the miscibility of acetone in water, isolation of DBP-degrading bacteria might be enhanced by increasing the dispersion of DBP in solution, making it more accessible to microbial attack. One problem with this approach however, is that acetone itself is biodegradable (Taylor *et al.,* 1980) and under the conditions provided, microbes able to degrade acetone may also be selected for. This problem may also arise using surfactants such as Tween 80. Both DBP and acetone share the structural feature of a central carbonyl group hence it was possible that

microorganisms able to attack the carbonyl group of acetone would also be active against DBP. Acetone degraders were selected for and screened for ability to degrade BP or DBP (see 3.2.1).

Sources of inocula were soils from sites known to be contaminated with chlorinated compounds. It is possible that organisms from these sites may have developed the enzymatic systems capable of degrading DBP due to previous exposure to similar chlorinated pesticides. Cultures were maintained by monthly subculture into fresh PSM containing DBP and after the month of incubation, extracted and analysed for their DBP content.

Of the five samples used, B16 of the AgResearch Winchmore Research Station and soil from the DowElanco Agricultural Research Farm, showed significant reductions in the levels of DBP hence they were assumed to contain microbes able to degrade DBP (Table 3.1.1).

Table 3.1.1. DBP removal in soil slurries.

Source	Subculture	DBP/DPM (peak area ratio)	% Removal
control (n=6)		0.643 sd=0.172	0%
Sludge	2	0.349	
Dow Elanco	2	0.042*	93%
B6 Winchmore	2	0.827	
B9	2	0.661	
B16	2	0.197*	69%
Sludge	3	0.520	
Dow Elanco	3	0.452	29%
B6	3	0.893	
B9	3	0.534	
B16	3	0.303*	53%

* = significant to 2 sd.

DBP degradation activity in these samples showing degradation, rapidly decreased with each subculture. Subculture 4 showed no reductions in DBP levels. The inability to sustain DBP degradative activity may be due to many factors. DBP degrading bacteria may require growth factors which were not provided in the enrichment cultures. Although these factors may have initially been available, due to the dilution effects upon subculturing the original soil components present were lost from the medium. Another alternative is that these DBP degrading microorganisms may have been growing co-metabolically and required an alternative substrate for growth, not acetone. Also the concentration of DBP employed may have been too low to induce sustained DBP degradation or alternatively the products of DBP degradation may have been inhibitory to microbial growth.

3.1.2. Time scale study of DBP degradation in soil

Soil slurries were re-established, this time using soil from B16 only, one of only two sites that had shown some apparent degradative activity against DBP. Flasks were extracted after 1, 2, 4, 8, 12 and 24 week periods to detect any DBP degradation and the rates at which this occurred. No degradation of DBP was detected in these cultures over the 24 week period even though DBP degradation had been detected in this soil previously. The only variation between this experiment and the set up provided in 3.1.1 where DBP removal was demonstrated may have been the state of the B16 soil sample

used. The soil sample used was not collected myself and the actual date of collection and consequent length of storage is unknown. Subsequent soil samples used were collected by myself and used fresh. These results shows that DBP is very stable in soil under the conditions provided.

3.1.3 Establishment of enrichment cultures

Enrichment cultures were established, using freshly collected B16 soil only. The DBP concentration was increased to 0.2 mM and added to hot medium to volatilise off the acetone in an attempt to avoid the selection of acetone-degraders. These cultures were subcultured monthly into PSM media with DBP as sole added carbon source or additionally supplemented with YE supplied as a source of growth factors or YE plus Sal to enhance the selection of microbes with the enzymatic capability to cleave aromatic compounds. Sal is a single ringed aromatic compound and its presence in the media may have helped activate ring cleaving enzymes and possibly act as a cometabolite for the degradation of DBP.

Enrichment cultures with BP, the nonchlorinated analogue of DBP were also established under the same conditions as for DBP. As a non-chlorinated analogue of DBP, BP could be more readily degraded than DBP. Some bacteria able to degrade biphenyl are also able to degrade chlorinated biphenyls. It was also postulated that microbes able to degrade BP may also be active against DBP.

BP proved to be readily utilised by the subcultures (approx. 90% depleted), whether as sole carbon source or with the addition of YE or YE plus Sal. Degradative capabilities were maintained in these subcultures over a 12 month period (Table 3.1.3(a)).

Table 3.1.3(a) Typical removal of BP in enrichment cultures.

Flask (n=3)	BP/DPM (peak area ratio)	BP Remaining (mM)	% Removal
Subculture 6			
control	0.914	0.20	0.0%
BP only	0.083	0.018	91.0%
BP + YE	0.090	0.020	90.0%
BP + YE + Sal	0.033	0.007	96.5%
Subculture 7			
control	0.228	0.20	0.0%
BP only	0.020	0.018	91.0%
BP + YE	0.017	0.014	93.0%
BP + YE + Sal	0.008	0.007	96.5%

Despite the large scale degradation of BP in the BP consortia, the DBP consortia failed to show any reductions in DBP concentrations through the first 12 subcultures, although some growth was observed in the flasks. The growth observed in the flasks may be attributed to acetone degraders or the presence of CO_2 -fixing organisms in the flasks. An attempt was made to screen the developing consortias for their bacterial composition but this proved impossible due to the overgrowth of fungi on plates used. The addition of 0.001% cycloheximide to the flasks to suppress fungal growth allowed the bacteria to develop and since then large reductions in DBP levels have been recorded (Table

3.1.3(b)). Subcultures without the addition of cycloheximide failed to show any reduction in DBP levels and were discontinued.

Table 3.1.3(b). Removal of DBP in enrichment cultures.

Flask (n=3)	DBP/DPM (peak area ratio)	DBP Remaining (mM)	% Removal
Subculture 13			
control	11.768 *	0.20	0.0%
DBP only	5.682	0.096	52.0%
DBP + YE	2.031	0.034	83.0%
DBP + YE + Sal	1.030	0.017	91.5%
Subculture 14			
control	12.349 *	0.20	0.0%
DBP only	9.137	0.148	26.0%
DBP + YE	5.583	0.090	55.0%
DBP + YE + Sal	5.255	0.085	57.5%
Subculture 16			
control	2.041*	0.20	0.0%
DBP only	1.597	0.156	22.0%
DBP + YE	1.482	0.145	27.5%
DBP + YE + Sal	1.117	0.109	45.5%

*the large variations shown in the DBP/DPM peak area ratio is due to problems associated with stock solutions of DPM. The variations shown between subcultures does not however, effect the concentrations of DPM within each subculture.

These results indicate DBP can be degraded by a consortia of organisms developed from naturally occurring soil bacteria through selection pressure. It appears that fungi growing in the experimental flasks were inhibitory to bacterial growth and it was only upon the addition of cycloheximide that the bacterial consortium were able to grow unrepressed. The addition of YE and YE plus Sal increased the amounts of DBP

degraded as was seen for BP. Media containing YE or YE plus Sal provide a more nutrient rich environment for bacterial growth, thus the increased rates of DBP removal could be due to increased bacterial numbers able to develop under nutrient rich conditions. Alternatively, YE or YE plus Sal may provide the essential co-metabolites necessary to degrade DBP. As observed with the soil cultures (see 3.1.1) the amount of DBP removed declined with each subculture. Samples from subculture 16 were visualised by spread plating, demonstrating the re-establishment of fungi had occurred, which was initially impeding the degradation of DBP by the bacteria in the culture vessels. The reoccurrence of the fungi would explain the reduction in levels of DBP removed. It also suggests that a fungal suppressant, such as cycloheximide, was probably required for each subculture.

3.1.4 Screening for metabolites of DBP degradation

No known metabolites were detected in the analysis of the DBP subcultures by GC suggesting that the first step in the degradation of DBP is the rate limiting step in the degradative pathway. Once this step is achieved, the resulting products can be readily degraded, hence no metabolites detectable. This, unfortunately, gives no indication of the nature of the first degradation step. If dechlorination occurs initially, one might expect to detect the presence of chlorobenzophenone or benzophenone. Alternatively, if ring cleavage occurs first the presence of *p*-chlorobenzoic acid, PCPA or similar compounds

might be expected. None of these compounds were detected by GC analysis, although if PCPA was present in the extract, its peak would have been masked by the peak corresponding to the internal standard DPM (see 2.1.4).

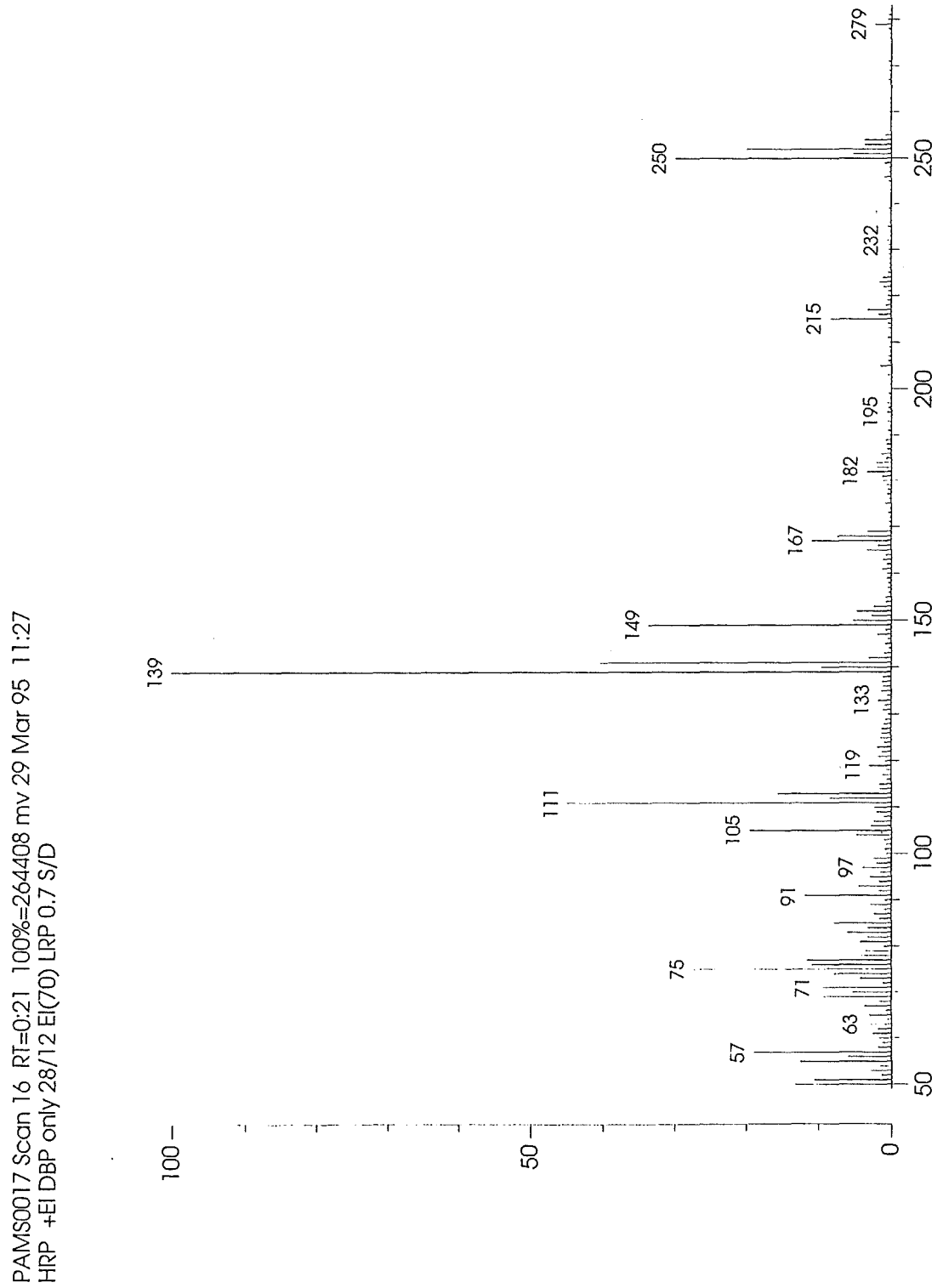
GC-MS analysis (Fig 3.1.4) of the DBP/YE/Sal subculture 13 did not detect the presence of either CBP or BP, as these are possible cleavage products of DBP by MS. *p*-Chlorobenzoic acid was also not detected. Two potential metabolites were detected at very low levels with the listed possibilities (from a PBM search using the Wileynbs library) including PCPA. This could not however be confirmed due to unavailability of PCPA to be used as control at the time GC-MS analysis was conducted.

TLC was used to concentrate compounds extracted from the enrichment cultures and in particular search for the presence of PCPA. Samples from the three DBP consortias (DBP only, DBP/YE and DBP/YE/Sal, subculture 16) were spotted onto the TLC plates alongside the control compounds DPM, DBP, CBP, BP, PCPA and *p*-chlorobenzoic acid. A band appearing from the extracts of all three of the DBP consortia with a Rf. value of 0.113 was noted. This band had an identical Rf value to the band produced by a control sample of PCPA. To confirm the identification of the compound isolated on the TLC plates bands were scraped directly from the TLC plates, extracted and analysed by GC. The compound extracted of the TLC plates showed identical retention times to PCPA standards when analysed by GC and coinjection off PCPA and the unknown produced a single peak. PCPA is therefore a metabolite of the degradation of DBP.

This confirms that the reductions in DBP levels noted in the enrichment cultures (see Table 3.1.3(b)) was due to bacterial degradation of the compound.

Some evidence for free chlorine ion release from the degradation of DBP was obtained by the method of Krockel and Focht (1987). Unfortunately the results obtained were inconsistent and therefore were not considered for presentation. An alternative method for the detection of free chloride ions, possibly using a chlorine specific electrode, needs to be employed to accurately detect the release of chlorine ions from the degradation of DBP.

Fig 3.1.4. GCMS analysis of enrichment culture extracts.



3.1.5 Study of the microbial consortia in the enrichment cultures

Microorganisms present in all three DBP consortia were studied in an attempt to determine whether the decreased amounts of DBP in the media containing YE or YE plus Sal (see 3.1.3) was due solely to the nutritional conditions provided or as a result of different bacterial compositions of the consortias selected for by the nutrient conditions.

Spread plates were prepared of all three consortia (subculture 13) and individual colonies isolated from these plates. Isolated organism were presumptively identified and comparisons made of the bacterial constituents of the three consortia.

Plate counts revealed an average of 5×10^6 colony forming units per ml in the DBP only consortium compared to 3.4×10^7 and 7.6×10^7 in the DBP/YE and DBP/YE/Sal consortia. Analysis of the bacterial compositions in the three consortia revealed little difference between the three with the DBP/YE and DBP/YE/Sal consortia having only two additional bacteria isolated, neither of which showed degradative ability against DBP. A core of six different bacteria were present in all three consortia. These were isolated and inoculated onto DBP overlay plates and two of the six showed zones of clearing around colonies. Both of these bacteria were presumptively identified as *Pseudomonas vesicularis* by API 20 NE biochemical strips although only one had the yellow pigmentation characteristic of *P. vesicularis* while the other was white and further tests will be needed to correctly identify this isolate. No *Streptomyces* sp. were isolated.

It appears that the increased rates of DBP degradation observed in the consortia

developed with the addition of YE or YE plus Sal is due to more favourable condition supporting growth rather than the bacteria selected for.

3.1.6 The effects of B16 soil conditions on the degradative capabilities of the developed consortia

The effects of B16 soil on the degradative abilities of the three DBP-degrading consortia were investigated. Of interest was the bioavailability of DBP to microbial attack considering possible adsorption to the soil particles in B16 soil, effects of competition from other organisms and the ability of the consortia to utilise DBP in the presence of other carbon and energy sources, as is the case in B16 soil. Flasks were prepared in triplicate and inoculated with 5 ml of the DBP only consortium (subculture 15). These were then extracted and analysed alongside subculture 16 of the DBP only consortium (see Table 3.1.6).

Table 3.1.6. Effects of B16 soil on DBP degradation by developed consortia.

Flask (n=3)	DBP/DPM (peak area ratio)	DBP Remaining (mM)	% Removal
DBP control (subculture 16)	2.0413	0.20	0.0%
Sterile soil (uninoculated)	1.7891	0.175	12.4%
Sterile soil (inoculated)	1.5306	0.15	25.0%
total removal due to microbial action in sterile soil = 12.6%			
Non-sterile soil (uninoculated)	1.9980	0.196	2.1%
Non-sterile soil (inoculated)	1.7792	0.174	12.8%
total removal due to microbial action in non-sterile soil = 10.7%			

These result show that there is very little DBP in a non-recoverable form due to adsorption to soil particles in uninoculated soil (2.1%), but quite surprisingly 12.4% of DBP added to the sterile, uninoculated soil was non-recoverable. The process of autoclaving must have resulted in some physical/chemical changes in soil composition allowing DBP to strongly bind to soil particles. This suggests that the chemical composition of various soils could have significant effects on the bioavailabilty of DBP to microorganisms, depending on the absorption of DBP to soil particles. The DBP only consortium was able to achieve some degree of DBP degradation in the soil environment with a net reduction in DBP levels of 10.7 % and 12.6 % respectively in the non-sterile and the sterile soil. This compares to the 21.7% reduction in DBP levels achieved by the DBP-only consortium in subculture 16 grown in PSM, where DBP was the sole provided carbon source. It follows that the availability of alternative carbon sources provided by the soil reduced the effectiveness of the consortia to degrade DBP. Competition effects of microorganisms already present in the Border 16 soil had minimal significance on the amount of DBP removed in this particular experiment but as seen in 3.1.3 fungal growth can be a major hindrance to the development of the DBP enrichment cultures.

These results have implications for possible bioremediation efforts. A consortia of bacteria with degradative capabilities, developed from naturally occurring soil organisms could possibly be used to degrade DBP or similar compounds present in soil. Unfortunately, as the results above have shown, the success of any bioremediation effort will depend on the soil's chemical composition with respect to the bioavailability of the compound, and the presence of other organisms which may hinder growth of DBP

degrading bacteria as seen by fungi in 3.1.3. In addition preferential utilisation of alternative carbon and energy sources in the soil will probably occur and unless the residue levels in the soil are extremely high, the addition of such consortia to contaminated soils will probably have minimal effect in reducing the levels of contamination.

3.1.7 Ability of consortias to degrade potential DBP metabolites

The fate of structurally related compounds can often give clues to the metabolic fate of a compound. DDM and DBH both undergo dechlorination before ring cleavage occurs (Focht & Alexander1970a, 1970b, 1971, Subba-Rao & Alexander 1977, Golovleva & Skyrabin 1981) but *p,p*'-dichlorobiphenyl undergoes ring cleavage before any dechlorination occurs (Adriaens *et al.,* 1989). This may be a reflection of the particular organisms used or the conditions provided.

The fates of three potential metabolites of DBP were investigated using PSM containing either 0.2 mM BP, 0.2 mM CBP or 0.4 mM *p*-chlorbenzoic acid. The media was inoculated with 5 ml of the DBP-only consortia (subculture 15) and incubated for two weeks. CBP, BP and *p*-chlorobenzoic acid, all showed extensive reductions in concentration under the three conditions surveyed (Table 3.1.7).

Table 3.1.7. Ability of consortias to degrade potential DBP metabolites

Flask (n=3)	CBP/DPM (peak area ratio)	CBP Remaining (mM)	% Removal
control	0.915	0.2	0.0%
CBP only	0.037	0.008	95.9%
CBP + YE	0.007	0.002	99.2%
CBP + YE+ Sal	0.013	0.003	98.5%
	BP/DPM (peak area ratio)	BP Remaining (mM)	% Removal
control	0.668	0.20	0.0%
BP only	0.008	0.003	98.7%
BP + YE	0.006	0.002	99.1%
BP + YE + Sal	ND	0.000	100%
	Cl-Ben/DPM (peak area ration)	Cl-Ben remaining (mM)	% Removal
control	1.351	0.40	0.0%
Cl-Ben only	ND	0.000	100%
Cl-Ben + YE	ND	0.000	100%
Cl-Ben + YE + Sal	ND	0.000	100%

The results highlight difficulties found in the detection of metabolites of DBP degradation. These three potential metabolites are extensively degraded in only two weeks, either as sole carbon source or with the addition of YE and YE plus Sal, compared to the four weeks it requires to achieve much lower rates of DBP degradation achieved by the same microbial consortium (see 3.1.3). It is possible that as soon as any of the products are formed (if any of these products are formed), they are quickly degraded to a readily utilisable carbon and energy source. Samples from the enrichment cultures must therefore be extracted at regular intervals early in the incubation stage and analysed in an to attempt to quantify metabolites before further degradation occurs.

3.2 Isolation of Microorganisms

3.2.1 Isolation of acetone degraders

Bacteria able to utilise acetone as sole source of carbon and energy were isolated from enrichments established in order to isolate DBP-degraders. These isolates were selected even when DBP, dissolved in acetone, was added to hot medium, indicating that the acetone had not been completely volatilised. This result could explain the growth observed in the early DBP enrichment subcultures even when no DBP was being utilised (see 3.1.3). Isolates were presumptively identified as *Pseudomonas maltophilia* (B16-2) and *Alcaligenes denitrificans* (B16-5) by use of API 20NE strips and with additional supplementary tests (Table 3.2.1). These identifications are supported by the electron micrographs shown in Fig 3.2.1(a) & (b). As expected *A. denitrificans* has peritrichous flagella whereas *P. maltophilia* has a single polar flagellum. Bacteria able to degrade acetone have been previously described (Taylor *et al.,* 1980, and Platen & Schink, 1989). Neither *P. maltophilia* or *A. denitrificans* showed any ability to degrade BP or DBP.

Table 3.2.1. Identification of acetone degraders.

	B16-2	**B16-5**
Source	Winchmore	Winchmore
Gram stain	- ve	- ve
Colour of colony	cream	clear
Morphology	rods	rods
Motility	+ ve	- ve
Spore stain	- ve	- ve
Oxidase reaction	+ ve	+ ve
Catalase reaction	+ ve	+ ve
Glucose utilisation	- ve	- ve
Flagella	single, polar	multiple, peritrichous
Florescence on Kings B	- ve	- ve
Degradation of	Acetone	Acetone
Presumptive Identification	*Pseudomonas maltophilia*	*Alcaligenes denitrificans*

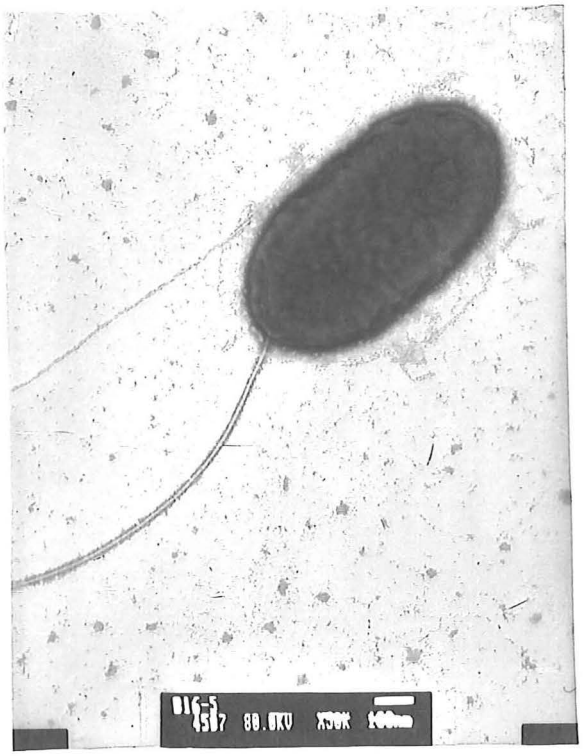


Fig 3.2.1(a) TEM of *Pseudomonas maltophilia.*

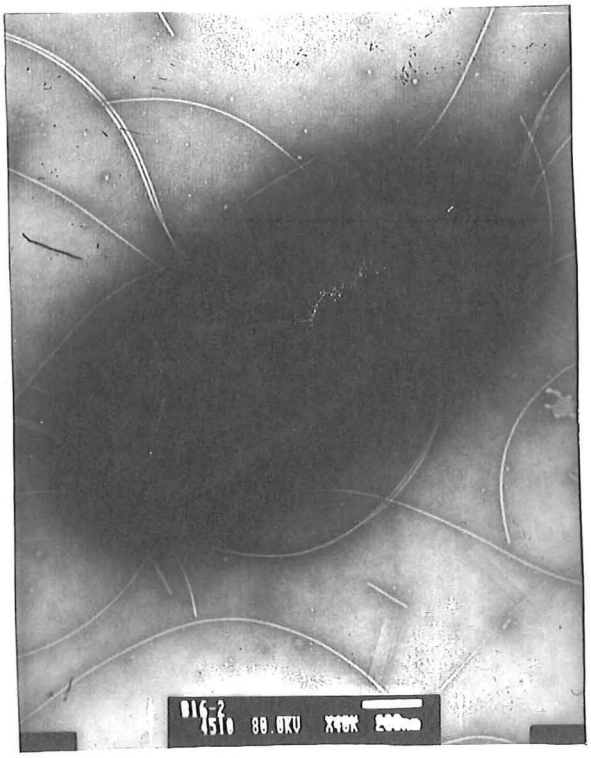


Fig 3.2.1(b) TEM of *Alcaligenes denitrificans.*

3.2.2 Isolation of BP Degraders

From the BP subcultures, organisms were isolated on 0.1 TSA and then plated onto solidified PSM with BP as sole added carbon source. Any colony showing growth on these enrichment plates was grown overnight and then inoculated into liquid culture with either BP as sole carbon source or with the addition of YE. After one month incubation the media was extracted and BP concentrations analysed using GC. Two isolates showed degradative capabilities either with BP as sole carbon source or with the addition of YE. Flasks containing YE had an increased amount of BP degraded (Table 3.2.2(a)).

Table 3.2.2(a). Degradation of BP in pure culture.

Flask	BP/DPM (peak area ratio)	BP Remaining (mM)	% Removal
control	0.200	0.20	0.0%
BP 6(BP only)	0.093	0.093	53.7%
BP 6 + YE	0.060	0.060	70.0%
BP 7(BP only)	0.088	0.088	56.2%
BP 7 + YE	0.062	0.062	68.9%

The two genera of bacteria were identified as a *Rhodococcus* sp. (BP 6) and a *Streptomyces* sp. (BP 7) (Table 3.2.2(b)) in a manner similar to the acetone-degrading bacteria. Degradation of BP as sole carbon source by bacteria has not been previously demonstrated. Neither of these bacteria were capable of utilising DBP. That two

organisms isolated could degrade BP but had no effect on DBP indicates that the chlorine's in the *para* position on DBP results in its recalcitrance, with the chlorine presumably inhibiting ring cleaving enzymes.

Table 3.2.2(b). Identification of BP degraders.

	BP 6	**BP 7**
Source	Winchmore	Winchmore
Gram stain	+ ve	+ ve
Colour of colony	creamy/yellow	cream colonies with grey mycelia
Morphology	filamentous rods becoming cocci	filamentous rods
Diffusable pigments	- ve	- ve
Motility	- ve	-ve
Spore stain	- ve	- ve
Acid fast	- ve	- ve
Oxidase reaction	- ve	+ ve
Catalase reaction	+ ve	+ ve
Glucose utilisation	oxidative	oxidative
Flagella	ND	ND
Florescence on Kings B	- ve	- ve
Degradation of	Benzophenone	Benzophenone
Presumptive Identification	*Rhodococcus sp.*	*Streptomyces sp.*

The genus *Rhodococcus* is a unique taxon consisting of microorganisms that exhibit broad metabolic diversity, particularly to hydrophobic compounds such as hydrocarbons, chlorinated phenolics, steroids, lignin and petroleum (Finnerty, 1992). It is not surprising, therefore, that this isolate was active against BP. Presumptive BP degraders were maintained on 0.1 TSA during the procedures used to confirm degradative capabilities. Maintenance on 0.1 TSA resulted in the loss of degradative capabilities. Unfortunately no stock cultures had been generated so the results obtained were not reproducible. Screening of bacteria in the subcultures failed to result in any additional organisms capable of degrading BP as sole carbon source being isolated. The inability to isolate organisms capable of degrading BP in pure culture from the consortia that were extensively degrading BP is not that unexpected. Consortias are complex mixtures of bacteria working on synergistic reactions such as the degradation of BP. Thus it is not unusual that none of the organisms involved have the capabilities to degrade BP on their own, without the enzymatic assistance of other members of the consortia.

The inability to isolate a rhodococcus/streptomycete with degradative capabilities from the consortia indicates that over time these organisms are not as successful in liquid culture as they may have been in the original B16 soil sample, possibly due to the loss of a plasmid.

3.2.3 Isolation of DBP Degraders

A plate assay was developed to directly screen for presumptive DBP degraders using a method similar to that of Bogardt *et al.,* (1992). DBP to be added to the plates was first dissolved in DMSO and mixed into an agar overlay which gave the appearance of a white precipitate when overlaid on agar plates. Presumptive degraders were detected by noting zones of clearing around the colonies (Fig 3.2.3(a)). From B16 soil, three presumptive DBP-degraders were obtained. These isolates are described in Table 3.2.3(a). DBP6 and DBP9 were Gram-positive, filamentous, branching organisms that produced abundant aerial mycelia and were presumptively identified as two different species of *Streptomyces.* The third isolate DBP1, was identified as *Pseudomonas vesicularis* by the use of API NE20 biochemical strips. A similar, but distinct isolate of *P. vesicularis* identified by the use of API NE20 strips also with the halo producing ability on the DBP overlay plates was isolated from Dow Elanco soil. No growth was seen on plates with DMSO as the sole carbon source.

Fig 3.2.3(a) Zones of clearing around presumptive DBP degraders on DBP overlay plates.

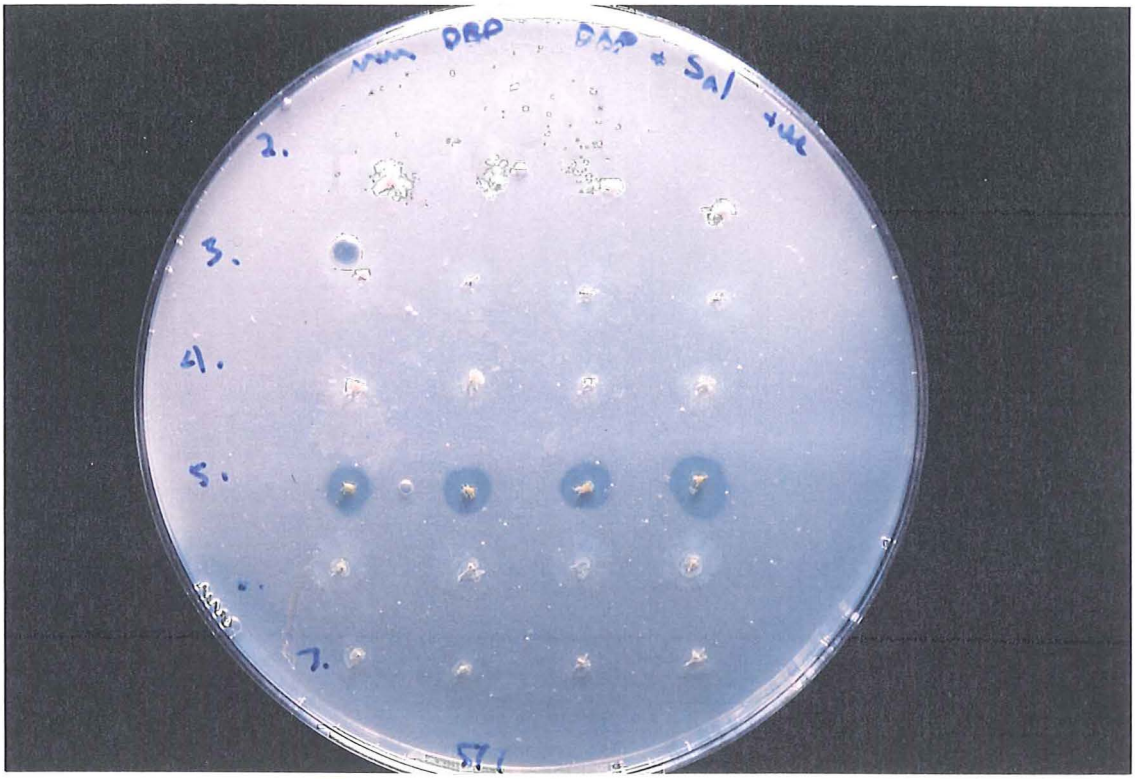


Table3.2.3(a). Identification of DBP degraders.

	DBP1	DBP6	DBP9
Source	Winchmore	Winchmore	Winchmore
Gram stain	- ve	+ve	+ve
Colour of colony	yellow	grey mycelia	white mycelia becoming grey
Morphology	rods	filamentous rods	filamentous rods
Diffusable pigments	-ve	-ve	+ve brown
Motility	+ve	-ve	-ve
Spore stain	-ve	-ve	-ve
Acid fast	-ve	-ve	-ve
Oxidase reaction	+ve	+ve	-ve
Catalase reaction	+ve	+ve	+ve
Glucose utilisation	?	oxidative	?
Flagella	single polar	?	?
Florescence on Kings B	-ve	-ve	-ve
Degradation of	Dichloro-benzophenone	Dichloro-benzophenone	Dichloro-benzophenone
Presumptive Identification	*Pseudomonas vesicularis*	*Streptomyces* sp.	*Streptomyces* sp.

The question arose with the appearance of the haloes of clearing around colonies on DBP overlay plates, as to whether they represented degradation and usage of DBP as a

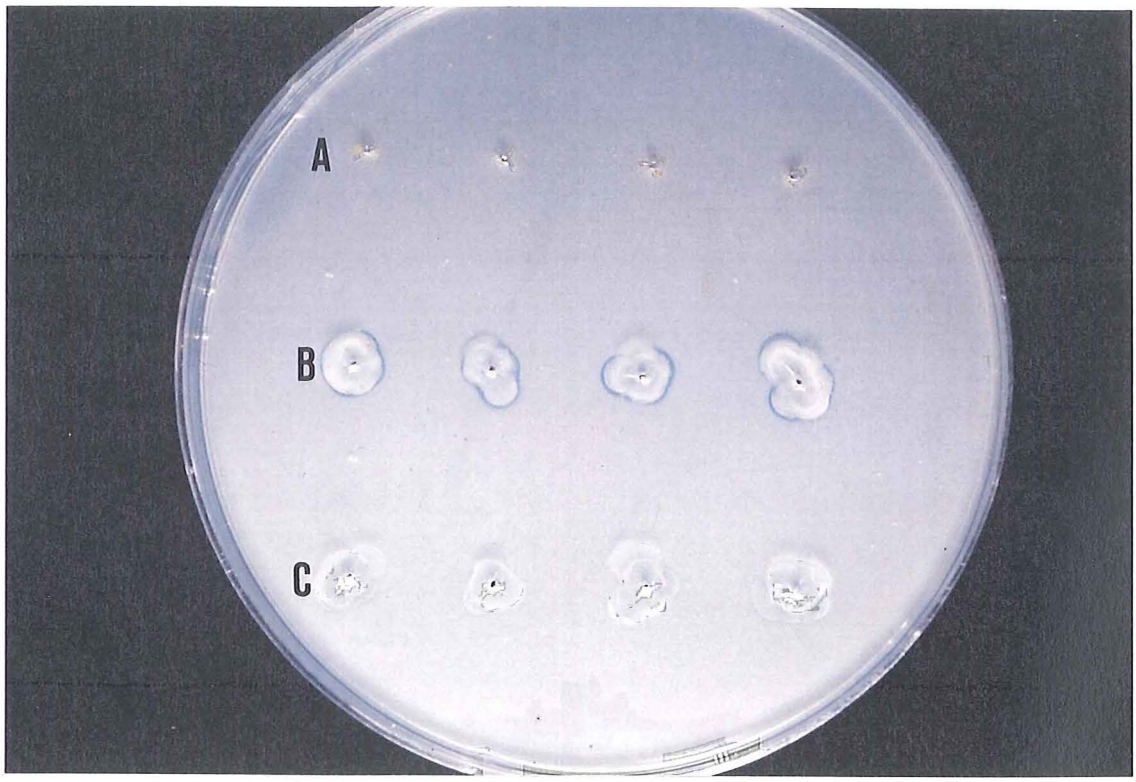
carbon and energy source, conversion of DBP for detoxification purposes (or otherwise) not supporting growth, or whether these organisms simply produced a natural surfactant like compound which could solubilise DBP, with no conversion taking place. Microbially produced surfactants do exist, such as the compound hydroxypropyl-ß-cyclodextrin which has been shown to increase the water solubility of low polarity compounds such as trichloroethene, chlorobenzene, naphthalene, anthracene and DDT (Wang & Brusseau 1993). Both *Streptomyces* sp. produced colony's of approximately 5 mm in diameter over two weeks with the halo extending another 1 mm beyond the colony's boundary in the case of DBP6 and ending on the colony boundary for DBP9 (Fig 3.2.3(b)). This compares to the *P. vesicularis* which showed little or no growth on the plates with a relatively small halo extending out from the point of inoculation. The appearance of the *P.vesicularis* halo was time dependent; initial halo formation required at least one week (Fig 3.2.3(b), and haloes of approximately 2 mm in diameter took up to a month. The inoculum remained viable and could be used as a source of inocula up to six months after inoculation on the DBP assay plate. Both growth and halo-expansion occurred during this time.

To rule out the surfactant possibility, agar medium was prepared containing DDT as the sole carbon source added in the same way as for DBP. None of the presumptive DBP degraders showed any clearing or growth on these plates. This result suggests that the microbes are not simply producing a surfactant-like compound, as no growth or halo production comparable to that observed on the DBP plates was recorded even though DBP and DDT are structurally similar and with similar solubilities.

Fig 3.2.3(b) Three presumptive DBP degraders.

A=DBP1, B=DBP6, C=DBP9

NB: plate is one week old.



Initial attempts to demonstrate DBP degradation by these three microorganisms in liquid culture studies, failed. The organisms were grown overnight in 0.1 TSB, cells washed and inoculated into PSM amended with DBP as sole carbon source , with the addition of 0.001% YE, or with YE and 0.05% Sal. Neither growth in experimental flasks was observed nor a reduction in DBP concentrations detected in extracts.

Inoculation into 0.1 TSB resulted in flocculant growth of the two *Streptomyces* sp. This might be due to accessibility of DBP, as due to its insolubility, DBP tends to stay on the surface of the liquid media. In the case of the pseudomonad the halo-producing phenotype is unstable and may have been lost when growing the organisms in nutrient broth, as had been observed for BP-degrading cultures maintained on 0.1 TSA plates. This observation is supported by the findings of Hussain *et al.*,(1994) who isolated an organism identified as *P. vesicularis* that was capable of degrading pentachlorophenol (PCP), but upon subculturing on nutrient media the organism lost all degradative capabilities, which were unrecoverable. This could be due to the degradative activity being plasmid based. Plasmids can be relatively unstable and easily lost when not selected for or required for growth which may have been the case with BP6, BP7 and the *P.vesicularis* when growing on 0.1 TSA or 0.1 TSB.

Liquid cultures were re-established for DBP 1, 6 and 9 using agar plugs containing the colony and halo from DBP assay plates, as the source of inocula. The agar plugs allow a solid platform for the organisms to grow on, possibly reducing the flocculation effect. No growth or reduction in DBP levels was achieved with the two streptomyces either as sole carbon source or with the addition of YE and YE plus Sal.

The *P. vesicularis* did, however, show growth and reduction of DBP levels when YE and Sal were added (Table 3.2.3(b)). *P.vesicularis* is reported in the literature to require the growth factors biotin, cyanocobalamin and pantothenate. The addition of these compounds to flasks with the pseudomonad failed to enhance growth and had no effect on the degradation of DBP achieved in liquid culture. This was also observable on solid media where DBP overlay plates amended with the growth factors for *P.vesicularis* failed to show any clearing when inoculated with DBP1.

It is possible that *P. vesicularis* was only capable of degrading DBP in a nutrient starved state and when the required growth factors are made available the DBP degrading activity is no longer required for survival.

Table 3.2.3(b). Degradation of DBP in pure culture.

Flask (n=3) DBP1	DBP/DPM (peak area ratio)	DBP Remaining (mM)	% Removal
control	1.184	0.200	0.0%
DBP + YE + Sal	0.911	0.154	23.0%
DBP + YE + Sal	0.795	0.134	32.8%
DBP + YE + Sal	0.786	0.133	33.6%

These results confirm that the *P. vesicularis* (DBP1) isolated can co-metabolise DBP. *Pseudomonas* species have been previously reported in the literature to degrade a wide range of aromatic compounds including polychlorinated biphenyls (Gibson *et al.*, 1993 and Bedard & Haberl 1990), carbazole (Ouchiyama 1993), chlorinated

acetophenones (Higson & Focht 1990), fluoranthene (Mueller *et al.,* 1990), and diphenyl methane and other nonchlorinated analogues of DDT metabolites (Francis *et al.,* 1976). Particularly active is *P. putida* which is reported to degrade naphthalene, fluorene, phenanthrene (Yang *et al.,* 1994), chlorobenzoates (Hernandez *et al.,* 1991), styrene (O'Conner *et al.,*1995) and several metabolites of DDT including converting DDM and DBH to DBP (Subba-Rao & Alexander 1977a, 1977b). No conversion of DBP by *P. putida* was reported. Pseudomonads have also been genetically engineered that are capable of degrading dicofol (Golovleva *et al.,* 1988).

P. vesicularis is a unique pseudomonad taxonomically assigned to the rRNA homology group IV, often referred to as the diminuta group, which is comprised of only two species *P. vesicularis* and *P. diminuta* (Ballard *et al.,* 1968, Segers *et al.,* 1994). Members of the diminuta group are distinct from other pseudomonads in that its members require the growth factors biotin, pantothenate and cyanocobalamin. They have also been isolated from a wide range of environments, including human clinical specimens (Ballard *et al.,* 1968). However results of DNA-rRNA hybridisation studies showed that the diminuta group belongs to a separate genus in the alpha subclass (rRNA superfamily IV) of the *Proteobacteria,* different from all other pseudomonads and Segers *et al.,*(1994) proposed that they be reclassified with the new names of *Brevundimonas diminuta* and *Brevundimonas vesicularis.*

3.2.4 Analysis of zones of clearing exhibited by presumptive DBP degraders on DBP overlay plates.

A method for extracting the halos produced from inoculation of the three presumptive degraders onto DBP plates and subsequent analysis of the DBP concentration within the halos was developed based on the method of Yang *et al.*, (1994). Spread and streak plating failed to produce growth in large enough quantities to allow extraction of the zones of clearing on plates. It was only by stab inoculating that suitably sized halos were produced. By increasing the number of inocula per plate or more precisely by reducing the proximity of inocula on the plate, the size of the subsequent halo produced was severely reduced. Hence it was not practical to extract an entire plate which had only approximately 12 % maximum of its surface area cleared.

Both of the *Streptomyces* sp. (DBP6 and DBP9) appeared to inhibit their neighbouring colonies. This was observed on 0.1 TSA where large amounts of inocula in close proximity such as growth on a streak plate failed to produce aerial mycelium (see Fig 3.2.4(a)). A reduction in the size of colonies produced was observable on both 0.1 TSA and DBP plates when stab inoculation occurred close to another site of inoculation. On DBP overlay plates both colony and halo size were reduced (see Fig 3.2.4(b)). This phenomenon of self inhibition may also explain the lack of growth and degradation of DBP by DBP6 and DBP9 in liquid culture. Since an entire cleared plate could not be obtained, individual halos were extracted for each of the three presumptive DBP

degraders. Controls were included using 5 mm plugs of DBP agar from an area on the same plate where no clearing had occured.

Fig 3.2.4(a) DBP6 exhibiting self inhibition.

Streak plating on 0.1 TSA produced areas with little or no aerial mycelia (A) where streaks of inocula were in close proximity. Streaks without neighbouring streaks in close proximity were able to produce aerial mycelia(B).

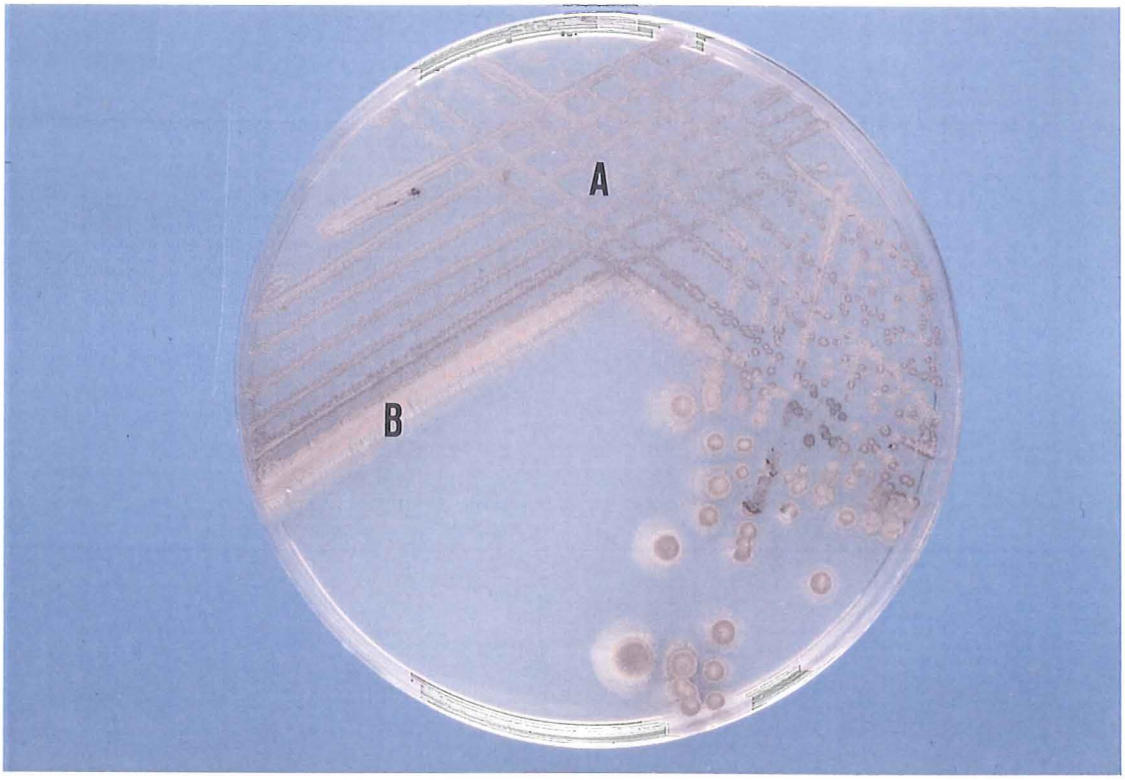
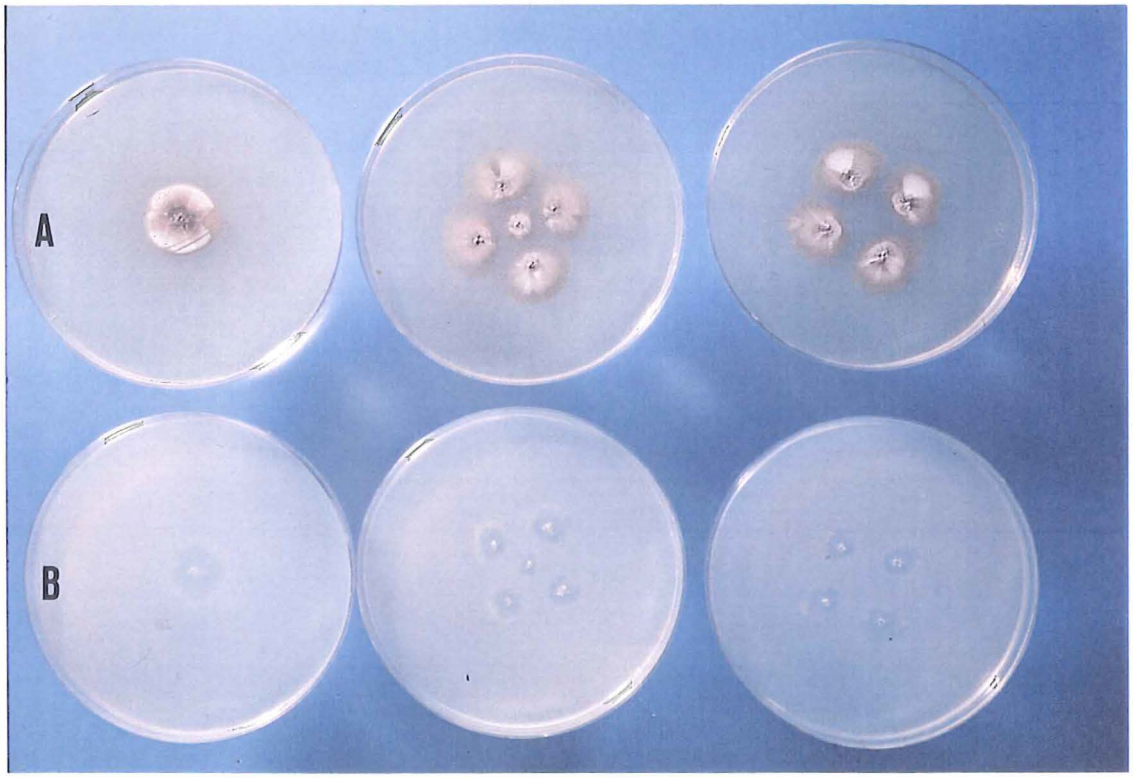


Fig 3.2.4(b) DBP9 exhibiting self inhibition.

Reductions in the amount of growth produced by stab inoculation onto both 0.1 TSA plates (A) and DBP overlay plates (B) was observed.



Analysis of the DBP concentrations in these halos showed significant reductions in DBP levels in comparison to the controls (see Table 3.2.4).

Table 3.2.4. Analysis of zones of clearing exhibited by presumptive DBP degraders on DBP overlay plates.

Organism	DBP/DPM (peak area ratio)	% Removal
DBP 1		
control 1	0.04248	0.0%
halo 1	0.03000	29.4%
control 2	0.03919	0.0%
halo 2	0.03107	20.7%
control 3	0.03120	0.0%
halo 3	0.02679	14.1%
DBP 6		
control 1.	0.04254	0.0%
halo 1	0.00871	79.5%
control 2	0.11781	0.0%
halo 2	0.02137	81.9%
control 3	0.16900	0.0%
halo 3	0.02478	85.3%
DBP 9		
control 1	0.04030	0.0%
halo 1	0.01338	66.8%
control 2	0.02677	0.0%
halo 2	0.00668	75.0%
control	0.03503	0.0%
halo 3	0.01292	63.1%

The variation in the reduction of DBP levels for the three isolates (14-29% for DBP1, 79-85% for DBP6 and 63-75% for DBP9) is not surprising considering the variation in sizes of individual haloes and with the 5 mm agar plugs including uncleared areas of the DBP plate surrounding the halo. This is particularly the case with DBP1 which produced very small halos of approximately 2 mm diameter. What should be noted from these results is not how much was removed but that the clearing around the colonies on the DBP overlay plates correlates directly with a removal of DBP from these areas.

3.2.5 Substrate specificity of presumptive DBP degraders

Each of the three presumptive DBP degraders were used to inoculate PSM medium enriched with either CBP, BP or Cl-benzoic acid as sole carbon source. No growth or degradation of these compounds was achieved in any of the experimental samples. As for the lack of growth and degradation seen when DBP1, 6 and 9 were inoculated into liquid medium containing DBP, it is difficult to determine if these organisms are not capable of utilising these compounds as sole carbon source or if the conditions provided were not suitable.

3.2.6 Plasmid study of the presumptive DBP degrader DBP1

Attempts to cure DBP1 of its plasmids resulted in death to the cells so unfortunately a plasmid cured strain could not be obtained. This suggests that one or more of the plasmids present in DBP1 is essential to survival and would make it difficult to determine if any of the apparent DBP degradative abilities, expressed by DBP1, is plasmid based as individual plasmids will need to be cured from the organism.

A plasmid preparation was made of DBP1 to screen for plasmid/plasmids present in the organism. At least one plasmid with approximate size of 15 kilobases was observed. This does not mean it is the only plasmid present in DBP1 as plasmids with low copy number are often hard to detect. Further studies on the genetics of DBP1 needs to be carried out in order to determine how degradation of DBP by this organism occurs and if indeed it is plasmid encoded. If DBP degradation is plasmid encoded it may be possible to conjugatively transfer the plasmid to an alternative host. This may already be occurring as seen with the detection of the bacteria isolated from the enrichment cultures which exhibited clearing on DBP overlay plates (3.1.5). This yet properly identified organism was not originally isolated when screening for presumptive DBP degraders (3.2.3), so therefore may have gained the observed degradative capabilities from a *P. vesicularis* when the two were growing together in enrichment cultures.

Section 4:

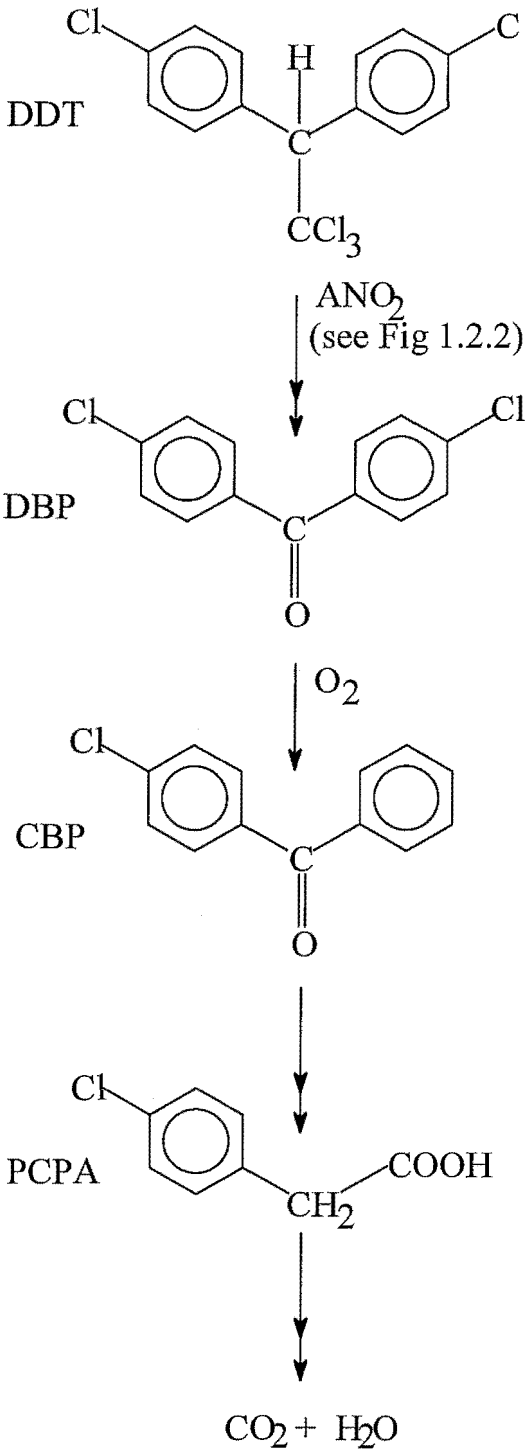
Summary

Soil slurries established with soil from the Dow Elanco Agricultural Farm and form the AgResearch Winchmore Research Station which had been previously exposed to the chlorinated compounds 2,4,5-T and DDT respectively showed degradative capabilities against DBP. GC analysis showed large reductions in DBP concentrations in the early subcultures established with these soils. Degradative ability was not sustained however, and by the fourth subculture no reductions in DBP levels were detected. A time scale study using B16 soil from the Winchmore site showed no reductions in DBP levels over a 24 week period which shows that DBP is generally very stable under soil conditions.

Enrichment cultures were established to select for and develop BP and DBP degraders. BP proved to be readily degraded with approximately 90% removal in all culture vessels whether BP was sole added carbon source or with the addition of YE and YE plus Sal. No degradation of DBP was achieved in the enrichment cultures through the first 12 subcultures and it was only after the addition of the fungal suppressant cycloheximide that DBP degradation was observed. A maximum reduction in DBP levels of 90% was observed in cultures provided with YE and Sal. Degradation rates in cultures provided with DBP as sole carbon source were much lower with a maximum of 52% DBP removed. Screening of the developed bacterial consortia in the enrichment cultures indicated that the enhanced rate of DBP removal in media containing YE or Sal was due to the more favourable conditions provided rather than the microorganisms selected for. GC analysis of extracts from these enrichment cultures failed to detect any metabolites and it was postulated that any metabolite formed was readily degraded by the developed

consortia in the enrichment cultures and hence difficult to detect. This was backed up by the findings that the consortium extensively degraded three potential metabolites of DBP with 96-100% of the added CBP, BP or *p*-chlorobenzoic acid degraded, in half the time required to achieve much lower rates of DBP degradation. GCMS analysis detected two possible metabolites with the listed possibilities including PCPA. This however could not be confirmed at the time. TLC was finally employed to study the extracts from enrichment cultures and by this method a metabolite was detected and identified as PCPA. PCPA is a known metabolite of DDT (Pfaender and Alexander 1972) and has shown to be formed from DDM. This is the first time sustainable degradation of DBP has been demonstrated and PCPA detected as a metabolite of DBP degradation. This indicates that BP is not a metabolite of DBP, which has previously been assumed (Golovleva and Skryabin 1981) and it is more likely that only a single dechlorination step occurs before ring cleavage. The mono chlorinated analogue of DBP, CBP has been detected as a metabolite of DBP previously (Subba-Rao and Alexander 1985) and has therefore been included in the proposed pathway of DDT degradation occurring via DBP (Fig 4).

Fig 4 Proposed degradation pathway of DDT occurring via DBP.



DBP was added to experimental vessels dissolved in acetone. It was hoped that the structural similarity of a central carbonyl group present in both compounds would aid in the development of DBP degraders. This proved not to be the case as acetone degraders were being selected for which had no degradative capabilities against either BP or DBP. Two acetone degraders were isolated and identified as a *Pseudomonas maltophilia* and an *Alcaligenes denitrificans*. As a consequence DBP dissolved in acetone was added to hot media to volatilise the acetone. Two organisms identified as a *Rhodococcus* sp. and a *Streptomyces* sp. were isolated that were able to degrade BP as sole carbon source which had not previously been demonstrated. Unfortunately the degradative capabilities of these two organisms was lost upon subculturing on a nutrient agar and were not recoverable.

A plate assay was developed to select for DBP degraders and three organisms were isolated that exhibited clearing on these DBP overlay plates. GC analysis of the zones of clearing on the assay plates showed a removal of DBP in the areas of clearing. The three organisms were identified as two separate *Streptomyces* sp. and a *Pseudomonas vesicularis*. None of the three were able to degrade DBP as sole carbon source in liquid culture although the *P. vesicularis* could co-metabolise DBP when YE and Sal were added to the media. The *P. vesicularis* was reisolated from enrichment cultures grown on DBP after 12 subcultures and still exhibited the clearing seen on DBP overlay plates. No *Streptomyces* sp. were reisolated from the enrichment cultures. Preliminary genetical studies of the *P. vesicularis* isolated showed that plasmid curing resulted in death to the cells. One plasmid of approximately 15 kilobases was observed using a small scale

plasmid preparation. Further study on this organism and its genetical capabilities is required to determine if degradation capabilities are plasmid encoded and if so an this plasmid be used as a tool to degrade DBP and possibly other chlorinated aromatics.

DBP has previously been shown to be very recalcitrant to biodegradation and the successful degradation of DBP achieved in these experiments may represent bacterial evolution in action. As discussed previously DDT is a xenobiotic compound and hence microorganisms may have had to develope the genetical systems required to degrade it. Consequently some of the metabolites of DDT degradation, including DBP are also xenobiotic compounds. As a metabolite of DDT, DBP has probably not been in the environment as long as its parent compound and certainly in far lower concentrations. Hence the processes required to degrade DBP may still be developing and it is only now that these processes are becoming evident.

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