IDENTIFICATION, DOCUMENTATION AND CONTROL OF BIOLOGICAL CONTAMINATION IN MIDDLE DISTILLATE FUEL

by

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ABSTRACT

The present study was initiated under a contract with the New Zealand Defence Scientific Establishment to document the nature, extent and possible sources of microbial contamination of middle distillate fuel ('Dieso') held in storage tanks at Devonport, Auckland, and to study possible methods for control of such contamination.

Thirty-one fungal species and five bacteria were isolated during the period 1982-1984. The principal contaminants were the fungi *Cladosporium resinae* (the anamorph of *Amorphotheca resinae* Parbery), *Penicillium corylophilum* and *Paecilomyces variotii*. All three fungi produced dark mycelial mats at the water/diesel fuel interface in laboratory studies. Interactions between these fungi were observed. In the presence of Bushnell-Haas mineral salts/diesel fuel phases *Cladosporium resinae* predominated while in seawater/diesel fuel phases *Penicillium corylophilum* predominated. All New Zealand and Australian isolates of *C. resinae* grew profusely in Bushnell-Haas mineral salts/diesel fuel phases.

The biostatic/biocidal effects of chemicals on the predominant fungi in diesel fuel were studied in laboratory and field tests during 1984-1985. The most effective biocides in controlling *C. resinae* were benomyl, imazalil and Kathon 886. Imazalil had no effect on *Paecilomyces variotii* but when used in combination with benomyl a synergistic effect occurred at 100 ppm. Biobor JF, DEGME and EGME performed poorly in laboratory tests regardless of the amount of water present, but gave temporary inhibition of *C. resinae* in the field tests. Isolates from tanks treated with Biobor JF and DEGME grew well in the presence of these compounds in the laboratory. DML-7 and Proxel AS inhibited *C. resinae* and *Penicillium* spp. in both laboratory and field tests at a high dose of 300 ppm but were less effective against *P. variotii*. The effects of the biocides on engine performance and carbon deposits on engine components were studied.

Recommendations for control of microbiological contamination of stored diesel fuel are given. In electron microscope studies no difference was observed in the intracellular structures between jet and diesel fuel isolates of *C. resinae* and the non-hydrocarbon utilizing *Cladosporium cladosporioides*.

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1 GENERAL INTRODUCTION AND AIMS OF STUDY

1.1 Studies on microbiological contamination of hydrocarbon fuels.

All heterotrophic micro-organisms, whether bacteria or fungi, have similar general requirements for growth. They require a source of organic carbon to supply both carbon skeletons for the formation of new cell constituents and to provide the necessary energy following partial or complete oxidation. Such organisms have been called *chemoorganotrophs* (Hawker and Linton, 1979). Hydrocarbon fuels contain between 80% and 89% carbon (King and McKenzie, 1977) and thus should be able to provide an excellent source of carbon energy. However they vary in their susceptibility to microbial degradation.

Certain organic materials which are now quite common environmental contaminants were not always so common, and the hydrocarbons which comprise the various types of modern liquid fuels are prime examples. In recent years due to the rapid development in industry, transportation and naval activity large quantities of fuel are stored for long periods providing a man-made environment where micro-organisms can thrive. Many other man-made organic molecules such as plastics and other synthetic materials widely used by man provide even more recent additions to the environment and may be capable of forming a substrate for microbial growth. The ability of micro-organisms to metabolise hydrocarbons was demonstrated long ago by Miyoshi (1895). More recently it has been shown that a range of microbial species have the capacity to break down various hydrocarbons (ZoBell, 1946; Beerstecher, 1954; Foster, 1962).

Aliphatic and long chain hydrocarbons appear to be more susceptible to microbial attack than aromatic and short chain hydrocarbons. Molecules containing from 10 to 18 carbons are attacked more frequently and rapidly, and they support abundant microbial growth (Lukins, 1962). The reason fuel containing aromatic hydrocarbons is less readily attacked, may perhaps be due to toxicity or the requirement for more specialised enzymes to degrade such compounds (ZoBell, 1950).

Other nutrients namely nitrogen, phosphorus, potassium and trace elements are less abundant in fuel, although their levels may be supplemented by fuel additives, rubber, paints, human waste and seawater (Hill and Hughes, 1969).

It is therefore not surprising to find micro-organisms associated with aviation fuels (gasoline and kerosene in both subsonic and supersonic aircraft and in storage tanks), diesel fuel (middle distillate fuel) in storage, in road and rail vehicles, diesel generators, central heating fuel and diesel fuel onboard ships, particularly where water displacement is used in order to maintain a consistent 'trim'. Green *et al.* (1967) reported that rocket propellants were also susceptible to microbial attack. The hydrocarbons are predominantly aliphatic but in some fuels, a high content of aromatic hydrocarbon may be present, for example some jet fuel contains 25% aromatics. The additives which may be present in the aviation fuels include anti-oxidants, metal deactivators, corrosion inhibitors, antistatics and anti-icing agents. The term 'diesel fuel' covers a wide range of differing hydrocarbon blends, some of which may also contain cetane number improvers and corrosion inhibitors (Genner and Hill, 1981). The navy uses a special type of marine diesel called 'dieso'.

All organisms including hydrocarbon utilizing organisms depend upon a certain amount of water in various forms. Growth of micro-organisms appears to occur in the water phase which is nearly always found in commercial hydrocarbons. For example hydrocarbon fuels will absorb water from the atmosphere to the extent of 100 ppm and this tends to separate out to generate the free water phase when subjected to temperature or pressure fluctuations. If hydrocarbon fuels are completely dry, it would appear that growth cannot occur but some contaminants may survive. Survival of cells can vary considerably (Sharpley, 1966). Bacteria may survive for only a few hours while resistant stages such as the conidia of *Cladosporium resinae* (anamorph of *Amorphotheca resinae*) are known to survive for prolonged periods (Hill, Evans and Davies, 1967). Survival of microbial spores in dry hydrocarbons also appears to vary depending on temperature (Sharpley, 1966).

1.1.1 Early reports of microbial attack

The earliest published reports on bacterial attack of fuels and oils were in the 1930's. Tausson and Aleshina (1932) reported attack on fuel by anaerobic sulphate-reducing bacteria. Thaysen (1939) investigated the explosion of a kerosene storage tank and found that growth of sulphate-reducing bacteria was responsible due to evolution of gas during their growth. The first report of yeasts assimilating hydrocarbons was by Tausson (1939). He reported that species of *Debaryomyces*, *Endomyces*, *Hansenula*, *Torulopsis* and *Monilia* were capable of growth on hydrocarbons.

Bushnell and Haas (1941) studied the micro-organisms present in the water bottoms of aircraft storage tanks, and found that many of the bacteria present were capable of hydrocarbon oxidation and could utilize gasoline and kerosene. Allen (1945) also studied the effects of bacterial growth on gasoline in storage tanks. He concluded that a major factor in the escalation of this problem was due to the increased storage at higher temperatures and this had increased the incidence of microbial growth in the fuel tank water bottoms.

Evidence that corrosion could result from microbial growth on hydrocarbons was presented by Ganser (1940). He reported that sulphate-reducing bacteria were responsible for corrosion in pipelines. In the 1960's aviation gasoline and jet fuel on aircraft carriers and amphibious assault ships became corrosive from fuel soluble sulfide generated from reduction of sulphate by anaerobic bacteria in the seawater stored in the bottom of storage tanks. (Kelley, 1967; Klemme, 1968).

The first reports of problems arising from bacterial attack of jet aircraft kerosene were published in the late 1950s. Bakanauskas (1958) found that fuel-filter clogging in aircraft was due to the bacterial slime formed at the fuel/water interface in the tanks of the aircraft and that fungi were not present. However, the mechanical strength of these bacterial slimes was subsequently thought to be insufficient to cause filter blockage implying that fungi were present.

De Gray and Killian (1960) reported that bacterial slimes occurring in fuel-storage tanks could increase corrosion of tanks and pipelines, due to the production of low molecular-weight organic acids which lowered the pH value of the aqueous phase. These acids could also react with mineral salts in solution to produce more corrosive acids.

The first report of fungal activity in jet fuel systems was by Klemme and Leonard (1960) who found that *Hormodendrum resinae* (later known as *Cladosporium resinae*) was the predominant fungus present. Hazzard (1961) reported that fuel tank corrosion in Lockheed aircraft was associated with fungal contamination of the fuel. He found that fungal growth was present in a high proportion of samples taken from storage tanks, aircraft tanks, tankers, refuelling vehicles and pipelines. Thereafter careful, extensive and comprehensive studies were carried out by many researches on the history of the problem of microbial contamination of aviation fuel and the development of control measures (Stormont, 1961; Parbery, 1971; Sheridan, Nelson and Tan, 1971; and Genner and Hill, 1981).

Cladosporium resinae possibly appeared in diesel fuel at the same time as in the aviation fuel, but because the fuel was used in less sophisticated diesel engines it probably passed unnoticed until its presence in jet fuel caused concern. A comprehensive survey by Liggett (1976) of marine, rail and road diesel fuels, tractor vapourizing oil, paraffin, gas oils and central-heating fuels gave a clear indication of the widespread nature of the microbial contamination. This survey also reported associated system malfunctions, particularly filter plugging and lowering of interfacial tension between fuel and water. Hostetler and Powers (1963) have demonstrated that injector failure in diesel locomotives can be attributed to contamination of the fuel and they quote figures indicating that biocides minimize formation of 'gum' sediments and filter plugging in heating oils, rail and road vehicle fuels.

Cladosporium resinae was found to be present in 'dieso' and moreover this fungus was reported to constitute the bulk of the 'biomass' (Houghton and Gage, 1979; Neihof, 1980; Bruce, 1982; Arnold, 1983).

Some bacteria and fungi isolated from hydrocarbons, do not survive in fuel for as long as the fuel-degrading organisms. This suggests that these are chance contaminants introduced through inadequate fuel sampling techniques or through recent fuel system contamination (Cooney and Kula, 1970).

The micro-organisms found in fuel can originate from a variety of sources. Bacteria and fungi may find their way from air, water and soil into storage tanks, oil-fired furnaces, diesel locomotives, trucks, aircraft and ground storage tanks. *C. resinae*, the common contaminant of kerosene and diesel, is known to be widespread in soils and as airborne spores in Australia, British Isles and Europe (Parbery, 1969) and New Zealand (Sheridan, 1972). The ubiquitous presence of *C. resinae* suggests that it will eventually find its way into fuel tanks but such contamination may be harmless unless water is present to provide the ideal habitat for growth (Sharpley, 1966).

As this study is focussed on the microbiological contamination of diesel fuel used mainly by frigates, it is appropriate to discuss the type of tanks used in warships and how microbiological contamination could affect the fuel system. Warship fuel systems provide basically two different microbial environments. These are as follows:

(i) Seawater-displaced fuel tanks (water compensated tanks) may contain a high ratio of water to fuel (refer Figure 1.1) and offer a wide range of nutrients, in addition to those already in the fuel. The nature and quantity of these supplementary nutrients will depend on the type of water used to fill the header tanks, which may range from highly saline deep-seawater to estuarine water. Factors such as sewage content and trace metal contamination of the water may affect the extent of colonization (Neihof and May, 1983).

(ii) Undisplaced fuel tanks (non-water compensated tanks) contain a low ratio of water to fuel and generally less particulate organic matter. Figure 1.2 illustrates such a system. Nutrient levels will be determined by the previous history of the fuel and subsequent factors such as deterioration of tank linings and seals.

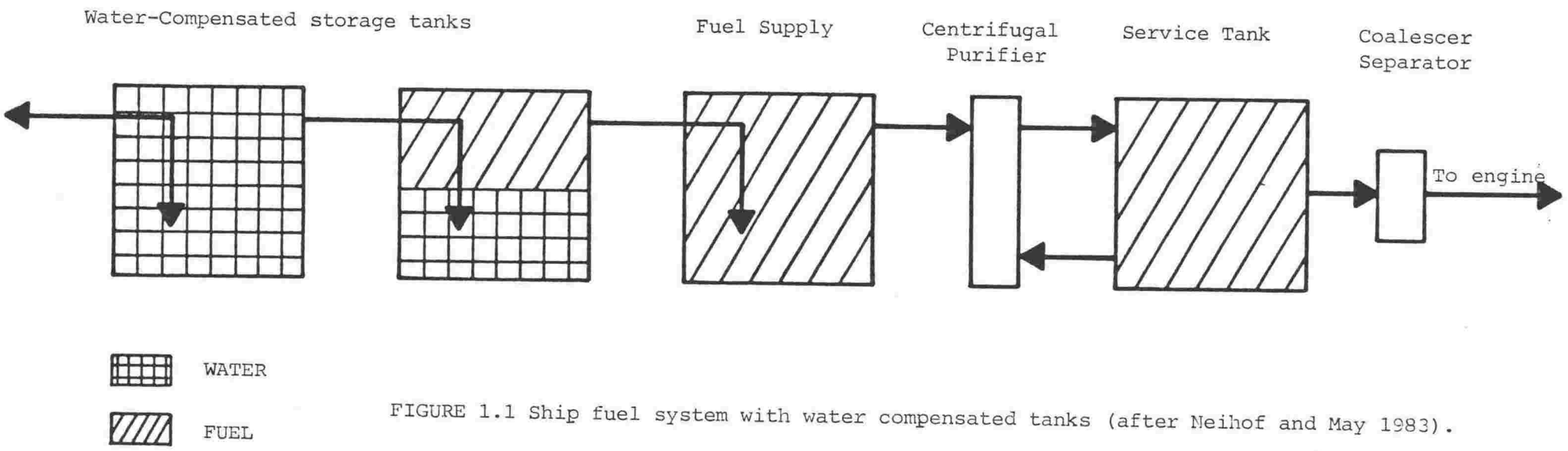


FIGURE 1.1 Ship fuel system with water compensated tanks (after Neihof and May 1983).

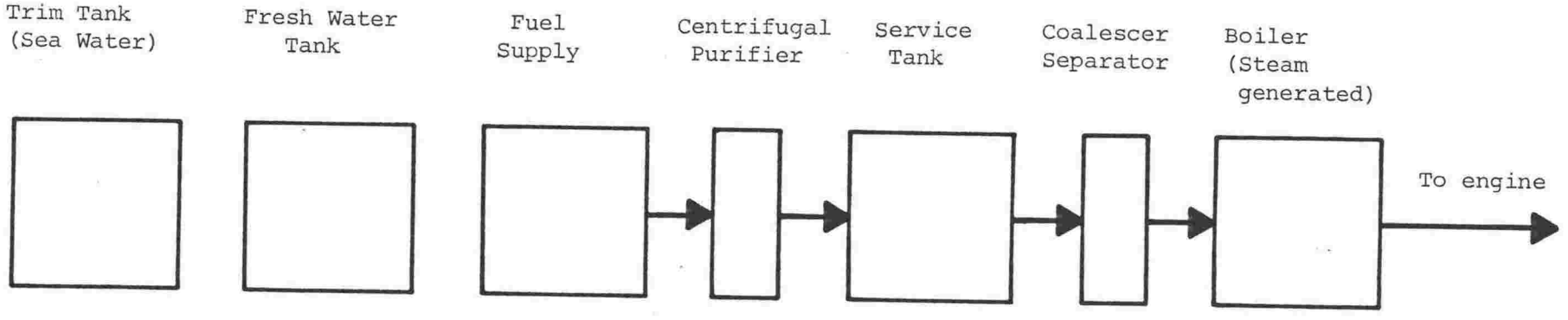


FIGURE 1.2 Ship fuel system-steam turbine powered engine with non-water compensated tanks (note boiler is only present in steam turbine ships).

Both the above systems may use the coalescer to effect separation of entrained water. In the coalescer elements a continuous flow of fuel and water passes over ensnared organisms, supplying their nutrient requirements and removing their metabolic products. Degradation of the cotton, used in the construction of the coalescer elements, may supply alternative carbon sources. During the fuel's journey through the system, the concentration of some nutrients decreases, while levels of metabolic and cell lysis products may increase (Turner, Eaton and Jones, 1983).

The sea-water displaced system of both steam and gas turbine powered ships was proved to be vulnerable to fuel system blockages resulting from microbial contamination of fuel. Prior to delivery to engines the solid matter and water content of the 'dieso' could be reduced by filtration and water separation techniques. High levels of fuel sludge result in premature failure of filters and water-separator systems and may cause acute fuel starvation (Turner, Eaton and Jones, 1983). The fuel temperature reflects the external sea temperature and hence can be expected to vary from 4°C to 25°C, which supports microbial growth.

According to Genner and Hill (1981), microbial growth in fuels can result in several types of problems which can be grouped into two major classes, whether they are caused by metabolism of organisms or by the physical presence of organisms.

Both corrosive substances and gas are known to be produced during microbial metabolism. For example it has been found that four carboxylic acids were produced by *C. resinae* (Rivers, 1973; McKenzie, Akbar and Miller, 1976) and they were identified as dodecanoic, acetic, glycollic and glyoxylic acids (Siporin and Cooney, 1975). Studies of the corrosive effect of *C. resinae* have centred on aluminium and its alloy: pitting, surface exfoliation, blistering of the oxide layer, and intergranular corrosion have commonly been reported, as well as general weight loss (Hedrick *et al.*, 1965; Parbery, 1968; Rivers 1973; Al-Haidary, 1977; Hansen, Tighe-Ford and George, 1981).

The acid metabolites increased corrosion by lowering the pitting potential of aluminium and aluminium-copper alloy (de Male, Salvarezza and Videla, 1979; de Meybaum and de Schiapparelli, 1980; de Schiapparelli and de Meybaum, 1980). Clearly, meshed fungal mycelium would lead to physical blockages of pipeline filters and drain holes and malfunction of coalescers and fuel gauge probes.

The technical innovation of using steam and gas turbines as power units for naval vessels has been accompanied by predictable microbial problems. Diesel fuelled gas-turbine powered engines, most common in American and British frigates, use water displaced tanks which are likely to be affected by the consequences of microbial growth. Here seawater displaces fuel as it is used to eliminate vapour space in the tanks. The Royal N.Z. Navy ships have steam turbine-powered engines using both displaced and undisplaced fuel tanks systems.

In the gas-turbine powered engines, the diesel is ignited to produce gas which drives the turbine. It is operated on a similar principle to a jet aircraft engine. In the steam-turbine powered engine, the diesel is injected into the boiler. Water is constantly circulated through tubes in the boiler, where the diesel is fired to convert the water into steam. Thus the steam drives the turbine. It works on the same principle as the steam driven turbine in a thermal power station.

Ships using water displacement systems have encountered occasional difficulties from rapid accumulation of particulate matter in centrifugal purifiers and coalescer filters (Neihof and May, 1982). A centrifugal purifier is designed to remove most of the particulate matter and entrained water before the fuel enters the service tank. There are no centrifugal purifiers in the fuel system in N.Z. frigates (Defence Scientific Establishment (DSE) per. comm.). Coalescer filters remove residual water before the fuel goes to the engine. The ground storage tanks used by the New Zealand Navy acquire water bottoms by rain-water seepage and condensation of water carried in the fuel. The microbial growth occurs at the interface of this aqueous phase.

1.1.2 Morphology of *Cladosporium resinae*.

Cladosporium resinae (Lindau) de Vries is the anamorph of the 'kerosene fungus' *Amorphotheca resinae* Parbery. It has recently been renamed *Hormoconis resinae* (Lindau) Von Arx and de Vries (1973). The reason for transferring the 'kerosene fungus' to the genus *Hormoconis* is based on the lack of prominent basal scars on the conidia, diagnostic of the genus *Cladosporium*. Since *C. resinae* is widely used and accepted, this name is used throughout this study. Four forms have been identified. Two forms have morphologically distinct reproductive structures - *f. avellaneum* and *f. resinae*. An albino may possess either morphology - *f. albidum* and there is a sterile form - *f. sterile* (de Vries, 1952; Parbery, 1969; Sheridan, Tan and Nelson, 1972). Intermediates and variants of each form exist (Tan, 1972). This variability, together with variation in the ability to grow on petroleum products such as aviation kerosene, diesel and in other substrates including creosoted wood (Christensen *et al.*, 1942; Marsden, 1954), wood treated with copper-chrome or arsenic preservatives (Nilsson and Henningsson, 1977), Coniferous resin and asphalt pavements have stimulated research interest in this fungus. These substances are normally considered to be fungistatic or fungicidal, yet *C. resinae* can utilize these as a source of carbon. Studies of anatomy and ultrastructure using both the scanning and electron microscope have been attempted to explain the properties of this most unusual fungus (Tan, 1972; Sheridan and Troughton, 1973; Soteros, 1973; Cooney, Siporin and Smucker, 1980; Smucker and Cooney, 1981; Smucker and Cooney, 1983).

1.2 Control of microbiological contamination in hydrocarbon fuel.

Solutions to the problem of microbial growth in diesel fuel systems have been sought by various groups (Landsdown, 1965; Edmonds, 1966; Hill *et al.*, 1967; Hendey *et al.*, 1971; Siporin and Cooney, 1975; Upsher, 1976; Houghton and Gage, 1979; Turner, 1981; Wycislik and Allsopp, 1983). The general conclusion from these studies is that there are several ways to prevent microbiological growth in fuel systems: good housekeeping, the addition of biocides and the use of tank linings.

Good housekeeping and biocides can be used to minimize both contamination and growth, while tank linings can minimize corrosion should contamination occur. The control of microbial contamination by application of heat directly to the fuel on ships or in land storage depots has been considered (Upsher, 1976; Houghton and Gage, 1979; Wycislik and Allsopp, 1983).

There have been reports of a novel type of control in New Zealand, called the 'hydromag' (news report Anon, 1986). This device is described as a small inexpensive filter containing circular ceramic magnetic disc plates through which the fuel passes on its way to conventional filters. These are now installed in trial Auckland Regional Authority buses (ARA pers. comm.). The preliminary results were reported to be favourable but inconclusive. The use of 'hydromag' may not be practical in large volume storage tanks at ground installations or in the frigates. The corrosion problems associated with microbial contaminants during storage, and in the fuel lines may not be eliminated by the use of this device.

Good housekeeping primarily involves removal of free water from ground storage tanks and onboard ship tanks thereby minimizing the likelihood of microbial growths becoming established. In underground fuel installations such as those of the N.Z. Navy at Devonport, free water cannot be entirely removed because of the poor design of the storage tanks system. Inevitably, water is present and the use of biocides must be considered. In Australia, the tanks are designed to have conical bottoms to drain water readily (Sheridan per. comm.). Corrosion is not a problem in the underground storage tanks because the walls are made of concrete. It could be a problem, however, further downstream particularly in tank ships. The fungus metabolizes fuel by beta-oxidation to produce carboxylic acids (Cooney and Proby, 1971; Walker and Cooney, 1973). Large volume and rapid turnover prevents any significant change in composition of fuel (Hill, 1978); but the acid production can cause a drop in pH of water bottom and this can lead to increased corrosion of metal tanks. Good fuel maintenance is not however the complete solution, it appears that the only practical method for control of *C. resinae* and other predominant fungi is use of appropriate biocides.

The effect of addition of biocides to jet fuel has been widely studied (Klemme and Leonard, 1960; Hitzman, Shotton and Alquist, 1963; Hendey, 1964; Hedrick and Carroll, 1966; Elphick and Hunter, 1968; Rogers and Kaplan, 1968; Rabotnova *et al.*, 1970; Park, 1973; Miller, Mohan and Strickland, 1975; Klemme and Neihof, 1976; Neihof and Bailey, 1978). Few studies have been made on diesel fuel (Stormont, 1962; Hostetler and Powers, 1963; Smith and Crook, 1980; Smith and Crook, 1983). Little work has been done on the effects of biocides on engine performance and the components of the combustion chamber.

Singer (1976) has described a method for recognizing biocidal activity in fuel. Elphick and Hunter (1968) and Rogers and Kaplan (1968) have described experiments which differentiated between biocidal and biostatic properties; but these were only qualitative. Smith and Crook (1980) described experiments which gave quantitative results on the effects of biocides in contaminated fuel.

According to Miller, Mohan and Strickland (1975) studies to identify biocides for the inhibition of growth of micro-organisms in 'dieso'-water systems have indicated that in addition to in vitro broad spectrum biocidal activity, such compounds must exhibit high solubility in the fuel coupled with an effective partitioning between the fuel and the water phase where the growth of micro-organisms occur. The water to fuel ratio can markedly influence the effectiveness of the biocide, a 1:40 ratio being considered ideal for micro-organism growth (Bennett, 1974). The narrower the water to fuel ratio, the smaller is the concentration of biocide required to inhibit the fungal growth.

1.2.1 Criteria for the choice of fuel biocides

The choice of a fuel biocide is restricted due to stringent specifications enforced by engine manufacturers. According to Hill (1982) the desirable properties of a biocide are as follows.
It should:

- be combustible, with no residual ash
- not interfere with fuel properties or the combustion process, or any other aspects of engine performance

- be soluble (or very miscible) in fuel but preferentially soluble in water
- present no health hazard during handling or combustion
- not be corrosive
- preferably achieve a total 'kill' of micro-organisms at minimum dose.

To this list could be added that it must be environmentally acceptable especially where seawater is used for displacement. No such biocide has been developed for commercial use. However a few biocides have achieved market penetration, although deficient in some of the listed characteristics. These are Biobor J.F., EGME and DEGME widely used in jet fuel.

Numerous reports in the literature indicate the effectiveness of EGME (Hitzman, 1964; London, Finefrock and Killian, 1964) and organoboron compounds in jet fuel (De Gray and Fitzgibbons, 1966; Rogers and Kaplan, 1968; Elphick and Hunter, 1968). Elphick and Hunter (1968) focussed on the possible effectiveness of these materials when used as intermittent treatments for jet fuel systems while the others referred to the exposure of micro-organisms to biocides as continuous additives to jet fuel.

It is important to consider the intermittent use of the available chemicals, because factors may arise which militate against the use of biocides as continuous additives to fuel. There may be physical or economic reasons associated with the incorporation of biocide in fuel at a number of different ports where a ship may be required to refuel. A ship may be treated with biocide-containing fuel, and allowed to stand thus fuelled during routine maintenance periods. At the end of these periods this fuel may either be drained off and the ship refuelled with untreated fuel, or the biocide-containing fuel may be used during the next sail, subsequent refuelling being with untreated fuel. Alternatively, or in addition to this procedure, a ship may receive biocide-containing fuel at more or less regular intervals during service, e.g. at one or more of its regular refuelling points.

Biobor JF (U.S. Borax & Chemical Corporation), a mixture of two organoboron compounds, was recommended for use at 270 ppm for disinfection and 136 ppm for continuous dosage in jet fuel. It has been found, however that Biobor JF when used at more than ten times the recommended dose rate in jet fuel still allowed *C. resinae* to remain viable for up to six weeks (Berner and Ahearn, 1977) and a dose rate of 5000 ppm was necessary to kill vegetative hyphae within two days (Singer, 1976). The use of Biobor JF has been criticized by Klemme and Neihof (1976) who found that 20,000 ppm was needed in the water phase to control sulphate-reducing bacteria. Moreover, certain engine manufacturers currently discourage the use of organoboron on a regular basis in fuel because it leaves an ash on combustion and takes several days to be completely effective biologically (Genner and Hill, 1981; Hill, 1982).

Ethylene glycol monomethyl ether (EGME) is used mainly in military aircraft as an anti-icing additive in the fuel to prevent ice formation from entrained water. It was reported by Hitzman *et al.* (1963) and Rogers and Kaplan (1968) to be effective in controlling microbiological contamination in kerosene type fuels. EGME at 1500 ppm (0.15%) is used to provide both anti-icing and antimicrobial activity. The major deficiency, however, is that at low concentrations it is readily metabolised by micro-organisms and hence, if used intermittently, there is a real risk that growth will be promoted (Hill, 1982). EGME affects the flash point of the fuel and there are moves to replace it with a related compound, DEGME (di-ethylene glycol monomethyl ether). This was reported to be a suitable replacement as it was effective as a biocide at 10,000 - 20,000 ppm (1-2%) in aqueous phase compared with 100,000 - 170,000 ppm (10-17%) for EGME (Neihof and Bailey, 1978). The dose rates of both biocides are relatively high and neither was likely to be very effective when the ratio of water to fuel was greater than 1:400 (Hill, 1982). Since 1983 DEGME has been used widely in the Australian Navy (Sheridan pers. comm.).

There are reports (Genner and Hill, 1981; Hill, 1982) of attempts to prevent the emergence of resistant organisms by using mixtures of biocides at different concentration. Walters (1971) suggested that constant concentration treatment with biocides may cause difficulties under practical condition through the appearance of resistant microbiological mutants.

Kathon 886 (active ingredients are 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was found to be biologically active in fuel when used in combination with EGME at the very low concentration of about 1 ppm (Thomas and Hill, 1977). Benomyl and imazalil are agricultural fungicides and the former was found to be effective in suppressing growth of *C. resinae* at a concentration of 1 ppm (W/U) in a 1:1 'dieso' fuel/water ratio (Smith and Crook, 1983). Imazalil has been suggested as a potential biocide for diesel fuel (Janssen Pharmaceutica Ltd. pers. comm.) but no published material is available.

Biocides may present some hazard to ships' personnel and can have unfavourable environmental effects when they are off-loaded (Neihof *et al.*, 1979). Recently Neihof and May (1983) discouraged the routine use of a biocide in ships operated on diesel fuel with a water compensated system. It has been observed that fuel contamination has originated before the ships leave the shipyard because of the introduction of particulate matter in silt laden, polluted water into the tanks (Neihof and May, 1982). This imposed a burden on the centrifugal purifiers and provided a favourable environment for subsequent microbial growth. Most of the particulate matter would appear to be the fuel insolubles formed from inherently unstable fuels and an array of particles introduced with the ballast water. These workers have made recommendations for minimizing these sources of contamination.

Anti-microbial activity depends upon effective contact between the chemical and the micro-organism and the mechanism involves disruptive interaction with a biochemical or physical component of the organism which is essential to its structure or metabolism (Kostenbauder, 1977; Albert, 1979).

The target may be a single enzyme, a cell membrane, a more generalised aspect of the cell or a combination of these and the nature of the action is dependent on the organism and the environment in which the interaction occurs as well as on the anti-microbial agent. For example quaternary ammonium agents are known to be surface active agents which may influence the cytoplasmic membrane and thereby upset the osmotic balance and permeability that are essential to the life of the cell. In fuel, quaternary ammonium compounds appeared to be particularly suitable, some resulting in sterilization after two hours exposure, and also they had good cleaning properties (Genner and Hill, 1981). However, they may lower the interfacial tension between fuel and water and become non-persistent and lose their activity progressively in emulsions, often within a few days and particularly above ambient temperature (Russell, 1982). Benomyl is known to interfere with mitosis by disrupting the normal assembly of the mitotic spindle (Davidse, 1973; Hammerschlag and Sisler, 1973). In aqueous solution, benomyl hydrolyses rapidly to form two compounds, carbendazim and butyl isocyanate, both of which show antifungal properties (Clemons and Sisler, 1969; Hammerschlag and Sisler, 1973; Chiba and Doornbos, 1974).

In New Zealand, major problems with diesel fuel blockages and fuel gauge failures have occurred in navy ships, in the 'Bounty' replica (pers. comm.), and in Auckland Regional Authority buses (News report-Anon, 1981). Little work has been carried out to establish the main cause of these problems. In the last decade the Royal N.Z. Navy has encountered continuous problems in frigates using water displacement tanks (DSE pers. comm.). The major problems involved fuel flow blockages and engine failures.

1.3 Aims of Study.

The present study was initiated under a contract with the New Zealand Defence Scientific Establishment. This concerned

(i) documentation of the nature, extent and possible sources of microbiological contamination of middle distillate fuel ('dieso') held in storage tanks at the RNZ naval base at Devonport, Auckland.

(ii) study of possible methods of control of contamination using biocides. The effects of biocides on fuel effectiveness and engine corrosion were also considered.

Methods for detection of micro-organisms in diesel fuel held in the large underground storage tanks were evaluated. The most efficient method was used to document the micro-organisms present. Changes in the microflora were studied over a two year period. The influence of factors such as container type, transport, shaking and time on micro-organisms in samples after collection was investigated. Interactions between the predominant fungal contaminants were studied.

Commercially available and experimental biocides were added to diesel fuel and their effects in inhibiting growth of micro-organisms in the laboratory and in field tanks (25,500 litres fuel capacity) were studied. The aim was to find an effective biocide which did not have a deleterious effect on engine performance and life.

The morphology and ultrastructure of the major contaminants were also studied to extend previous observations of peculiarities that may explain their unusual metabolic capabilities.

PART 1

STUDIES ON MICROBIOLOGICAL CONTAMINATION OF DIESEL FUEL:

2. MATERIALS AND METHODS.

2.1 Sampling of storage tanks.

2.1.1 Tank description.

Two cliff storage tanks containing diesel, at the Royal naval base, Devonport, Auckland, were investigated for microbiological contamination. The horizontally placed, cylindrical tanks (numbers 2 and 3) are approximately 115 m long and 9 m deep. The fuel tanks and the fuel reticulation system are illustrated in figure 2.1. Underneath each cliff tank there are 'field tiles' consisting of a spaced series of short round, coarse ceramic pipes (300 mm long x 76 mm wide). These pipes are designed to absorb any diesel that soaks through the tank wall and also the ground water seepage. The liquids are channelled into a small sullage tank (2.4 m high x 1.8 m diameter), one for each cliff tank. Within the bottom of each cliff tank, there is a sullage pipe that drains the water and some bottom fuel into the sullage tank. The sullage tank contents are pumped into the separating tank. Everyday as the fuel rises in the separating tank, it cascades over and is pumped directly into tank 3 while the water is pumped into the sea. There is no filter system in between tank 3 and the separating tank.

The tanks are adequately ventilated as shown in figure 2.2. Tank 3 was selected for fuel testing because it was the only tank where the fuel was constantly circulated. The fuel in tank 2 was frequently used by frigates and the fuel usage was higher than from tank 3. The fuel from all the cliff tanks flowed through the same pipeline to the wharf.

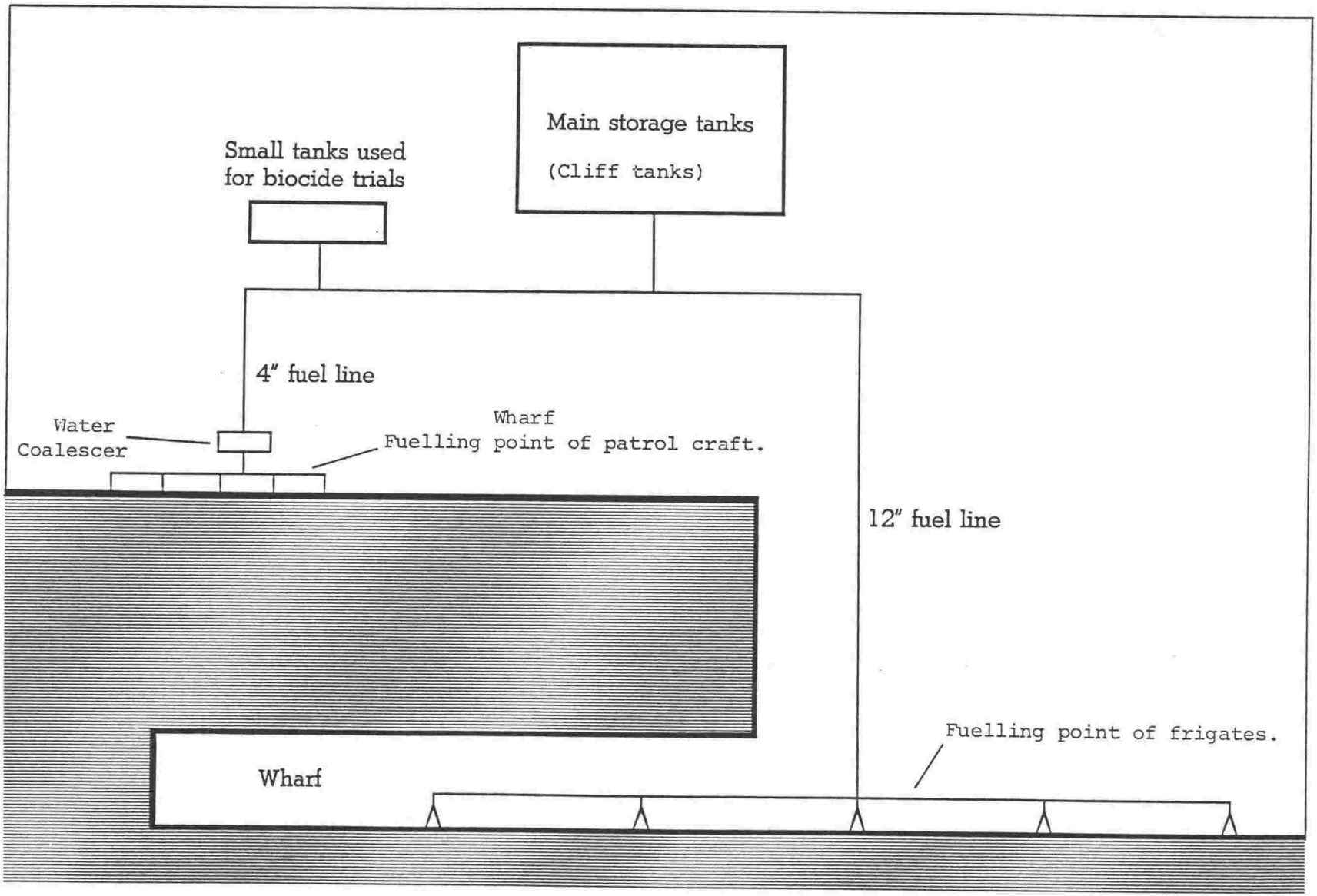


Figure 2.1 The fuel tanks and the fuel reticulation system.

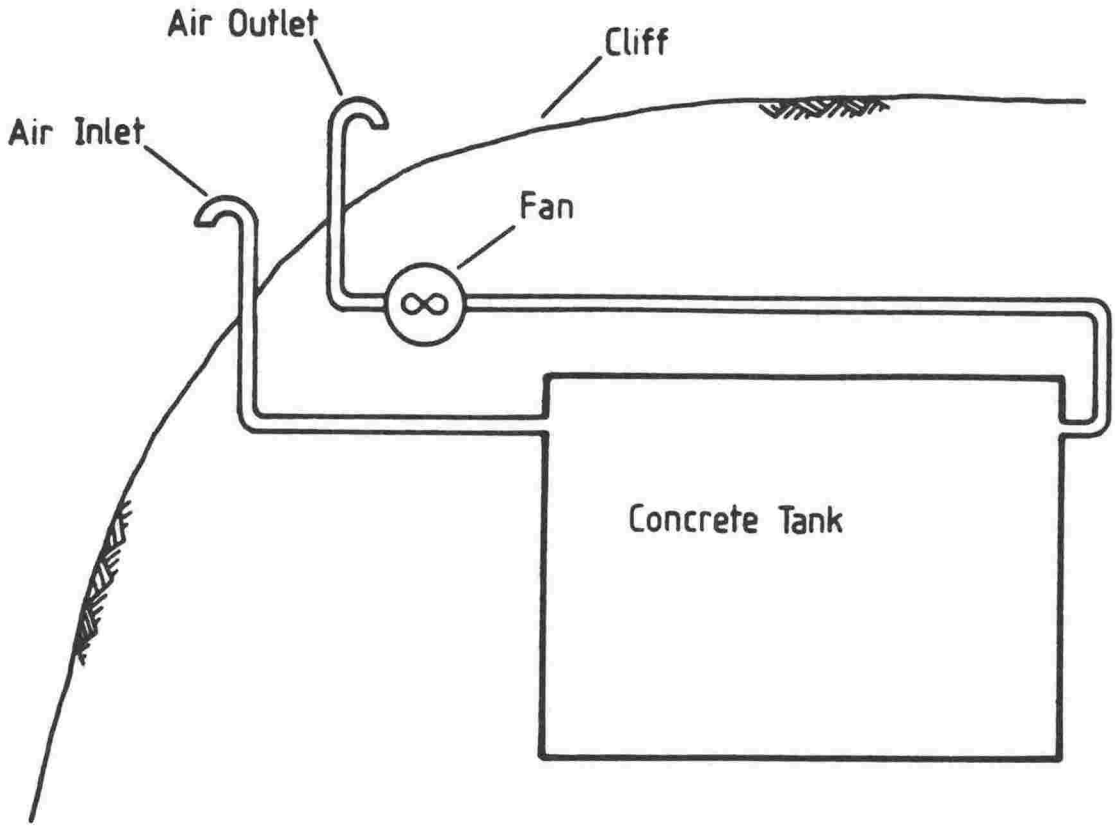
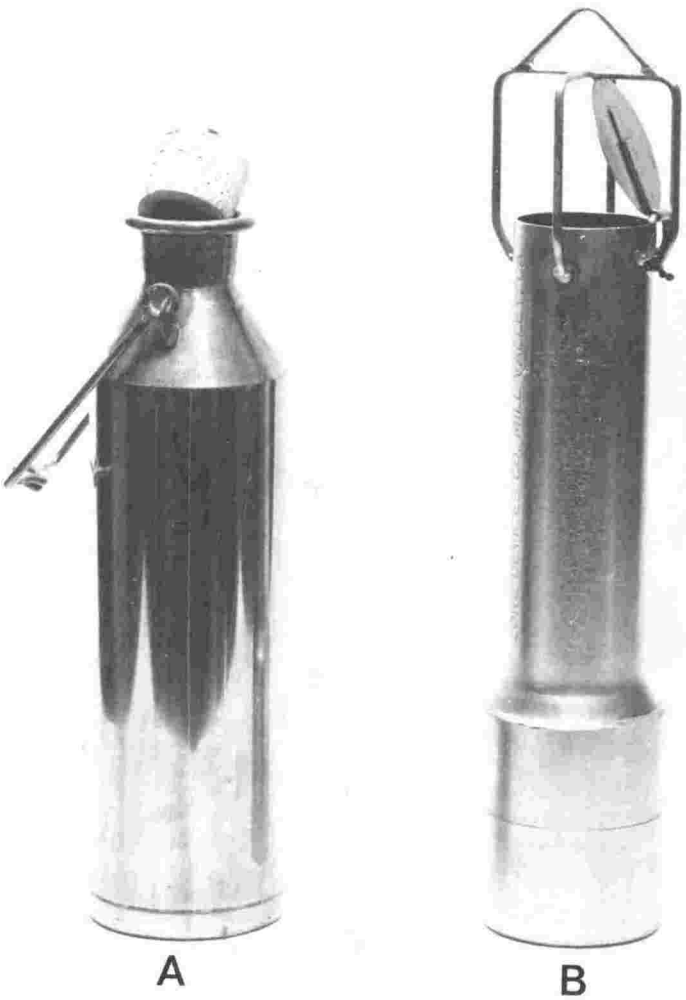


Figure 2.2 Sectional view of the cliff storage tank showing ventilation system. (tank not to scale)



2.1.2 Collection of samples

A total of ninety three fuel samples from the top, middle, bottom and 'very bottom' of the two cliff storage tanks (tanks 2 and 3) were tested between May 1982 - December 1984.

In addition the following fuel samples were tested:

(i) Fuel and water sumps were collected separately from the separating tank to observe the contamination level in the fuel re-entering tank 3.

(ii) Fuel from wharf samples was collected from the water coalescer (water separator) before the fuel is pumped into the patrol craft. This water coalescer (refer figure 2.1) is made of cotton and inside there is fine brass gauze. Later it became evident that it was important to test the fuel entering the patrol craft (after and not before the water coalescer) for biological contamination level. The fuel for the frigates is pumped directly from the cliff tank and not through the above water coalescer. Onboard frigates, before the fuel goes into the engine system, it is filtered through a coalescer which consists of glass fibre elements with a hydrophilic coating.

(iii) A single fuel sample was obtained from the Shell storage tank (situated directly on the opposite side of the Devonport wharf). Shell was the supplier of fuel to the base.

(iv) A single fuel sample from the tank cleaning vessel (TCV) was collected. TCV was used for cleaning and maintenance of the shipboard tanks. It also collects fuel from the Shell tanks and pumps the fuel into the cliff tank and occasionally pumps the fuel from the frigates to the cliff tanks.

These samples were important because they might establish the source of contamination in the ground installation tank. Due to management difficulties, it was impossible to obtain fuel samples from all of the above positions regularly. It was not possible to sample the fuel along the fuel line, since most of the pipes are buried underground.

2.1.3 Sampling methods

Fuel from the top (0.6 m from the top of the tank ceiling), middle (2 m below the surface of the fuel), bottom (6 m below the surface of the fuel) of the storage tanks was sampled by a weighted sampling can (figure 2.3A). This is a brass bottle with a capacity of one litre. It fills with fuel after being lowered to the appropriate level where the stopper is removed by the captive rope.

The 'very bottom' of the tank was sampled by a zone sampler also sometimes called the 'thief' (figure 2.3B). This sampler allows the fuel to pass through as it descends through the fuel in the tank. This is made possible by the top and bottom valves opening due to the pressure of the fuel against the descending sampler. When the sampler contacts the very bottom of the tank, the valves close and the sample is retained as the sampler is retrieved to the surface. This fuel sample is thus from one mm from the very bottom of the tank to 300 mm to the top of the sampler.

The temperature of the fuel was recorded by dipping a thermometer into the fuel sample as soon as it was brought to the surface.

2.1.4 Sampling pattern

The fuel samples were immediately transferred into clean, sterile containers in such a manner as to minimize contamination from the environment and transported to Wellington, where the microbiological examination was carried out. Initially two types of containers: glass preserving jars and tin cans were used, to observe whether container type had any effect on the recovery of micro-organism. It was found that glass bottles and tin cans were equally suitable. However the former were preferred because they do not corrode and are easier to clean and sterilize. For routine use the sampling cans and glass preserving jars were washed in detergent, rinsed with 95% alcohol and sterilized at 200°C in a hot air oven for three hours before collection of the diesel fuel.

Fungal and bacterial assays were carried out on the samples both immediately and twenty-four hours after sampling.

Later this was extended to 4-5 days in order to detect any change in the composition and relative abundance of the microflora in the sample over time. As the time of testing of samples after collection did not appear to be critical, immediate testing in Auckland was discontinued. The fuel was always visually examined before subjecting it to microbiological procedures.

2.2 Detection of micro-organisms in the fuel samples

2.2.1 Culture media

2% malt agar without peptone and dextrose and V-8 juice agar (Sheridan, Steel and Knox, 1971) were used for growth and isolation of fungi. Sterile Petri-dishes containing V-8 juice agar with creosote were used selectively to isolate *C. resinae* from the atmosphere (Sheridan and Nelson, 1971). Water agar was used on settle plates to trap air spora. Czapek-Dox agar (Pitt, 1979) was used as an identification medium for *Penicillium* spp. Nutrient and blood agar base (Collins and Lyne, 1976) were used to isolate bacteria. Selective media such as Starkey, Baar's agar (Sharpley, 1966), Bacto sulfate API broth with and without sodium chloride (Hill, 1975) were used to isolate sulphur bacteria. MacConkey medium (Collins and Lyne, 1976) was used for the detection of *E. coli*. Bushnell-Haas mineral salt medium was prepared according to Parbery and Thistlewaite (1973) and used in conjunction with a selected hydrocarbon as carbon source. All the media were prepared by dissolving the agar before the constitutents were added and mixed. Sterilization was by autoclaving at 103 kPa (121°C) for 15 mins, except in the following cases:

(i) for the blood agar, the sterile defibrinated horse blood was added to the autoclaved medium at 48°C;

(ii) when creosote was used it was autoclaved and added aseptically to sterile molten media (55°C) just before pouring the plates.

2.2.2 Membrane filtration

The fuel sample was shaken thoroughly before filtering through a membrane filter. The size of the fuel samples tested varied between 50 cm^3 - 200 cm^3 depending upon the contamination level.

Sterile cellulose acetate filters with a pore size 0.45 µm, 47 mm diameter (0.45 µm, filters, type HAW 0047 sl) and with a pore size 0.22 µm (0.22 µm filter GSWP 04700) were used to isolate fungi and bacteria respectively. Suction was applied by a mechanical vacuum pump for 30 seconds after the last drops of the sample had passed through the membrane. A trap was always used between the pump and the membrane, to prevent any fuel entering the pump. The pump was then disconnected and the membrane transferred, using sterile forceps, to the centre of a sterile Petri-dish. The selective medium (at 55°C) was poured over the filter membrane and the Petri-dishes were incubated at 25°C in the dark for 5 days.

Whenever the samples exuded the strong 'rotten egg smell' characteristic of sulphur bacteria, or if there was an increase in water at the tank water bottom, the water sumps and the 'very bottom' samples from the cliff tanks were tested for sulphur bacteria. The samples were filtered using 0.22 µm filters and these were plated on sterile Petri-dishes. The selective medium (see culture media) was poured over filters. These plates were incubated in gaspak anaerobic jars at 35°C for 5 days.

On the 19th April 1983, it was suspected that sewage could have leaked into the tank. Thus the samples were tested for the presence of *Escherichia coli (E. coli)*. The water samples from the very bottom of tanks 2 and 3 and the separating tank were filtered using 0.22 µm filters. The MacConkey medium was poured over the membranes and the plates were incubated at 37°C for 24 hours. The bacteria isolated from the water samples were tested for breakdown of lactose and the subsequent production of acid and gas at 44°C, a test which is diagnostic for *E. coli* (Collins and Lyne, 1976).

Quantitative estimation of the level of contamination in the tanks was made by recording the number of colonies of each fungus on each plate and the number of samples in which a colony forming unit of a particular fungus occurred. Each discrete colony formed by a fungus on a culture plate was recorded as one colony forming unit (CFU).

For each sample assayed five aliquots were filtered and the counts of CFU recorded are the mean number of colonies from these five filters for each fungus.

2.2.3 Direct Observation

Duplicate 100 cm^3 of each sample were filtered separately using a 0.45 µm filter. The filters were then transferred aseptically to sterile Petri-dishes. The spores were either stained with cotton blue or mounted in colourless lactophenol and examined under the phase contrast microscope immediately to record the range of spore types present.

2.2.4 Pour plate method

Duplicate samples of 5 cm^3 from water sumps, from the separating tank and sometimes water from the 'very bottom' sample from tanks 2 and 3 were aseptically pipetted directly into sterile Petri-dishes. Molten selective culture media at 45°C was poured over the sample and mixed by rotating the dish clockwise and anticlockwise to disperse the organisms. After incubation for 5 days the colonies were identified and counted.

2.2.5 Spread plates

Samples of fuel or water bottom were vigorously shaken and duplicate samples of 5 cm^3 were pipetted onto a selective agar medium and a sterile bent glass rod was used to spread the sample over the entire surface of the plate. To observe the growth of anaerobic sulphur bacteria, the plates were incubated in an anaerobic gaspak jar at 35°C for 5 days.

2.3 Detection of micro-organisms in air and soil

Initially the air spora in the immediate vicinity of the storage tanks was examined by the settle plate method. Water agar plates were exposed in duplicate inside and outside the area surrounding the tanks at different times during the day. In the laboratory, V-8 juice agar with and without 0.1% creosote was poured over the water agar plates. The creosote was used to selectively isolate creosote tolerant fungi (cf. Section II).

In order to obtain data on the seasonal variation of *C. resinae* and other creosote tolerant fungi, the atmosphere around the storage tanks was continuously monitored by a seven day Kramer-Collins spore sampler (Kramer *et al.*, 1976) over a period of 24 months. The spore sampler was situated on the top of the cliff tanks, near the air inlet vent of the tank. In the laboratory the strips of double-sided cellotape coated with sterile vaseline were cut from the cassette and placed in sterile Petri-dishes. V-8 juice agar containing 0.1% creosote was poured over them and plates were then incubated at 25°C for 5 days before observation. Because the amount of air entering the trap and the rate of movement of the tape past the orifice is known, the concentration of the fungal spores in the air together with the time at which they landed can be calculated.

The soils were examined for the presence of *C. resinae* by the modified creosote matchstick method (Sheridan, Steel and Knox, 1971). The soils were collected from around the tanks into clean plastic bags using a sterile spoon. These soils were compared with a standard Brooklyn soil which had previously given positive results for the presence of *C. resinae*. All tests were set up in duplicate.

In November 1983, soil samples from the vicinity of the cliff tanks were collected (as above) and tested by the dilution plate method described below to determine the species of fungi present for comparison with those found in the fuel.

2.3.1 Dilution plating

5 grams from each of 22 soil samples were weighed accurately into 50 cm^3 of sterile water and shaken on a mechanical shaker for 1 hour (100 rotation per minute). Nine dilutions from 10^1 to 10^9 were made in standard test-tubes. 1 cm^3 aliquots from the undiluted suspension, the x 10, x 10^2, x 10^5 and x 10^9 dilution were separately added to 15 cm^3 of cooled (45°C) malt extract agar, contained in sterile 90 mm diameter plastic Petri-dishes. The dishes were rotated gently to disperse the spores in the agar. The colonies were observed after 5 or 6 days incubation at 25°C. The *Penicillium* spp. isolated from the plates were re-inoculated into malt extract and Czapek-Dox agar media to identify the species.

2.4 Identification of micro-organisms

Fungi were examined microscopically and identified using available keys. The Commonwealth Mycological Institute, Kew, England identified or confirmed identification of a number of species.

2.5 Laboratory studies using fungi isolated from diesel fuel

2.5.1 Growth studies

Growth studies of fungi isolated from fuel, air and soil in this study and of a range of fungi isolated from other sources were carried out using conidial suspensions as inoculum into aqueous/diesel phases. Growth and interactions were studied by observing the growth of mixed inocula and also by inoculating conidial suspensions into aqueous/diesel fuel phases recovered after six weeks growth of the same or different fungi. The selectivity of fungi was studied further by comparing growth obtained from diesel and aviation fuel as carbon source. In addition to growth measurements (dry weight) the survival of colony forming units (spores) or other fragments was also determined after the growth period.

(i) Single species culture (Table 2.1)

Growth was initiated by inoculating 100 cm^3 of the culture media contained in a 200 cm^3 medical flat bottle, with a spore suspension. The culture media varied between experiments, an initial set comparing diesel fuel alone with a 1:1 mixture of seawater/fuel, distilled water/fuel, tapwater/fuel and Bushnell-Haas mineral salt medium (BH)/fuel.

Seawater was collected from the Zoology Marine laboratory, Wellington, left in the dark for several weeks before use in order to kill off any photosynthetic organisms and subsequently sterilized by filtration (using 0.22 μm filters) in order to preserve the nutrient constitutents. Diesel fuel was also filter-sterilized. All the other aqueous media was autoclaved at 103 kPa (121°C) for 15 minutes.

Table 2.1 Growth Studies of single species in various media

Fungal Species	Growth Media					
	Diesel Fuel only	Seawater/ Diesel	Distilled Water/Diesel	Tapwater/ Diesel	BH/ Diesel	BH/ Jet Fuel
1. N.Z. Isolates						
*C. resinae** ex diesel	X	X	X	X	X	
*C. resinae** ex soil (S82)	X	X	X	X	X	
*C. resinae** ex air (A82)	X	X	X	X	X	
C. resinae ex jet fuel (K10f)	X	X	X	X	X	
*Paecilomyces** *variotii* ex diesel (P82)	X	X	X	X	X	
*Penicillium** *corylophilum* ex diesel (IMI 280045)	X	X	X	X	X	
2. Australian Isolates						
C. resinae ex Army culture (25/5/83)					X	
C. resinae ex Tasmania (24/6/83)					X	
C. resinae ex Cook Island (1/8/83)					X	
C. resinae F. albidum ex Australia (28/9/83)					X	
C. resinae ex HMAS Cerebus (4/7/83)					X	
*C. resinae** ex diesel (G82)					X	
C. resinae ex Jet Fuel (K10f)					X	
3. *Penicillium* spp. from diesel						
*P. chrysogenum** (IMI277572)					X	X
*P. corylophilum**					X	X
*P. echinulatum** (IMI277570)					X	X

Table 2.1(cont.) Growth Studies of single species in various media

Fungal Species	Growth Media				
	Diesel Fuel only	Seawater/ Diesel	Distilled Water/Diesel	Tapwater/ Diesel	BH/ Diesel	BH/ Jet Fuel
*P. expansum** (IMI282480)					X	X
*P. digitatum** (IMI277577)					X	X
*P. frequentans** (IMI277573)					X	X
*P. spinulosum** (IMI277571)					X	X
*C. resinae** ex diesel					X	X
C. resinae ex jet fuel (K10f)					X	X
*Paecilomyces variotii**					X	X
4. Soil Isolates						
*Penicillium aculeatum** (IMI282471)	X				X	
*P. brevicompactum** (IMI282467)	X				X	
P. funiculosum* (IMI282477)	X				X	
*P. loliense** (IMI282469)	X				X	
*P. nigricans** (IMI282460)	X				X	
*P. verrucosum** (IMI282470)	X				X	
*Trichoderma harzianum** (IMI282478)	X				X	
*T. hamatum** (IMI282475)	X				X	

* These species were isolated from this study

The conidial suspensions were prepared from two week-old cultures grown on V-8 juice agar at 25°C. 20 cm^3 of BH medium was shaken over the V-8 juice agar culture and the resulting suspension centrifuged in a 15 cm^3 sterile glass centrifuge tube at 1520x g for 20 minutes. The conidia were resuspended into 20 cm^3 BH medium and 0.1 cm^3 containing approximately 5 x 10^6 spores/cm^3 was inoculated into the BH/fuel. The tests were set up in 200 cm^3 medical flat bottles (previously sterilized in an oven at 200°C for 3 hours). The aluminium caps with the rubber liner removed, were screwed on loosely and the bottles incubated at 25°C for 6 weeks. At the end of this period, the growth was assessed visually. Regardless of growth or no growth, the contents of the bottles were filtered through 0.45 µm membrane filters, partially wet in sterile water and diesel fuel. The filter membranes were dried to constant weight at 80°C in a hot-air oven. Constant weight was achieved in two days in the case of growths from diesel fuel. The dry weights of the membranes were recorded in mg. The pH of the aqueous phase was determined initially and at weekly intervals throughout the 12 weeks. (For each determination 5 cm^3 of the aqueous phase was removed with a sterile pasteur pipette and returned to the remaining growth medium after pH determination).

(ii) Growth in mixed cultures

The same method used in (i) was used in the following growth experiments.

Two series of experiments were carried out to determine the effects of fungal growth upon the ability of media to support and maintain viability of subsequent spore inoculum.

(a) *P. corylophilum* ex diesel and *C. resinae* ex diesel were grown as mixed inoculum on seawater/Diesel(D. fuel), distilled water/D. fuel, tapwater/D. fuel, Bushnell-Haas/D. fuel and D. fuel only. After 6 weeks, growth was measured. Samples of 5 cm^3 were removed from the interface and used for pour plate on V-8 juice medium and observed for growth.

The aqueous phase was also examined in the light microscope to determine the extent of germination.

(b) Growth of *P. corylophilum* and *Paecilomyces variotii* was determined after 6 weeks growth in media which had previously been used for growth of *C. resinae* for 6 weeks. *C. resinae* was grown on seawater/D. fuel, distilled water/D. fuel, Bushnell-Haas/D. fuel at 1:1 ratio at room temperature, and harvested after 6 weeks and dry weights being recorded. The filtered media were then inoculated with other fungi *P. corylophilum* and *P. variotii* separately. The pH of the aqueous phase was determined initially and at weekly intervals throughout the 12 weeks. The initial pH of seawater was 8.3; of BH —7, tapwater —7 and distilled water —7. In order to establish whether germination had occurred, in doubtful cases, small amounts of water at the fuel-water interface were removed for microscopic examination. The microbial growth was observed visually and a rating system employed as follows:

0 - no germination nor growth
\+ - thin spore mat, very little growth
++ - very thin colourless mycelial mat
+++ - thick dark mycelial mat.

(c) A second more extensive set of mixed spore cultures including more isolates were established as follows: *Candida albicans* (404) was grown. Alone and with each of the following fungi separately: *C. resinae*, *Penicillium corylophilum*, *P. expansum*, *Paecilomyces variotii*.

C. resinae was grown alone and with *P. corylophilum*, *P. expansum*, and *P. variotii*.

P. corylophilum was grown alone and with *P. expansum*, and *P. variotii*.

P. expansum alone and with *P. variotii*.

P. variotii grown alone.

The above fungi were grown for 6 weeks in seawater, tapwater, BH, seawater/Diesel, tapwater/Diesel, BH/Diesel. After 6 weeks, where there was no visual growth, the bottles were shaken thoroughly before removing 5 cm^3 of the suspension for pour plate, to check the viability of the conidia. In the case of growth, the mycelial mat was harvested as mentioned previously and dry weights recorded and pH was monitored at weekly intervals.

(d) In a separate experiment medium in which *P. corylophilum* had grown for 6 weeks was inoculated with *C. resinae*, medium in which *C. resinae* had grown was inoculated with *P. variotii* and medium on which *P. variotii* had grown was inoculated with *P. corylophilum*. Growth was measured after the first 6 weeks and the second 6 weeks of culture. The growth media were filtered before the second batch of inoculation. Growth media used were seawater/diesel fuel, seawater, tapwater/fuel, tapwater, BH/fuel and BH only. pH was monitored weekly throughout the experiment.

2.5.2 Effect of creosote on fungal growth

Isolates from soil around the cliff tanks, diesel fuel in the tanks, Australian diesel, jet fuel and *Cladosporium* spp. from other sources were compared to determine their ability to grow in the presence of a range of creosote concentrations (table 2.2).

The concentrations of creosote used on V-8 juice agar were 0.05%, 0.1%, 0.3%, 0.5%, 0.7%, 0.9%, 1%. Agar plugs of 0.5 mm were taken from 7 day old V-8 juice agar cultures of the above fungi and inoculated centrally on the duplicate creosote plates. Plates were incubated at 25°C for 14 days. At two days interval, the growth diameter was measured.

Table 2.2 Isolates of fungi scanned for their tolerance to creosote

Soil	N.Z. Diesel	Australian Diesel	Jet Fuel	Other Sources
Trichoderma harzianum	*Penicillium expansum*	*C. resinae*	*C. resinae f. resinae* (IMI 49620)	*C. cladosporioides* ex air (A1/82)
T. hamatum	*P. corylophilum*	*C. resinae* ex Army culture	*C. resinae f. avellaneum*	*C. resinae f. resinae* ex feather
P. verrucosum	*P. frequentans*	*C. resinae* ex HMAS Cerebus	*C. spheros-perum* (WU 27/72)	*Cladosporium cladosporioides* ex tomato (WU 217)
P. funiculosum	*P. chrysogenum*	*C. resinae f. albidum*	*Alternaria alternata* (WU 25/72)	*Alternaria alternata* (ex air spora)
P. velutinum	*P. spinulosum*	*C. resinae* ex Cook Island		
P. aculeatum	*P. digitatum*	*C. resinae* ex Tasmania		
P. brevicom-pactum	*C. resinae*			
P. loliense	*P. variotii*			
P. nigricans	*Cladosporium cladosporioides* (C82)			
P. janthin-ellum	*Alternaria alternata*			
C. resinae				

2.5.3 Shaking and transport effects on spore viability in fuel

The possibility that transporting fuel samples from Auckland to Wellington caused changes in the recovery of microbial contaminants, was examined in a series of simple tests.

(i) Filter-sterilized diesel fuel, 150 cm^3 in sterile 200 cm^3 medical flats was inoculated with spore suspensions in BH medium. 0.1 cm^3 containing different concentrations (5 x 10^2 and 5 x 10^5 spore/cm^3) of *C. resinae*, *P. corylophilum* and *P. variotii* (all diesel isolates) singly and as a mixture of all three were added to a duplicate set of bottles. Each bottle was thoroughly hand shaken one hundred times and shaken by mechanical agitation for up to 8 hours, before reisolating the spores by filtration through a millipore filter (0.45 µm pore size). The filters were transferred aseptically to sterile Petri-dishes, covered with V-8 juice agar and incubated at 25°C for 5 days.

(ii) Further samples of *C. resinae* were inoculated into filter sterilized fuels (500 cm^3) in one litre tin cans and treated as indicated in table 2.3 in order to determine the effects of road transportation on viability.

Viable spores remaining were determined by membrane filtration and growth on V-8 juice agar as described above.

2.6 Fine structure studies on hydrocarbon-utilizing fungi

C. resinae F. avellaneum (ex jet fuel), *C. resinae F. avellaneum*, *Paecilomyces variotii*, *Penicillium corylophilum* (ex diesel fuel) and *Cladosporium cladosporioides* ex soil (V28) were used throughout this study. They were maintained on V-8 juice agar prior to examination. 5 day old cultures were used after growth at 25°C.

2.6.1 Light microscopy study

Portions of a 5 day culture from the actively growing edge were mounted in lactophenol and photomicrographs were obtained using a Carl Zeiss plano-apo x40 oil immersion objective on a photomicroscope.

Table 2.3 The effects of road transportation on spore viability

Spore source and concentration in inoculum		Treatment	Replication
C. resinae ex diesel	37 x 10^6/cm^3	Diesel fuel A	X5
C. resinae ex diesel	37 x 10^6/cm^3	Diesel fuel B	X5
C. resinae ex diesel	40 x 10^6/cm^3	Diesel fuel C	X2
C. resinae ex jet fuel	40 x 10^6/cm^3	Diesel fuel C	X2
C. resinae ex diesel	40 x 10^6/cm^3	Kerosene C	X2
C. resinae ex jet fuel	40 x 10^6/cm^3	Kerosene C	X2
C. resinae ex jet fuel	40 x 10^6/cm^3	Diesel fuel D	X2
C. resinae ex diesel	40 x 10^6/cm^3	Kerosene D	X2

A - Tins were kept stationary in the laboratory over 3 days
B - Tins transported intermittently over 300 km during 3 days
C - Tins transported intermittently over 500 km during 2 days
D - replicates inoculated and filtered/analysed without shaking or transport.

2.6.2 Scanning electron microscopy (SEM)

Because of the delicate nature of the above mentioned fungal material, it was necessary to use a preparatory procedure which preserved the original shape of the cells as well as the structural relationship between conidial chain and conidiogenous cells. The critical point drying method has demonstrated very good preservation of conidia and conidiogenous cells of deuteromycetous fungi (Cole, 1973, 1974, 1975, 1976) and has been used exclusively in this study.

A sporulating culture was first flooded with a mixture of equal volumes of 6% glutaraldehyde and 1% osmium tetroxide (OsO_4), each prepared at 4°C in 0.1 m sodium cacodylate buffer (pH 7.1 - 7.4). Small blocks (approximately 50 mm square) of agar and mycelium were then excised from the flooded culture and placed in a vial containing fresh, cold fixative solution and left in the dark for 2 hours. The samples were then washed in buffer (10 times) and placed in 1% OsO_4 buffered as above for 2 hours. This is considered to be an important step in the preparation of soft biological material for the SEM (Cole & Samson, 1979). Kelly *et al.* (1973) have shown that osmium forms a thin coat (40-200 A° in thickness) over the surface of cells which provides the specimen with some resistance to contamination from electron beam during long term observation in the SEM. The specimens were subsequently washed in buffer (10 times) and then dehydrated in a graded acetone series (30, 50, 70, 90, 95, 100%).

The blocks of mycelium were then placed in Beem capsules whose ends were cut and replaced with fine mesh screens permitting free flow of liquid through the capsules while retaining the fungal samples. The Beem capsules were loaded with agar blocks in a small Petri-dish filled with absolute acetone. Extreme care was taken to prevent the specimens from drying out. The loaded capsules were then quickly placed in the bomb of the Polaran critical point drying apparatus and the latter was filled immediately with liquid carbon dioxide (CO_2). The bomb was flushed with liquid CO_2 for 2 minutes and then filled and sealed off for 1 hour. It was then partially emptied (pressure in bomb reduced to approximately 250 p.s.i.). Finally, the bomb was sealed and its pressure increased to 1200 p.s.i. by heating with water at approximately 32°C.

The CO_2 gas was subsequently released and the dried specimens were removed from the Beem capsules and mounted on aluminium stubs which were coated with conductive silver paint. The stubs were placed on an omnirotary table in a Dynavac Sputter Coater Model Sc 150 high vacuum evaporator and coated with about 100 A^o (Russ and Kabaya, 1970) of gold palladium. The material was examined in a Phillips SEM 505. The scanning electron micrographs were recorded with PAN-F medium speed film.

2.6.3 Transmission electron microscopy (TEM)

C. resinae (ex jet and diesel) and *C. cladosporioides* (ex soil) cultures were used for this study. Small blocks (approximately 5-10 mm square) of agar and mycelium were excised from culture. A number of fixation procedures were used in this study and these are detailed on table 2.4.

After fixation, the material was dehydrated in an ethanol series (30, 40, 50, 70, 90, 95, 100%). Routinely, specimens were post-stained in 2% uranyl acetate in the 70% ethanol stage during dehydration for 45 minutes. The material was then placed in 25% Spurr's low viscosity resin (1969) (obtained from Polyscience Inc.) in absolute ethanol for 1 hour, with continuous, gentle agitation on a rotating stage. The samples were then transferred to a 50% resin solution for 4 hours, to 80% resin for 4 hours and 100% resin overnight. The material was then removed from the vials, placed in fresh 100% resin in capsules and allowed to polymerise at 70°C overnight.

The specimens were sectioned using standard techniques on an LKB8800 ultratome III. Thin sections were picked up on copper grids (400 mesh). Before electron microscopy, grid mounted sections were further stained with 0.5% aqueous uranyl acetate (20 minutes) and Reynold's (1963) lead citrate 7 minutes. The material was examined in a Philips EM420 twin Transmission Electron Microscope (TEM).

Table 2.4 Summary of fixation procedures for TEM studies

Pre-fixatives[1]	Buffer used	Rinses	Post fixatives	Rinses
1. Mixture of equal volumes of 6% glutaraldehyde and 1% OsO_4, each prepared at 4°C and specimens left at 4°C for 2 hours.	0.1 m sodium cacodylate (pH 7.4)	In buffer 10 times each wash 3 minutes.	1% buffered OsO_4 for 2 hours at 4°C.	Buffer wash 10 times
2. 2.5% glutaraldehyde[2] for 2 hours at room temperature.	0.05 m sodium cacodylate (pH 7.2)	Buffer wash 3 times each wash 5 minutes.	1% buffered OsO_4 for 4 hrs at room temperature and then for 15 hours at 4°C.	Buffer wash 3 times.
3. 3% glutaraldehyde for 1 1/2 hours at room temperature.	0.1 m sodium cacodylate (pH 7.2)	3 changes of buffer each for 30 minutes.	1% OsO_4 for 2 hours at 5°C.	3 changes of buffer each for 30 minutes.
4. 3% glutaraldehyde for 1 1/2 hours at room temperature.	0.05 m sodium phosphate (pH 6.9)	Buffer wash 3 times.	1% OsO_4 at room temperature for 2 hours	3 changes of buffer each wash 5 minutes.
5. 4% glutaraldehyde[3] in .01 m $CaCl_2$ and 3% Triton x 100 for 3 hours at 4°C. Fixed for further 15 hours in glutaraldehyde without detergent at 4°C.	0.1 m sodium cacodylate (pH 7)	Washed twice in collidine buffer pH 7.4.	1% OsO_4 in collidine buffer for 12 hours at 4°C.	Washed twice in collidine buffer.
6. 3% glutaraldehyde[4] +2% formaldehyde The tissues were treated differently at this stage. (a)Vacuum infiltrated 5 mins stored in fixative at room temperature (23°C) (b)Detergent treated (0.05% saponin) in fixative for 2 hours at room temperature. (c)Directly stored in the primary fixative at for 24 hours at 4°C.	0.1 m phosphate (pH 7.2)	3 buffer washes in 30 minutes.	1% OsO_4 for 3 hours at 4°C.	3 buffer washes in 30 minutes.

1 Cole & Samson (1979)
2 Cooney, Siporin and Smucker (1980)
3 Hayat (1981)
4 Karnovsky (1965)

2.7 Determination of water in diesel fuel samples

The total water (free and dissolved water) in the fuel sample was determined at the Defence Scientific Establishment by the Karl Fisher method (ASTMD 1744-64).

2.8 Meteorological records

A Thermohygrograph was set up within the entrance corridor leading to Cliff tank 2 to provide a continuous record of temperature and humidity during these studies. Rainfall data for Auckland was obtained from the Metereological Office at Wellington.

3. EXPERIMENTAL RESULTS : SECTION I - FIELD STUDIES

3.1 Micro-organisms occurring in diesel fuel.

Thirty-one species of viable fungi were isolated from 'dieso' fuel samples collected from fuel storage tanks at the naval base at Devonport, Auckland. Most belonged to the Hyphomycetes but Zygomycetes, Ascomycetes and Basidiomycetes were also represented. Only five bacterial isolates were obtained. These included a sulphur-reducing bacterium. Fungi and bacteria isolated and identified over a 24 month period are listed in table 3.1.

Tables 3.2 and 3.3 present a summation of the quantitative data (refer appendices 3.1, 3.2 and 3.3), obtained from the assay of the monthly samples for each of the two storage tanks, the separating tank, the Shell tank and the wharf sampling point. Mean numbers of CFU were obtained for each fungus and for each separate position analysed. The tables also list the frequency of isolation of each fungus as a percentage of the total samples analysed.

The most frequently isolated contaminants were the fungi *Cladosporium resinae*, *Penicillium corylophilum* and *Paecilomyces variotii* (table 3.2). These were isolated from both storage tanks at each monthly sampling date over the 24 month period. Tank 3 was more heavily contaminated than tank 2. The mean number of CFU tended to be higher at the bottom or 'very bottom' of the tanks. The above three fungi were also the predominant contaminants found in the fuel samples from the separating tank, wharf sampling point, Shell tank and tank cleaning vessel (table 3.3). The highest mean number of CFU for *C. resinae* were found in the tank cleaning vessel (372 CFU/litre) and Shell tank sample (320 CFU/litre).

Table 3.1 Fungi isolated and identified from N.Z. stored diesel fuel (1982-1984)

ZYGOMYCETES

Mucor sp.
Rhizopus sp.

ASCOMYCETES
Amorphotheca resinae Parbery
Chaetomium globosum Kunze

BASIDIOMYCETES

Unidentified isolates with clamp connexions.

HYPHOMYCETES

Alternaria alternata (Fr.) Keissler
Aureobasidium pullulans (de Bary) Arnaud
*Aspergillus fumigatus** Fres.
*Aspergillus sejunctus** Bainier and Sartory
*Aspergillus versicolor** (Vuill.) Tiraboschi
Aspergillus niger Van Tiegh
Botrytis sp.
Cladosporium resinae (Lindau) de Vries state of *Amorphotheca resinae*.
Cladosporium cladosporioides (Fresen.) de Vries
Epicoccum purpurascens Ehrenb. ex Schlecht
Fusarium sp.
Gliocladium roseum Corda
Paecilomyces variotii Bainier
*Paecilomyces lilacinus** (Thom) Samson
*Penicillium corylophilum** Dierckx
*Penicillium digitatum** (Pers. ex Fr.) Sacc.
*Penicillium echinulatum** Raper & Thom ex Fassatiova
*Penicillium chrysogenum** Thom
*Penicillium frequentans** Westling
*Penicillium spinulosum** Thom
*Penicillium expansum** Link
Phomopsis sp.
Trichoderma viride Pers. ex S.F. Gray
*Trichoderma hamatum** (Bonorden) Bainier
*Trichoderma harzianum** Rifai
Ulocladium sp.
Yeasts

Bacteria

Bacillus sp.
Pseudomonas sp.
Micrococcus sp.
Desulfovibrio desulfuricans

* Identified or identification confirmed by the Commonwealth Mycological Institute, Kew, England.

Table 3.2 Mean number of colony forming units (CFU) and frequency of isolation of fungi from diesel fuel stored in tanks 2 and 3 over a 24 month sampling period.

Fungus	Tank 2						Tank 3					
	CFU/litre*				Frequency**		CFU/litre*				Frequency**	
	T	M	B	VB	(%)		T	M	B	VB	(%)	
Cladosporium resinae	17	31	27	29	88		95	135	199	290	81	
Penicillium corylophilum	27	18	28	45	50)		105	112	88	163	60)	
P. digitatum	17	15	14	37	20)		46	31	29	60	20)	
P. echinulatum	9	11	12	10	5)		15	12	30	15	5)	
P. chrysogenum	12	14	10	7	7)	100	17	10	11	13	5)	100
P. frequentans	10	8	9	7	5)		13	8	14	4	3)	
P. spinulosum	14	12	17	21	3)		21	6	4	5	5)	
P. expansum	14	4	14	5	10)		9	3	6	2	2)	
Paecilomyces variotii	138	66	26	134	52	55	45	35	85	45	59	
P. lilacinus	0	5	6	3	3		0	0	0	0	0	
Mucor sp.	144	15	175	50	15		60	13	68	28	15	
Trichoderma viride	125	74	25	92	3)		46	30	30	73	3)	
T. hamatum	17	3	1	0	3)	18	0	0	0	0	0)	10
T. harzianum	175	87	37	271	12)		200	42	197	168	7)	
Yeast	20	3	7	38	9		8	2	4	81	12	
Aspergillus fumigatus	0	10	0	0	1)		0	0	0	0	0)	
A. sejunctus	14	9	10	3	7)	13	17	20	2	3	12)	20
A. versicolor	10	6	3	2	3)		4	10	3	4	7)	
A. niger	0	0	20	2	2)		0	0	0	2	1)	
Alternaria sp.	3	4	0	10	4		0	0	6	2	3	
Fusarium sp.	20	0	12	15	5		9	115	14	45	13	
Rhizopus sp.	100	0	30	0	3		0	525	3	0	3	
Basidiomycete	10	4	0	0	3		0	0	0	0	0	
Chaetomium globosum	5	3	0	0	2		0	15	20	0	3	
Cladosporium cladosporioides	0	40	0	0	1		30	16	30	37	6	
Aureobasidium pullulans	0	0	0	0	0		14	0	1	2	4	
Phomopsis sp.	0	0	0	12	1		0	0	0	60	1	
Epicoccum purpurascens	0	10	0	0	1		2	0	1	2	3	
Gliocladium roseum	0	0	0	10	1		10	0	0	40	2	
Ulocladium sp.	0	0	0	0	0		0	5	0	0	1	
Botrytis sp.	0	0	0	0	0		0	0	0	1	1	

* Numbers of CFU obtained per litre of fuel sampled from the 24 samples listed in appendices 3.1 and 3.2

** The percentage of samples (93) from which the fungus was isolated
T = Top ; M = Middle ; B = Bottom ; VB = Very Bottom

Table 3.3 Mean Number of Colony Forming Units (CFU) and frequency of isolation of fungi from the separating tank, Shell tank, tank cleaning vessel and wharf sampling point

Fungus	Separation Tank		Shell Tank	Cleaning Vessel	Wharf	
	CFU/l+	F(%)*	CFU/l+	CFU/l+	CFU/l+	F(%)*
C. resinae	238	63	320	372	222	100
Penicillium spp.	415	79	170	103	315	100
Paecilomyces variotii	38	17	453	30	165	100
Yeast	52	21	-	-	9	67
Aspergillus sp.	11	17	10	-	-	-
Fusarium sp.	5	13	-	-	18	33
Alternaria sp.	1	4	-	-	-	-
Trichoderma sp.	4	4	-	-	-	-
Rhizopus sp.	-	-	10	-	-	-
Mucor sp.	-	-	-	-	20	67

\+ CFU/litre

* Frequency (%)

The total water (dissolved and free water) was usually more than 130 ppm in tank 3 while tank 2 had less than 105 ppm. Sometimes three-quarters of the 'very bottom' of tank 3 sample and the separating tank sample contained water. With excessive amounts of water, the pH usually exceeded 8 and the conditions became more favourable for anaerobic bacteria. During the day the flow of fuel in the separating tank from all the cliff tanks was monitored and only fuel was pumped back into tanks. At night, however, both fuel and water were automatically pumped into tank 3 causing an increase in the water level and possibly contamination levels.

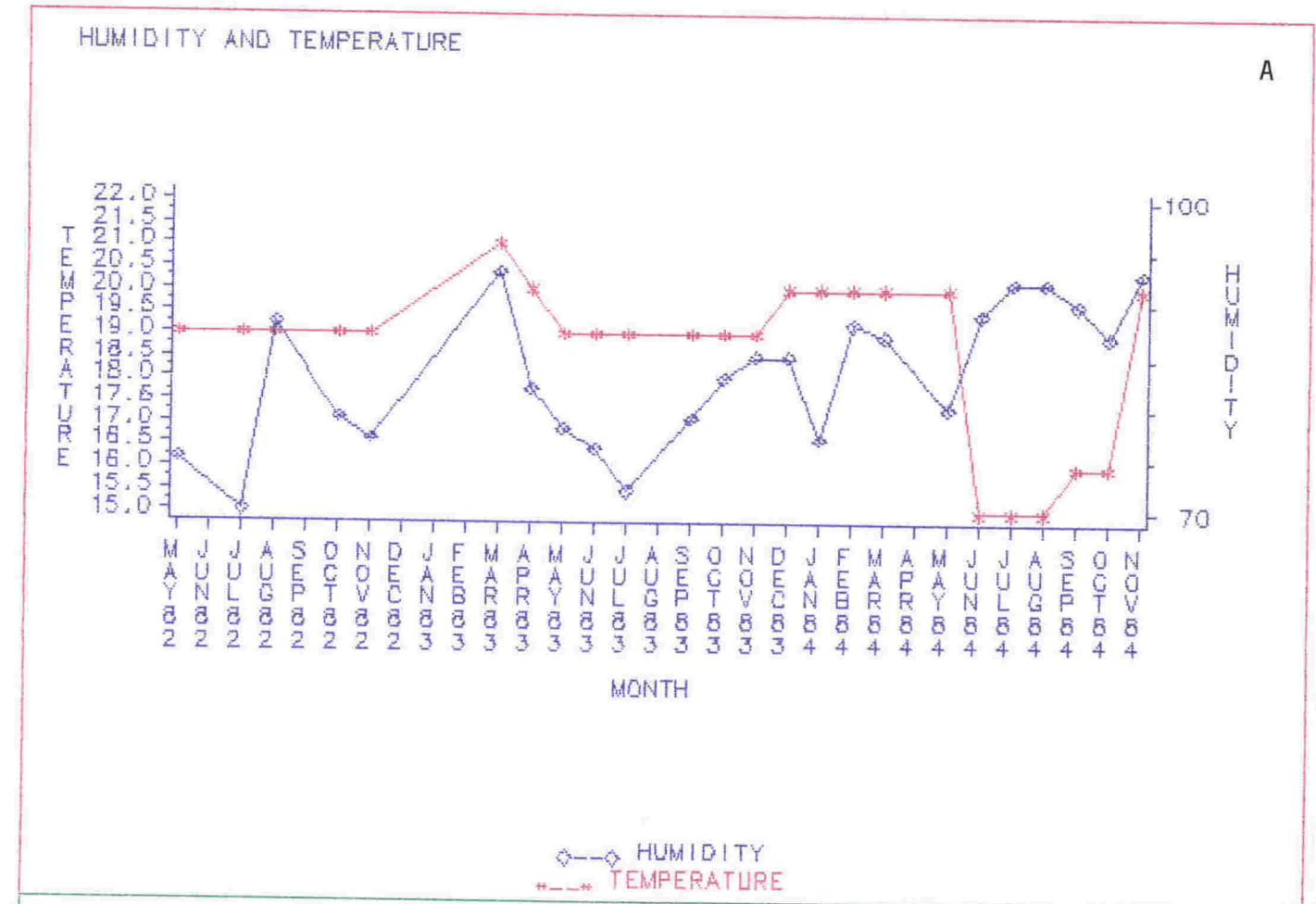
Seven species of *Penicillium* were isolated from the storage tanks, the most frequent being *P. corylophilum* and *P. digitatum*. The others which occurred less frequently and in low numbers were *P. echinulatum*, *P. chrysogenum*, *P. frequentans*, *P. spinulosum* and *P. expansum*. *Paecilomyces variotii* occurred more frequently than *P. lilacinus*. *Trichoderma viride*, *T. hamatum* and *T. harzianum* were found occasionally in the tanks when the predominant fungi were in low numbers. They did not grow in fuel but remained as viable spores (see section II).

The data in figures 3.1A and 3.1B show the fluctuation of temperature, humidity and the major fungi in the storage tanks over the twenty-four months. The air temperature (15°C - 20°C) and the humidity (70 - 95%) were ideal for the growth of most of the fuel fungi. The surface fuel temperature was the same as the surrounding air temperature and it was almost constant with depth in the tank apart from an occasional difference of 1°C.

No regular seasonal pattern was observed for the predominant fungi. *C. resinae*, occurred in greater numbers between May '82 - April '83 and December '83 - February '84 while *P. variotii* occurred frequently in low numbers compared to *C. resinae* and *Penicillium* spp. *Penicillium* spp. numbers were consistent throughout the two years of sampling and peaked during September '84.

Figures 3.2A, B and C shows the variation in the CFU of the three major fungi in the separating tank, and tanks 2 and 3 over twelve months. Between July 1982 and September 1983, the separating tank was contaminated with high numbers of *C. resinae* which decreased during the subsequent months. During the same period, the CFU of *C. resinae* in tank 3 were low initially and then increased in number. In tank 2, *C. resinae* remained low compared to the other two tanks. *Penicillium* spp. were persistent in all the tanks.

Bacteria occurred in low numbers in the diesel fuel. Sulphur bacteria became a problem when the very bottom of tank 3 and the separating tank contained an excessive amount of water.


HUMIDITY AND TEMPERATURE
A
TEMPERATURE
22.0
21.5
21.0
20.5
20.0
19.5
19.0
18.5
18.0
17.5
17.0
16.5
16.0
15.5
15.0
100
70
HUMIDITY
MAY82 JUN82 JUL82 AUG82 SEP82 OCT82 NOV82 DEC82 JAN83 FEB83 MAR83 APR83 MAY83 JUN83 JUL83 AUG83 SEP83 OCT83 NOV83 DEC83 JAN84 FEB84 MAR84 APR84 MAY84 JUN84 JUL84 AUG84 SEP84 OCT84 NOV84
MONTH
HUMIDITY
TEMPERATURE

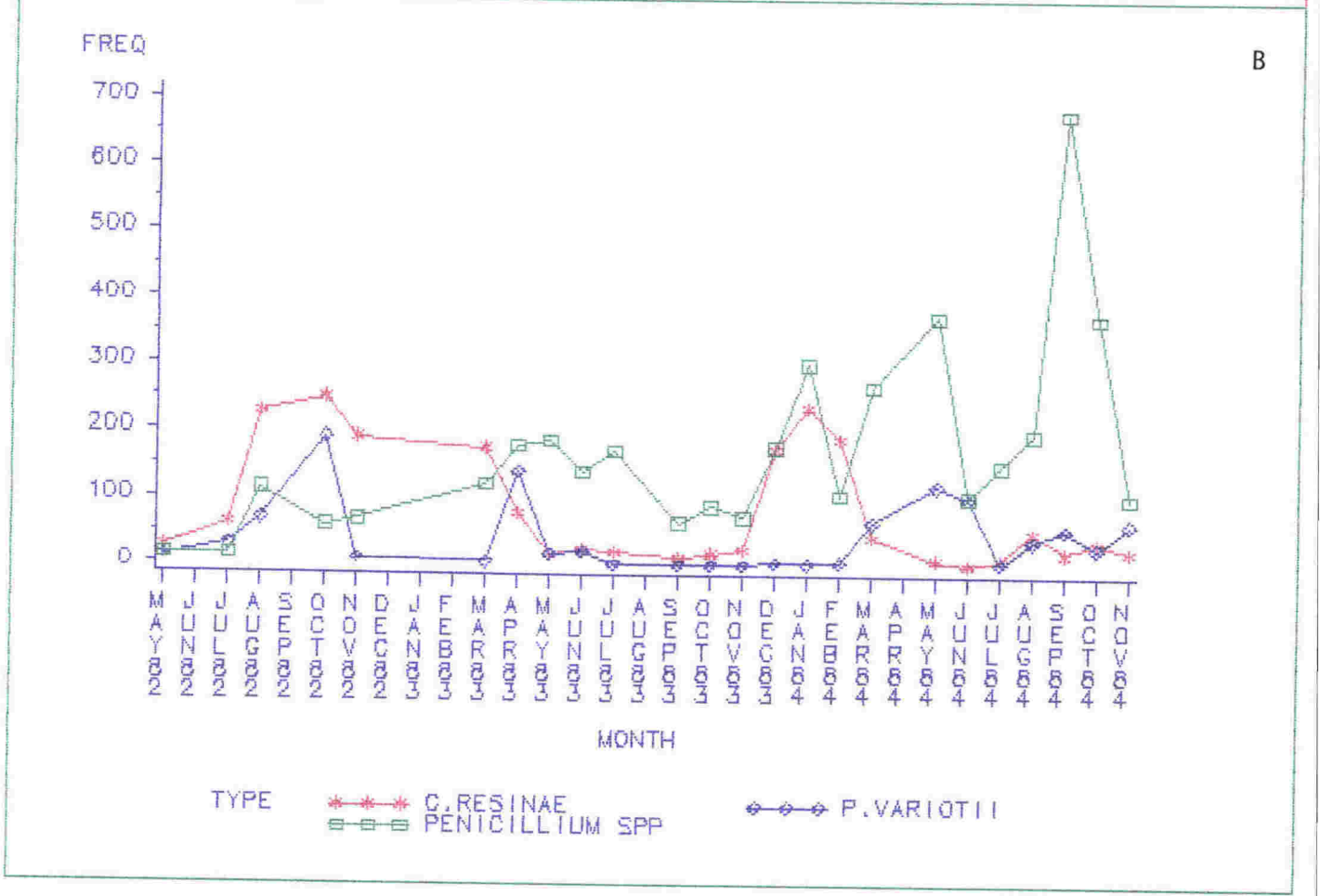
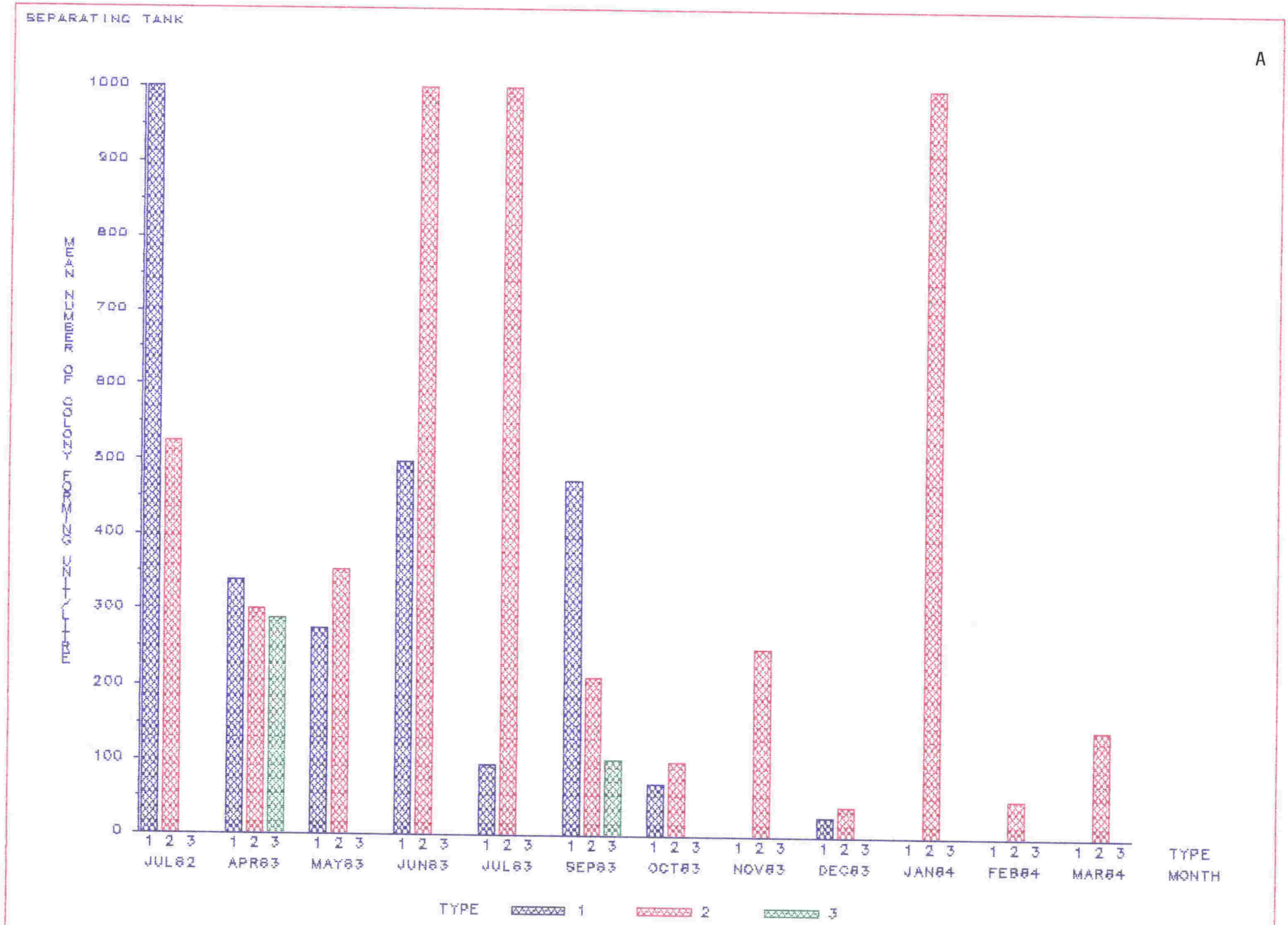

FREQ
B
700
600
500
400
300
200
100
0
MAY82 JUN82 JUL82 AUG82 SEP82 OCT82 NOV82 DEC82 JAN83 FEB83 MAR83 APR83 MAY83 JUN83 JUL83 AUG83 SEP83 OCT83 NOV83 DEC83 JAN84 FEB84 MAR84 APR84 MAY84 JUN84 JUL84 AUG84 SEP84 OCT84 NOV84
MONTH
TYPE
C.RESINAE
PENICILLIUM SPP
P.VARIOTII

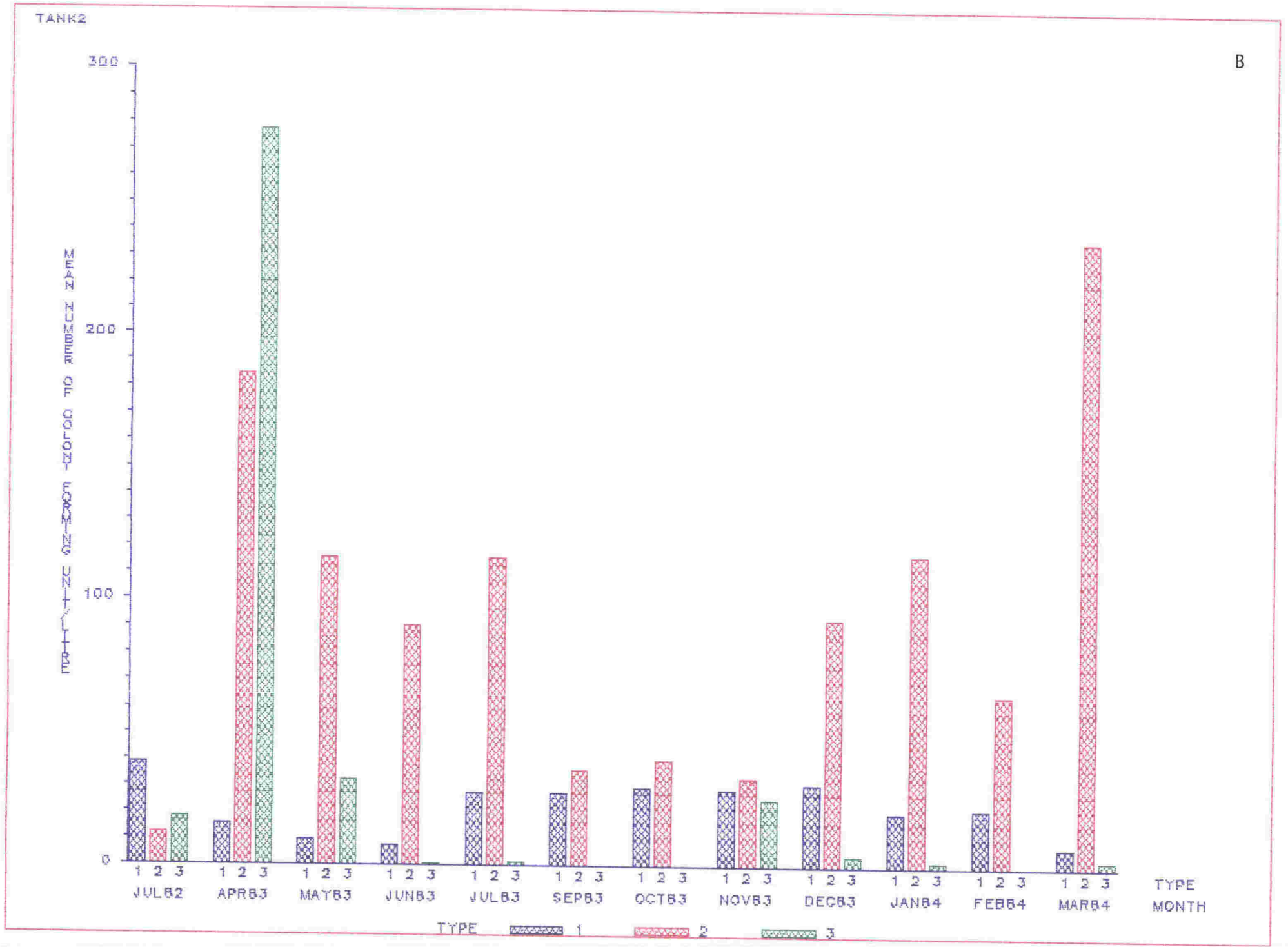
Figure 3.2 Comparing the occurrence of the predominant fungi in the separating tank with that of tanks 2 and 3 (1982-1984)

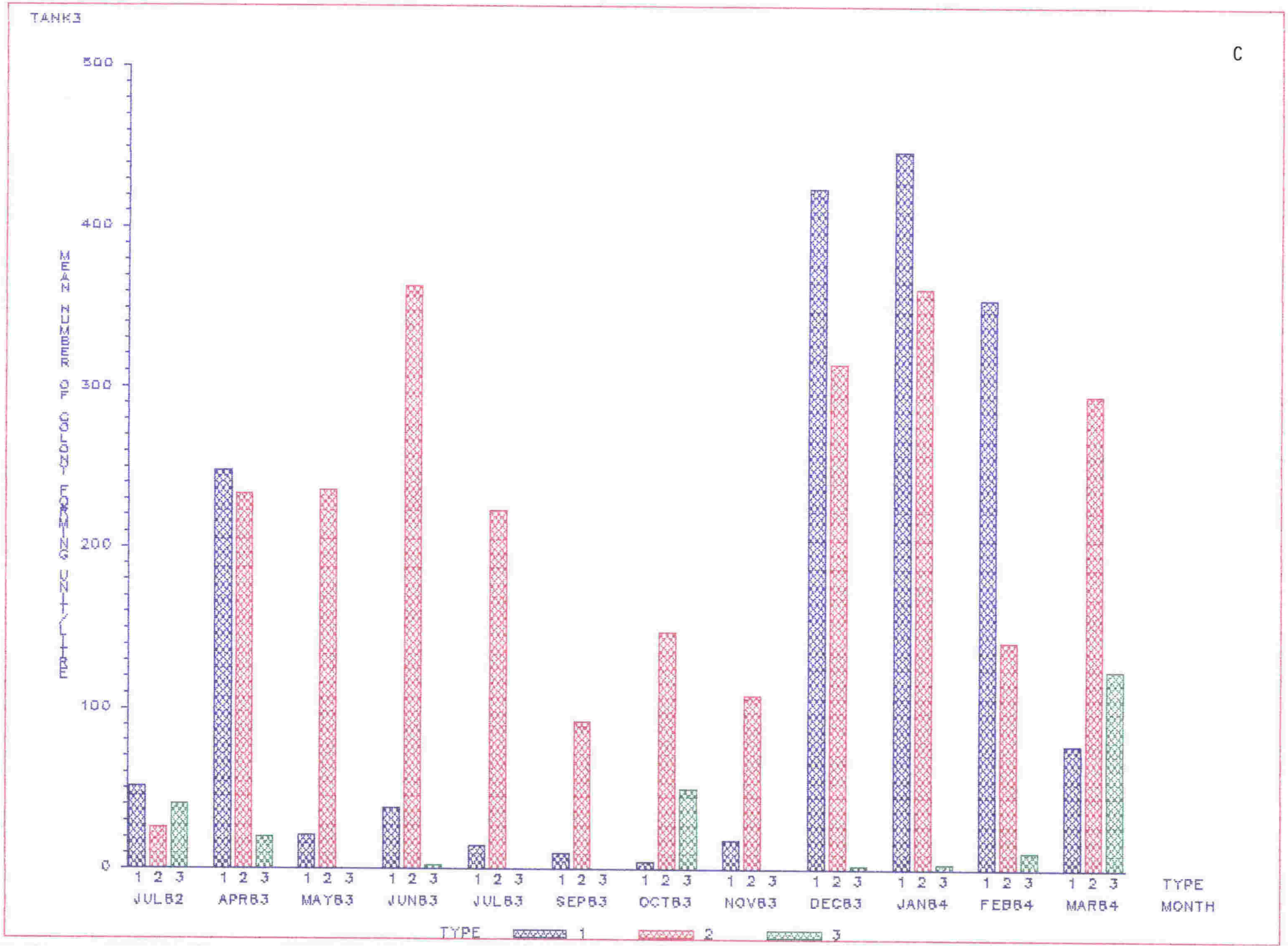
A. Mean number of CFU/litre of fungi occurring in the separating tank

B. Mean number of CFU/litre of fungi occurring in the storage tank 2 (page 46)

C. Mean number of CFU/litre of fungi occurring in the storage tank 3 (page 47)


SEPARATING TANK
A
MEAN NUMBER OF OIL DROPLETS PER MILLILITRE
1000
900
800
700
600
500
400
300
200
100
0
1 2 3
JUL82
APR83
MAY83
JUN83
JUL83
SEP83
OCT83
NOV83
DEC83
JAN84
FEB84
MAR84
TYPE
MONTH
TYPE 1 2 3


TANK2
B
300
200
100
0
MEAN NUMBER OF ROOT-KNOT NEMATODES
1 2 3
JUL82
APR83
MAY83
JUN83
JUL83
SEP83
OCT83
NOV83
DEC83
JAN84
FEB84
MAR84
TYPE
MONTH
TYPE 1 2 3


TANK3
C
500
400
300
200
100
0
MEAN NUMBER OF GONOCYTES/CYST/FEMALE
1 2 3
JUL82
APR83
MAY83
JUN83
JUL83
SEP83
OCT83
NOV83
DEC83
JAN84
FEB84
MAR84
TYPE
MONTH
TYPE 1 2 3

The commonly isolated bacteria were *Pseudomonas* spp., *Micrococcus* spp. and *Bacillus* spp. (table 3.4 and 3.5). The only sulphur-reducing bacterium isolated was *Desulfovibrio desulfuricans* from the water bottoms. Isolation of this organism coincided with an obvious smell of hydrogen sulfide emanating from the fuel. On Baar's medium and API broth and agar the presence of *Desulfovibrio* was indicated by the medium turning black. Attempts to isolate *E. coli* failed indicating that sewage contamination of the stored fuel had not occurred. Colonies on MacConkey medium were sticky and showed confluent growth. The organisms were Gram negative, rod-shaped, non-sporing and non-lactose fermenting and therefore not *E. coli*. The number of bacteria occurring in diesel fuel decreased with storage.

Table 3.4 Incidence and levels of bacteria found in diesel fuel at different testing dates.

Date and time of sampling	Number of Bacterial Colonies per litre					
	Micrococcus sp.		*Pseudomonas* sp.		*Bacillus* sp.	
	Tank 2	Tank 3	Tank 2	Tank 3	Tank 2	Tank 3
July 1982						
Immediate	0	0	0	0	1	22
24 Hours later	4	0	0	0	20	13
August 1982						
Immediate	142	60	0	0	0	52
24 Hours later	40	72	27	0	30	67
48 Hours later	107	93	7	0	67	0
72 Hours later	27	27	0	0	7	0
96 Hours later	53	0	0	0	7	0

Table 3.5 Numbers of bacterial colonies per litre obtained from Tanks 2 and 3 and the separating tank (1982–1984)

Bacteria	Jul 82	Aug 82	Oct 82	Mar 83	Apr 83	Jun 83	Jul 83	Sep 83	Oct 83	Nov 83	Dec 83	Feb 84	Mar 84	Jun 84
Tank 2														
Bacillus sp.	21	53	114	0	3	29	18	3	0	0	15	28	0	11
Pseudomonas sp.	0	33	98	0	123	26	52	15	13	30	63	30	5	56
Micrococcus sp.	3	118	30	0	20	5	3	8	5	3	3	0	0	3
Desulfovibrio desulfuricans	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tank 3														
Bacillus sp.	35	118	38	0*	0	23	25	0	3	8	28	0	6	22
Pseudomonas sp.	0	0	69	0	145	75	120	3	38	53	70	63	38	84
Micrococcus sp.	17	70	15	0	0	5	13	8	13	10	0	8	3	5
Desulfovibrio desulfuricans	0	0	0	250	0	0	0	375	250	250	250	0	0	0
Separating Tank														
Bacillus sp.	50	–	–	–	0	40	0	0	15	0	50	0	0	–
Pseudomonas sp.	110	–	–	–	350	353	125	138	10	239	150	0	50	–
Micrococcus sp.	0	–	–	–	0	23	45	75	139	5	331	0	13	–
Desulfovibrio desulfuricans	0	–	–	–	0	0	0	0	0	500	500	1000	500	–

* March 1983 - sampled only for sulphur bacteria

– Not tested

3.2 Comparison of isolation methods

Direct observation of filters showed a wide variety of fungi existing in fuel including *Alternaria*, *Drechslera*, *Epicoccum*, *Trichoderma* and *Ulocladium* and many unidentified spores. This method was tedious because of the large amounts of inert non-biological material present and the difficulties of distinguishing fungal spores. Furthermore the majority of these fungi failed to sporulate on culture plates.

Pour and spread plate methods were used when fuel samples were saturated with water or contained a distinct water layer. As a routine the membrane filtration technique was used because it allowed both qualitative and quantitative studies. A number of modifications were examined. The one giving consistently highest counts was adopted i.e. medium poured over the filter membrane (see table 3.6). V-8 juice agar medium gave reliable and consistent results and was used throughout.

Table 3.6 Comparison of two media and various modified membrane filter techniques for two separate fuel samples. (10 replicates)

Fungus	CFU/litre				
	Sample I			Sample II	
	V-8	Malt		V-8	
	O	O	S	O	U
C. resinae	*601*	*300*	*19*	*142*	*212*
Penicillium spp.	*149*	*104*	*16*	*308*	*139*
Paecilomyces variotii	*166*	*4*	*1*	*159*	*10*
Fusarium sp.	*2*	*11*	*0*	*9*	*2*

S = Filter placed on the surface of the medium
O = Medium poured over the filter in the dish
U = Filter inverted on the surface of the medium

3.3 Comparison of container type used for collecting samples (Table 3.7)

Generally the bottle counts of *C. resinae* were lower than the counts from tin stored samples. The CFU for both container types fluctuated during the different sampling period. However, the former were preferred because they do not corrode and are easier to sterilize.

3.4 Effect of time of collection and testing of samples on the recovery of *C. resinae* (Table 3.7)

Initial tests were carried out with a minimum delay to avoid changes in the composition and relative abundance of the microflora. Subsequent tests were carried out at intervals up to five days after collection. In jet fuel, delayed testing resulted in failure to recover *C. resinae* (Sheridan pers. comm.). The July '82 samples were tested immediately and after 24 hours only. Mean counts of *C. resinae* in tank 2 decreased from 62 CFU/litre to 28 CFU/litre within 24 hours. The apparent drop in tank 3 was due to very low figure obtained for the bottle samples which were not tested immediately after collection. In the August sampling this drop did not occur. Further sampling in October and November showed less marked differences on the recovery of *C. resinae* within each tank, with the different time of testing.

3.5 Sources of fuel contamination

The occurrence of *C. resinae*, *Penicillium* spp. and *Paecilomyces variotii* in the surrounding atmosphere near the tanks can be seen in figures 3.3A, B and C. *P. corylophilum*, *P. digitatum* and *P. expansum* frequently occurred in the air spora and showed a corresponding high incidence in the fuel storage tanks. But no such observation was made for *C. resinae* and *Paecilomyces variotii*. Most of the *C. resinae* and *P. variotii* spores were trapped in summer and autumn and occurred in small numbers. In tank 2 among the three fungi *Penicillium* spp. and *P. variotii* showed high peaks in April 1983 and May 1984 after low numbers in the preceding months.

Table 3.7 Effect of time of testing of samples and container types on recovery of *C. resinae*

Date and time of testing	CFU/litre				
	Tank 2+	Tank 3+	Wharf	Tin*	Bottle*
July 1982					
Immediate	62	1000	1000	598	-
24 Hours later	28	533	536	451	183
August 1982					
Immediate	25	225	115	124	-
24 Hours later	25	307	458	178	237
48 Hours later	173	351	510	546	282
72 Hours later	70	321	25	212	111
96 Hours later	73	438	28	286	160
October 1982					
24 Hours later	13	288	285	231	162
48 Hours later	7	397	255	231	163
72 Hours later	10	403	215	227	165
96 Hours later	13	410	295	257	162
120 Hours later	62	745	630	422	461
November 1982					
24 Hours later	23	372	-	209	246
48 Hours later	15	246	-	158	136
72 Hours later	7	295	-	183	163
96 Hours later	11	513	-	347	253
120 Hours later	7	111	-	99	14

- not tested

* mean for tanks 2 and 3

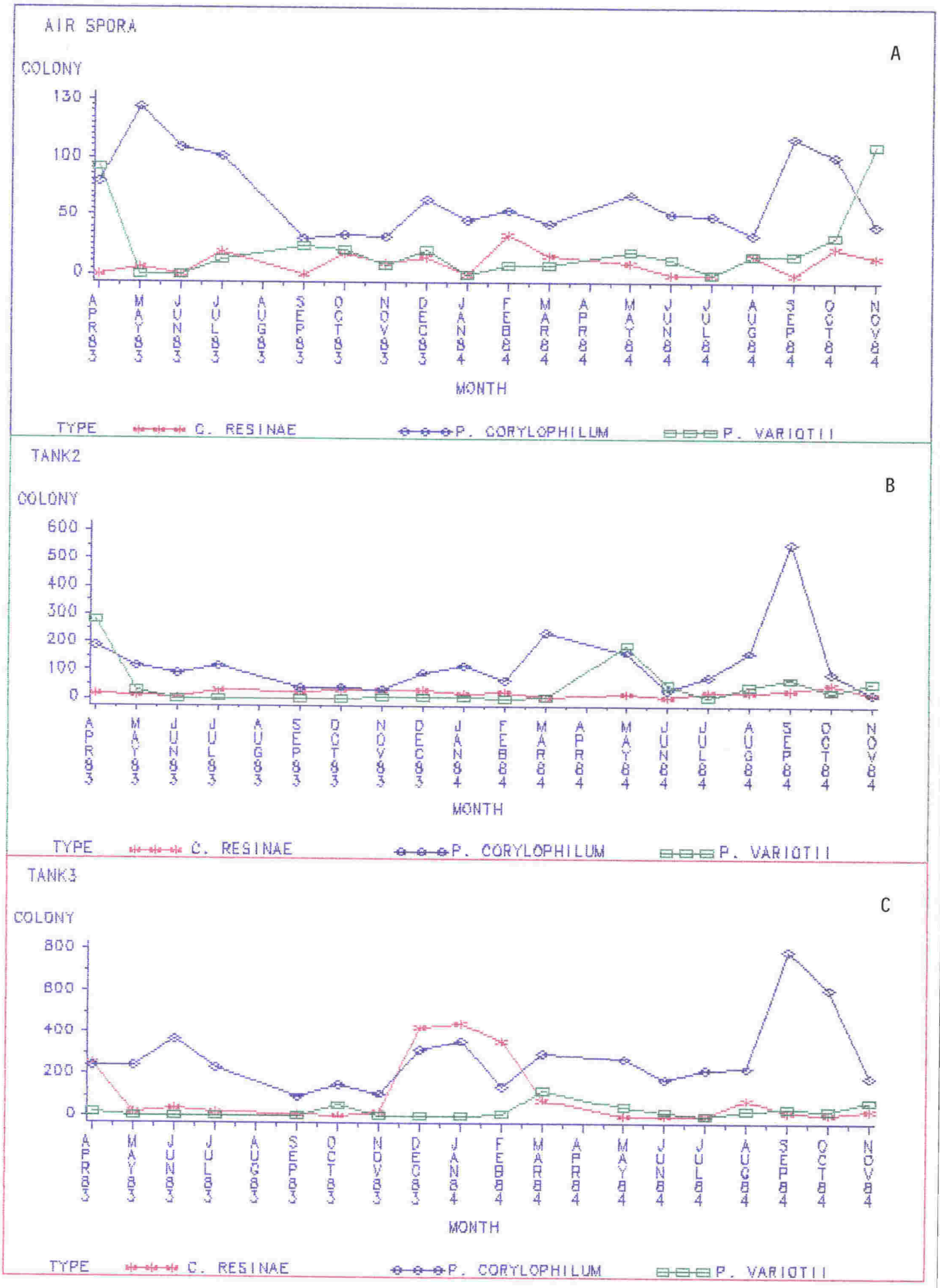
+ mean for tin and bottle samples

Figure 3.3 The occurrence of the predominant fungi in the atmosphere and the storage tanks 2 and 3 (1982-1984)

A. Mean number of colonies/m^3 of fungi occurring in the air spora

B. Mean number of CFU/litre of fungi occurring in the storage tank 2

C. Mean number of CFU/litre of fungi occurring in the storage tank 3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COLONY
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100
50
0
APR83
MAY83
JUN83
JUL83
AUG83
SEP83
OCT83
NOV83
DEC83
JAN84
FEB84
MAR84
APR84
MAY84
JUN84
JUL84
AUG84
SEP84
OCT84
NOV84
MONTH
TYPE
C. RESINAE
P. CORYLOPHILUM
P. VARIOTII
TANK2
B
COLONY
600
500
400
300
200
100
0
APR83
MAY83
JUN83
JUL83
AUG83
SEP83
OCT83
NOV83
DEC83
JAN84
FEB84
MAR84
APR84
MAY84
JUN84
JUL84
AUG84
SEP84
OCT84
NOV84
MONTH
TYPE
C. RESINAE
P. CORYLOPHILUM
P. VARIOTII
TANK3
C
COLONY
800
600
400
200
0
APR83
MAY83
JUN83
JUL83
AUG83
SEP83
OCT83
NOV83
DEC83
JAN84
FEB84
MAR84
APR84
MAY84
JUN84
JUL84
AUG84
SEP84
OCT84
NOV84
MONTH
TYPE
C. RESINAE
P. CORYLOPHILUM
P. VARIOTII

This could be due to the occasional pumping of fuel from tank 2 into the frigate and its replenishment from the tank cleaning vessel (TCV) may have contributed to the fluctuation of the number of fungi.

Table 3.8 lists the fungi isolated using the Kramer Collin's airsampler, and indicates their tolerance to creosote (0.1%). *Fusarium* sp., *Ulocladium* sp., *Aspergillus* spp., *Chaetomium globosum*, *Botrytis* sp., *Mucor* sp., *Rhizopus*, *Cladosporium cladosporioides* and *Aureobasidium* sp. grew on culture plates exposed in the storage tanks indicating that they were air borne. *Alternaria* sp. occurred in abundance on filters through which fuel had been passed and also on the air spora plates with and without creosote. This fungus was only infrequently isolated from the fuels and it could be suppressed by other dominant fungi in fuel.

The fungi isolated from the soils surrounding the tanks are listed in table 2.1. *C. resinae* was isolated from soils surrounding the tanks. Most of the *Penicillium* spp. occurring in the fuel were isolated from the air spora plates but rarely from the soil. Among the different *Penicillium* spp. isolated from the soil by serial dilution and direct plating of the soil on selective media, *P. expansum* was the only species isolated from both the sources. *P. variotii* was not isolated from soil. All the species of *Trichoderma* occurring in the fuel were isolated from the soil.

Table 3.8 Fungi* isolated from the atmosphere by the Kramer Collins spore sampler during survey period 1983-84, growing on V-8 juice agar containing 0.1% creosote.

Sampling Months	*Penicillium* spp.	*Basidiomycetes*	*Paecilomyces* spp.	*Alternaria* spp.	*Aspergillus* sp.	*Cladosporium resinae*	*Epicoccum* sp.	*Mucor* spp.	*Fusarium* spp.	*Trichoderma* spp.	*Aureobasidium* sp.	*Rhizopus* sp.	*Cladosporium cladosporioides*	Yeast	*Nigrospora* sp.	*Gliocladium roseum*	*Aspergillus niger*	*Ulocladium* sp.	*Pestalotiopsis* sp.	*Stemphylium* sp.	*Aspergillus fumigatus*
April 1983	68	12	80	50	58		10	12	19	20		10				10					
May	125	62		51		5						100									
June	95	25		40					30	77			15								
July	87	11	10	76	50	16	21			55				7			13				
August	55	18	18	20	7	15		10	27	5			18	4				15			
September	26	46	20	15	9		7		10												
October	28	23	17	22	17	15	3		7												
November	27	8	6	15	5	8															
December	55	40	18	16	12	12	5			20	7								6		
January 1984	40	55			21		10		11		4									7	
February	46	23	7	20	7	28			5												
March	37	29	6	28	9	14	5	5													
April	57	18	112		8	7															
May	59	31	16	10		8		6	6		12		7		3						2
June	44	25	11				8								4						
July	42	17		32	5			15			7	25		8							
August	28	23	13			14		50													
September	102		13					30													
October	86	14	27	17	7	19				20	10										
November 1984	36	25	96	7		12		95													

* Mean no. of colonies/m^3 per month (i.e. total daily count divided by number of days in the month)

3.6 Discussion

In this study of micro-organisms associated with the diesel fuel storage tanks system at Devonport, thirty-one fungal species were isolated during a twenty-four month period in 1982-1984. The most frequently isolated fungi were *C. resinae*, *Penicillium* spp. and *Paecilomyces variotii*. These could be classified as 'high activity' organisms capable of good growth in fuel. A similar range of organisms had been isolated from jet fuel (Sheridan and Soteros, 1974). Although *C. resinae* is a significant diesel contaminant, *Penicillium* spp. may be equally important as observed in jet fuel distribution systems (Soteros, 1973) and in diesel (Bruce, 1982). In both tanks, *Penicillium* spp. were found to be persistent and sometimes in greater number than *C. resinae*. *Penicillium* spp. also increased markedly with corresponding peak occurrence in the surrounding atmosphere. Most of the *Penicillium* spp. found in the fuel have been reported to be soil inhabitants (Pitt, 1979) but in this study, most of the species isolated from diesel fuel were present in the air spora.

The *Paecilomyces* spp., *Penicillium* spp., *Fusarium* spp., *Alternaria* spp., *Trichoderma* spp., *Rhizopus* sp., *Mucor* sp., *Aureobasidium* sp., *Aspergillus* spp., *Epicoccum* sp., and *Botrytis* sp. were known to be soil borne and air borne and have been reported to play a vital role in biodegrading different substrates (ZoBell, 1946; Miyoshi, 1895; Hendey, 1964; Lonsane, Singh and Baruah, 1975; Klausmeier and Andrews, 1981). They may have entered into the fuel from the atmosphere and remained as casual colonizers. The ability of some of these fungi to utilize diesel fuel is discussed in chapter 4.

If microbial fuel problems are directly related to the numbers of colony forming units able to be isolated from samples, then it is clear that both *Penicillium* and *Paecilomyces* must be included as well as *C. resinae* when considering causes and cures for problems due to micro-organisms in diesel fuel. It is not surprising that with time more micro-organisms will grow and become genetically adapted to the prevailing conditions in the fuel.

In the main storage tanks there was a gradient in contamination levels of *C. resinae*. The highest concentration of CFU occurred at the bottom and 'very bottom' of the tanks. Data obtained from laboratory experiments conclusively showed a greater occurrence of *C. resinae* at the fuel/water interface and in water bottoms. It appears that the spores of *C. resinae* were suspended at the fuel-water interface. *C. resinae* spores are larger (3-7 x 2-4 µm) than *Penicillium* spp. spores (2.5-3 µm). *C. resinae* spores possibly have greater affinity for water and the settling out process may be more rapid. There was a higher incidence of microbiological contamination in tank 3 than in tank 2. The 'very bottom' of tank 3 constantly contained abundant water because all the water bottoms from the main storage tanks were usually drained into a separating tank. As the fuel partitions from the water in the separating tank, the fuel is pumped back into tank 3 and water to the sea, thereby unknowingly maintaining the contamination level in tank 3.

The temperature and humidity of air entering the tanks through ventilation provide favourable conditions for the growth of the major fungi. These conditions would allow continuous condensation and accumulation of water particularly on the exposed tank wall and fuel surface. Diesel being viscous, water will settle out slowly and has a tendency to form and maintain a stable water-fuel interface.

Bacteria do not appear to be major diesel fuel contaminants. The detection of *Pseudomonas* sp., *Micrococcus* sp. and *Bacillus* sp. in the water bottom of the separating tank and the 'very bottom' of tank 3, appears to show the necessity for free water for growth of bacteria in fuel tanks. Fass and Miller (1980) have suggested that bacteria die at a much faster rate than fungi in fuel. All the genera isolated in this study are known to contain hydrocarbon utilizers (Sohngen, 1913; ZoBell, 1946). The aerobic bacteria in the separating tank have been reported to first lower the redox potential and this promotes suitable conditions for the growth of anaerobic sulphate reducers such as *Desulfovibrio desulfuricans* (Walter, 1971; Isenberg and Bennett, 1959).

The pH of the environment is one of the controlling parameters for microbial growth and probably affects the stages of succession. It is known that bacteria and yeasts secrete low molecular weight fatty acids (Landsdown, 1965) which will subsequently lower the pH of the aqueous environment making it suitable for fungal colonization.

An alternative source of contamination which needs to be considered before any control measures for the storage tanks can be implemented is the fuel as supplied by the 'Shell' and TCV. There is some degree of correspondence between the contamination detected in 'Shell' and TCV as compared with tanks 2 and 3. Clearly any attempts at a general reduction must take this into account. The high level of microbial contamination in samples from TCV are of concern in that this could provide large contaminant inocula if this diesel is ever returned to vessels and storage tanks before being properly cleaned or filtered.

Techniques and sampling devices used for the isolation of bacteria have been in use for a considerable time (Sharpley, 1966; Hill, Davies, Pritchard and Byron, 1967; Hill, 1970) but there is a lack in the development of techniques for estimating viable fungal spores from hydrocarbon fuels. There has been little advancement over the last twenty years since Hazard (1963) developed the membrane filter technique. There is a need for the development of a better technique to isolate the increasing number of fuel contaminants. Among the methods used in this study, Hazzard's method proved to be the best. Hill (1970) described a rapid method for determining the level of contamination in aviation fuel condensate samples by testing the phosphatases, a group of enzymes widely distributed in micro-organism. They split off phosphate from organic phosphate compounds which can readily be detected. The problem with such a chemical method is that it gives no indication about the identity of the potential contaminants.

Most methods used were found to have distinct disadvantages, some were unpredictable in that they did not give consistent and reproducible recoveries of viable organisms.

Apart from the filtration method the other techniques have limitations which are reflected by the sample size used, and they are dependent upon the degree of contamination of the sample for them to be effective. Methods for the detection of viable organisms do not give accurate quantitative estimates of the number of organisms present in contaminated diesel fuel, as only viable organisms that grow on the culture medium are counted. Although counts have been expressed quantitatively they should be considered to be only qualitative. This is because a single fungal hypha may be fragmented during sampling and could give rise to a number of colonies from the fragments on culturing thereby indicating a count higher than the true one. Conversely, an aggregation of spores may be counted as one colony by giving rise to one mycelial mass.

The culture method of pouring the medium over the membrane inverted on the medium gave greatest numbers of the three major species. But placing the membrane on the medium with the right side up was understandably less valid. This method gave low recoveries of *C. resinae*. It may be that the hydrophobic nature of the membrane does not allow adequate nutrients to pass through for the spores to grow. The disadvantage of placing the membrane inverted on the medium is that the colonies growing on the surface of the medium, underneath the membrane are crowded, antagonism and other interactions between the organisms may have affected the number of viable counts. However using the modified Hazzard's method (1963) described in this work the above disadvantages are mainly overcome by pouring the medium over the membrane. In this way the spores are dispersed by the medium and the masking and suppression effects from over-crowding are reduced. This gave consistent and reproducible counts of viable organisms.

The pour and spread plate used for water bottom samples gave accurate and reliable counts when the sample of fuel was heavily contaminated. They were not satisfactory, when aliquots of fuel sample larger than 1 cm^3 floated on the top of the medium. Growths submerged in fuel were difficult to see and the colonies appeared darker in colour.

Time of analysis of the fuel samples was standardized to twenty-four hours despite some difference in the number of CFU of fungi recovered from zero to twenty-four hours. This difference was insignificant for this delay period. It was impossible to apply statistics to the colony numbers recovered due to the variable nature of the CFU which included spores and mycelial fragments, each having its own characteristic viability and multiplication rate. To have filtered two single, say 500 cm^3 samples from each litre would have seriously underestimated contamination in most samples. While filtering twenty 50 cm^3 samples could perhaps have resulted in a reasonably accurate estimate of viable CFU at that time. To test such numbers of replicates was impossible given the financial and human resources available. Thus the data obtained from the filters cultured from each sample will have some limitations because high numbers of colonies of a particular fungus may obscure some potential CFU of another fungus and the variation of this nature of the CFU makes statistical treatment irrelevant.

Although container type, analysis time, number of filters assayed were standardized, it was recognized that different individual fungi may have responded differently after removal from diesel fuel. Thus the results presented can only be discussed in rather general terms in order to attempt a better understanding of the source of and cure for microbial contamination. The aim throughout has been to obtain trends which may then be subjected to further laboratory and field testing under more defined conditions, hopefully giving some significant findings.

Direct observation of filters revealed a wide variety of fungal spore types in stored diesel fuel. Air spora studies suggest that many such as *Alternaria*, *Epicoccum*, *Ulocladium*, *Trichoderma*, *Fusarium*, *C. cladosporioides* and others could have entered the tanks from the surrounding air. But these fungi could not utilize the fuel, for they were highly selective as to the type of substrate that they would degrade. *C. resinae*, *Penicillium* spp. and *P. variotii* occurred in the surrounding atmosphere near the tanks. However, CFU may arise from any of these potential contaminants as soon as there is water in the fuel.

But the time lag in detecting the fungi in the 'very bottom' will depend upon the settling rate of the individual contaminant. Also the time lag between contamination and causing engine problems will depend on spores getting to the diesel/water interface, and subsequent laboratory experiments showed spores of all three major species can be detected as CFU on V-8 juice agar, immediately after inoculation at various periods of time.

Sharpley (1966) suggested a possible correlation between the seasonal variation of *C. resinae* in air and that in fuel. In the present study, *Penicillium* spp. were observed to occur in high levels in the atmosphere and a corresponding increase in CFU in the fuel, but this was less evident for *C. resinae* and *Paecilomyces variotii*. But air must still be recognised as a source of fuel contamination. Sheridan and Nelson (1971) and Sheridan (1974) monitored the atmosphere for *C. resinae* in Wellington and found similar autumn and summer peaks. But they did not correlate the seasonal incidence of *C. resinae* from the atmosphere to its incidence in fuel. It appears that contamination of fuel is a complex problem.

According to Reichart (1983) fungal spores are always present in the atmosphere and therefore will eventually find their way into fuel tanks but these are harmless unless water is present to provide the ideal habitat for growth. Once established the fungus produces water as a by product of growth, which is probably the reason for its rapid proliferation.

One obvious source of the air spora isolated near the tanks in this study was analysed by determining the soil microflora and it is clear that both *C. resinae* and a number of *Penicillium* spp. were abundant adjacent to the tanks. *C. resinae* was found to be widespread in soil in New Zealand (Sheridan and Knox, 1970; Sheridan, 1974).

The possible sources and causes of high levels of microbial contamination in the storage tanks at Devonport may be summarised as follows:

- The major fuel-utilizing contaminants were *C. resinae*, *Penicillium corylophilum* and *Paecilomyces variotii*.

- Tank 3 had some continuous re-inoculation by the contaminants because of the regular return of fuel and water from the separation tank.

- Fuel as supplied is seldom (if ever) free of microbial contamination.

- Fungal spores from the air were a continuous source of inoculum and this was significant for *Penicillium* spp., compared with *C. resinae* and *P. variotii*.

- Any source of inoculum will only present a problem if the fungi can grow and this requires a free aqueous phase which is commonly present.

4 EXPERIMENTAL RESULTS: SECTION II - LABORATORY STUDIES

4.1 Growth studies

4.1.1 Single species culture

A) Isolates from this study:

Figure 4.1 shows the growth of diesel isolates of *C. resinae*, *P. variotii* and *P. corylophilum* after 6 weeks in medical flats containing equal volumes of Bushnell-Haas (BH) and diesel in the laboratory. These fungi formed mycelial mats about 5 mm thick from an inoculum of 5×10^6 spores/cm^3 when grown at 25°C. Sporulation could be perceived after one week with spores being in the diesel phase. Mycelial debris were present in the aqueous phase from 4 weeks onwards.

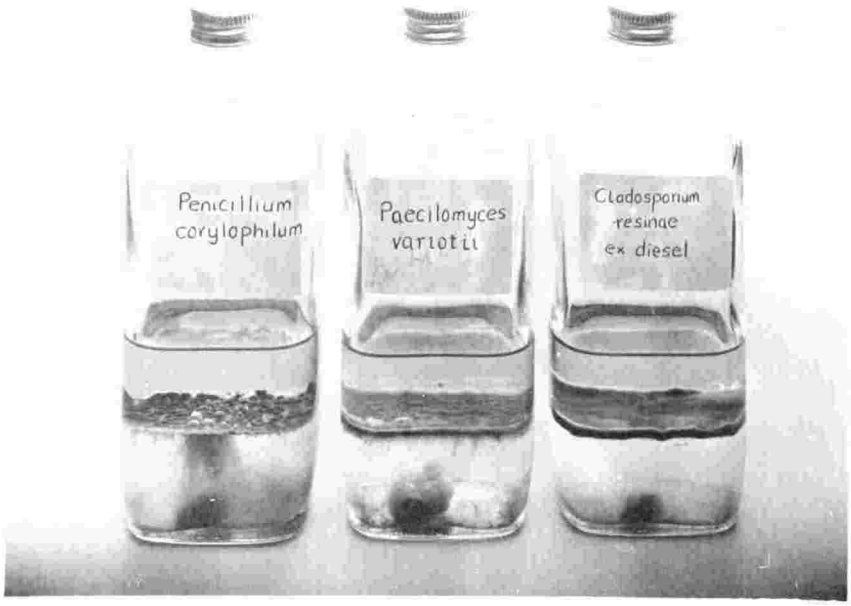
Table 4.1A and figure 4.2 show that all isolates of *C. resinae*, *P. corylophilum* and *P. variotii* grew well in Bushnell-Haas/diesel phases and showed a high degree of viability in subsequent culture on V-8 juice agar (table 4.1B). Figure 4.3 shows the growth of these fungi in different aqueous phases apart from in BH/fuel phases. On seawater/diesel phases only *P. corylophilum* grew well. In the tapwater/diesel phases *P. variotii* however appeared to survive to a higher degree. It did not produce as many viable spores in the distilled water/diesel phases. In diesel alone none of the fungi tested grew significantly although spores of all fungi except for *P. corylophilum* remained viable. The pH of the aqueous phases remained unchanged. There was a time lag period of 2-3 days for *C. resinae* and *P. variotii*.

B) Isolates from other sources:

Australian isolates of *C. resinae* and the NZ diesel isolate showed similar growth on the BH/diesel phases producing thick dark mycelial mats. The white form of *C. resinae* from Australia produced only a thin white mycelial mat. The dry weight yields of fungal material after 6 weeks growth in the 100 cm^3 culture were as follows:

Figure 4.1. Growth of *Cladosporium resinae*, *Paecilomyces variotii* and *Penicillium corylophilum* (ex diesel fuel) in Bushnell-Haas/diesel fuel interface after six weeks.
Note:- Mycelial debris in the aqueous phase.

Figure 4.3. Growth of various types on aqueous/diesel fuel phases a expressed in table 4.1A.
Left:- Some spore germination, very little growth (+)
Centre:- Spore layer (inoculum) unchanged (0).
Right:- Very thin colourless mycelial mat (++).


Penicillium
corylophilum
Paecilomyces
variotii
Cladosporium
resinae
ex diesel

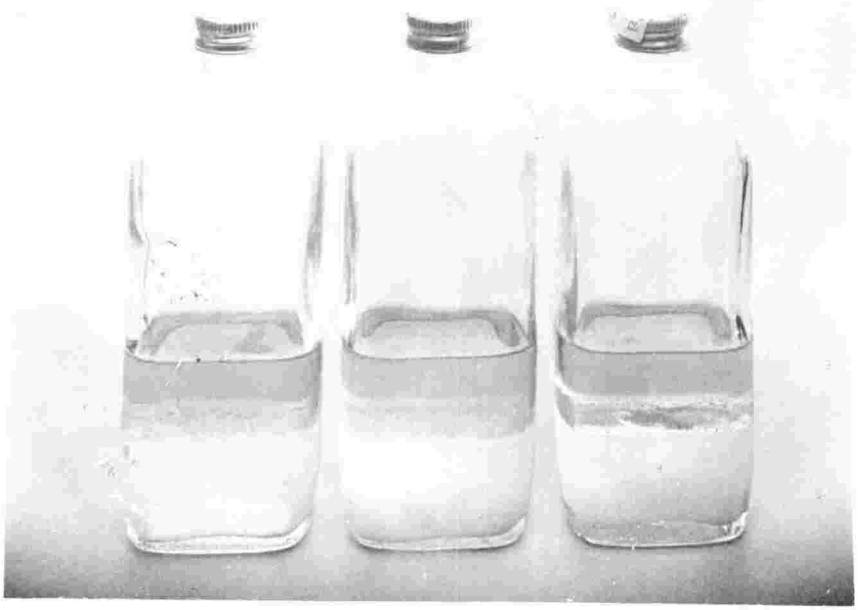


Figure 4.2. Growth* of fungal isolates on various aqueous/diesel fuel phases (1:1).

Type 1 - *C. resinae* ex diesel fuel.
2 - *C. resinae* ex jet fuel.
3 - *C. resinae* ex Air.
4 - *C. resinae* ex soil.
5 - *Penicillium corylophilum*.
6 - *Paecilomyces variotii*.

* Growth is expressed as the mean dry weight of fungus recovered by filtration from duplicate 100 cm^3 cultures inoculated with 5 x 10^6 spores/cm^3.

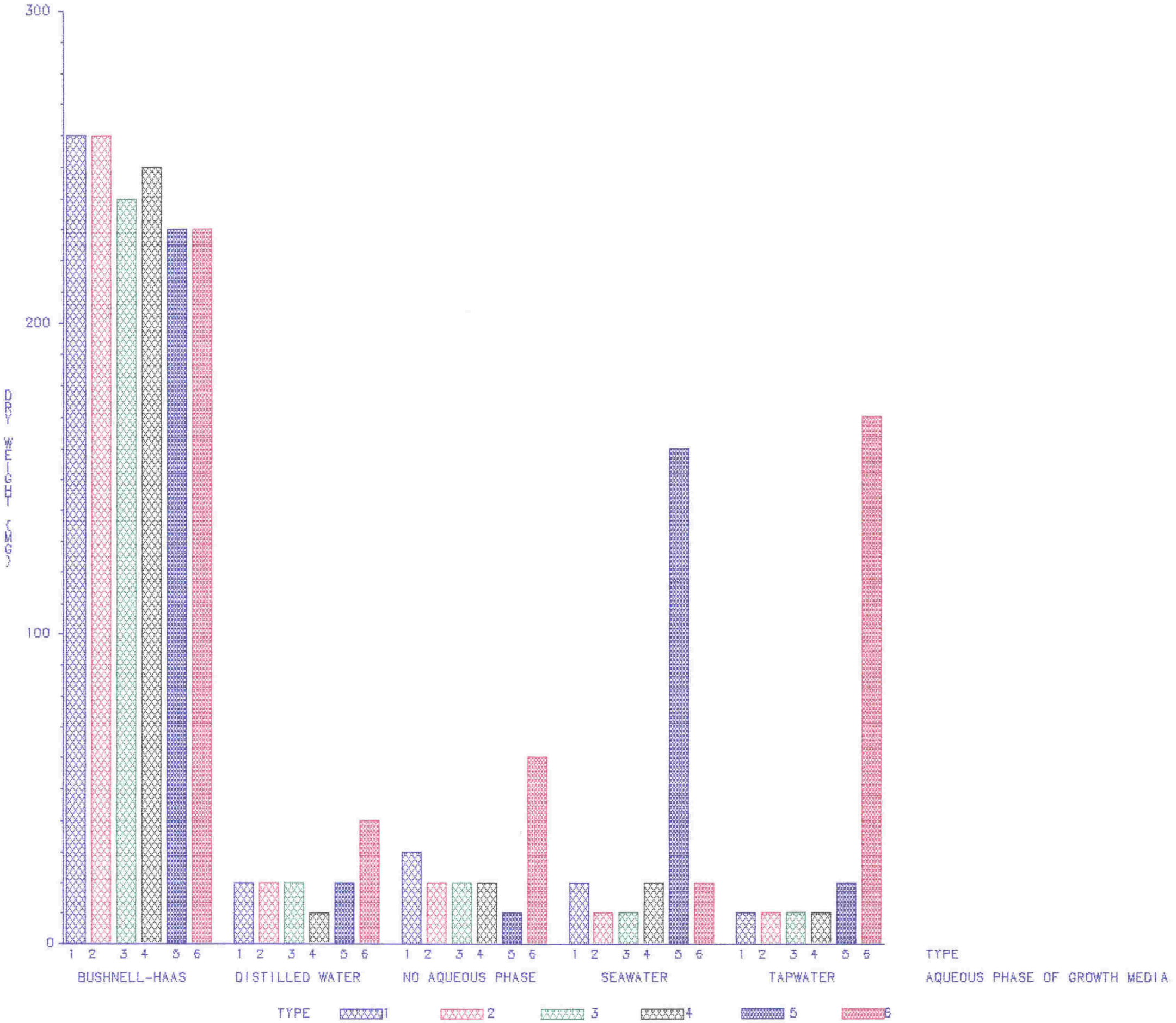

300
200
100
0
DRY WEIGHT (MG)
1 2 3 4 5 6
BUSHNELL-HAAS
DISTILLED WATER
NO AQUEOUS PHASE
SEAWATER
TAPWATER
TYPE
AQUEOUS PHASE OF GROWTH MEDIA
TYPE 1 2 3 4 5 6

Table 4.1A Growth* of principal microbial contaminants in the laboratory on various aqueous/diesel fuel phases.

Fungus	Bushnell-Haas/Diesel	Distilled water/Diesel	Diesel only	Seawater/ Diesel	Tapwater/ Diesel
C. resinae (ex diesel)	+++	+	0	0	+
C. resinae (ex jet fuel)	+++	+	0	0	+
C. resinae (ex soil)	+++	+	0	0	+
C. resinae (ex air)	+++	+	0	0	+
Penicillium corylophilium	+++	+	0	++	+
Paecilomyces variotii	+++	+	0	0	+

* Growth is expressed as follows from observations made after 6 weeks and shown in figure 4.3

0 Spore layer (inoculum) unchanged

\+ Some spore germination, very little growth

++ Very thin colourless mycelial mat

+++ Thick dark mycelial mat

Table 4.1B Viability[+] of fungi/spores isolated after 6 weeks and grown on V-8 juice agar.

Fungus	Bushnell-Haas/Diesel	Distilled water/Diesel	Diesel only	Seawater/ Diesel	Tapwater/ Diesel
C. resinae (ex diesel)	+++	+	+	0	+
C. resinae (ex jet fuel)	+++	+	+	0	+
C. resinae (ex soil)	+++	+	+	0	++
C. resinae (ex air)	+++	+	+	0	+
Penicillium corylophilum	+++	+	0	++	+
Paecilomyces variotii	+++	+	+	0	+++

[+] Viability of fungi, colony frequency, 5 days after pour plate inoculation.

0 no colonies

\+ 10 colonies/cm^3 or less

++ 10-50 colonies/cm^3

+++ > 50 colonies/cm^3

C. resinae ex Australian Army: 410 mg,
ex HMAS Cerebus (Australia): 330 mg,
ex Tasmania: 490 mg,
ex Australia: 450 mg,
ex Australia (white form): 120 mg,
ex Devonport, N.Z.: 450 mg.

Table 4.2 compares the growth of the seven species of *Penicillium* isolated from diesel fuel with that of *P. variotii* and various *C. resinae* isolates on both Bushnell-Haas/diesel and BH/jet fuel. Only *P. corylophilum* grew well compared to the other *Penicillium* spp., *P. expansum* and *P. spinulosum* showed little growth. Spores of all *Penicillium* spp. except *P. digitatum* appeared to retain full viability after 6 weeks in the BH/diesel fuel phases.

C) Soil isolates:

Among the soil isolates obtained from around the cliff tanks, only *Penicillium nigricans* and *P. janthinellum* showed any signs of growth on BH/diesel fuel phases. This growth was very limited producing 80 mg and 50 mg dry weight respectively after six weeks. No growth occurred in BH without fuel or in diesel fuel alone.

4.1.2 Growth in mixed cultures

i) When *P. corylophilum* and *C. resinae* were grown as a mixed culture in the Bushnell-Haas/diesel phases, a thick dark mycelial mat was produced after 6 weeks. *P. corylophilum* spores were not visible in samples of the well shaken cultures and on plating out on V-8 juice agar, only colonies of *C. resinae* were produced. By contrast in seawater/diesel growth medium, no visible growth had occurred after 6 weeks. Microscopic observation indicated that *P. corylophilum* spores germinated but not *C. resinae* spores. When the seawater/diesel fuel sample was plated onto V-8 juice agar only *P. corylophilum* colonies were obtained. Little growth occurred in tapwater/diesel phases but spores of both fungi retained viability for 6 weeks after mixed spores were inoculated.

Table 4.2 Growth and survival of *Penicillium*[*] spp., *C. resinae*[+] and *P. variotii*[#] on Bushnell-Haas (BH) plus diesel or jet fuel.

Fungi	Growth[1] after 6 weeks in BH/diesel or jet fuel	Fungal viability[2]$_x$
P. echinulatum	±	100
P. chrysogenum	±	100
P. frequentans	±	100
P. spinulosum	+	100
P. corylophilum	+++	100
P. expansum	+	100
P. digitatum	±	50
C. resinae (ex jet fuel)	+++	100
C. resinae	+++	100
C. resinae	+++	100
C. resinae	+++	100
P. variotii	+++	100

* The seven *Penicillium* spp were all isolated from diesel fuel in this study.

\+ *C. resinae* isolated from diesel fuel, soil and air in this study and one previously isolated from jet fuel were grown.

\# *P. variotii* isolated from diesel fuel in this study.

1 Identical growth responses were obtained regardless of hydrocarbon type.

± Very thin spore mat

\+ Thick spore mat, very thin colourless mycelial mat.

+++ Thick dark mycelial mat.

2 Identical growth patterns were observed regardless of hydrocarbon type.

x Viability after 6 weeks growth was determined by the pour plate method using 5 cm^3 sample from shaken cultures and is expressed simply as a proportion of Petri-dish covered by fungal growth after 5 days.

In diesel fuel without aqueous phase, no growth occurred of either fungus but spores of *C. resinae* retained some viability.

ii) The effect of metabolic changes brought about by growth of *C. resinae* on subsequent growth of *Penicillium* and *Paecilomyces* is shown in table 4.3. *P. corylophilum* and *P. variotii* grew well following growth of *C. resinae* on BH/diesel producing just as much dry weight after the 6 weeks growth period as had been produced on fresh BH/diesel phases. No significantly increased growth was observed on distilled water/diesel and diesel alone. Only *P. corylophilum* grew on seawater/diesel fuel phases. Both *P. corylophilum* and *P. variotii* produced little growth on tapwater. There was only a slight shift in the pH (8.3 - 8.1) and (7 - 6.8) in the seawater/diesel fuel and BH/diesel fuel respectively after *P. corylophilum* and *P. variotii* growth period. No significant change in pH was observed after *C. resinae* was grown in above mentioned media. The other aqueous phases remained neutral (pH 7). As shown in table 4.4A, in a more extensive study of the effect of the presence of one fungus upon the growth of another, little growth was observed in the absence of diesel fuel. All cultures containing *P. corylophilum* alone or as a mixed inoculum showed growth after 6 weeks in seawater.

Survival of ungerminated and germinated spores in aqueous phases alone and aqueous/diesel fuel phases is shown by the data in table 4.4B. *Candida* did not survive on tapwater, alone or as a mixed spore inoculum; while its survival in BH was affected by the accompanying fungus. Germinating *C. resinae*, *P. corylophilum* and *Paecilomyces variotii* spores appeared to make *Candida* spores non-viable after 6 weeks. *Candida* retained some viability after 6 weeks in seawater. Again this was reduced by the presence of other fungi and was eliminated by *C. resinae*, *Penicillium corylophilum*, *P. expansum* and *Paecilomyces variotii*. In tapwater *C. resinae* suppressed the viability of *P. expansum*.

ollowed by growth of *P. corylophilum* (B) and *P.*
arious aqueous/diesel phases.

ry t. g)	Seawater/ Fuel	Dry wt. (mg)	Distilled water/fuel	Dry wt. (mg)	Fuel only	Dry wt. (mg)
10	0	20	0	10	0	10
30	++	120	0	10	0	10
70	0	10	0	40	+	60

owth

Table 4.4A Growth of *Candida albicans*, *Cladosporium resinae*, *Penicillium corylophilum*, *Penicillium expansum*, *Paecilomyces variotii* as single and various mixed cultures on aqueous and aqueous/diesel fuel phases.

Fungi	Seawater only	Dry wt. (mg)	Tapwater only	Dry wt. (mg)	Bushnell-Haas only	Dry wt. (mg)	Seawater/ Fuel	Dry wt. (mg)	Tapwater /fuel	Dry wt. (mg)	Bushnell-Haas/fuel	Dry wt. (mg)
Candida albicans	0	30	0	20	0	10	+	70	0	20	++	120
Candida + C. resinae	+	60	0	10	+	80	0	13	+	60	+++	290
Candida + P. corylophilum	+	90	0	10	0	10	++	170	+	50	+++	240
Candida + P.expansum	0	30	0	10	+	80	0	30	+	60	++	100
Candida + P. variotii	0	30	+	80	0	20	0	20	+	50	+++	340
Cladosporium resinae	0	30	+	50	+	80	+	70	0	40	+++	330
C. resinae + P. corylophilum	++	110	+	60	0	10	++	130	0	20	+++	610
C. resinae + P. expansum	0	40	0	10	0	30	0	20	0	20	+++	540
C. resinae + P. variotii	0	40	+	70	0	10	+	60	+	50	+++	700
Penicillium corylophilum	+	90	0	30	0	10	++	180	+	80	+++	250
P. corylophilum + P. expansum	+	85	+	80	0	10	++	200	0	30	+++	420
P. corylophilum + P. variotii	++	106	+	70	0	20	++	130	+	70	+++	500
Penicillium expansum	+	80	+	90	0	10	+	70	+	50	+++	270
P. expansum + P. variotii	+	90	+	50	0	30	++	140	+	60	+++	380
Paecilomyces variotii	0	10	0	40	0	10	+	60	+	90	+++	280

0 Spore layer (inoculum) unchanged

\+ Some spore germination, very little growth

++ Very thin colourless mycelial mat

+++ Thick dark mycelial mat

* Viability is expressed as the number of colonies observed 5 days after plating 5 cm^3 sample on V-8 juice agar.

0 Absence of colonies

<50 less than 50% viable spores

>50 more than 50% viable spores

Table 4.4B Viability* of fungi in samples from 6 weeks old cultures described in Table 4.4A

Fungi	Seawater only	Tapwater only	Bushnell-Haas only	Seawater/ Fuel	Tapwater /fuel	Bushnell-Haas /fuel
Candida albicans (C)	<50	0	>50	<50	0	>50
Candida + C. resinae (C.R.)	0	>50	>50	0	>50	>50
Candida + P. corylophilum (P.C.)	>50	C.R. only >50	C.R. only >50	>50	C.R. only <50 & >50	C.R. only >50
Candida + P.expansum (P. exp)	P.C. only 0	P.C. only >50	P.C. only >50	P.C. only 0	C & P.C. >50	P.C. only >50
Candida + P. variotii (P.V.)	0	P. exp only >50	both >50	0	both >50	both >50
Cladosporium resinae	0	P.V. only >50	P.V.only >50	0	both >50	P.V. only >50
C. resinae + P. corylophilum	>50	>50	>50	>50	>50	>50
C. resinae + P. expansum	P.C. only 0	both >50	both >50	P.C. only 0	both >50	both >50
C. resinae + P. variotii	0	C.R. only >50	both >50	0	C.R. only >50	C.R. only >50
Penicillium corylophilum	>50	both >50	both >50	>50	both >50	C.R. only >50
P. corylophilum + P. expansum	>50	>50	>50	>50	>50	>50
P. corylophilum + P. variotii	P.C. only >50	both >50	both >50	P.C. only >50	both >50	both >50
Penicillium expansum	P.C. only 0	both >50	both >50	P.C. only 0	both >50	both >50
P. expansum + P. variotii	0	>50	>50	0	>50	>50
Paecilomyces variotii	0	both >50	both >50	0	both >50	both >50

In tapwater/diesel fuel phases, *Candida* showed least evidence of growth. In seawater/diesel, *P. corylophilum* showed some growth and this appeared unaffected by the presence of other fungi nor was the viability of *P. corylophilum* reduced after 6 weeks in mixed culture. *Candida* retained viability after 6 weeks in seawater/diesel but the viability was completely suppressed by *P. corylophilum*.

As already shown BH/diesel phases supported considerable growth of fungi, even of *Candida*. The total growth of *Candida* and *P. expansum* was considerably less than that of either fungus alone but viability of either fungus appeared to be unaffected when grown alone or as a mixed inoculum. When *C. resinae*, *P. corylophilum* and *P. variotii* were growing profusely in the presence of *Candida*, *Candida* could no longer be recovered after 6 weeks growth in BH/diesel. Similarly, *C. resinae* appeared to eliminate viable spores of *P. expansum* and *P. variotii* after 6 weeks of growth.

(iii) The data in Table 4.5 show *P. corylophilum*, *C. resinae* and *P. variotii* produced thick dark mycelial mats in BH/diesel phases. *P. corylophilum* produced little growth in seawater/diesel and no significant change in growth pattern was observed in other aqueous phases.

After the first 6 weeks, when *C. resinae*, *P. variotii* and *P. corylophilum* were inoculated in *P. corylophilum*, *C. resinae* and *P. variotii* grown media respectively, they continued to form dark mycelial mats in the BH/fuel. The media did not seem to have any limiting effect on the growth of the second batch of fungi. But in the seawater/fuel, *C. resinae* formed a very thin colourless mycelial mat after *P. corylophilum* had grown on the same media. When *C. resinae* was grown initially in seawater/fuel, it produced only a spore layer and the dry weight was insignificant. *P. corylophilum* grown media may have undergone some metabolic changes enhancing *C. resinae* survival in seawater/fuel and seawater only. By contrast there was a change in pH 8.3 to 8.

Table 4.5 The effects of growing[#] selected fungi on the growth of other fungi.

Fungi	Bushnell-Haas/fuel	Dry wt. (mg)	Bushnell-Haas	Dry wt. (mg)	Seawater/Fuel	Dry wt. (mg)	Seawater	Dry wt. (mg)	Tapwater/Fuel	Dry wt. (mg)	Tapwater	Dry wt. (mg)
C. resinae	+++	400	+	50	0	40	0	10	0	20	0	40
P. corylophilum	+++	340	0	10	++	160	+	80	+	60	0	20
P. variotii	+++	360	0	10	0	40	0	5	++	100	0	30
Second 6 weeks												
P. variotii	+++	390	0	20	0	15	0	10	+	70	0	25
C. resinae	+++	410	0	10	++	110	+	50	0	40	0	10
P. corylophilum	+++	370	0	20	++	130	+	60	0	30	0	10

0 Spore layer (inoculum) unchanged

\+ Some spore germination, very little growth

++ Very thin colourless mycelial mat

+++ Thick dark mycelial mat

[#] The growth of *C. resinae*, *P. corylophilum* and *Paecilomyces variotii* for 6 weeks followed by the growth of *C. resinae* on *P. corylophilum* grown media, *P. corylophilum* on *P. variotii* media and *P. variotii* on *C. resinae* media for another 6 weeks on the various aqueous/diesel phases.

P. corylophilum grew singly and regardless of the other type of fungi grown in its media.

4.2 Creosote tolerance studies

The effects of creosote on the growth of fungi is shown by the data in figures 4.4A, B, C, D and E. The *C. resinae* isolates (ex diesel, jet fuel, soil and air) and *P. corylophilum* and *P. variotii* (ex diesel) grew on V-8 juice agar in the presence of creosote concentration up to 1%. No growth occurred at concentrations of 2% creosote. *C. resinae f. albidum* (ex Australia) also tolerated 1% creosote but the N.Z. isolate of this form appeared slightly less tolerant. *P. expansum*, *P. digitatum*, *P. frequentans*, *P. chrysogenum*, *P. echinulatum* and *Cladosporium cladosporioides* from diesel were able to grow and tolerate up to 0.5% creosote. *Alternaria alternata* ex air, ex diesel and ex jet fuel tolerated up to 0.3% creosote. Except for *P. brevicompactum* and *Trichoderma hamatum*, the other fungi isolated from soil and other sources in this study showed growth in the presence of creosote but only up to a concentration of 0.3%.

4.3 Effects of shaking and transport on spore viability

Spores of *C. resinae*, *Penicillium corylophilum* and *Paecilomyces variotii* appeared to be completely unaffected by being shaken in diesel fuel, by hand for up to 30 minutes, or by mechanical agitation for up to 8 hours, whether as single or mixed inocula when viability was tested by the millipore filtration method.

The effect of carrying inoculated diesel or jet fuel samples by car over various distances (300–500 km) during 2–3 days, equally was shown to have no influence on the viability of *C. resinae* isolated from either diesel or jet fuel. 100% spore germination recovery was obtained from both the diesel and jet fuel.

Figure 4.4. Growth of the various isolates on V-8 juice agar containing different concentrations of creosote (after 14 days at 25°C).

A. Soil Isolates

Type *1. C. resinae. 2. Trichoderma harzianum.*
3. Penicillium aculeatum. 4. P. funiculosum.
5. P. janthinellum. 6. P. loliense. 7. P. nigricans.
8. P. verrucosum. 9. P. velutinum.

B. Diesel Isolates (Page 78)

Type *1. C. resinae f. albidum. 2. C. resinae.*
3. Paecilomyces variotii. 4. Penicillium corylophilum.
5. P. chrysogenum. 6. P. digitatum. 7. P. expansum.
8. P. echinulatum. 9. Alternaria alternata.
10. Penicillium frequentans. 11. P. spinulosum.
12. Cladosporium cladosporioides.

C. Jet Fuel Isolates (Page 79)

Type *1. C. resinae f. resinae. 2. C. resinae f. avellaneum. 3. C. sphaerospermum. 4. Alternaria alternata.*

D. Australian Diesel Fuel Isolates (Page 80)

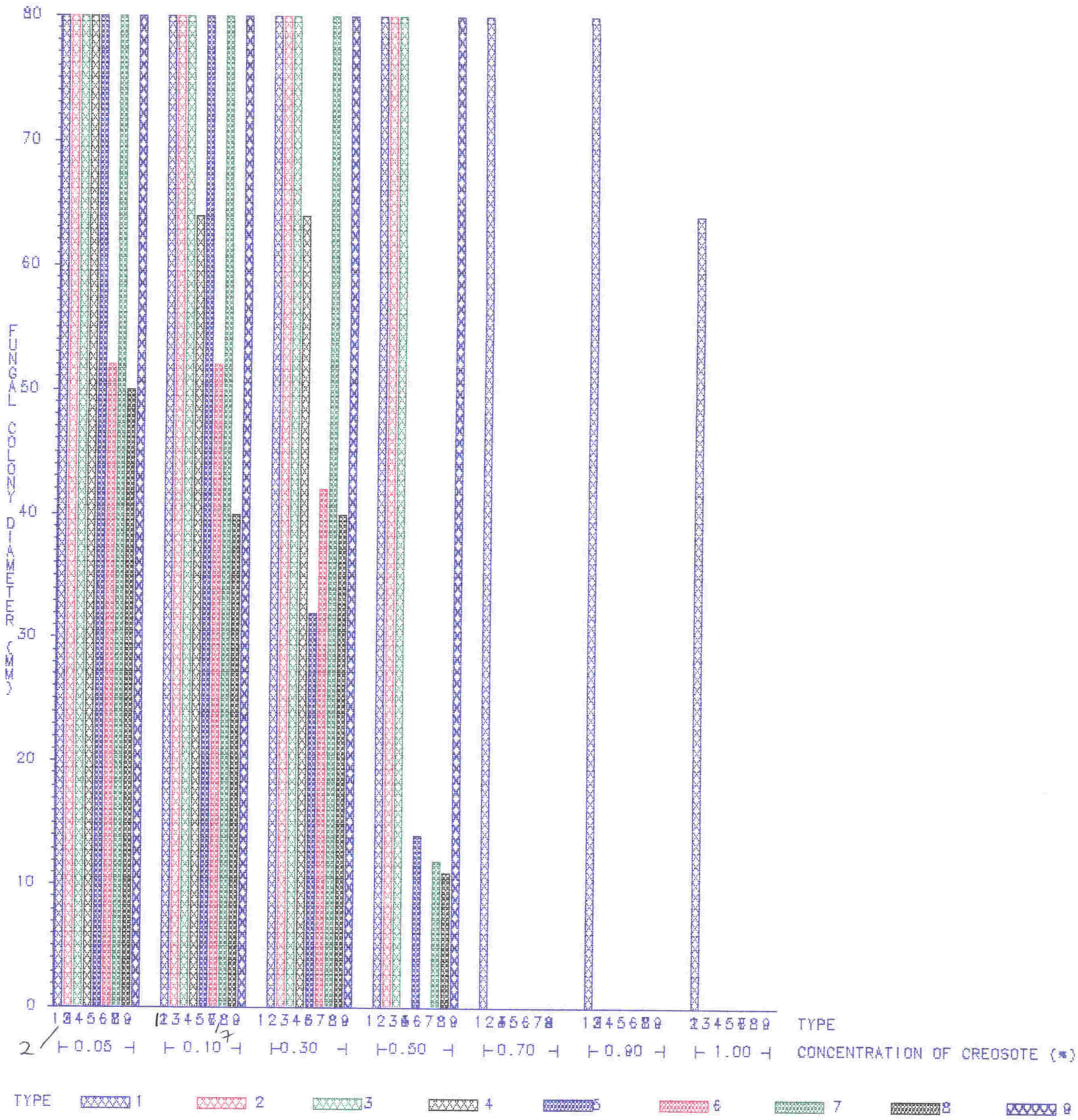
Type *1. C. resinae* ex *albidum. 2. C. resinae* ex Australia. *3. C. resinae* ex Army. *4. C. resinae* ex Cook Island. *5. C. resinae* ex HMAS Cerebus. *6. C. resinae* ex Tasmania.

E. Others (Page 81)

Type *1. Cladosporium cladosporioides* ex Air.
2. C. resinae f. resinae ex feather.
3. C. cladosporioides ex tomato.
4. Alternaria alternata.

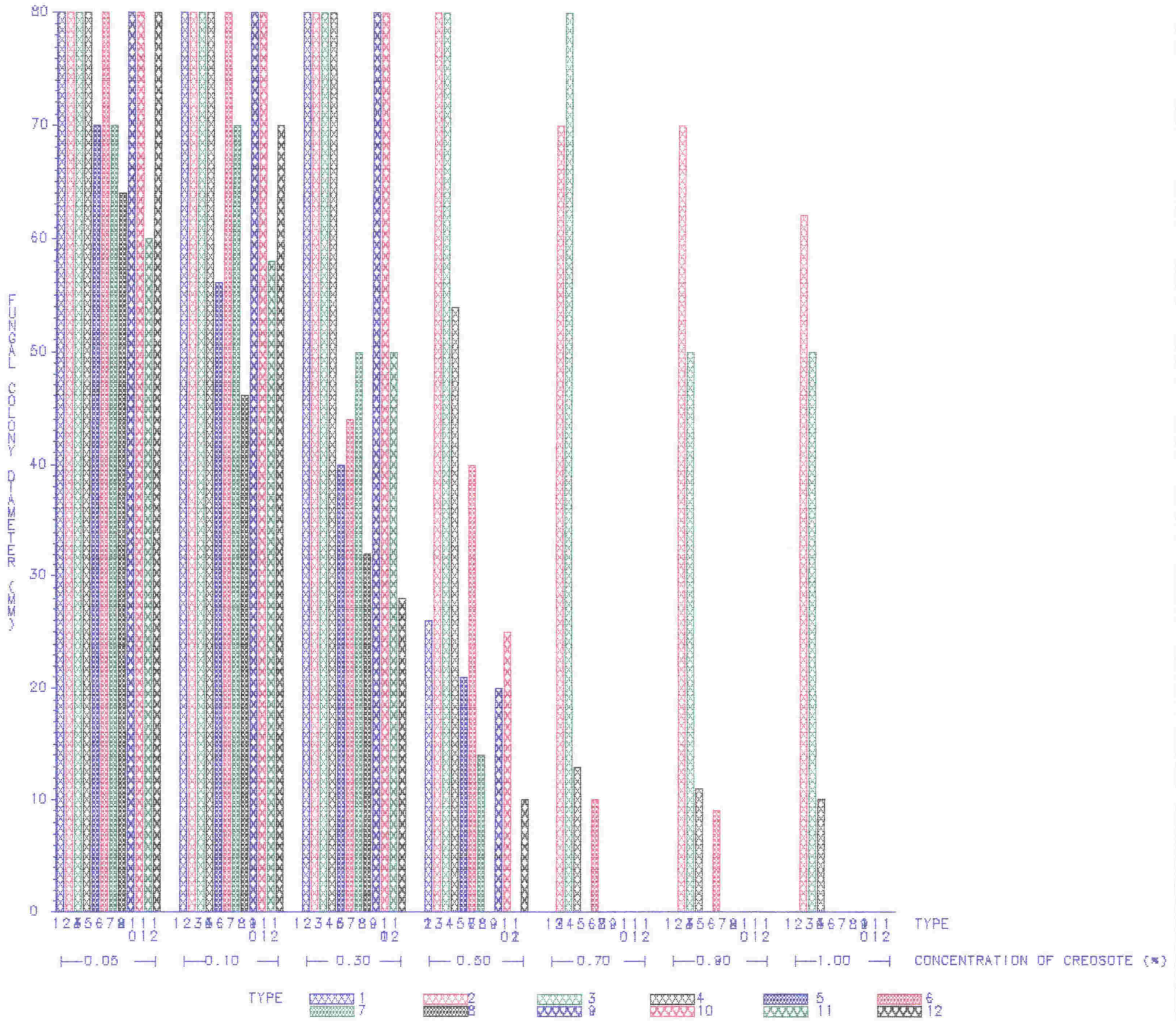
SOIL ISOLATES

A

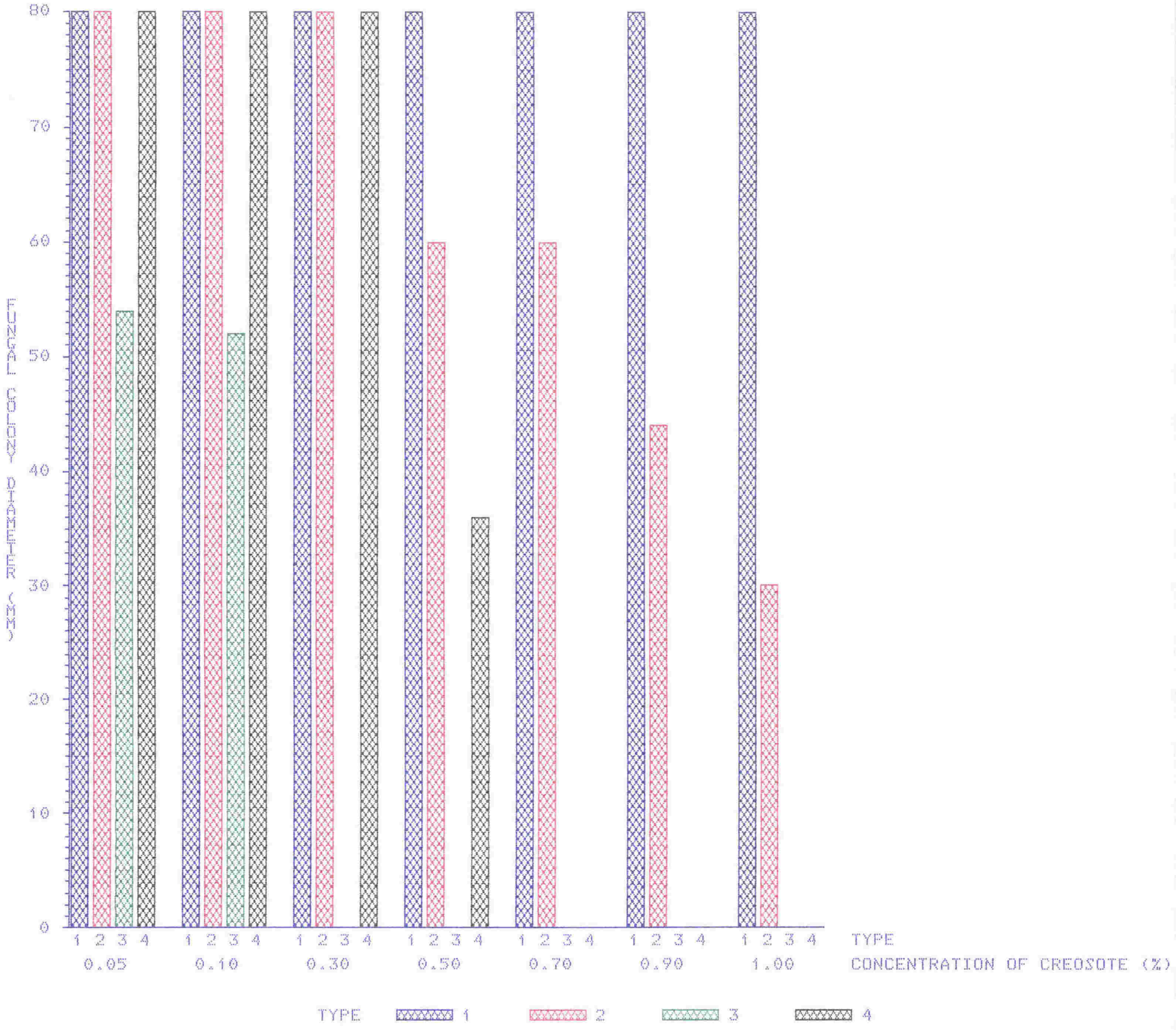


DIESEL ISOLATES

B

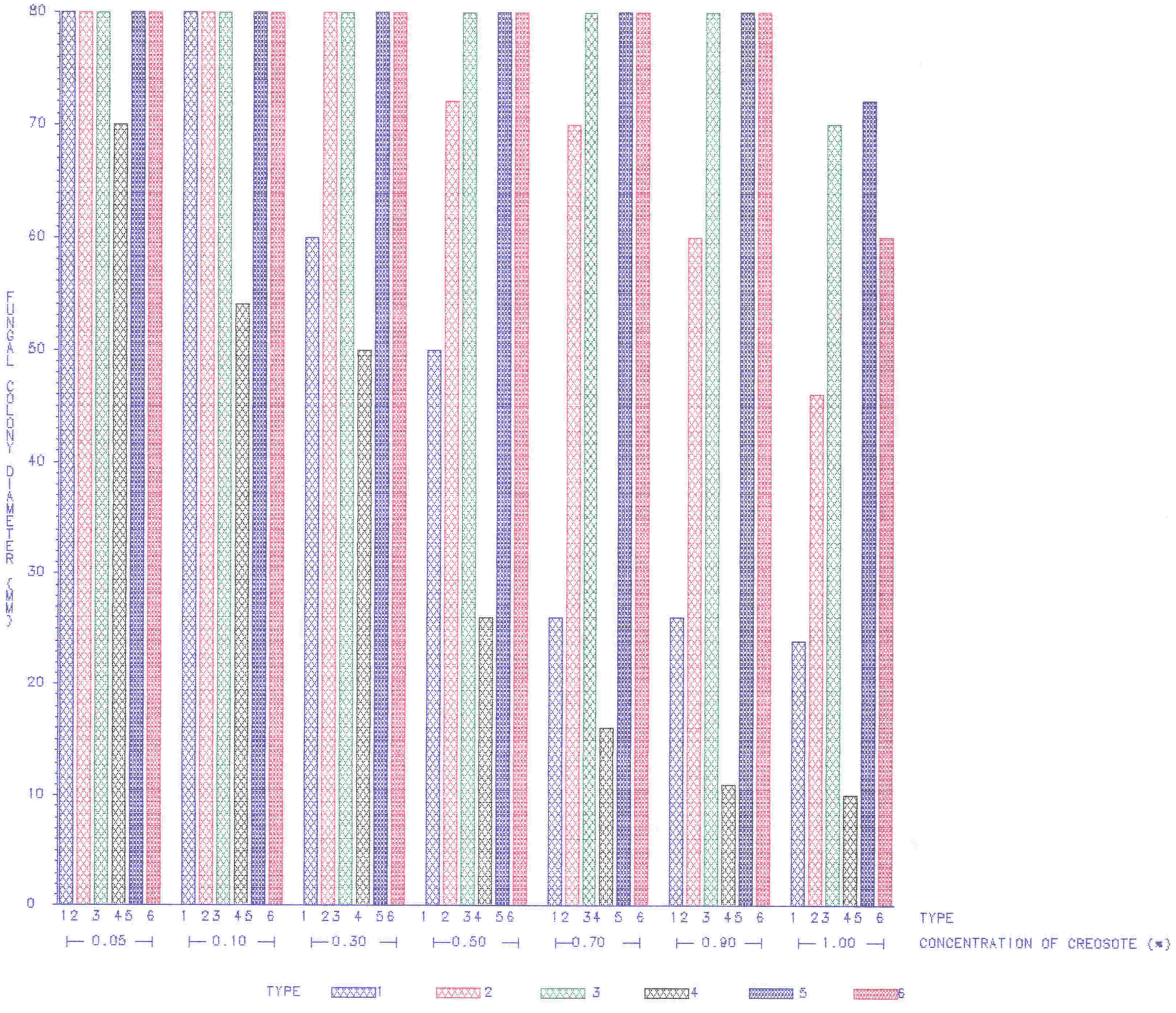


JET FUEL ISOLATES C

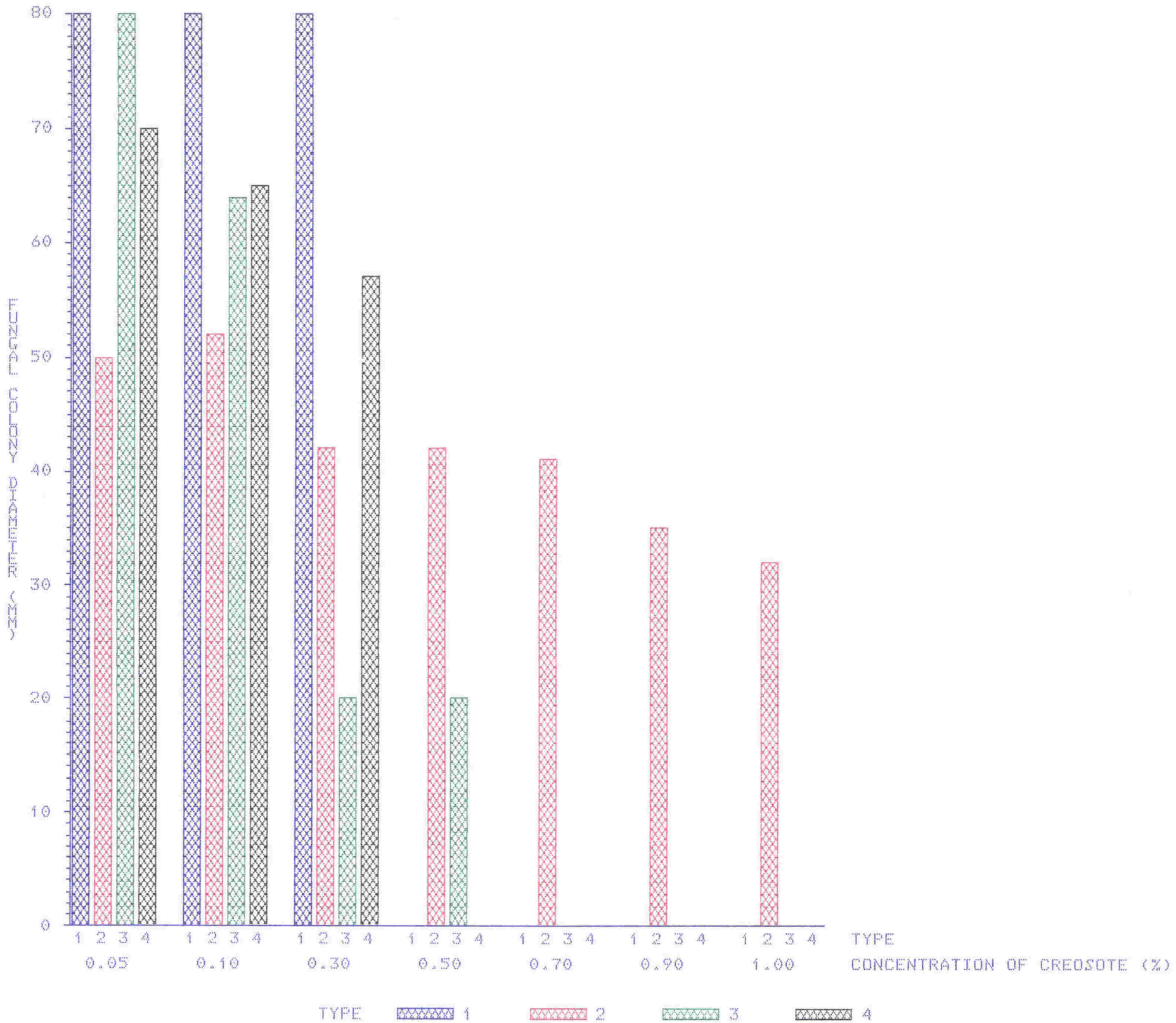


AUSTRALIAN ISOLATES

D



E


80
70
60
50
40
30
20
10
0
FUNGAL COLONY DIAMETER (MM)
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
TYPE
0.05 0.10 0.30 0.50 0.70 0.90 1.00
CONCENTRATION OF CREOSOTE (%)
TYPE 1 2 3 4

4.4 Morphology and ultrastructure studies

Five fungi *C. resinae* ex jet, ex diesel, *Paecilomyces variotii*, *Penicillium corylophilum* and *Cladosporium cladosporioides* were studied using light and scanning electron microscopy (SEM) in order to see if any surface features could be identified as being common to those able to use hydrocarbon. Representative photographs of conidiophores and conidia of these fungi in the light and scanning electron microscopy are represented in figures 4.5a, b, c, d and e and 4.6a, b, c, d and e.

The conidiophores and conidia of *C. cladosporioides* were rough-walled, but for the other fungi, they appeared smooth-walled in light microscopy. In SEM, the conidiophore, ramoconidia and blastospores of *C. resinae* (ex jet fuel) appeared smooth, whereas in *C. resinae* ex diesel, the above structures appeared 'crinkled'. Both forms usually had 2-3 projections on the ramoconidia. It was also evident in the SEM micrographs that the conidia of *C. resinae* were not separated from each other by prominent scars, hence the name *Hormoconis resinae* (Lindau) Von Arx and de Vries is acceptable.However *C. cladosporioides* conidia had scars and the conidiophore was less ornamented than the conidia. The sporulating structures of *P. variotii* were smooth. The phialides were drawn out to produce the conidia in chains.

In contrast to the light micrographs, the stipe of *P. corylophilum* appeared warted but the phialides and conidia were smooth-walled. No single characteristic can clearly be associated with those fungi capable of utilizing diesel fuel.

Techniques for fixing and staining material for ultrastructural characteristics using transmission electron microscopy of the sections varied considerably in their effectiveness. Figures 4.7-1, 2 and 3 show the appearance of sections of conidia of *C. resinae* (ex diesel) which have been prepared using a range of techniques. Many of these techniques show inadequate details in the conidia ultrastructure.

In methods 1-4, stated in Table 2.4, the cell wall appeared disintegrated and the cytoplasm and cellular components were dark (figure 4.7-1).

Figure 4.5. Light micrographs of conidiophores and conidia.

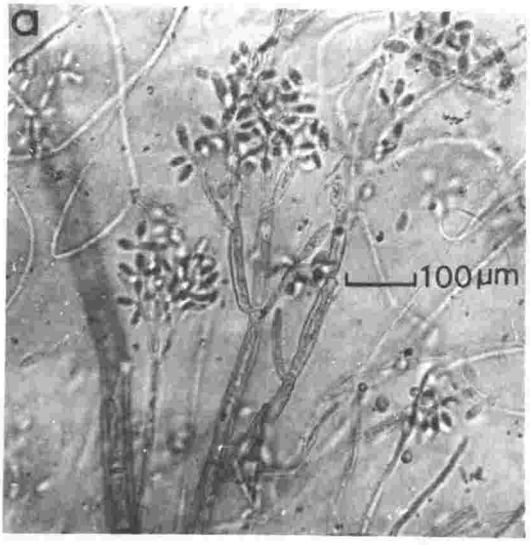
a. *Cladosporium resinae f. avellaneum.*

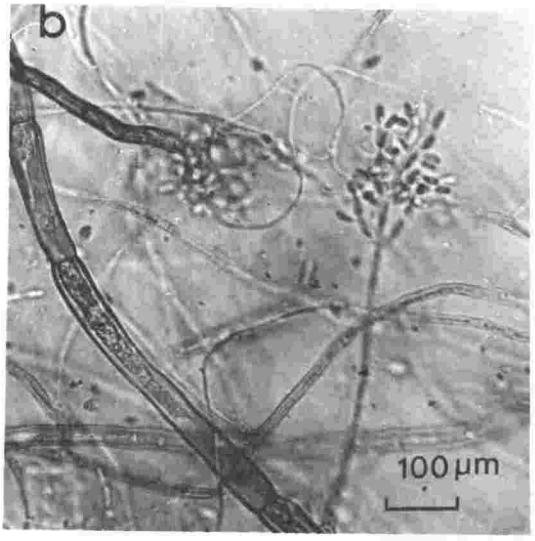
b. *Cladosporium resinae f. avellaneum* ex jet fuel.

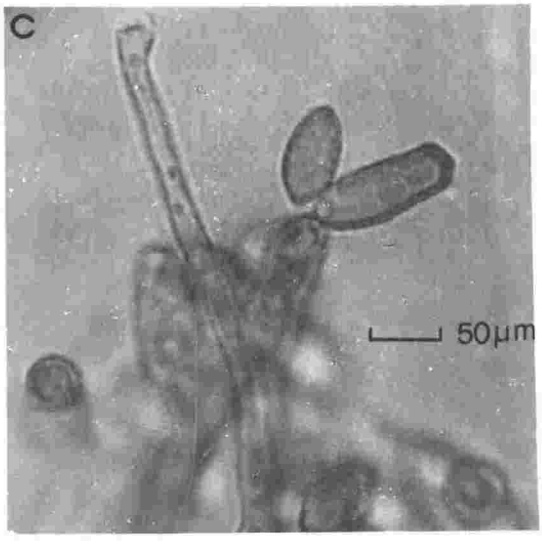
c. *Cladosporium cladosporioides* ex soil.

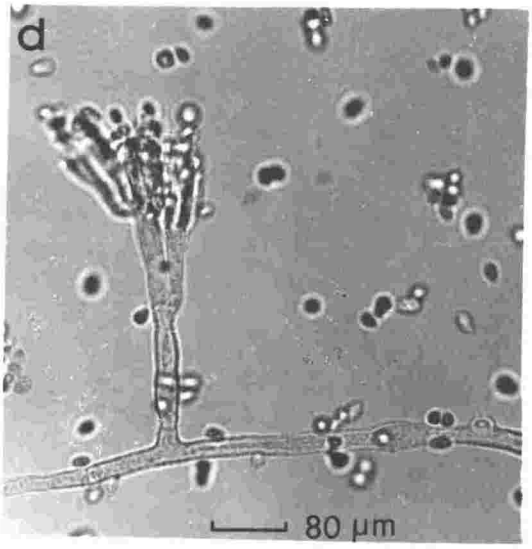
d. *Paecilomyces variotii* ex diesel fuel.

e. *Penicillium corylophilum* ex diesel fuel.


a
100 µm


b
100 µm


c
50 µm


d
80 µm

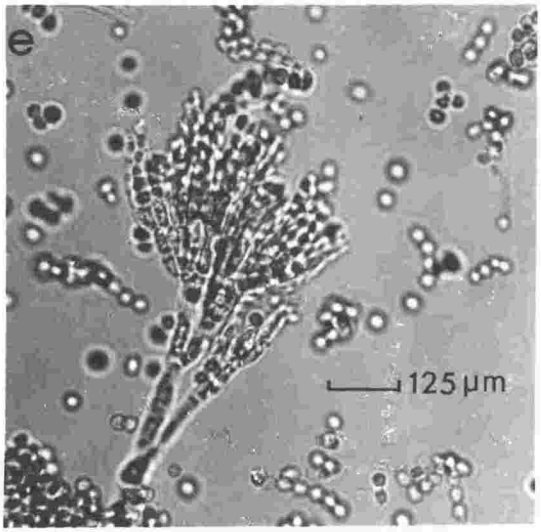

e
125 µm

Figure 4.6. Scanning electron micrographs (SEM) of conidiophores and conidia.

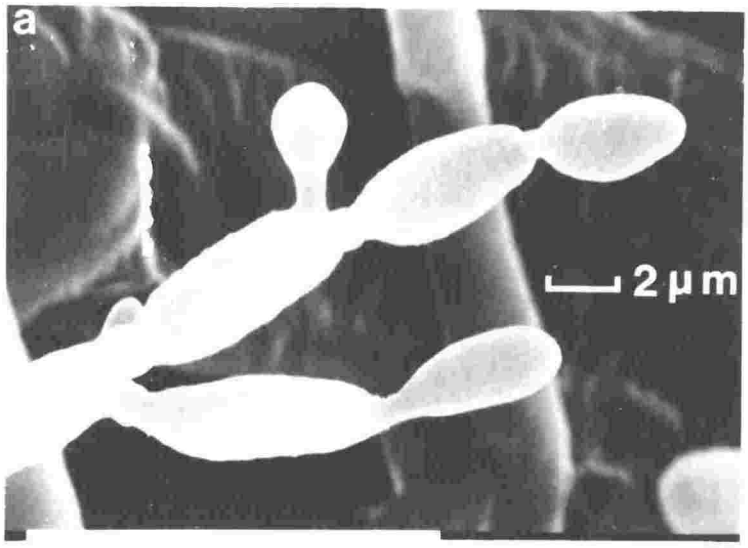
a. *Cladosporium resinae f. avellaneum* ex diesel fuel.

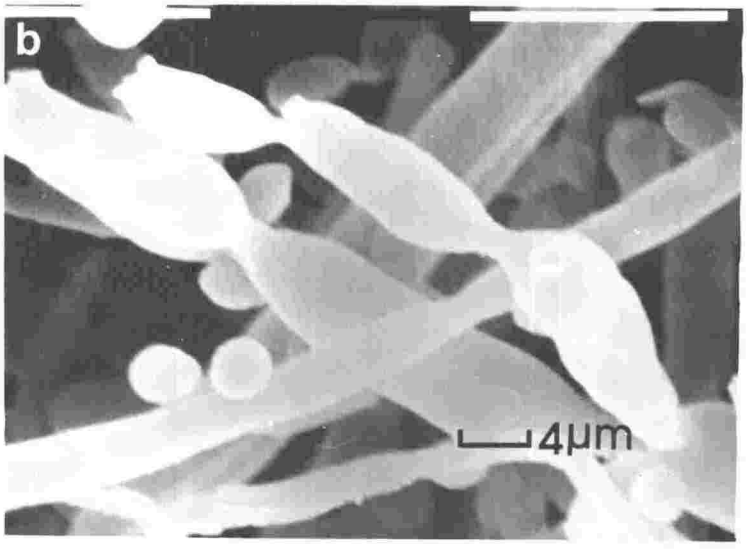
b. *Cladosporium resinae f. avellaneum* ex jet fuel.

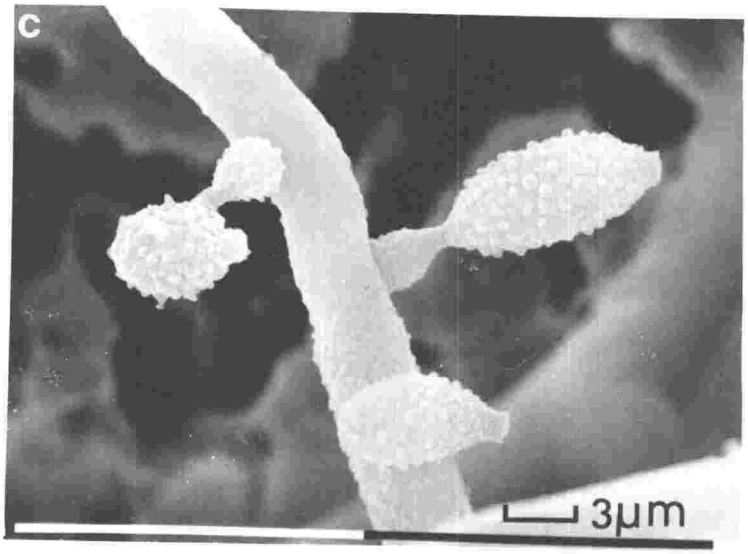
c. *Cladosporium cladosporioides* ex soil.

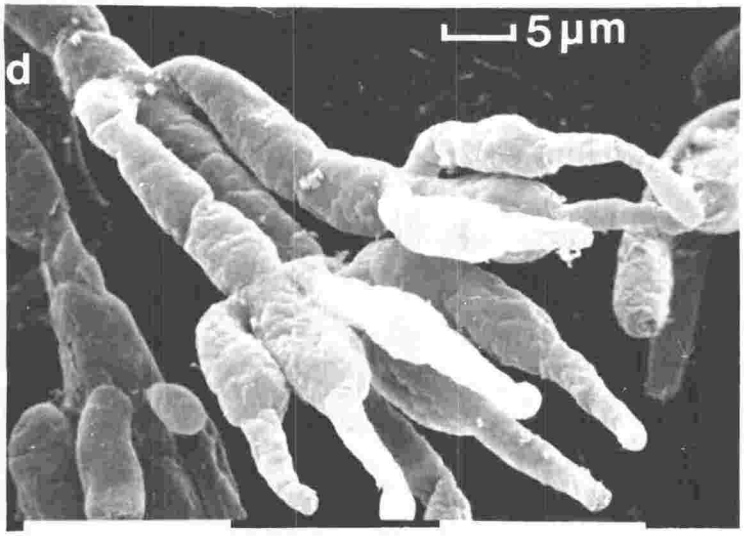
d. *Paecilomyces variotii* ex diesel fuel.

e. *Penicillium corylophilum* ex diesel fuel.


a
2 µm


b
4µm


c
3µm


d
5µm

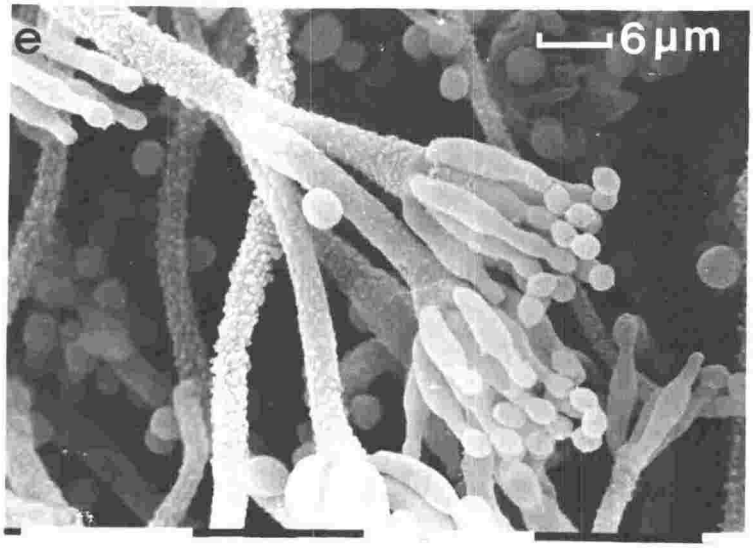

e
6µm

Figure 4.7. Transmission electron micrographs showing the conidia of *Cladosporium resinae* (ex diesel) prepared using different techniques.

1. Mixtures of glutaraldehyde and osmium tetroxide fixative resulted in cell wall disintegration and the intracellular components appeared dark and granular.

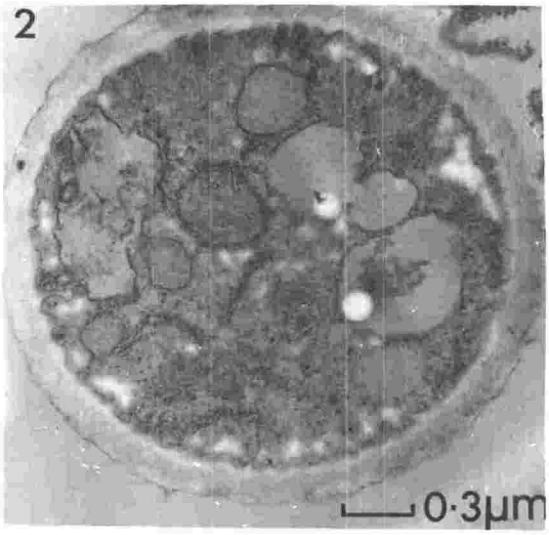
2. Sections fixed in collidine buffer, cell wall was preserved but not the cellular details.

3. Glutaraldehyde and formaldehyde fixative provided more details of the cell wall and intracellular structure.

1

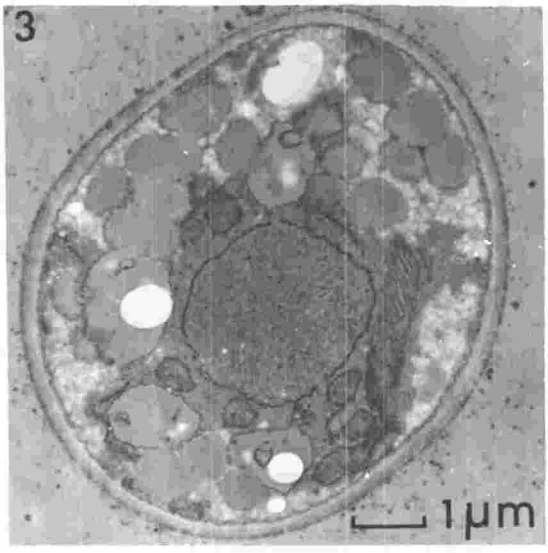
0·2 µm

2



0·3µm

3



1µm

In the sections fixed in collidine buffer, the cell wall was preserved but not the cellular details (figure 4.7-2), but the glutaraldehyde and formaldehyde fixative provided more details of the cell wall and intracellular structure (figure 4.7-3).

Ultrastructure is compared using cultures grown on V-8 juice agar rather than fungal fragments recovered from diesel. A general feature of low power transmission electron micrographs of three *Cladosporium* spp isolates are shown in figure 4.8a, b and c. Conidia have a relatively thick wall within which a substructure can be seen (figure 4.9a, b and c). The cell wall (CW) consists of three distinct layers, an outer electron-transparent layer (A) which can vary in thickness from 600 nm - 750 nm, a central electron dense layer (B) (750 nm - 900 nm) and a thick granular inner layer (C) (120 nm - 300 nm) which in some cases is closely appressed to the plasma membrane from which it is difficult at times to distinguish.

There are clearly variations in the thickness of each layer but there are no consistent differences between the three species. The plasma membrane (PM) shows frequent irregular invaginations and can be seen to possess the trilamellar structure (figure 4.9b and c). Some of the membrane irregularities extended quite considerably into the cytoplasm, and structures which may be called lomasomes (LO) appeared as double or multiple membrane surrounded vesicles between the PM and the cell wall (figure 4.9c). Such structures were not restricted to any one species. The cytoplasm is dense and contains all of the expected organelles and structures. The nucleus (N), globose to subglobose was bounded by a nuclear envelope (NF) of two membranes that is frequently continuous with endoplasmic reticulum (ER) (figures 4.10a and b). Pores (NP) in the nuclear envelope were common. No nuclear division was observed in germinating spores. Other structures observed included numerous mitochondria which varied in sectional view from round to oval. Mitochondria (M) vary little in apparent size, are randomly distributed within the conidia of each spp. and appear to possess plate-like cristae (figures 4.10a and b). Endoplasmic reticulum (ER) was sparse but nearly always observed (figure 4.10a).

Figure 4.8. Low power transmission electron micrographs of:-

a. *Cladosporium resinae* ex diesel fuel.

b. *Cladosporium resinae* ex jet fuel.

c. *Cladosporium cladosporioides* ex soil.

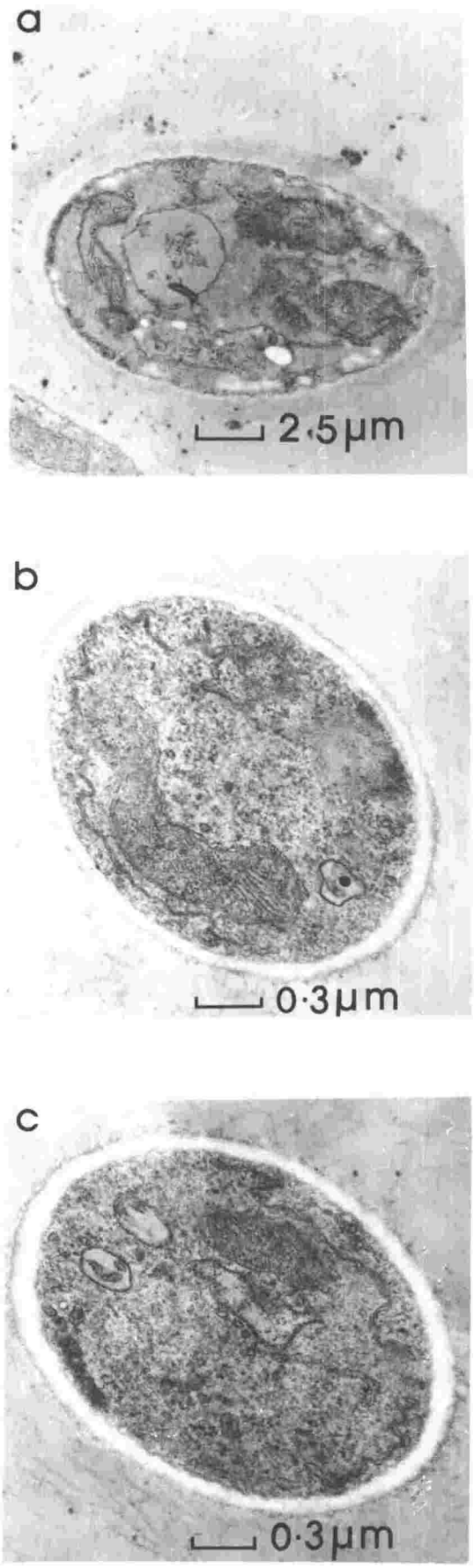
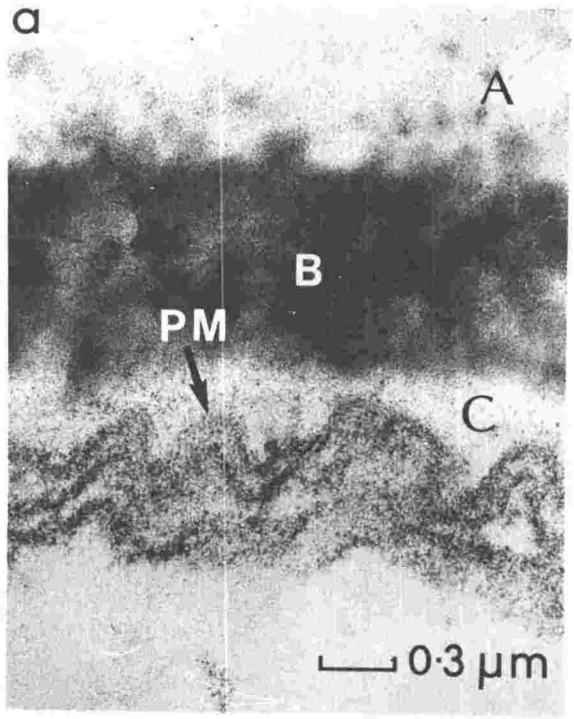

a
2·5µm
b
0·3µm
c
0·3µm

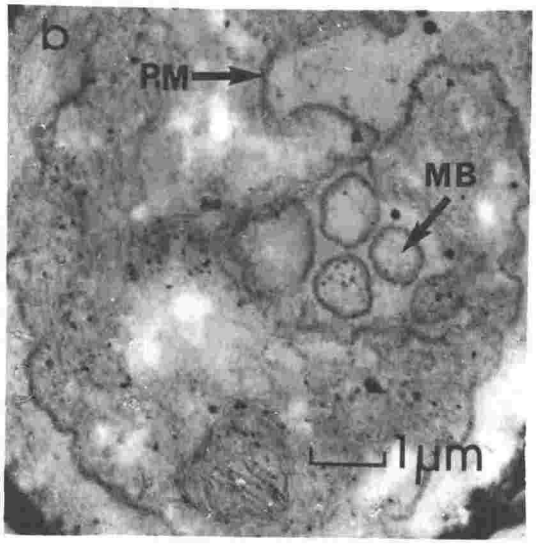
Figure 4.9. a. Transmission electron micrograph showing the cell wall structures of *Cladosporium resinae*.

b & c. Electron micrograpahs of plasma membrane of *Cladosporium resinae*.

Key:

A = outer electron-transparent layer;
B = central electron-dense layer;
C = thick granular inner layer;
PM = plasma membrane;
CW = cell wall;
MB = microbody;
LO = lomasomes.


a
A
B
PM
C
0·3 µm


b
PM
MB
1µm

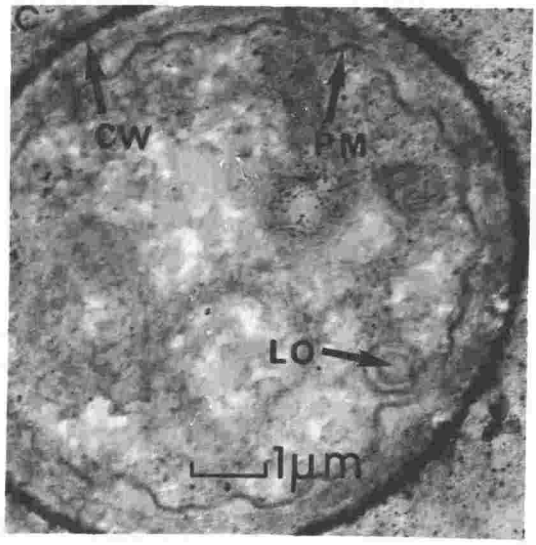
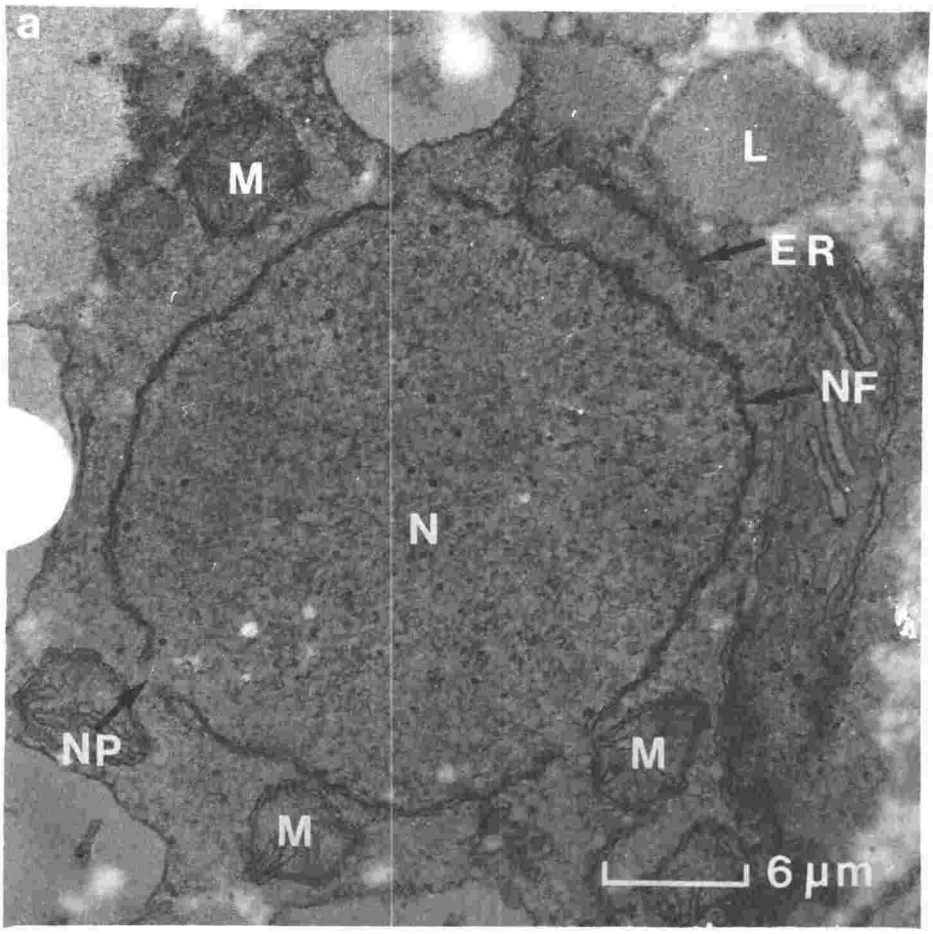
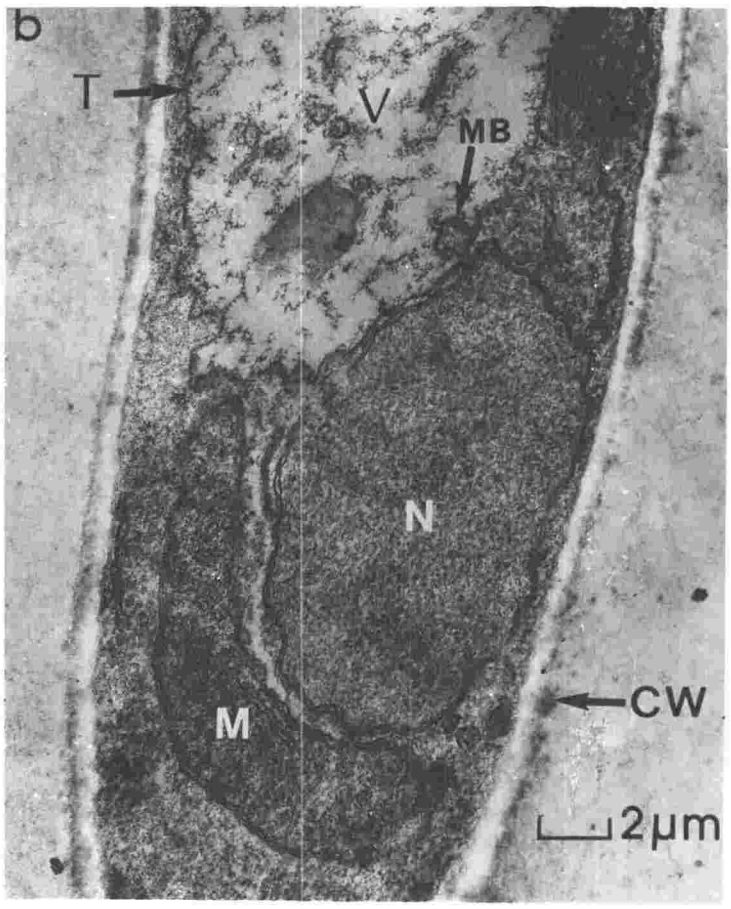

c
CW
PM
LO
1µm

Figure 4.10. a & b. Electron micrograph of the intracellular conidial structure of *Cladosporium resinae*.

Key:

ER = endoplasmic reticulum;
L = lipid material;
M = mitochondria;
N = nucleus;
NF = nuclear envelope;
NP = nuclear pore;
T = tonoplast;
V = vacuole.


a
M
L
ER
NF
N
NP
M
M
6 µm


b
T
V
MB
N
M
CW
2µm

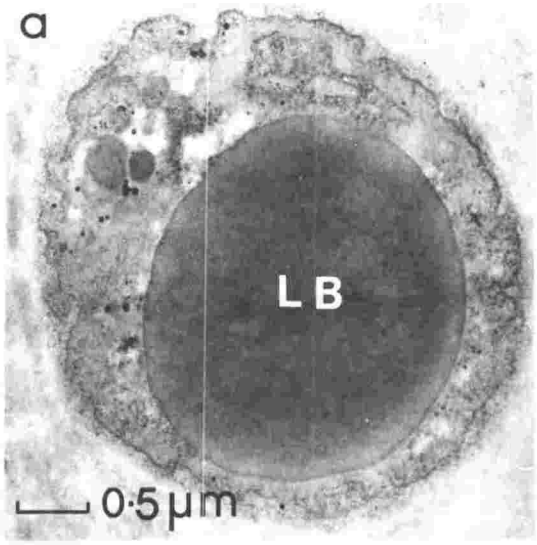
A feature seen in many sections was a highly electron dense cytoplasmic inclusion (LB) which appeared not to be bounded by a membrane and the significance of these bodies is not known (figures 4.11a and b). In a few cases lipid material (L) nearly filled the entire content of the conidia (figure 4.11b). Other similar bodies to lipids may contain proteins and small electron dense particles may be glycogen or other storage granules (arrowed). The conidia of all the *Cladosporium* spp. showed small intensively stained round bodies in vacuoles and adjacent to septum and these inclusions may be microbodies (MB) (figures 4.12a and b). In the vegetative hyphae, adjacent to the septum (S), two to three inclusions, which were surrounded by a membrane, appeared similar to woronin bodies (WB) (figure 4.12b). The vacuoles (V) were well-defined by a tonoplast (T) and were particularly evident in germinating spores (figure 4.10b). Sections of the blastospore, ramoconidia and hyphae of all the three species revealed analogous structure. Ramoconidia were typically clavate or cylindrical and contained a greater number of reserve substances (figure 4.11b).

Figure 4.11. a. Conidia of *Cladosporium resinae* with the large electron-dense body.

b. Electron micrograph of the ramoconidia of *Cladosporium resinae*, showing the storage granules (arrowed).

Key:

LB = electron-dense body.


a
LB
0·5 µm

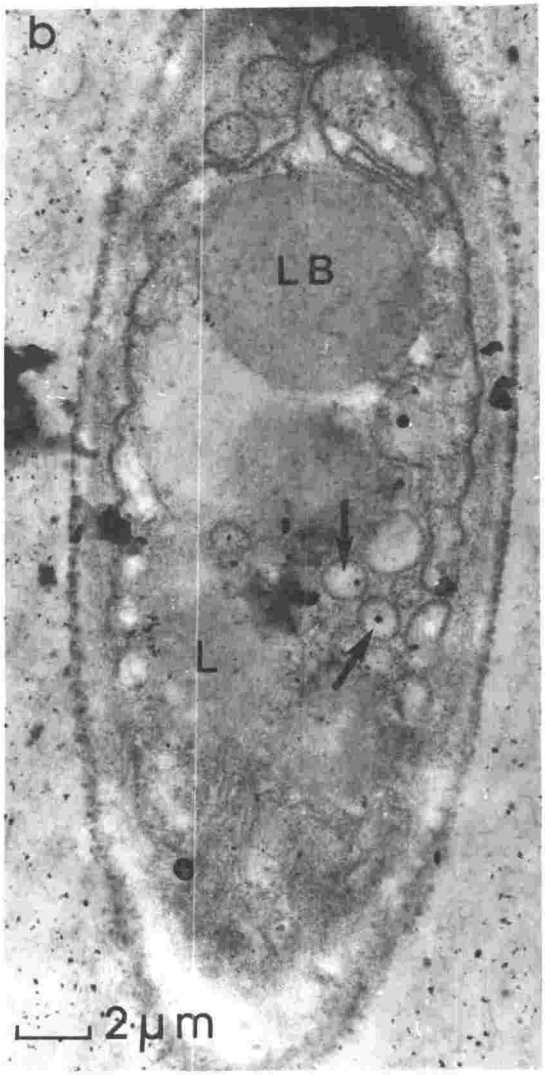
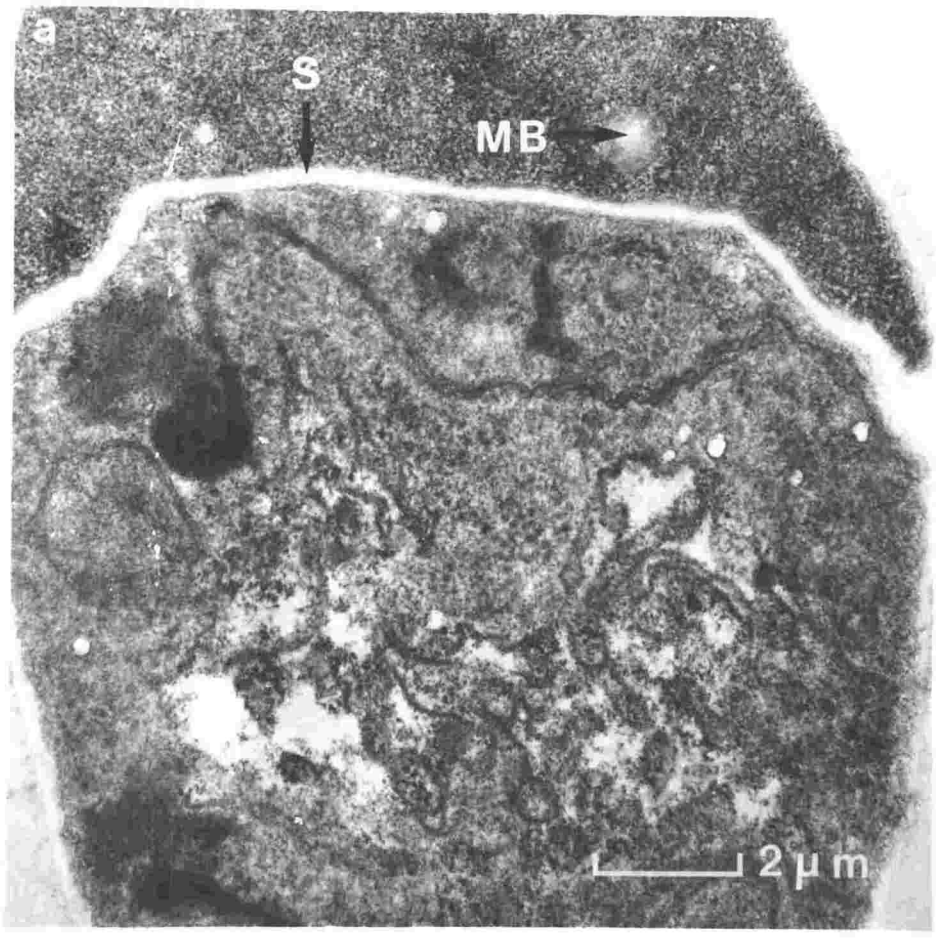

b
LB
L
2·µm

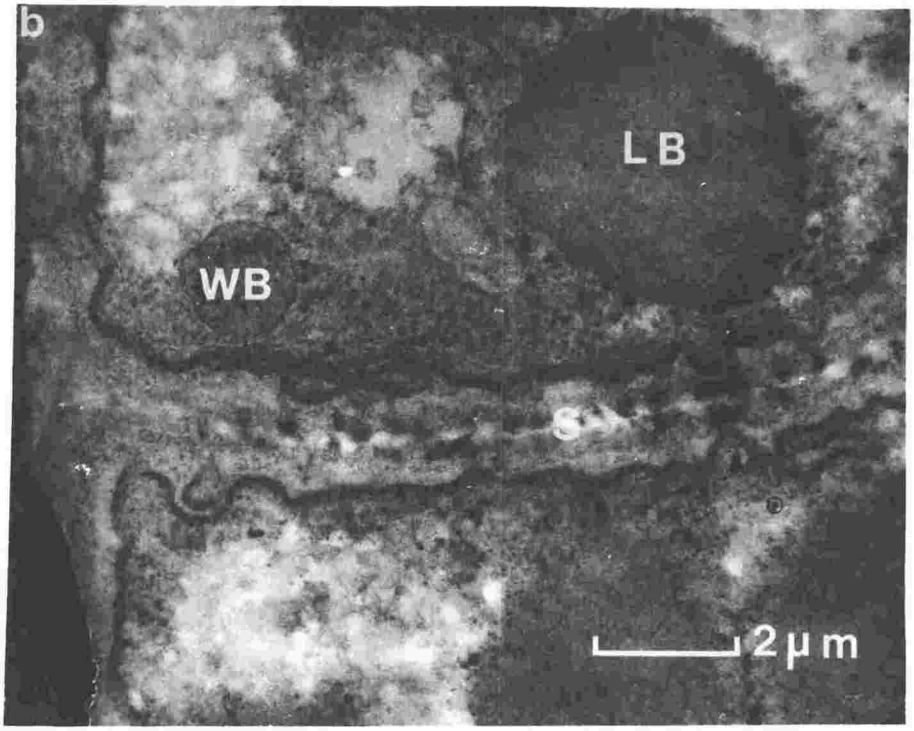
Figure 4.12. a. Microbody adjacent to the septum.

b. Woronin body and electron-dense bodies adjacent to the septum.

Key:

MB = microbody;
S = septum;
WB = woronin body.


a
S
MB
2 µ m


b
LB
WB
S
2 µ m

4.5 Discussion

The observations made of microbial contamination of the storage tanks were somewhat difficult to interpret due to the uncontrolled nature of many variables such as fungal inoculum, water soluble nutrients, the nature of the fuel hydrocarbon among others. In the laboratory studies of microbial growth, some factors were deliberately controlled to relate to field situations. However there may be some unidentified variables that have not been controlled.

The predominant fungi isolated from diesel fuel in this study were *C. resinae*, *P. variotii* and *P. corylophilum* with other *Penicillium* spp. occurring frequently. In all of the laboratory studies the inoculum has been standardized as 10^6 spores per 100 cm^3 of hydrocarbon with or without an equal volume of various aqueous phases in glass containers about 50% full. In all cases these 10^6 spores were added not as an aerial suspension but suspended in 0.1 cm^3 of BH and thus the spores were fully wetted. All fungal growth occurs at the aqueous/fuel interface and this is not surprising since the spores will settle here, as they float in water and water is more dense than hydrocarbon. With the hydrocarbon as the only source of carbon and energy and the aqueous phase as the only source of water-soluble nutrients in the laboratory growth studies, the appearance of extensive mycelium is restricted to this interface. When the aqueous phase contains balanced nutrients as in Bushnell-Haas, growth of all *C. resinae* isolates and of the other fungi is considerable. These growth studies show that apart from *C. resinae*, both *P. variotii* and various *Penicillium* spp. were able to grow in diesel and in the presence of creosote and to utilize these as a source of carbon and energy. Parbery (1970) reported the presence of colonies of *P. variotii* and *Penicillium* spp. from soil isolates on media containing creosote but no further growth studies were performed. During growth studies in this work a lag phase of two days was observed for *C. resinae* and *P. variotii*. Parbery (1971) reports that this may represent a period of adaptation and that increasing the inoculum may reduce the lag phase but this suggestion has not been followed experimentally.

Water enters fuel storage systems in various ways and may be 'pure', contaminated with various soluble materials or be primarily seawater. The results of varying the aqueous phase in these controlled growth studies show clearly how important water-soluble mineral nutrients are and how different fungi require different levels of nutrients. *C. resinae* appeared to grow only when adequate nutrients were available i.e. in BH/diesel, and was inviable in seawater/diesel after 6 weeks but the prolonged immersion did not affect *P. corylophilum*. *P. variotii* survived in tapwater/diesel, where inadequate mineral nutrient was present. Nicot and Zakartchenko (1966) have noted prolonged (30 months) immersion in a seawater/kerosene phases depressed sporulation and changed conidial morphology. Although with distilled water, where negligible or minimal amount of nutrients were present except for those entrained in the diesel fuel, little if any growth was observed but spores of all the test fungi retained viability for at least 6 weeks.

Since fuel tanks were shown to contain more than one fungal contaminant, the interaction between the predominant or other fungi has been examined with respect to growth and spore viability. In the mixed inoculum studies, growth was observed in the Bushnell-Haas/diesel and seawater/diesel. When *C. resinae* and *P. corylophilum* were combined, *P. corylophilum* did not survive in BH/diesel and conversely *C. resinae* did not grow in seawater/diesel at the same time. This study has revealed that *C. resinae* grew in seawater/diesel after *P. corylophilum* had been grown on the same medium. Again, *C. resinae* was observed to have no metabolic effect on the growth medium especially the BH/diesel medium as the other fungi grew on the same medium when *C. resinae* was eliminated. In some of the growth experiments the final pH in the aqueous phase dropped marginally. Even a small drop in pH may play a vital role in the interaction of *C. resinae* with *P. corylophilum*. In other studies, *Candida* was reported to be present in seawater used in ballasting fuel tanks on ships which produce sufficient acidic metabolic products to lower the pH to levels where *C. resinae* could grow (May and Neihof, 1981). Possibly, similar mutual benefits exist between *Penicillium corylophilum* and *C. resinae* in seawater/diesel fuel.

The *Candida albicans* isolate used in this work may not have been necessarily isolated from seawater showed viability in the various aqueous/diesel phases as single inoculum but was reduced or even eliminated by the presence of *C. resinae*, *P. corylophilum* and *P. variotii*. Clearly *Candida* was unable to interact with the predominant fuel fungi.

C. resinae is widely known as the 'creosote fungus' for it grows on wood impregnated with creosote and utilizes it as food (Christensen *et al.*, 1942; Sheridan, Tan and Nelson, 1972; Parbery, 1971). Few have studied the effect of hydrocarbon fuels and creosote on *Penicillium* spp, *P. variotii* and other fungi. It became evident in this study that, like *C. resinae* isolates, *P. corylophilum* and *P. variotii* were able to tolerate creosote up to 1%, including *C. resinae f. resinae* ex jet fuel which had not been tested before. Most other *Penicillium* spp ex diesel were also creosote tolerant and suggest their potential to grow in diesel, provided the favourable conditions exist. Sheridan and Soteros (1974) did not recover any creosote tolerant *Alternaria* spp. from soil and air in N.Z., but in this study, *Alternaria* spp. were isolated from air and diesel and both isolates grew in the presence of creosote in V-8 juice agar up to 0.3%. The tolerance of *Alternaria alternata* and *Cladosporium cladosporioides* to creosote has not been recorded before.

According to Sheridan (per. comm.) delayed sampling of jet fuel affected the viability of *C. resinae* spores. This adverse effect was thought to be due to shaking of the fuel samples during transportation. In these studies *C. resinae* spores were viable after shaking and transportation regardless of the type of hydrocarbon fuel. Delayed testing and long term agitation did not affect the recovery of the spores from diesel and jet fuel.

The SEM has confirmed the picture seen in the light microscope with regard to surface structures of conidiophores and conidia of the fungi, except for *P. corylophilum* where the stipe appeared warted and not smooth (Pitt, 1979).

Sheridan and Troughton (1973) observed warted conidiophores and ramoconidia in *C. resinae F. avellaneum* (ex jet fuel), but in this study no such surface ornamentation was obvious. No new fine structures were observed in the SEM. Fungal spores with ornate microscopic surface features can be fully documented by the use of SEM compared with light microscopy.

In the ultrastructural studies, cultures grown on V-8 juice agar provided the source of spores which were used in the laboratory growth studies and these simple observations were to determine possible fine structural features which might explain the ability of spores to survive or to compete in diesel. The buffer solutions used in the study were important to prevent damage and preserve the fine structures of the specimens. The effect of specific ions of the buffer on the fine structures of *Cladosporium* spp. are not known. Presumably ions in the buffer interact with certain chemical groupings within the specimen which appeared to affect the quality of fixation. Studies with different buffer systems have demonstrated that variations in the specific constitution of the buffer produce significant variations in the staining and appearance of cells and organelles (Wood and Luft, 1965). The efficiency of the buffer system in the present study varied at the different pH levels. It is now recognized that the osmolarity of a fixative has a direct effect on the appearance of the fixed specimen (Hayat, 1970). The various structures would also differ in the degree of their response to the difference in ion balance between the fixing solution and the organisms' normal environment.

The fixation techniques employed for *Cladosporium* spp. gave variable results. However, a double fixation by glutaraldehyde and formaldehyde appeared to be more effective in maintaining the constitutents. Mixtures of glutaraldehyde and Osmium tetroxide tend to distort the cell wall and the intracellular structures were dark and granular. But this fixation was ideal for SEM. At present, the knowledge of chemical reaction between the fixing agents and specific cellular constitutents is rather meagre in fungi and interpretation of electron micrographs is dependent upon a better understanding of the chemistry of fixation.

Studies on the fine structures of *Cladosporium* spp. showed a thick cell wall composed of several successive layers. Underlying the cell wall, the dark convoluted 'zigzag' membrane (Tan, 1972; Soteros, 1973) was resolved to be a trilamellar plasma membrane. The 'zigzag' membrane may be common to most fungi (Bracker, 1967). The cytoplasmic granular matrix contains organelles characteristic of other fungi.

The nuclear condition of all spores and hyphae of *Cladosporium* spp. was consistent containing a single nucleus. The pores on the nuclear envelope appear to be potential avenues for exchange of substances between nuclei and cytoplasm (Fawcett, 1966).

The membranous systems of the lomasomes are characteristic structures of fungal cells (Moore, 1965). They arise from the plasma membrane and are not thought to be associated with cell wall formation (Brushaber and Jenkins, 1971). The membranes of lomasomes and the cytoplasmic invagination displayed a trilamellar structure characteristic of the plasma membrane. Since it appears that lomasomes are structures involved in membrane formation, it is considered that the membranous systems of *Cladosporium* spp. and the invaginations of the plasma membrane may be unique to fungi and not only to hydrocarbon utilizing fungi as suggested by Sheridan, Tan and Nelson (1972).

Cooney, Siporin and Smucker (1980), Smucker and Cooney (1981) compared cells grown on hydrocarbon to those grown in glucose. They observed small spherical, electron-dense bodies in the vacuoles of hydrocarbon grown cells and not in glucose medium, and suggested they were microbodies, the site of initial oxidation of hydrocarbon. They also pointed out the occurrence of large electron-dense bodies in vacuoles of glucose and kerosene grown cells. In this study, such structures were evident in all the *Cladosporium* spp., despite spores not being processed directly from hydrocarbon medium but from culture medium. The above workers have not made any intracellular comparison between the hydrocarbon and non-hydrocarbon utilizing fungi.

According to Brenner and Carroll (1968), woronin bodies may function to block the septal pores and regulate or prevent passage of cellular organelles or materials and may also function to occlude the pores. McKeen (1971) ascertained the chemical composition and fine structure of woronin membrane bodies and showed that they occurred in definite location adjacent to septal pores. Cole and Samson (1979) indicated Woronin bodies develop within microbodies as homogenous, electron-opaque inclusions, then move into invaginations of the latter and are pinched off by constriction and fusion of the encompassing membrane. Wergin (1973) and Heath (1977) have pointed out that microbodies are found in animals and plants (Vigil, 1973; Frederick, Gruber and Newcomb, 1975), and fungi (Avers, 1971) have been credited with enzymatic activity, and woronin bodies may possess similar activity. Thus this study did not recognize any intracellular structural difference among the *Cladosporium* spp.

PART 2

CONTROL OF MICROBIOLOGICAL CONTAMINATION IN DIESEL FUEL

This part is concerned with the use of biocides for controlling microbiological contamination in diesel ('dieso') fuel.

5. MATERIALS and METHODS

5.1 Laboratory studies on fuel biocides

The effects of biocides on spore germination, fungal growth rate and survival were determined using the following techniques.

5.1.1 Agar/diesel slide technique

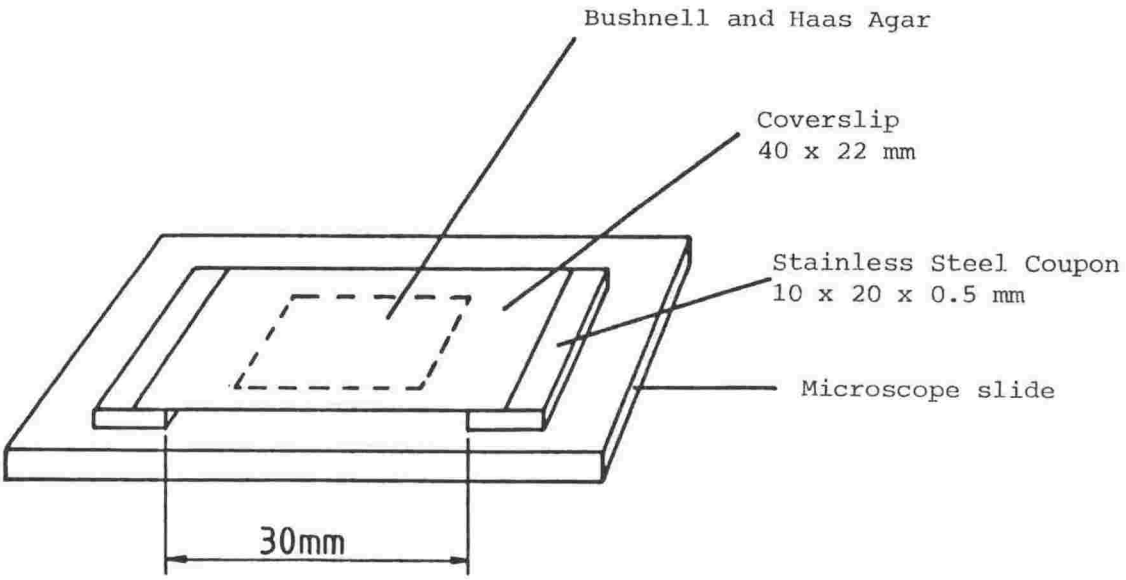
This is a modification of the method described by Smith and Crook (1980) using suspensions of conidia of *Cladosporium resinae*, *Penicillium corylophilum* and *Paecilomyces variotii* on shallow nutrient agar platforms which were then overlaid with diesel (figure 5.1). The agar platforms were placed on the slide in the following manner. Two sterile coverslips 18 x 18 mm and each 100 µm thick were fixed one above the other in the centre of a normal microscope slide so that it had a raised area of 200 µm high. A small volume (0.75 cm^3) of molten Bushnell-Haas medium solidified with 2% agar at 50°C was then placed on another culture slide between two stainless steel coupons. Stainless steel 500 µm thick was cut into 20 x 10 mm coupons and the two coupons were fixed 30 mm apart across the slide using petroleum jelly. The slide bearing the two coverslips was laid firmly across the two sterile steel coupons with the two coverslips on the underside. When the agar had set the top slide was carefully removed leaving a layer of agar 500 µm deep containing a depression, 18 mm square and 300 µm deep, between the steel coupons. The agar surrounding the square depression was then cut away aspetically leaving a platform of 18 mm square and 300 µm deep; the surface was smooth and permitted fungal conidia and hyphae to be observed with ease.

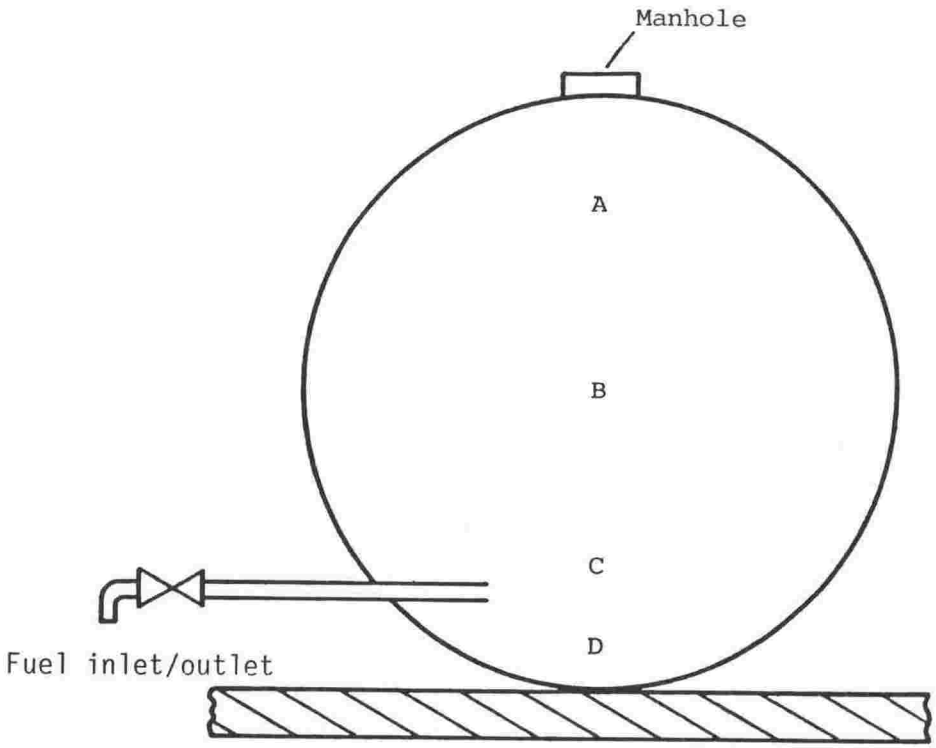
Figure 5.1. Apparatus for agar slide culture technique (after Smith and Crook, 1980).

Figure 5.2. Sectional view of the small steel tank showing the sampling points.

Key:

A = top;
B = middle;
C = bottom;
D = very bottom.


Bushnell and Haas Agar
Coverslip
40 x 22 mm
Stainless Steel Coupon
10 x 20 x 0.5 mm
Microscope slide
30mm


Manhole
A
B
C
D
Fuel inlet/outlet

This agar platform was then inoculated with 0.01 cm^3 drop (containing 10^3 spores/cm^3) of the appropriate conidial suspension (see chapter 2 for details) which was spread on the agar surface. The slide was then left for 10 minutes to allow the agar to absorb the free water. A sterile 40 x 22 mm No. 1 coverslip was then placed across the steel coupons and about 0.33 cm^3 of filter-sterilized diesel fuel containing the various biocides was allowed to run under the coverslip using a sterile Pasteur pipette. The agar cultures (duplicate slides of each fungus per biocide concentration) were placed in covered sterile Petri-dishes and incubated at 25°C. Germination was determined microscopically daily up to 7 days, being scored on a total of 1000 spores for each treatment and recorded as positive when a developing germ tube had emerged for a distance equal to the width of the spores.

The biocides benomyl, Biobor JF, DML-7, imazalil, Kathon 886 and Proxel AS were used at a final concentration in diesel of 0, 5, 10, 50 and 100 ppm and DEGME at concentrations of 0, 500, 1000, 2000, 3000, 4000, 5000 ppm. Imazalil and benomyl were solids and all the other products were liquids. A 500 ppm (W/V) stock solution of each in filter-sterilized diesel fuel was prepared and diluted as required. Imazalil was heated to 50°C in a water bath before adding the diesel when making up the stock solution. Benomyl required a different procedure due to its low solubility in both water and diesel. It is soluble in both dimethyl formamide (DMF) and dimethyl sulphoxide (DMSO) (Smith, per. comm.) but the latter was not used because it was thought that the sulphur content might stimulate sulphur bacteria in the tanks. The stock solution of benomyl was prepared by dissolving 25 mg in 0.5 cm^3 of DMF, mixing with 5 cm^3 of diesel and making up to a volume of 50 cm^3 with diesel to give the 500 ppm solution required.

5.1.2 Poison plate test (Growth rate)

The effect of biocides, incorporated into nutrient agar on the growth of colonies of *C. resinae*, *P. variotii*, *Penicillium corylophilum*, *P. chrysogenum*, *P. digitatum*, *P. frequentans* and *P. spinulosum* was determined over a 7 day period.

The additives were incorporated immediately into the sterile molten malt extract agar (2%) at 50°C at concentrations of 0, 10, 50, 100, 200 and 300 ppm except for DEGME and EGME. These were used at the higher concentrations of 1000, 2000, 3000, 4000, 5000 ppm (0.1 - 0.5%). The mixtures were gently shaken to distribute the biocides and plates poured. 5 mm plugs from the periphery of 2 week old actively growing cultures were placed upside down centrally in each Petri-dish. Colony diameter from five replicate dishes was measured daily for each treatment.

The effect of biocides on the following seven isolates of *C. resinae* was studied: *C. resinae f. avellaneum* ex soil (C1); R5 ex feather; ex Air at Devonport; ex untreated diesel (isolated in this study); ex jet fuel (K10F); ex Diesel (D1); ex Australian army culture. Isolates of *C. resinae* and *P. variotii* from the two diesel tanks obtained during and after treatment for up to 5 months with Biobor JF and DEGME were also studied.

The effect of mixtures of benomyl (B) and imazalil (I) on the growth of *C. resinae* and *P. variotii* was tested as follows:

- total concentration 5 ppm with B decreasing from 5 to 0 while I increased from 0 to 5 in steps of 1 ppm.

- total concentration 10 ppm with B decreasing from 10 to 0, while I increased from 0 to 10 in steps of 1 ppm.

- total concentration 50 ppm with I at 5, 10, 20, 25, 30, 40, 45 and 50 and B decreasing in proportion.

- total concentration 100 ppm with B decreasing from 100 to 0 ppm while I increased from 0 to 100 ppm in steps of 10 ppm.

- total concentration 200 ppm with B decreasing from 200 to 0 while I increased from 0 to 200 in steps of 10 ppm.

Growth was assessed by measuring the colony diameter after 8 days.

5.1.3 The effects of biocides on fungal growth and survival in BH/diesel phases

The activities of the biocides listed in 5.1.1 were tested as follows:

(i) To determine the growth and survival of mixed spore inoculum. Mixed spore inoculum of *C. resinae*, *P. variotii*, *Penicillium corylophilum*, *P. chrysogenum*, *P. spinulosum* and *P. digitatum* was inoculated in (100 cm^3) media consisting of autoclaved Bushnell-Haas mineral medium and filter-sterilized diesel fuel in the following proportions 5:0, 5:1, 5:2, 5:4, 5:5, 4:5, 3:5, 2:5, 1:5 and 0:5. The tests were set up in 200 cm^3 medical flat bottles (sterilized in an oven at 200°C for 3 hours). Benomyl (dissolved in DMF), DML-7, imazalil and Proxel AS were dissolved in diesel and added to give final concentrations of 0, 50, 200, 300 ppm. Biobor JF and DEGME concentrations were 0, 125, 170 and 270 ppm (commercially recommended) and 0, 1000, 2000 and 3000 ppm respectively. After shaking, 0.1 cm^3 of fungal spore suspension (containing approximately 10^5 spores/cm^3) in BH was inoculated into each medical flat. The bottles were shaken again and the aluminium caps with the rubber liner removed, were screwed on loosely and the bottles incubated at 25°C for 6 weeks.

Where growth had occurred, the mycelial mat produced by the fungi was harvested by filtering using sterile No. 1 Whatman filter papers (50 mm diameter). Where there was no mycelial mat formation, cultures were shaken, 5 cm^3 of the BH/diesel sample was placed in duplicate sterile Petri-dishes and V-8 juice agar poured over the sample and shaken gently. The plates were incubated at 25°C and scored for the number of fungal colonies produced.

(ii) To observe the effect of EGME and DEGME on C. resinae at reduced temperatures

The sterile BH/diesel fuel was used at 1:1 ratio in sterile 200 cm^3 medical flat bottles as described in (i). The BH/fuel was inoculated with 0.1 cm^3 *C. resinae* spore suspension in BH (approximately 17 x 10^5 spores/cm^3). EGME and DEGME were added to give concentrations of 0, 300, 500, 1000 10,000 ppm (0.03 - 1%). The bottles were incubated at 4°C for 4 weeks and then transferred to laboratory temperature (18-19°C) and kept for a further 4 weeks to observe whether vigorous growth had occurred by the end of the 8 weeks. The mycelial mat produced by the fungus was harvested and assessed as above.

(iii) To determine whether batches of DEGME varied in biocidal activity

An Australian diesel fuel isolate, a N.Z. diesel fuel isolate and a N.Z. jet fuel isolate of *C. resinae* were found to be insensitive to the DEGME sample used in the laboratory. The new batch of DEGME purchased for the field trial was tested using this culture method against these *C. resinae* isolates to investigate whether the response of the test organism varied with different samples of DEGME.

5.1.4 Large volume liquid fuel : biocide tests

Fuel (2 litres) obtained from the main storage tank 2 was placed in sterile 'Winchester quart' bottles (capacity 2.25 litres). Before the addition of the biocides, three 50 ml samples of the fuel were filtered to discover the level of microbial contamination. The biocides dissolved in sterile diesel were added to the fuel to give a final concentration at 0, 50, 100, 200, 300 ppm for DML-7, Proxel AS, imazalil, benomyl, Biobor JF, Kathon 886 but for EGME and DEGME at 0, 500, 1000, 2000, 3000 ppm (0.05-0.3%). 24 hours after the incorporation of the biocide, three 50 cm^3 samples of fuel were filtered. This was repeated daily for seven days using 0.45 µm membrane filters. The membrane filters were placed in sterile Petri-dishes and V-8 juice agar poured over the filters. The plates were incubated at 25°C for 5 days before colonies were counted.

5.1.5 Intermittent exposure of the three common fuel fungi to biocides in diesel fuel

The method was described by Elphick and Hunter (1968) for jet fuel and modifications were made whenever necessary. 30 mm circles were cut from no. 1 Whatman filter paper. The filter paper circles were autoclaved for 15 minutes at 103 kPa (15 p.s.i.). To these sterile circles were applied 0.3 cm^3 of a mixed spore suspension (approximately 10^7 spores/cm^3) of *C. resinae*, *P. variotii* and *P. corylophilum* in sterile water, a volume sufficient just to saturate the circles. The circles were placed in sterile 140 mm Petri-dishes, arranged concentrically without overlapping, and a 5 mm deep layer of filter-sterilized fuel carefully poured over them. The dishes were incubated at 25°C. After 7 days, a further 0.3 cm^3 of sterile water was added to each filter paper. After a further 14 days incubation, vigorous mixed colonies of the fungi were growing at the centre of each circle.

The mixed colonies were then immersed in fuel containing the biocides for 24 hours, held in 200 cm^3 beakers and covered with sterile aluminium foil. The products used were Biobor JF, benomyl, DML-7, imazalil, Kathon 886, Proxel AS at concentrations of 0, 50, 100, 200, 300 ppm. Filter paper circles were removed after 24 hours and, without washing, placed in untreated sterile fuel for 6 days. They were then removed and immersed once more in biocide-treated fuel for a second 24 hour period, the process being repeated to give a maximum of six immersion periods in biocide-treated fuel, a total of 144 hours over a 6 week period. Three filter paper circles carrying colonies were removed after each 6 day period in untreated fuel, washed with sterile water and placed in sterile Petri-dishes. Malt extract agar was poured over the filters. They were assessed for relative growth after 5 days incubation at 25°C.

5.2 Field evaluation of the fuel biocides

5.2.1 Biocide effects on microbial contamination

(i) Tank description

Uncoated steel tanks at Devonport Naval Base, Auckland, were used for the large scale biocide testing. Each of the cylindrical tanks was approximately 4.57 m long and 2.74 m wide, with a capacity of 25,500 litres of diesel fuel. They faced south and were thus completely sheltered by the cliffs behind from direct sun. Usually three tanks were used, two treated and the third as an untreated control. The tanks were drained, biocides added and then refilled with diesel which completely mixed the biocides with the fuel.

(ii) Biocides

DEGME and Biobor JF were added to give final tank concentrations of 3000 ppm (0.3%) and 270 ppm (0.027%) respectively, DML-7 and Proxel AS at 300 ppm; benomyl at 50 ppm and imazalil at 200 ppm. Benomyl was dissolved in DMF, all others were added at the required final concentration directly to the tanks. Benomyl was used at a low concentration because it was found to be controlling the predominant fuel contaminants between 10 ppm and 50 ppm in the laboratory.

(iii) Sampling

Samples were collected and transferred into sterile preserving jars as described in Part 1. Samples were collected before (the untreated fuel) and about 2 1/2 hours after addition of biocides (allowing time for the product to settle) and tested immediately in Auckland and after 24 hours in Wellington for the viable fungal contaminants in the DEGME and Biobor JF treated samples. The treated samples were tested immediately and after 24 hours, to observe whether there was any change in the fungal contamination level. Later it was found, that delayed sampling did not have any effect on the microbiological contaminants.

In the case of DML-7, Proxel AS, benomyl and imazalil, the untreated fuel samples were collected and tested immediately in Auckland. The treated tanks were allowed to settle for 24 hours before sampling, and samples were tested in Wellington. The source of fuel was from the main storage tank 2 and it was sampled and tested before pumping into the tanks. The subsequent samples from the treated and untreated tanks were collected fortnightly from the top (A), middle (B), bottom (C) and infrequently from the very bottom (D) as shown in figure 5.2. The samples were tested in Wellington for a period of from 16 to 20 weeks after the addition of biocides. The tanks have the sullage bottom (D) (undrainable bottom) which were only drained manually and extended 305 mm high from the very bottom to the point at which fuel is normally withdrawn (C).

Initially only a single sample (1000 cm^3) was collected from the above levels. Later, it was noticed that the number of colony forming units fluctuated greatly between levels in the tanks at the same sampling time, so triplicate samples (1000 cm^3) were collected separately at each level.

5.2.2 Microbial detection

The fuel was thoroughly hand shaken before subjecting to the membrane filtration technique (refer Part 1). The size of the 10 replicate fuel samples filtered was 100 cm^3.

Since the presence of biocide on the filters or in fungal fragments may affect the detection of viable fungi, the following tests were performed on filters before covering with the V-8 juice agar, each being replicated 15 times.

(a) Filters plated without washing.

(b) Filters were washed with sterile distilled water before plating.

(c) The filters were washed with a detergent triton X-100 (20 cm^3 sterile distilled water mixed with 0.1 cm^3 triton X-100), mainly to remove any residue of DEGME and Biobor JF.

(d) V-8 juice agar containing 0.1% creosote was poured over the unwashed filters.

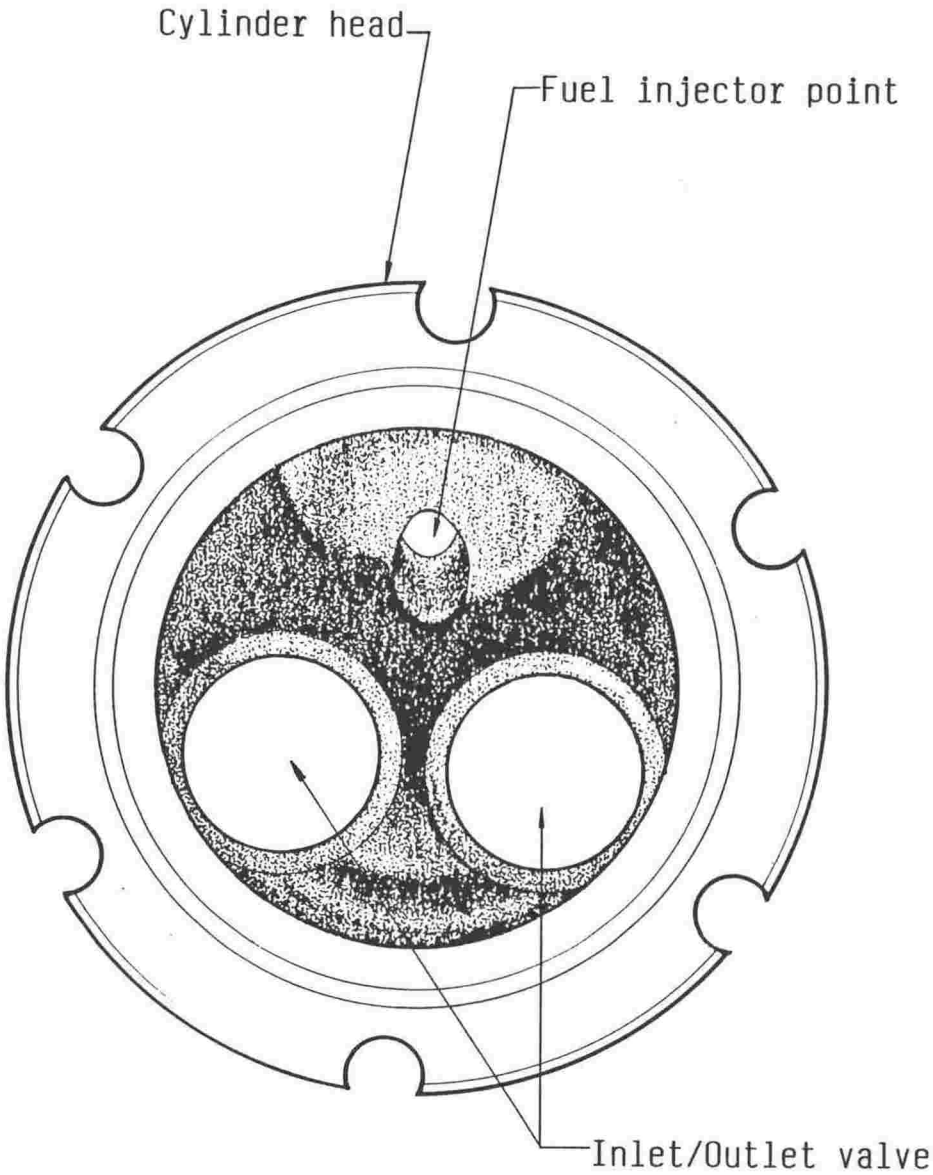
The plates were incubated at 25°C and observed after 5 days for fungal colonies. There were only minor differences between treatments, with treatment 'C' appearing most satisfactory. This detergent wash was used for all subsequent samples. Whenever free water was observed in the very bottom samples, the pH was read using the pH meter.

5.2.3 Effects of biocides

5.2.3.1 Engine performance and combustion chamber components

The object of engine running tests with dosed fuel was to ascertain whether the chemical products under test had any effect on the performance of diesel engines and engine components. The engine trials were run at the Defence Scientific Establishment (D.S.E.) at Devonport, using one single cylinder Enfield VSI engine. The motor drives an electrical alternator which is off-loaded onto a bank of resistance heaters. The engine was rated to run at a constant speed of 1400 rpm and it developed 4474 Watts (6 hp). The alternator generated a maximum of 2780 Volt Amps (VA) which could be translated into 2780 Watts if used to power a bank of electrical resistance heaters, because of the unity power-factor under this type of loading. To the test diesel generator was fitted 2 AC meters, one to read 0-250 Volts and the other to read 0-20 Amps. The test engine was placed in a weather-proof bay and an additional metering tank (snap tank) was connected in parallel with the main fuel tank. This permitted the recording of fuel consumption rate while the engine was running at a fixed power output. Any changes to the thermal efficiency of fuel caused by the additive was measured by this method. After each major fuel/biocide trial, the cylinder head (see figure 5.3) was removed so that deposits could be visually examined and any relevant measurements made.

Figure 5.3. The cylinder head of the Enfield VS1 engine.


Cylinder head
Fuel injector point
Inlet/Outlet valve

Each biocide/fuel mixture was tested over a two or three day continuous running period and fuel consumption rate and oil pressure measured hourly. All biocides used in the field trial including Kathon 886 were tested in the engine and in the following corrosion test.

5.2.3.2 Corrosion test

A standard method for detection of copper corrosion from petroleum products by the copper strip tarnish test, as described in the IP 154/84, ASTM D130-83 was followed.

5.2.4 Water and biocide content of fuel samples

5.2.4.1 Determination of water content

The total water (free and dissolved water) in most of the fuel samples from the three tanks was determined at D.S.E. by the Karl Fisher method (ASTM D17 44-64).

5.2.4.2 Determination of Partition Coefficient

Biobor JF - Boron in Biobor products in diesel was determined by the potentiometric method (Park, 1975) at B.P. N.Z. Ltd.

DEGME - the method used was a standard technique for Icing Inhibitor using a refractometer, DEF (Aust.) 5240.

Proxel AS was analysed by high performance liquid chromatography (HPLC) at Chemistry Division, DSIR as follows:

Ten millilitres of diesel sample and 10 cm^3 of distilled water were shaken together vigorously for one minute in a 25 cm^3 vial and the two phases were allowed to separate completely. The aqueous phase was then analysed by HPLC on a Hichrom C18 reverse phase column (50 mm x 8 mm) using methanol-water-orthophosphoric acid 50:50:0.25 V/V/V at 3 cm^3/min as mobile phase. 500 μl injections were made and Proxel AS was detected by UV absorbance at 240 nm. Concentrations were calculated using peak heights calibrated using standards. Analyses were reproducible to within $\pm$2%, and were carried out in duplicate.

Experiments using authentic mixtures showed that in a 50:50 mixture of diesel and water, (80±1)% of the Proxel was in the aqueous phase and only 20% in the diesel.

DML-7 - HPLC and capillary gas chromatography (GLC) were used for quantitation of active components from DML-7 in aqueous and hydrocarbon phases at Industrial Processing Division (IPD), DSIR. The major components identified were:

- Aromatic Hydrocarbon Solvent (Boiling Range 160°C→220°C)
- Unknown Compound (water soluble, Boiling Point ~ 120°C)
- Diethylene glycol-mono methyl ether (Boiling Point 194°C)
- Methylene bis thiocyanate (melting Point 103°C).

The first three components could be quantified in both hydrocarbon and aqueous phases using GLC, but the methylene bis thiocyanate required aqueous extraction followed by HPLC.

Once the composition of the DML-7 was determined, it was decided that methylene bis thiocyanate was the only component worth quantifying. It was analysed as follows in the 200 cm^3 diesel fuel from a 25,500 litre storage tank initially dosed with 300 ppm of DML-7.

The DML-7 sample was analysed by GLC using a non-polar capillary column with flame ionisation detection. Water was added to the DML-7 and both the hydrocarbon phase and the aqueous phase analysed. A sample of the biocide was washed with water and pentane to extract the partially soluble solid component. The melting point of the crystals was determined. A sample of the diesel fuel was analysed by capillary GLC. DML-7 standards were prepared by adding 300 ppm of biocide to diesel, consequently methylene bis thiocyanate results are reported as ppm biocide because the methylene bis thiocyanate content is not accurately known. Some of the extracted crystals were used to estimate the amount of methylene bis thiocyanate in the DML-7 biocide. The result indicated the biocide contained ~ 8% mass/mass of methylene bis thiocyanate, however this value is not precise due to interference from other biocide components.

40 grams of the submitted fuel samples were extracted with 3 x 4 ml of distilled water and the combined extracts analysed by reverse phase HPLC using ultraviolet absorption detection at 254 nm. Two fuel samples contained small quantities of separated aqueous phase and these volumes were measured using a microlitre syringe. The significant concentrations of solid corrosion products in the aqueous phase prevented HPLC analysis.

The extraction of biocide from diesel into water was measured by adding 1 part water to 250 parts of diesel containing 300 ppm DML-7. The mixture was stirred for 4 hours. The extraction was repeated on the same sample.

Imazalil content was not measured specifically in fuel samples but since imazalil is a non-polar substance (evident from the chemical formula) one can predict it to be more soluble in fuel than in water (R.D. Wilson, Chemistry Division, DSIR, per. comm.). A manufacturer representative (Janssen Pharmaceutica, per. comm.) advised that imazalil was more soluble in octanol than in water phases from their determination of partition coefficient. (See appendix 5.1 for octanol-water partition coefficient).

Benomyl - Smith and Crook (1983) using 14C labelled benomyl found a partition ratio of 10:90 water to diesel fuel after equilibration.

6. EXPERIMENTAL RESULTS

In this study eight chemical compounds were tested as biocides in the laboratory and six in the field. Their overall performance is summarised in table 6.1.

6.1 SECTION I - Laboratory studies of biocide effects

6.1.1 Agar/diesel slide technique

Table 6.2 shows that germination of conidia of the three major fungal contaminants was evident within 24 hours when placed onto the interface between BH mineral salt agar and diesel. The germination was complete within 48 hours.

Neither DEGME nor Biobor JF affected germination significantly although the latter gave a slight delay in germination of *C. resinae* at the higher concentrations.

DML-7 at all concentrations and Proxel AS at the higher concentrations delayed germination of *C. resinae* and *P. corylophilum* but not *Paecilomyces variotii*. By 96 hours germination was 100% for all three fungi.

At or above 100 ppm, benomyl inhibited germination completely in all three fungi. *C. resinae* was most sensitive to benomyl being completely inhibited at 10 ppm with delayed germination at 5 ppm.

Imazalil at or above 50 ppm inhibited germination in *C. resinae* and *P. corylophilum*. *Paecilomyces variotii* was very insensitive with complete germination after 96 hours at all concentrations.

The agar slide technique permitted observation of germination and growth of spores as they produce individual colonies. Hyphal growth in untreated diesel resulted in an interwoven mycelial mat in which no individual colonies could be distinguished within 48 hours. The type of hyphal growth and the formation of conidiophores and conidia could be readily observed using this technique.

Table 6.1 Summary of biocide screening results.

No.	Compound	Producer	Solubility Fuel or Water	Minimum inhibitory Concentration (mic) *C. resinae*	*Penicillium* spp.	*P. variotii*	Mixed Inoculum
1	Biobor JF 2,2'-oxybis(4,4,6-trimethyl-1,3,2-dioxaborinane) 2,2'-(1-methyltrimethylenedioxy) bis-(4-methyl-1,3,2-dioxaborinane)	U.S. Borax	Fuel & Water	None	None	None	None
2	DEGME Diethylene glycol monomethyl ether (2-beta-methyl-"Carbitol"); methoxyethoxyethanol $CH_3OCH_2CH_2OCH_2CH_2OH$		Fuel & Water	None	None	None	None
3	EGME ethylene glycol monomethyl ether (2-methoxy-ethanol) $CH_3OCH_2CH_2OH$	U.S. Borax	Fuel & Water	None	None	None	None
4	DML-7 Diethylene glycol-mono methyl ether and methylene bis thiocyanate	Gamlen	Fuel & Water	300 ppm	300 ppm	300 ppm	300 ppm

Table 6.1(cont.) Summary of biocide screening results.

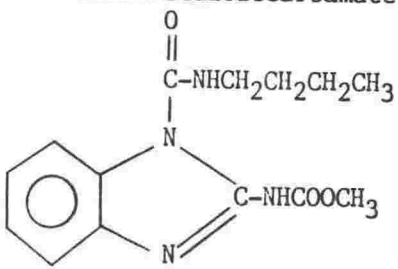
No.	Compound	Producer	Solubility Fuel or Water	Minimum inhibitory Concentration (mic) *C. resinae*	*Penicillium* spp.	*P. variotii*	Mixed Inoculum
5	Proxel AS 1,2-benzisothiazolin-3-one and 33% aqueous glycol solution of a quartenary ammonium salt	ICI	Fuel & Water	300 ppm	300 ppm	300 ppm	300 ppm
6	Benomyl methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate 	Du Pont	None	10 ppm	10 ppm	50 ppm	50 ppm
7	Imazalil 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy) ethyl]-1H-imidazole	Janssen Pharmaceutical Ltd	Fuel	50 ppm	50 ppm	300 ppm	200 ppm
8	Kathon 886 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one	Rohm Haas Ltd	Fuel & Water	50 ppm	50 ppm	300 ppm	100 ppm

Table 6.1(cont.) Summary of biocide screening results.

	Chemical Stability	Corrosion Test	Engine Test	Potential Useful biocide
1)	Cloudy soln.	"	"	No
2)	Cloudy soln.	"	"	No
3)	Cloudy soln.	"	"	No
4)	Cloudy soln.	"	"	Yes an inhibitor
5)	Cloudy soln.	"	"	Yes an inhibitor
6)	ppt	Same as a freshly polished copper	No significant effect on the performance of engine. 70 μm of carbon was recorded as in the control engine.	Yes
7)	Solution	"	"	Yes
8)	Solution	"	"	Yes

C.R. - *Cladosporium resinae*

P.C. - *Penicillium corylophilum*

P.V. - *Paecilomyces variotii*

(a) The table shows the % germination and as a superscript (b), an indication of the type or extent of germ tube growth.

1 - Very isolated signs of germtube

2 - Distorted germtubes, very reduced growth,swollen hyphal tips, some bursting.

3 - Unbranched hyphae and no sporulation.

4 - Extensive branching but sporulation rare.

5 - Hyphae elongated, branching sporulation common.

Table 6.2 *The effects of biocides on conidial germination (a) and early growth (b) using the agar slide/diesel technique.*

Biocide Concentration	Number of 24 h periods exposure to biocide																				
	24 h			48 h			72 h			96 h			120 h			144 h			168 h		
(ppm)	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.
DEGME																					
0	70^3	57^3	88^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
500	53^3	45^3	87^3	100^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
1000	36^3	63^3	72^3	100^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
2000	32^3	75^3	85^3	100^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
3000	27^3	52^3	50^3	100^3	100^5	100^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
4000	29^3	52^3	47^3	100^3	100^5	100^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
5000	38^3	60^3	77^3	100^3	100^5	100^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
Biobor JF																					
0	57^3	100^3	75^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
5	44^3	91^3	60^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
10	60^3	77^3	51^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
50	53^3	74^3	53^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
100	46^3	60^3	57^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
200	57^3	55^3	40^3	97^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
300	51^3	64^3	46^3	86^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
DML-7																					
0	73^3	96^3	87^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
5	22^3	49^3	71^3	72^3	77^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
10	17^3	32^3	74^3	59^3	90^3	100^5	85^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
50	24^3	25^3	86^3	68^3	94^5	100^5	97^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
100	29^3	27^3	69^3	74^3	82^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
200	30^3	29^3	78^3	83^3	47^3	100^5	93^3	100^5	100^5	100^4	100^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5
300	15^3	15^3	92^3	38^3	46^3	100^4	81^3	96^5	100^5	100^4	100^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5
Proxel AS																					
0	57^3	71^3	69^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
5	49^3	55^3	72^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
10	44^3	52^3	44^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
50	46^3	67^3	78^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
100	31^3	40^3	62^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
200	27^3	45^3	51^3	77^4	85^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5
300	22^3	30^3	46^3	84^4	89^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5	100^4	100^4	100^5

Table 6.2(cont.) The effects of biocides on conidial germination (a) and early growth (b) using the agar slide/diesel technique.

Biocide Concentration	Number of 24 h periods exposure to biocide																	
	24 h			48 h			72 h			96 h			120 h			144 h			168 h		
(ppm)	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.
Benomyl																					
0	53^3	100^3	93^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
5	0^1	37^3	100^3	10^1	100^3	100^3	100^2	100^3	100^3	100^2	100^3	100^3	100^2	100^3	100^3	100^2	100^3	100^3	100^2	100^3	100^3
10	0	0	0	0	100^2	100^2	0	100^2	100^2	0	100^3	100^2	0	100^3	100^2	0	100^3	100^2	0	100^3	100^4
50	0	0	0^2	0	14^2	33	0	18^2	51^2	0	100^2	100^2	0	100^2	100^2	0	100^2	100^2	0	100^2	100^2
100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Imazalil																					
0	55^3	98^4	87^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
5	4^3	26^1	49^3	10^5	32^3	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
10	3^3	54^3	72^3	11^3	58^3	81^4	19^3	100^5	100^5	78^4	100^5	100^5	100^4	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
50	0	0	13^3	0	0	19^3	0	0	100^4	0	0	100^5	0	0	100^5	0	0	100^5	0	0	100^5
100	0	0	5^3	0	0	12^3	0	0	64^4	0	0	100^5	0	0	100^5	0	0	100^5	0	0	100^5
200	0	0	10^3	0	0	27^3	0	0	51^4	0	0	100^5	0	0	100^5	0	0	100^5	0	0	100^5
300	0	0	1^3	0	0	13^3	0	0	38^4	0	0	100^5	0	0	100^5	0	0	100^5	0	0	100^5
Kathon 886																					
0	77^3	100^3	100^3	89^4	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
5	0	100^3	100^3	24^3	100^3	100^5	39^4	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5	100^5
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Details of the normal rapid hyphal growth along the interface with frequent branching and sporulation occurring within 48 hours was observed in all control cultures. Hyphae which grew down into the solidified aqueous phase eventually produced conidiophores but conidia were produced profusely on the diesel side of the interwoven interfacial mat. Neither DEGME nor Biobor JF had any effect on this normal growth pattern. In addition to inhibiting germination completely, the effective biocides benomyl, imazalil and Kathon 886, resulted in the production of deformed germ tubes with limited growth potential. Benomyl for example caused swollen hyphae to form which soon stopped growing and in some cases cell walls and membranes appeared to be disrupted and cell contents leaked out.

With DML-7 and Proxel AS at the higher concentrations, both *C. resinae* and *P. corylophilum* failed to sporulate, indicating that even if these compounds were not biocidal, then some biostatic activity was evident.

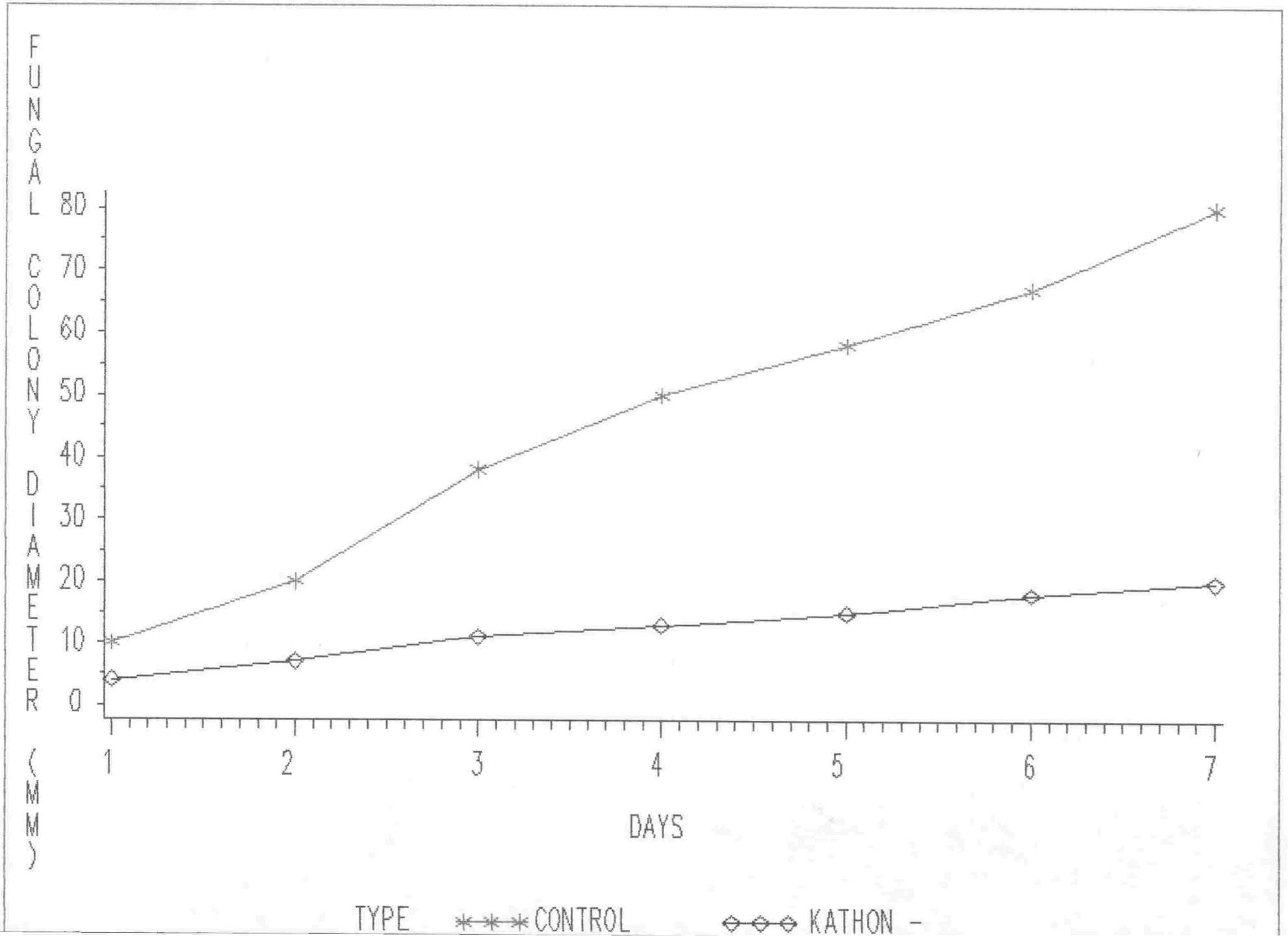
6.1.2 Poison Plate Test

When agar plugs carrying actively growing mycelia of *C. resinae*, *Paecilomyces variotii*, *Penicillium corylophilum* and the other *Penicillium* spp. isolated from diesel were placed on MEA the colony diameter increased in a linear fashion to cover the Petri-dish in 8 days, as shown in Figure 6.1. The growth rate was always uniform throughout the 8 days during which colony diameter was measured. Tables 6.3, 6.4 and 6.5 present the growth of these fungi as recorded after 8 days only and show the effects of the inclusion of the various biocides at a range of concentrations upon colony growth rate.

EGME, DEGME and Biobor JF (Table 6.3) had no effect on hyphal growth at any of the concentrations tested under these conditions. Of the other biocides only Kathon 886 inhibited growth of all three fungi more or less equally, being totally inhibitory above 50 ppm.

DML-7 and Proxel AS were less inhibitory, having no effect at all on *P. variotii*. Proxel AS was slightly inhibitory to the other fungi at 300 ppm while DML-7 gave some inhibition at 100 ppm affecting some of the fungi more than others.

Figure 6.1. The growth of treated and untreated fungal colony in the poison plate test.


FUNGAL COLONY DIAMETER (MM)
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
DAYS
TYPE
CONTROL
KATHON -

* Growth of fungus from 5 mm inoculum plug after 8 days recorded as follows :

0	No growth
+	Little growth (6-10 mm)
++	Moderate growth (11-30 mm)
+++	Profuse growth (31-80 mm)

** Identical growth responses were obtained from *C. resinae* and *P. variotii* when isolated from diesel fuel which had contained DEGME and Biobor JF for 5 months in the fuel tanks.

Table 6.3 Growth* of fungi isolated from diesel fuel on MEA containing biocides. (Poison Plate Test).

Fungi	Concentration of biocide (ppm)					
	0	1000	2000	3000	4000	5000
			EGME			
C. resinae	+++	+++	+++	+++	+++	+++
P. variotii	+++	+++	+++	+++	+++	+++
P. chrysogenum	+++	+++	+++	+++	+++	+++
P. corylophilum	+++	+++	+++	+++	+++	+++
P. digitatum	+++	+++	+++	+++	+++	+++
P. frequentans	+++	+++	+++	+++	+++	+++
P. spinulosum	+++	+++	+++	+++	+++	+++
			DEGME			
*C. resinae***	+++	+++	+++	+++	+++	+++
*P. variotii***	+++	+++	+++	+++	+++	+++
P. chrysogenum	+++	+++	+++	+++	+++	+++
P. corylophilum	+++	+++	+++	+++	+++	+++
P. digitatum	+++	+++	+++	+++	+++	+++
P. frequentans	+++	+++	+++	+++	+++	+++
P. spinulosum	+++	+++	+++	+++	+++	+++
	0	10	50	100	200	300
			BIOBOR JF			
*C. resinae***	+++	+++	+++	+++	+++	+++
*P. variotii***	+++	+++	+++	+++	+++	+++
P. chrysogenum	+++	+++	+++	+++	+++	+++
P. corylophilum	+++	+++	+++	+++	+++	+++
P. digitatum	+++	+++	+++	+++	+++	+++
P. frequentans	+++	+++	+++	+++	+++	+++
P. spinulosum	+++	+++	+++	+++	+++	+++
			DML-7			
C. resinae	+++	+++	++	++	++	++
P. variotii	+++	+++	+++	+++	+++	+++
P. chrysogenum	+++	++	++	+	+	+
P. corylophilum	+++	++	++	+	+	+
P. digitatum	+++	+++	++	+	+	+
P. frequentans	+++	++	++	++	+	+
P. spinulosum	+++	++	++	++	+	+

Table 6.3(cont.) Growth* of fungi isolated from diesel fuel on MEA containing biocides. (Poison Plate Test).

Fungi	Concentration of biocide (ppm) 0	10	50	100	200	300
			PROXEL AS			
C. resinae	+++	+++	+++	+++	++	++
P. variotii	+++	+++	+++	+++	+++	+++
P. chrysogenum	+++	++	++	++	++	+
P. corylophilum	+++	+++	+++	+++	++	++
P. digitatum	+++	+++	+++	+++	++	++
P. frequentans	+++	+++	+++	+++	+++	++
P. spinulosum	+++	+++	+++	+++	+++	++
			BENOMYL			
C. resinae	+++	0	0	0	0	0
P. variotii	+++	+++	+	0	0	0
P. chrysogenum	+++	0	0	0	0	0
P. corylophilum	+++	0	0	0	0	0
P. digitatum	+++	+++	++	++	++	++
P. frequentans	+++	0	0	0	0	0
P. spinulosum	+++	0	0	0	0	0
			IMAZALIL			
C. resinae	+++	+	0	0	0	0
P. variotii	+++	+++	+++	+++	+++	+++
P. chrysogenum	+++	+	0	0	0	0
P. corylophilum	+++	0	0	0	0	0
P. digitatum	+++	0	0	0	0	0
P. frequentans	+++	0	0	0	0	0
P. spinulosum	+++	0	0	0	0	0
			KATHON 886			
C. resinae	+++	+	0	0	0	0
P. variotii	+++	++	0	0	0	0
P. chrysogenum	+++	++	+	0	0	0
P. corylophilum	+++	++	+	0	0	0
P. digitatum	+++	0	0	0	0	0
P. frequentans	+++	0	0	0	0	0
P. spinulosum	+++	0	0	0	0	0

Table 6.4 Growth* of various *C. resinae* isolates on MEA as affected by biocides. (Poison Plate Test).

Biocides concentrations (ppm)	*C. resinae* Isolates: ex feather (R5) 1972	ex Airspora 1983	*F. avellaneum* ex soil 1983	ex Diesel (D1.f) 1970 isolate	*F. avellaneum* ex jet fuel(K10f) 1972	*F. avellaneum* ex diesel 1982	ex diesel (Aust.) 1983
DEGME							
0	+++	+++	+++	+++	+++	+++	+++
1000	+++	+++	+++	+++	+++	+++	+++
2000	+++	+++	+++	+++	+++	+++	+++
3000	+++	+++	+++	+++	+++	+++	+++
4000	+++	+++	+++	+++	+++	+++	+++
5000	+++	+++	+++	+++	+++	+++	+++
BIOBOR JF							
0	+++	+++	+++	++	+++	+++	+++
10	+++	+++	+++	+++	+++	+++	+++
50	+++	+++	+++	+++	+++	+++	+++
100	+++	+++	+++	+++	+++	+++	+++
200	+++	+++	+++	+++	+++	+++	+++
300	+++	+++	+++	+++	+++	+++	+++
DML-7							
0	+++	+++	+++	+++	+++	+++	+++
10	+++	+++	+++	+++	+++	+++	+++
50	+++	+++	+++	+++	+++	+++	+++
100	+++	+++	+++	+++	+++	+++	+++
200	0	0	0	++	++	++	++
300	0	0	0	0	0	0	0
PROXEL AS							
0	+++	+++	+++	+++	+++	+++	+++
10	+++	+++	+++	+++	+++	+++	+++
50	+++	+++	+++	+++	+++	+++	+++
100	+++	+++	+++	+++	+++	+++	+++
200	++	++	++	++	++	++	++
300	++	++	++	++	++	++	++

Table 6.4(cont.) Growth* of various *C. resinae* isolates on MEA as affected by biocides. (Poison Plate Test).

Biocides concentrations (ppm)	*C. resinae* Isolates: ex feather (R5) 1972	ex Airspora 1983	*F. avellaneum* ex soil 1983	*F. avellaneum* ex Diesel (D1.f) 1970 isolate	*F. avellaneum* ex jet fuel(K10f) 1972	*F. avellaneum* ex diesel 1982	*F. avellaneum* ex diesel (Aust.) 1983
BENOMYL							
0	+++	+++	+++	+++	+++	+++	+++
10	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
IMAZALIL							
0	+++	+++	+++	+++	+++	+++	+++
10	++	++	++	++	++	++	++
50	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
KATHON 886							
0	+++	+++	+++	+++	+++	+++	+++
10	++	++	++	+++	+++	+++	+++
50	+	+	+	++	+	+	++
100	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0

* Growth of fungus from 5 mm inoculum plug after 5 days recorded as follows :

0 No growth
\+ Little growth (6-10 mm)
++ Moderate growth (11-30 mm)
+++ Profuse growth (31-80 mm)

Table 6.5 Determination of biocidal activity* of benomyl (B) and imazalil (I) alone or in mixture at varying concentrations in poison plates on *Paecilomyces variotii* (P.V.) and *Cladosporium resinae* (C.R.) after 8 days.

Biocide Concentration (ppm)	P.V.	C.R.	P.V.	C.R.	P.V.	C.R.	P.V.	C.R.	P.V.	C.R.	P.V.	C.R.	P.V.	C.R.	P.V.	C.R.	P.V.	C.R.	P.V.	C.R.	P.V.	C.R.	P.V.	C.R.
5 ppm	0		B 5		B+I 4+1		B+I 3+2		B+I 2+3		B+I 1+4		I 5											
	+++	+++	+++	0	+++	++	+++	++	+++	++	+++	++	+++	++										
10 ppm	0		B 10		B+I 9+1		B+I 8+2		B+I 7+3		B+I 6+4		B+I 5+5		B+I 4+6		B+I 3+7		B+I 2+8		B+I 1+9		I 10	
	+++	+++	+++	0	+++	0	+++	0	+++	0	+++	0	+++	0	+++	++	+++	++	+++	++	+++	++	+++	+++
50 ppm	0		B 50		B+I 45+ 5		B+I 40+10		B+I 30+20		B+I 25+25		B+I 20+30		B+I 10+40		B+I 5+45		I 50					
	+++	+++	0	0	0	0	0	0	++	0	0	0	+++	0	++	0	++	0	+++	0				
100 ppm	0		B 100		B+I 90+10		B+I 80+20		B+I 70+30		B+I 60+40		B+I 50+50		B+I 40+60		B+I 30+70		B+I 20+80		B+I 10+90		I 100	
	+++	+++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	0
200 ppm	0		B 200		B+I 190+10		B+I 180+20		B+I 170+30		B+I 160+40		B+I 150+50		B+I 140+60		B+I 130+70		B+I 120+80		B+I 110+90		B+I 100+100	
	+++	+++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			B+I 90+110		B+I 80+120		B+I 70+130		B+I 60+140		B+I 50+150		B+I 40+160		B+I 30+170		B+I 20+180		B+I 10+190		B+I 80+120		I 200	
			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	0

* Growth of fungus from 5mm diameter inoculum plug after 8 days recorded as :
0 - No growth
\+ - Little growth
++ - Moderate growth (11-30 mm diameter)
+++ - Profuse growth (31-80 mm diameter)

Benomyl at 10 ppm caused total inhibition of growth of all fungi except *P. digitatum*. This fungus continued to grow even in the presence of 300 ppm of benomyl.

Imazalil also proved to be very effective, inhibiting the growth of all fungi, except *P. variotii* at 10 ppm. *P. variotii* appears to be totally insensitive to this biocide.

Table 6.3 also includes the effects of the biocides on the hyphal growth of *C. resinae* and *P. variotii* isolated from the fuel tanks which had been treated with DEGME and Biobor JF for 5 months. These isolates were insensitive to DEGME and Biobor JF in the laboratory.

The data of Table 6.4 show that the various isolates of *C. resinae* showed an identical response to the different biocides with all of them including the 1960 and 1970 isolates being insensitive to DEGME and Biobor JF.

The two effective biocides, benomyl and imazalil when tested in combination over a range of concentrations gave the results as shown in table 6.5. At 50 ppm, *C. resinae* showed no growth in any proportion of the two, while *P. variotii* was unaffected by the presence of imazalil and totally controlled by benomyl. When the concentration of benomyl fell below 40 ppm, the effectiveness of benomyl was partly lost in the presence of the imazalil. Thus it would appear that imazalil somehow reduces the effectiveness of benomyl against *P. variotii*. But at equal proportions of 25 ppm, the biocides inhibited both the fungi. Similar interactive effects were apparent at the 200 ppm level where 200 ppm of imazalil was ineffective against *P. variotii* but only 10 ppm of benomyl plus 190 ppm of imazalil gave complete growth suppression.

6.1.3 The effects of biocides on fungal growth and survival in BH/diesel phases

The effects of biocides on spore germination and growth on Bushnell Haas/diesel phases are shown by the results in table 6.6. The most significant effect of the changes in the proportions of the aqueous and hydrocarbon phases were seen at the extremes.

In the absence of biocides, the mixed spore inoculum failed to grow on either the aqueous phase or on diesel alone. The changing proportions of the two phases in the mixtures had some effect on the amount of fungal growth. It was evident that greatest growth was obtained in liquid media containing almost equal volumes of the two phases and least when one phase was 20% of the total volume. Once again DEGME and Biobor JF had no significant effect on fungal growth. Both DML-7 and Proxel AS caused a significant reduction in fungal growth with the effect being the same at all three concentrations. No systematic variation in the degree of inhibition accompanied the changing proportions of aqueous/hydrocarbon phases. Both benomyl and imazalil almost completely prevented any fungal growth at all concentrations of biocide and in all proportions of Bushnell-Haas medium and diesel. When little growth was observed in the presence of DML-7 and Proxel AS, spores retained their viability when filtered out and placed on V-8 juice agar medium. After 6 weeks in medium containing benomyl at or above 50 ppm, all spores had lost their viability except for *Penicillium digitatum*. In the presence of imazalil, at or above 200 ppm, all fungal spores lost viability except for *P. variotii*.

The pH in all the test systems was between 5 and 7 at the end of 6 weeks. The aqueous phase in Proxel AS bottles appeared clear but all the others were cloudy. There was a deposit at the bottom of benomyl treated bottles.

A fresh batch of DEGME gave identical results under these test conditions and remained ineffective.

* Mixed spore inoculum was used in this test.

** Growth recorded after 6 weeks as follows:

0	No growth
+	Some spore germination, very little growth
++	Very thin colourless mycelial mat
+++	Thick dark mycelial mat

And as + Dry weight (mg) of fungal mycelia
The data in the ( ) is from the new batch of DEGME tested

Spore viability results:-

Benomyl completely controlled *C. resinae* and *P. variotii* at 50 ppm, and *Penicillium digitatum* survived but did not form a mycelial mat.

Imazalil controlled *C. resinae* and *Penicillium* spp. at 100 ppm but not *Paecilomyces variotii*. DML-7 and Proxel AS did not kill the spores at any concentration but inhibited growth.

Table 6.6 Spore* germination and growth** in Bushnell-Haas diesel fuel phases.

Bushnell-Haas : Fuel	Concentration of Biocide (ppm)																		
	0	50						200						300					
		Benomyl	Biobor JF	DEGME+	DML-7	Imazalil	Proxel AS	Benomyl	Biobor JF	DEGME+	DML-7	Imazalil	Proxel AS	Benomyl	Biobor JF	DEGME+	DML-7	Imazalil	Proxel AS
5:0	+ *23	0	+ 15	+ 58 (55)	+ 15	+ 15	+ 55	+ 7	+ 19	+ 40	+ 17	+ 11	+ 37	+ 12	+ 14	+ 45 (52)	+ 18	+ 13	+ 47
5:1	+++ 407	+ 11	+++ 249	+++ 262 (300)	++ 121	+ 27	++ 132	+ 9	+++ 349	+++ 395 (322)	+++ 138	+ 28	+++ 257	+ 10	+++ 260	+++ 375 (295)	++ 168	+ 32	++ 147
5:2	+++ 512	+ 15	+++ 357	+++ 425 (389)	++ 227	+ 58	++ 130	+ 12	+++ 357	+++ 471 (342)	++ 118	+ 58	++ 137	+ 18	+++ 291	+++ 427 (334)	++ 268	+ 47	++ 208
5:3	+++ 475	+ 17	+++ 493	+++ 300 (421)	+++ 105	+ 38	+++ 450	+ 15	+++ 328	+++ 379 (399)	++ 127	+ 15	++ 184	+ 20	+++ 283	+++ 400 (367)	++ 155	+ 51	++ 206
5:4	+++ 675	+ 22	+++ 418	+++ 455 (372)	++ 121	+ 40	+++ 185	+ 14	+++ 284	+++ 787 (435)	++ 246	+ 43	++ 173	+ 13	+++ 355	+++ 432 (311)	++ 177	+ 47	++ 210
5:5	+++ 673	+ 25	+++ 850	+++ 684 (713)	++ 125	+ 31	+++ 610	+ 17	+++ 663	+++ 1095 (589)	++ 220	+ 53	+++ 395	+ 22	+++ 755	+++ 831	++ 145	+ 52	++ 216
4:5	+++ 616	+ 15	+++ 540	+++ 734 (444)	++ 216	+ 12	+++ 635	+ 14	+++ 805	+++ 565 (631)	++ 212	+ 48	+++ 402	+ 11	+++ 612	+++ 601 (438)	++ 49	+ 50	++ 150
3:5	+++ 576	+ 19	+++ 595	+++ 952 (641)	++ 230	+ 19	++ 137	+ 19	+++ 605	+++ 623 (495)	++ 118	+ 30	++ 145	+ 16	+++ 553	+++ 596 (432)	++ 133	+ 48	++ 120
2:5	+++ 570	+ 21	+++ 645	+++ 578 (597)	++ 118	+ 27	++ 125	+ 23	+++ 450	+++ 402 (415)	++ 108	+ 41	++ 132	+ 14	+++ 631	+++ 514 (388)	++ 180	+ 20	++ 155
1:5	+++ 406	+ 18	+++ 630	+++ 470 (511)	++ 123	+ 38	++ 122	+ 21	+++ 325	+++ 375 (422)	++ 217	+ 23	+ 33	+ 15	+++ 408	+++ 325 (431)	++ 155	+ 57	++ 130
0:5	+ 40	+ 5	+ 14	+ 36 (21)	+ 31	0	+ 22	+ 3	+ 15	+ 58 (33)	+ 28	+ 13	+ 16	+ 2	+ 7	+ 49 (35)	+ 28	+ 18	+ 30

When a *C. resinae* spore suspension was inoculated into BH/diesel mixture, held at 4°C for 4 weeks and then returned to normal temperature, growth was reduced at the low temperature and was then vigorous at 18-19°C. Even after 4 weeks exposure to DEGME and EGME under slow growth conditions these biocides had no effect on subsequent growth.

6.1.4 Large volume liquid fuel : biocide tests

Addition of biocides to two litre bottles of diesel fuel taken directly from the storage tanks gave results similar to the other laboratory growth studies (table 6.7).

The number of CFU of *C. resinae*, *P. variotii* and *Penicillium* spp. showed some variation in the different two litre batches before addition of the biocides. *Penicillium* spp. occurred at a lower frequency compared to *C. resinae* and *P. variotii*.

Kathon 886 was biocidal after 2 days exposure at 300 ppm and after 3 days at 100 and 200 ppm to all the fungi. *C. resinae* was inhibited at 50 ppm after 6 days. *P. variotii* was slightly more tolerant being almost unaffected at 50 ppm.

Both benomyl and imazalil were effective against *C. resinae* but some CFU of *Penicillium* spp. managed to survive at 300 ppm after 5-6 days exposure to benomyl. Imazalil was ineffective against *P. variotii* but benomyl gave good control at all concentrations.

6.1.5 Intermittent exposure of the three common fuel fungi to biocides in diesel fuel

Intermittent exposure to biocides of a mixed spore proportion carried on sterile filter papers in diesel fuel for 24 hour periods separated by 6 day exposure in clean diesel fuel gave results shown in table 6.8. Biobor JF was biocidal to *Penicillium corylophilum* at 50 ppm after three 24 hour exposures but had no effect on *C. resinae* and *P. variotii*. DML-7 was effective against *C. resinae* after five 24 hour periods at 100 ppm but reduced viability almost to zero after two exposures at 200 and 300 ppm of all three fungi.

x C.r. - *C. resinae*
Pen. - *Penicillium* spp.
P.v. - *Paecilomyces variotii*

* Two litre samples were incubated with biocides at various concentrations

+ Numbers of colonies per litre are calculated from colonies counted after the fungi have been allowed to form colonies on V-8 juice agar plates for 5 days.

Table 6.7 Viable microbial contamination during biocide treatment of large volumes * of diesel fuel.

Hours Exposure/Biocide		Concentrations of Biocides (ppm)/colonies+ per litre of diesel fuel														
		0			50			100			200			300		
		C.r.x	Pen.x	P.v.x	C.r.	Pen.	P.v.	C.r.	Pen.	P.v.	C.r.	Pen.	P.v.	C.r.	Pen.	P.v.
Before biocide treatment:																
	Biobor JF	350	40	1000	850	550	1000	300	100	1000	850	25	1000	100	150	1000
	DEGME	1000	55	1000	400	1000	1000	1000	500	1000	500	1000	1000	600	1000	1000
	DML-7	40	25	1000	20	220	1000	0	800	1000	0	120	1000	474	120	550
	Proxel AS	1000	200	1000	717	280	1000	180	140	1000	1000	240	1000	800	360	1000
	Benomyl	1000	80	1000	1000	60	100	1000	40	100	1000	60	1000	1000	120	1000
	Imazalil	1000	400	1000	194	140	1000	1000	80	1000	1000	231	1000	1000	40	1000
	EGME	510	100	1000	730	20	680	270	200	1000	1000	50	1000	500	0	1000
	Kathon	380	260	1000	600	160	1000	1000	300	480	1000	600	1000	1000	860	1000
After biocide treatment:																
24 hrs	Biobor JF	1000	10	1000	185	100	1000	0	60	1000	500	55	730	180	10	1000
	DEGME	1000	50	1000	1000	20	900	180	120	630	300	400	500	100	1000	780
	DML-7	1000	60	905	1000	300	1000	592	22	817	80	0	160	212	0	607
	Proxel AS	1000	0	1000	1000	535	1000	40	100	60	20	40	0	0	40	611
	Benomyl	1000	0	1000	0	0	0	0	0	0	0	0	0	0	0	0
	Imazalil	1000	0	1000	0	0	1000	0	0	1000	0	0	200	0	0	507
	EGME	1000	20	1000	520	500	1000	1000	0	1000	1000	80	1000	1000	0	1000
	Kathon	400	0	1000	544	0	1000	0	0	0	100	140	200	20	0	40
48 hrs	Biobor JF	0	55	1000	1000	0	1000	0	1000	1000	85	500	1000	1000	500	1000
	DEGME	1000	15	1000	280	20	1000	250	300	1000	1000	500	1000	520	275	1000
	DML-7	1000	60	1000	1000	1000	524	140	520	286	140	260	60	260	300	1000
	Proxel AS	1000	1000	1000	1000	0	1000	80	20	20	0	20	280	20	0	20
	Benomyl	550	200	1000	0	0	0	0	20	80	0	40	0	0	0	0
	Imazalil	1000	300	1000	500	200	1000	0	0	1000	0	20	400	0	0	670
	EGME	1000	40	1000	600	0	1000	1000	500	1000	1000	100	1000	1000	60	1000
	Kathon	300	40	1000	60	0	1000	60	20	260	0	0	20	0	0	20
72 hrs	Biobor JF	220	100	1000	1000	0	1000	0	1000	1000	250	60	1000	0	1000	1000
	DEGME	1000	500	1000	550	100	1000	400	100	1000	20	200	1000	0	250	1000
	DML-7	1000	300	1000	1000	45	1000	811	604	330	514	100	0	380	40	400
	Proxel AS	1000	120	1000	1000	1000	1000	100	0	120	40	0	0	0	0	0
	Benomyl	1000	20	1000	0	0	0	0	0	0	0	40	0	0	0	0
	Imazalil	1000	0	1000	0	0	1000	0	0	1000	0	0	1000	0	0	1000
	Kathon	673	60	1000	80	20	517	0	0	0	0	0	0	0	0	0
	EGME	1000	0	1000	1000	40	1000	1000	0	1000	550	0	1000	400	0	1000

Table 6.7 (cont.) Viable microbial contamination during biocide treatment of large volumes * of diesel fuel.

Hours Exposure/Biocide		Concentrations of Biocides (ppm)/colonies+ per litre of diesel fuel														
		0			50			100			200			300		
		C.r.x	Pen.x	P.v.x	C.r.	Pen.	P.v.	C.r.	Pen.	P.v.	C.r.	Pen.	P.v.	C.r.	Pen.	P.v.
96 hrs	Biobor JF	525	63	1000	500	78	1000	100	150	1000	300	200	1000	300	28	1000
	DEGME	1000	50	1000	40	800	1000	100	50	1000	500	250	1000	120	45	100
	DML-7	1000	40	1000	1000	100	1000	320	0	100	267	0	211	120	0	20
	Proxel AS	1000	213	1000	599	544	361	80	20	20	20	0	80	45	0	375
	Benomyl	544	20	1000	0	0	0	0	0	0	0	0	0	0	20	0
	Imazalil	1000	0	1000	0	0	1000	0	0	1000	0	0	1000	0	0	1000
	Kathon	1000	468	1000	500	80	1000	100	20	1000	0	0	0	0	0	0
	EGME	1000	1000	1000	1000	250	1000	1000	300	1000	300	300	1000	1000	200	1000
120 hrs	Biobor JF	500	370	1000	1000	200	1000	0	50	1000	100	25	500	900	20	1000
	DEGME	600	500	1000	200	500	1000	1000	500	1000	0	500	1000	1000	50	1000
	DML-7	1000	20	1000	100	60	300	581	22	60	572	0	47	484	60	20
	Proxel AS	1000	0	1000	1000	594	579	100	0	0	20	0	175	20	40	541
	Benomyl	1000	200	1000	0	0	0	0	0	0	0	0	0	0	40	0
	Imazalil	1000	0	1000	0	60	1000	0	0	1000	0	0	1000	0	0	1000
	Kathon	390	40	1000	20	0	500	0	0	0	0	0	0	0	0	0
	EGME	1000	1000	1000	1000	100	1000	1000	140	1000	880	1000	1000	1000	200	1000
144 hrs	Biobor JF	550	500	1000	300	200	1000	250	300	1000	150	1000	1000	350	1000	30
	DEGME	150	1000	100	0	1000	250	20	300	100	60	300	1000	1000	500	1000
	DML-7	1000	0	1000	1000	0	1000	523	0	111	20	40	0	140	0	20
	Proxel AS	1000	0	10	1000	250	40	20	0	0	20	0	266	100	20	340
	Benomyl	1000	300	1000	0	140	0	0	80	0	0	260	0	0	0	0
	Imazalil	1000	0	1000	0	0	1000	0	0	1000	0	0	1000	0	0	1000
	Kathon	400	517	1000	0	140	40	0	0	0	0	0	0	0	0	0
	EGME	1000	160	1000	1000	350	990	1000	550	1000	1000	1000	1000	1000	1000	1000
168 hrs	Biobor JF	250	340	1000	350	1000	1000	300	150	1000	580	50	550	300	29	1000
	DEGME	50	1000	1000	280	1000	380	1000	150	400	1000	150	750	200	1000	450
	DML-7	1000	0	1000	1000	0	0	180	0	109	40	0	0	380	0	0
	Proxel AS	250	250	0	528	571	0	40	0	20	20	0	194	0	160	400
	Benomyl	1000	0	1000	0	0	0	0	20	0	0	60	0	0	0	0
	Imazalil	1000	0	1000	0	0	1000	0	0	1000	0	0	170	0	0	1000
	Kathon	1000	1000	1000	0	50	515	0	0	0	0	0	0	0	0	0
	EGME	1000	1000	1000	1000	500	1000	1000	570	1000	1000	650	1000	1000	450	1000
192 hrs	Biobor JF	1000	100	1000	500	50	100	440	1000	1000	500	50	1000	720	50	1000
	DEGME	1000	100	1000	500	1000	150	280	1000	300	20	1000	0	150	1000	1000
	DML-7	1000	0	1000	1000	41	20	1000	0	360	561	0	0	1000	0	100
	Proxel AS	1000	340	500	1000	780	500	60	0	100	20	80	200	40	180	420
	Benomyl	1000	40	1000	0	20	0	0	0	0	100	0	0	0	0	0
	Imazalil	1000	415	1000	0	0	1000	0	0	1000	0	0	120	0	0	210
	Kathon	720	60	1000	0	20	320	0	0	0	0	0	0	0	0	0
	EGME	1000	1000	1000	1000	720	1000	1000	1000	1000	1000	890	1000	1000	900	1000

x C.R. - *Cladosporium resinae*
P.C. - *Penicillium corylophilum*
P.V. - *Paecilomyces variotti*

* Figures shown are the total CFU score from 3 replicate filters counted after 5 days growth in V-8 juice agar.

+ Moist sterile filters carrying spores were exposed to diesel containing biocide for 24 hours followed by exposure to sterile diesel for 6 days before repeating up to 7 times, some filters were removed for determining viable CFU after each treatment.

0 - no growth or no colonies
1 - > 10 colonies/filter
2 - > 20 colonies/filter
3 - > 30 colonies/filter
4 - > 40 colonies/filter
5 - > 50 colonies/filter

Table 6.8 Colony forming units* surviving after exposure to biocides+ intermittently.

Concentration in fuel	Number of 24 hours periods exposure to biocide																				
	1			2			3			4			5			6			7		
	C.R.x	P.C.x	P.V.x	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.	C.R.	P.C.	P.V.
Biobor JF																					
0	15	15	15	15	10	15	15	15	15	15	10	15	15	10	15	15	0	12	15	0	12
50	15	15	15	15	0	15	15	10	15	15	0	15	15	0	15	15	0	15	15	0	15
100	15	15	15	15	0	15	15	15	15	15	0	15	15	0	15	15	0	15	12	0	2
200	15	15	15	15	5	15	10	5	15	15	0	15	15	10	15	15	0	15	15	0	5
300	15	15	15	15	15	15	15	15	15	15	0	15	15	0	15	15	0	15	15	0	15
DML-7																					
0	15	15	15	15	10	15	15	5	15	15	5	15	15	5	15	15	15	15	15	15	15
50	15	15	15	15	10	10	15	0	0	15	0	0	15	0	0	15	2	2	0	0	0
100	15	15	15	10	15	15	5	0	0	10	0	0	5	2	5	0	2	0	0	0	2
200	15	15	15	0	0	0	0	0	0	2	0	0	0	0	2	0	2	0	0	0	0
300	15	15	15	0	0	0	0	0	0	0	2	0	0	0	4	0	2	0	0	0	0
Proxel AS																					
0	15	15	15	15	10	15	15	15	15	15	10	15	15	10	15	15	5	15	15	15	15
50	15	10	15	15	10	15	15	0	15	15	0	0	15	15	0	15	8	15	15	0	15
100	15	15	15	4	2	0	0	0	0	8	2	0	0	2	2	4	6	0	2	0	2
200	15	5	10	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
300	15	10	15	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
Kathon 886																					
0	15	15	15	15	5	15	15	15	15	15	10	15	15	10	5	15	10	15	15	5	15
50	2	2	8	10	0	15	10	0	15	15	0	0	15	15	0	0	0	0	0	2	0
100	0	0	0	0	0	0	0	0	0	8	2	0	0	2	2	0	0	0	2	0	2
200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
Benomyl																					
0	15	10	15	15	10	15	15	15	15	15	5	15	15	15	15	15	15	15	15	10	15
50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Imazalil																					
0	15	15	15	15	10	15	15	15	15	15	15	15	15	15	15	15	10	15	15	0	15
50	15	15	15	8	8	15	15	15	15	15	0	15	15	0	10	15	2	5	15	0	2
100	15	15	15	15	0	15	5	0	15	8	0	5	5	0	15	10	0	0	15	0	15
200	15	15	15	15	0	15	0	0	15	5	0	10	0	5	12	0	0	0	0	0	0
300	15	15	15	15	0	15	15	5	15	10	10	10	0	8	2	0	0	0	0	0	0

Kathon 886 required three 24 hour exposures at 50 ppm but was biocidal to all three fungi after only one 24 hour exposure at 100 ppm. Benomyl was effective after a single 24 hour exposure, killing all fungi. Imazalil was effective against *C. resinae* after five 24 hour exposures and required six 24 hour exposures to inhibit *P. variotii* and *Penicillium corylophilum*.

6.2 SECTION II - Field studies of biocide effects

6.2.1 Effects of biocides on microbiological contamination.

The effects of biocides added to field storage tanks on microbiological contamination, corrosion and engine performance are reported here.

Table 6.9 presents the mean number of CFU isolated from the DEGME and Biobor JF treated and untreated tanks. Complete data on the effects of DEGME and Biobor JF at the different levels of the tanks in the field trials are given in Appendix 6.1.

DEGME and Biobor JF significantly reduced *C. resinae* but had little effect on *Paecilomyces variotii* and *Penicillium* spp. Numbers of *P. variotii*, *Penicillium* spp. and *Trichoderma* spp. were intermittently high in all the tanks. *P. variotii* was observed to peak only in late June, July and August 1984. The decrease of *Penicillium* spp. and *Trichoderma* sp. seemed to alternate with increase of *P. variotii*. The frequent occurrence of *Trichoderma* sp. in large numbers and a decrease in all fungi in early August suggest a seasonal influence. It was also notable in April that whenever a particular fungus occurred in high numbers, it remained high in all the sampling levels. The numbers fluctuated at the different levels of the tank only when the fungus occurred in small numbers.

Table 6.10 shows the distribution of DEGME, Biobor JF, DML-7 and Proxel AS mainly in the fuel phase. When excessive amounts of water were present on the very bottom of the tank, Proxel AS partitioned preferentially into the water phase. DEGME and Biobor JF were water soluble and as the water content increased they partitioned into the water phase.

Table 6.9 The effects of the additives DEGME and Biobor JF on microbiological growth in diesel fuel field trials at Devonport (April 1984 - August 1984): mean number of colony forming units* per litre of diesel fuel.

	UNTREATED CHECK (Tank 4)				DEGME (3000ppm) (Tank 5)				BIOBOR (270ppm) (Tank 6)			
DATE TESTED	*Cladosporium resinae* +1	*Paecilomyces variotii*	*Penicillium* spp.	*Trichoderma* spp.	*Cladosporium resinae* +2	*Paecilomyces variotii*	*Penicillium* spp.	*Trichoderma* sp.	*Cladosporium resinae* +3	*Paecilomyces variotii*	*Penicillium* spp.	*Trichoderma* sp.
17 April 1984	67	0	45	0	7	0	6	0	21	1	136	0
9 May 1984	76	0	391	0	1	12	23	0	0	3	15	0
25 May 1984	342	25	78	783	1	381	91	441	0	254	92	230
16 June 1984	106	26	26	296	24	14	41	107	4	6	11	111
28 June 1984	29	133	37	119	1	1	31	51	6	4	57	62
17 July 1984	78	10	107	166	0	0	458	0	0	46	242	0
27 July 1984	28	667	85	0	1	605	38	3	14	840	74	1
10 August 1984	18	1	18	0	2	5	2	0	1	7	1	0
28 August 1984	223	362	66	5	0	223	6	0	2	542	40	100

* Mean for top, middle and bottom of tanks 4, 5, 6

Using sign test +2 and +3 show a significant reduction in relationship to +1 ($p<0.01$)

Table 6.10 The partition coefficient of the compounds studied in the field and the water content in each of the treated tanks.

* DEGME - 3000 ppm)
* BIOBOR JF - 270 ppm) Concentrations added to the tanks
* DML-7 and PROXEL AS - 300 ppm)

Date tested	Samples analyed	DEGME* Conc. ppm in fuel	Water in DEGME treated tank (ppm)	Biobor* conc. ppm in fuel	Water in Biobor treated tank (ppm)	DML-7* Conc. ppm in fuel	Water in DML-7 treated tank (ppm)	Proxel AS* ppm in fuel	Water in Proxel treated tank (ppm)
25.05.84	Middle fuel	2000	84.2	24	107.5				
25.05.84	Bottom fuel	2000		24					
04.07.84	Bottom fuel	2000	79	23	109				
27.07.84	Bottom fuel	1900	79	12	701				
28.08.84	Bottom fuel	3700	158	13	190				
18.12.84	Bottom fuel							37±1	842
19.12.84	Bottom fuel							24±1	
07.02.84	Bottom fuel							7.5±0.5	
19.12.84	Middle fuel							2±0.5	
07.02.85	Middle fuel							1±0.5	
19.12.84	Top fuel							1±0.5	
07.02.85	Top fuel							1±0.5	
07.02.85	Bottom fuel (Fresh sample from the treated tank)							4±0.5	
07.02.85	Middle fuel							3±0.5	
07.02.85	Top fuel							3±0.5	
07.02.85	Water from tank bottom							1700±50	1197
29.11.84	Bottom fuel					250	130.8		
18.12.84	Bottom fuel					240	112.1		
23.01.85	Bottom fuel					220	255		
28.02.85	Bottom fuel					100	199.8		
27.03.85	Bottom fuel					150	86		
18.04.85	Bottom fuel					130	74.4		

Although 270 ppm of Biobor JF was added into the fuel, only 13-24 ppm remained in the fuel phase throughout the five months of sampling. But *C. resinae* numbers remained low in the treated tanks, regardless of the amount of water present in the tanks. The total water content fluctuated in both the untreated and treated tanks.

The data in table 6.11 show the effect of DML-7 and Proxel AS on microbiological growths in field tanks. Throughout the sampling period the untreated tank remained highly contaminated with *C. resinae* and *Penicillium* spp. *P. variotii* contamination fluctuated. There was an increase in the different species of fungi in tank 4 compared to the treated tanks (Appendix 6.7). In late November 1984 in the untreated tank, the presence of high numbers of *P. variotii* corresponded with a decrease in numbers of *C. resinae* and *Penicillium* spp. In December the decrease of *P. variotii* corresponded with high numbers of *C. resinae* and *Penicillium* spp. Probably, interaction occurred between the predominant fungi in the tanks, whereby each fungus was not able to tolerate the presence of another. The water bottom of the tanks was free from microbiological contamination in the presence of the high proportion of DML-7 and Proxel AS. In February and March 1985 high numbers of *C. resinae* occurred in tank 2 (containing DML-7) and in the untreated tank, suggesting Proxel AS to be more effective than DML-7. Generally both the compounds significantly inhibited *C. resinae* and *Penicillium* spp. in relation to the untreated tank. The numbers of the predominant fungi fluctuated highly, especially *P. variotii*. To determine the cause of the large fluctuation in May 1985, repeated samples were taken from each level in each tank (Appendix 6.3). It was found that when *C. resinae*, *Penicillium* spp. and *P. variotii* occurred in high numbers at a particular level, the number of CFU fluctuated between replicates. The presence of *Mucor*, *Rhizopus* and the other fungi listed in Appendix 6.2 were casual and occurred infrequently in high numbers.

Ninety-nine percent of the Proxel AS migrated to the water phase in the storage tank (table 6.10). A layer of water bottom was observed in the Proxel AS treated tank. The pH of the aqueous phase was between 5 and 6.5 throughout the trial period. Proxel AS was also unevenly distributed through the diesel in the storage tank.

Table 6.11 The effects of the biocides DML-7 and Proxel AS on microbiological growth in diesel fuel field trials at Devonport (October 1984 - April 1985): mean number of colony forming units* per litre of diesel fuel.

	UNTREATED CHECK (Tank 4)				DML-7 (300ppm) (Tank 2)				Proxel AS (300ppm) (Tank 3)			
DATE TESTED	*Cladosporium resinae* +1	*Paecilomyces variotii*	*Penicillium* spp. *4	Mean	*Cladosporium resinae* +2	*Paecilomyces variotii*	*Penicillium* spp. *5	Mean	*Cladosporium resinae* +3	*Paecilomyces variotii*	*Penicillium* spp. *6	Mean
31 October 1984	404	17	361	261	0	0	1	0	0	0	0	0
14 November 1984	229	47	270	182	0	0	1	0	0	1	8	3
29 November 1984	121	436	63	207	33	833	3	290	0	370	3	124
19 December 1984	158	0	115	91	1	58	27	29	0	83	89	57
24 January 1985	360	174	316	283	8	18	50	25	0	2	8	5
28 February 1985	623	5	417	348	183	18	52	84	5	15	42	21
7 March 1985	1000	0	392	464	156	0	74	77	0	25	9	11
19 April 1985	312	5	329	215	0	0	32	11	0	0	10	3
17 May 1985	108	32	231	124	1	6	6	4	1	2	8	4

* Mean for top, middle and bottom of tanks 2, 3, 4.

Using sign test# +2 and +3 show a highly significant reduction in relationship to +1 ($p<0.01$)

*5 and *6 show a significant reduction in relationship to +4 ($p<0.01$)

See Appendix 6.6

It was unstable in diesel and may have undergone oxidation by dissolved air. It was also evident from the table 6.9 that when Proxel AS was analysed in the same sample of fuel repeatedly at intervals in the laboratory, it was observed to partition continually into the aqueous phase.

The methylene bis thiocyanate, an active ingredient in DML-7, was found to be water soluble and migrated rapidly to the aqueous phase on vigorous shaking. Analyses carried out in the laboratory have demonstrated that in 1:250 water/diesel fuel system, stirred gently, DML-7 at 300 ppm in the fuel produced a level of methylene bis thiocyanate equivalent to 76% DML-7 in the water phase after 4 hours at room temperature. The rate at which the chemical compounds partition from fuel to water will clearly be a factor of great consequence to the treatment of fuel storage tanks, where agitation to increase the partition rate may be impracticable.

It was observed that imazalil and benomyl significantly controlled all the predominant fungi (table 6.12). The untreated tank was highly contaminated by *C. resinae* and *Penicillium* spp. However the *P. variotii* CFU occurred in low numbers and fluctuated in all three tanks. (In the laboratory, imazalil was not effective against *P. variotii*, but inhibited it in the field). Increased numbers of fungal species were isolated from all the three tanks (Appendix 6.4). The majority occurred in small numbers. *Zygorhynchus* sp. occurred infrequently in high numbers in all the tanks. The CFU between replicates did not show much variation (Appendix 6.5). The total water content in the untreated and treated tanks fluctuated slightly and remained below 115 ppm throughout the trial (Appendix 6.4). As benomyl and imazalil were more fuel than water soluble, they may partition slowly and decompose into the aqueous phase, maintaining an effective biocidal activity at water/diesel interface.

Table 6.12 The effects of the biocides benomyl and imazalil on microbiological growth in diesel fuel field trials at Devonport (July 1985 - November 1985): mean number of colony forming units* per litre of diesel fuel.

	UNTREATED CHECK (Tank 4)				benomyl (50ppm) (Tank 5)				imazalil (200ppm) (Tank 6)			
DATE TESTED	*Cladosporium resinae* +1	*Paecilomyces variotii* +a	*Penicillium* spp. *4	Mean	*Cladosporium resinae* +2	*Paecilomyces variotii* *b	*Penicillium* spp. *5	Mean	*Cladosporium resinae* +3	*Paecilomyces variotii* *c	*Penicillium* spp. *6	Mean
5 July 1985	392	185	185	254	8	2	28	13	7	20	61	29
30 July 1985	546	13	359	306	1	42	9	17	0	0	10	3
20 August 1985	121	45	41	69	0	28	3	10	0	0	3	1
3 September 1985	477	71	193	247	0	14	0	5	0	2	0	1
17 September 1985	264	37	368	223	0	0	3	1	0	0	9	3
1 October 1985	321	2	728	350	0	1	6	2	0	31	0	10
16 October 1985	392	10	141	181	0	75	0	25	0	1	1	1
31 October 1985	200	150	186	179	0	17	3	7	0	1	1	1
19 November 1985	500	38	253	264	0	21	8	10	0	2	0	1

* Mean for top, middle and bottom of tanks 4, 5, 6

Using sign test: +2, +3, *5 and *6 show a highly significant reduction in relationship to +1 and *4 ($p<0.01$)

*b and *c show a significant reduction in relationship to *a ($p=0.02$)

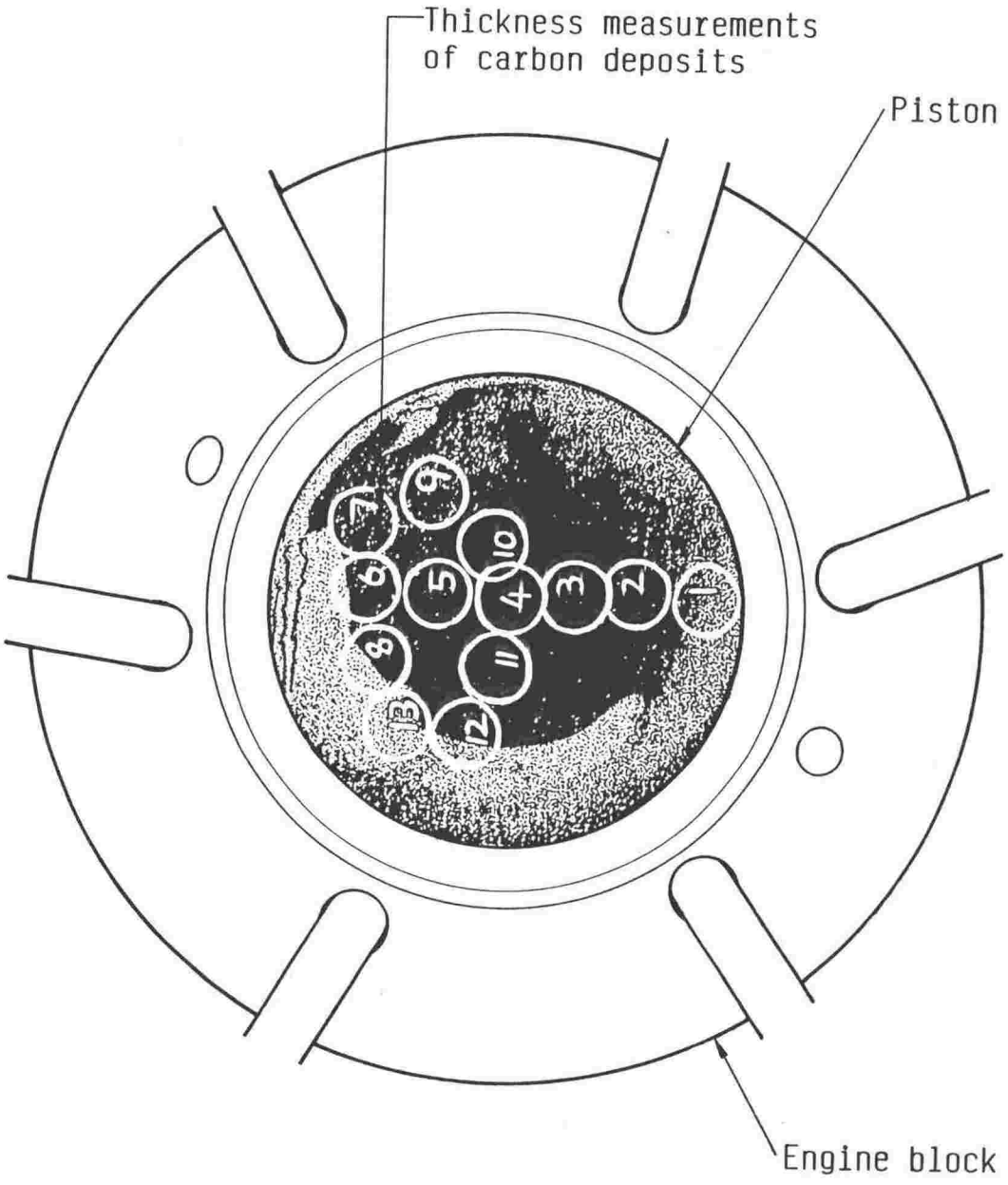
6.2.2 Test on engine performance

In the engine trial test, none of the compounds had a significant effect on the performance characteristics of the diesel fuel from thermodynamic aspects. Refer Appendix 6.7 for graphs on diesel engine additive trials.

The carbon deposit (ash residue) was measured in the form of black sooty deposit formed on the piston as in Figure 6.2. A depth of 70 μm of carbon was recorded when the treated and untreated fuel was used in the engine for all the compounds tested.

6.2.3 Corrosion test

In accordance with the IP154/84 corrosion test method, no significant corrosion was recorded on the copper strip for all the compounds. The test compounds proved to be "1a", almost the same as a freshly polished copper strip.


Thickness measurements of carbon deposits
Piston
Engine block
1
2
3
4
5
6
7
8
9
10
11
12
13

6.3 Discussion

Although *C. resinae* is generally considered to be the most troublesome contaminant of jet and diesel fuel (Hendey, 1964; Berner and Ahearn, 1977), *Paecilomyces variotii* and *Penicillium* spp. can also produce mycelial growths. In this study all the fungi were examined in biocide tests.

Most of the earlier workers (Klemme and Leonard, 1960; Elphick and Hunter, 1968; Rogers and Kaplan, 1968, Hendey *et al*, 1971; Neihof and Bailey, 1978) and the recent work by Smith and Crook (1983) have considered the effects of biocides on *C. resinae* alone.

DEGME, EGME and Biobor JF were included in this work because they were the only known commercial products widely used in the fuel industry. Ethylene glycol alkyl ethers have been known for many years to be bacteriacidal (Berry and Michaels, 1950) and EGME is the form that has become widely accepted for application to jet fuels and it is now commonly used. Neihof and Bailey (1978) reported that of the seven compounds tested, diethylene glycol monomethyl ether (DEGME) was the most suitable replacement since it was effective at 1-2% (in aqueous phase) compared with 10-17% required for EGME. However, most of the other compounds tested had less favourable partition coefficients than EGME and it would be more difficult to ensure that the required anti-microbial concentration accumulated in the water phase. Despite their short-comings, in particular their ability to stimulate growth at low concentration, the ethylene glycol derivatives were widely accepted as they combust completely, are freely fuel miscible, and are readily available.

The other biocidal compounds in wide use since the early 1960s are the organoborates. Borates have long been known to inhibit microbial and enzymic activity (Zittle, 1951) and this activity was found to be greater for organic borate compounds. The activity of organoborates has been investigated by DeGray and Fitzgibbons (1966). Some organoborate compounds were known to precipitate boric acid when treated fuel comes into contact with water and this may cause filter plugging.

One product which is widely used, Biobor JF, was found by Saunders, Wotring and Taylor (1966) not to undergo this precipitation. The recommended concentration for use of Biobor JF was between 135 ppm for continuous use and 270 ppm for shock treatment. Later it became evident that the anti-microbial activity of both EGME and Biobor JF was quite slow, a long exposure time being required for effective use and these compounds were found to stimulate microbial growth at low temperature (Genner and Hill, 1981) in addition to low concentrations.

In the present studies, laboratory tests showed that DEGME, EGME and Biobor JF were ineffective against all the predominant fungi at room temperature. Tests on agar plates showed no effect on hyphal growth. Effectiveness in the presence of diesel was studied and an attempt made to simulate the field conditions. Tests carried out on the agar/diesel culture confirmed that they were ineffective as they were in the BH/diesel medium and in the large volume liquid fuel.

The restriction of extensive hyphal growth along the water/diesel interface observed in the DEGME, EGME and Biobor JF treated and untreated agar slide culture in this study may be a nutritional effect (Smith and Crook, 1980) and may also be due to the high solubility of oxygen in the fuel (Teh and Lee, 1973). Hill, Evans and Davies (1967) have determined experimentally that kerosene in equilibrium with air would in conditions of modest turbulence pass oxygen to an aqueous bottom at a rate and to a level which would satisfy the needs of obligate aerobic micro-organisms. Swatek (1963) mentioned that liquid hydrocarbons like jet, rocket and diesel fuel have adequate oxygen absorbent qualities.

DEGME and EGME were ineffective at both low and high temperatures. All the *C. resinae* isolates from the environment and fuel sources (ex feather, soil, jet and diesel fuel from N.Z. and Australia) were tested in EGME and Biobor JF treated systems and appeared to be insensitive. It became evident that all *C. resinae* isolates (whether isolated in 1960's or 1970's) were insensitive to the compounds. Biobor JF was ineffective against *C. resinae* when used intermittently and in the various proportion of BH and diesel mixtures.

Hill (1978) reported that it was effective when used intermittently with fuel to water ratios of less than 400:1. The possibility that N.Z. *C. resinae* (ex diesel) used in this study was unusual in being insensitive to DEGME was investigated. But results showed all the Australian and N.Z. jet and diesel fuel isolates were all insensitive to DEGME.

DML-7 and Proxel AS were biostatic against *C. resinae* and *P. corylophilum* but not against *P. variotii*. They prevented the formation of a mycelial mat but a spore mat[1] was evident. They showed a relatively high degree of inhibition at a very high dose level of 300 ppm. When the fungal spores were exposed intermittently to Proxel AS and DML-7, *C. resinae* was inhibited at 100 ppm in contrast to 300 ppm during the continuous exposure. At above 50 ppm, Kathon 886 provided total control of *C. resinae*, *Penicillium* spp. and *P. variotii*.

Kathon 886 is fuel and water soluble and maintains an effective biocidal activity at the water/fuel interface. Recently, the Rolls-Royce Ltd. in England have given approval to the use of Kathon 886 as an additive to fuel for industrial, marine diesel engines and also gas turbines (Rohm and Haas Ltd. pers. comm.).

In the agar slide culture experiment, benomyl, imazalil and Kathon 886 in the fuel caused a large reduction in the viability of both spores and hyphae of *C. resinae* at concentration of between 10-50 ppm. Germination of spores was not suppressed but germ tube outgrowths was halted within 3 days of emergence. Suppression of germination in benomyl treated fuel was expected as a mode of action of the biocide is by interference with the mitotic process (Davidse, 1973) and the other compounds must have also acted upon the actively metabolizing cells. Abnormalities like leakage of the cell content were apparent in the benomyl treated conidia. Similar observations were made by Van Gestel (1986) when *Penicillium* spp. spores were treated with azole fungicides. *Paecilomyces variotii* and *Penicillium digitatum* were insensitive to imazalil and benomyl respectively.

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[1] viable concentration of spore inoculum at the fuel/water interface

But it was possible to control *P. variotii* when imazalil was used in combination with benomyl at a low concentration. Hence the efficiency of imazalil could be increased in the presence of benomyl.

Penicillium digitatum is known to cause citrus green mold and was found to be insensitive to control by benomyl in Australia and Japan (Wataru, 1975; Jones, 1977). It was controlled by imazalil at 20 ppm. *P. digitatum* occurred as spores in diesel fuel and was regularly isolated from the atmosphere surrounding the tanks.

The BH/diesel fuel growth method using medical flat bottles was a reliable method of screening active compounds which gave results in 6 weeks and the same results could be obtained within 8 days using conidial suspension in the agar slide/diesel mixture method. The agar slide method may also be used for dose response studies. In the poison plate test, the effect of increasing fungicide concentration on the growth rate of a known amount of fungal mycelia may be directly observed and measured. The 'natural' two litre fuel test system simulated the actual field condition and provided an opportunity to observe the effectiveness of the biocide in natural conditions.

Studies by Neihof and Bailey (1978) indicated that suitable fuel biocides should have water to fuel partitioning values as high as 1120:1. Rapid partitioning into any water present in the fuel system, would possibly cause biocidal loading to be depleted and provide inadequate microbiological protection by the time the fuel reaches the shipboard tanks. Except for benomyl and imazalil, all the other products tested were more water soluble than fuel soluble, thereby having the ability to partition rapidly into the aqueous phase with increasing amount of water. The partition coefficient of a compound is also affected by temperature and agitation (Hitzman, 1964; Elphick and Hunter, 1968). For example the coefficient of EGME in water to fuel at 18°C is 800:1 whereas at 27°C it is 200:1. Thus, at lower temperatures, EGME becomes more concentrated in the water phase and hence its anti-icing properties are enhanced. However, the increase in biocidal activity with increased temperature compensates for this change in partition and the net result is an increase in antifungal activity as temperature rises (Thomas and Hill, 1977).

However, when one considers the possible implication of these factors which may intervene in practical application of biocide to frigates, the situation becomes more complex. Agitation may not be a problem in the storage tank but agitation of the fuel and water phases during sailing will increase the rate of partitioning of the water soluble biocide. On the other hand dilution of biocide-treated fuel with untreated fuel at subsequent refuelling points and leaching of biocide from the fuel and dilution of biocide in the water phase by precipitated or condensed water will serve to diminish biocidal efficiency. Of perhaps even greater importance are the environmental factors which will dictate whether, and to what extent, microbial growth will occur in a particular ship. *C. resinae* and other micro-organisms isolated from diesel fuel systems are capable of rapid growth at normal room temperature, provided water and water soluble mineral nutrients are available.

Smith and Crook (1983) found using 14C labelled benomyl a partition ratio of water to fuel of 10:90. As the partition of benomyl into the aqueous phase was low, the depletion of the biocidal properties by loss of water present would occur only slowly. Imazalil may behave similarly to benomyl being more fuel soluble than water soluble and may have a similar partition ratio. Janssen Pharmaceutica Ltd., (the suppliers of imazalil) have obtained a partition coefficient in an octanol-water system of 4:1 at pH 9. The solubility of imazalil decreases at lower pH and the same effect may be observed in 'dieso'-water system.

The pH in the BH/diesel fuel phases was closely monitored, although none of the compounds affected the pH at the end of 6 weeks. Hill (1982) suggested that biocides particularly those functioning by virtue of a formalin release mechanism have little activity against yeasts and filamentous fungi, and these organisms may present a serious secondary infection hazard after the more vigorous bacteria have been suppressed, as the result of the change in pH in the last system. To guard against this contingency, a mixture of biocides may be used and also it is essential to achieve an initial high effective concentration to prevent micro-organisms developing resistance to inhibitors by natural selection.

Therefore Miller, Mohan and Strickland (1975) have suggested that a system in which biocide concentration is at least three times the minimum inhibitory concentration (MIC) would be expected to prevent such development of resistance.

In the field, the compounds tested showed variation in their effectiveness towards the predominant fungi, when compared with the results obtained in the laboratory. Biobor JF and DEGME showed significant reduction of *C. resinae* but not *Penicillium* spp. and *P. variotii*. Benomyl and imazalil controlled the major fuel contaminating fungi whereas Proxel AS and DML-7 were inhibitory against *C. resinae* and *Penicillium* spp. but not *P. variotii*.

It may be suggested that the crucial test of the effectiveness of a water/fuel soluble compound is a full scale field exposure evaluation and careful analysis under conditions which arise in practice. The field exposure test under conditions of expected use was the primary criterion for evaluating the effectiveness of biocide treatments and was the standard against which the laboratory biocide test method was calibrated. ~~The majority~~ Most of the published data on biocides have been collected from laboratory studies rather than based on field evaluation. Field trials may prove to be expensive and unpredictable due to the influence of uncontrollable factors. In this work even though the compounds were submitted to the field test after careful scrutiny in the laboratory, some variable results were obtained for DEGME, Biobor JF and imazalil.

Laboratory test data comprised the bulk of the information used in making the decision to proceed with the field test. It must be remembered that the laboratory evaluation of a microbial problem is carried out under conditions that attempt to simulate field conditions. These conditions necessarily differ from the wide spectrum of field conditions in order to meet workable and practical laboratory limitations. It is, therefore, not possible to accurately predict the performance of a biocide in the field on the basis of its laboratory performance.

DML-7 and Proxel AS have a biostatic effect against *C. resinae* and *Penicillium* spp. in the laboratory and field. A high proportion of both the compounds migrate to the aqueous phase and may decompose to form toxic compounds, to maintain a low level of contamination in water/diesel interface (DSIR per. comm.). It was obvious in the field study, that there was a large fluctuation in the number of CFU recovered from the treated tanks. The fluctuation in the fungal colonies was more evident in the case of biostats than biocides. For example imazalil and benomyl effectively controlled the predominant fungi and such fluctuation was not observed. The fluctuation in the fungal colonies in the tanks could be due to a number of factors: clumping of fungal mycelium and spores, settling effect, interaction among the dominant fungi, seasonal and biocide effect.

The interaction of all these factors and undoubtedly others, suggest that there may well be a very narrow borderline between a set of conditions under which a fuel tank will remain virtually free of microbiological growth and those under which significant growth will occur. When conditions near to this borderline prevail, intermittent use of even a relatively inefficient compound such as DEGME and Biobor JF may be sufficient to swing the balance in favour of trouble-free service. On the other hand, under really severe operating conditions, an even more efficient compound such as Proxel AS or DML-7 may fail to keep the problem in check as the biocide migrates rapidly into the water phase, therefore it may be necessary to be used very frequently. During situations of heavy microbial contamination, imazalil, benomyl and Kathon 886 may be recommended, as these compounds partition gradually into the aqueous phase, maintaining a sterile water bottom and frequent topping would not be necessary.

Micro-organisms can survive in fuel in a dormant state for months or even years without causing any problem, but if the fuel comes in contact with water, the micro-organisms can then proliferate and grow into large masses of mycelial growth which can cause some of the problems previously reported. With continued emphasis placed on good-housekeeping and good fuel-handling practices, it would appear that the microbial problem is subordinate and arises only after continued disregard for established and proven fuel-handling practices.

As none of the tested compounds showed any deleterious effect on engine performance, nor corrosion at fairly high concentration, probably all the seven compounds may be recommended for further vigorous testing.

Tolerance of micro-organisms to compounds like DEGME and Biobor JF may be suspected but there is no direct evidence. It is becoming increasingly important to have alternate biocides like Proxel AS, DML-7, imazalil, benomyl and Kathon 886 to combat the fuel contaminants. Each of these compounds may be selected based on the severity of the condition.

Benomyl and imazalil have many of the advantages of the ideal fuel biocides. They are agricultural fungicides, easily available, active in low concentration, present no corrosion or residue problems, and their by-products on decomposition may be less toxic than those of DML-7 and Proxel AS. Thus a higher concentration as a disinfectant treatment or lower concentration for continuous dosage on their own or possibly in combination may be used. They may be good candidates as an alternative to the fuel biocide used at present.

7.

SUMMARY

This study established that:

(i) The predominant contaminants of middle distillate fuel ('dieso') are *Cladosporium resinae*, *Paecilomyces variotii* and *Penicillium corylophilum*.

(ii) *Penicillium* spp. and *Paecilomyces variotii* are capable of growing in diesel fuel and have the potential to cause major problems in the fuel systems in addition to *Cladosporium resinae*.

(iii) Interactions exist among the predominant microbial contaminants.

(iv) Biocides effective against *C. resinae*, *Paecilomyces variotii* and *Penicillium* spp. are available.

The present investigation showed that the storage tanks surveyed at Devonport, Auckland were contaminated with micro-organisms. *Cladosporium resinae*, *Paecilomyces variotii* and *Penicillium* spp. were consistently isolated and were capable of forming dark mycelial mats at the water/diesel interface. Bacteria occurred infrequently and in low numbers.

Methods developed for the detection of fungi and bacteria showed that a filtration method using membrane filters gave higher colony counts and more reliable results. V-8 juice agar and malt extract agar media poured over the filters were found best for isolating *C. resinae* and other micro-organisms.

There are numerous sources of contamination of storage fuel tanks by *C. resinae* and other micro-organisms. Microbial contamination may occur through breather vents which allow air borne and soil borne spores to enter with rainwater or dust particles. Contamination may also occur from the introduction of contaminated fuel into the fuel distribution system especially from the supplier and the tank cleaning vessel.

It is evident, although growth of the major fungi was slow, that these fungi may obtain sufficient nutrients from diesel to grow in the presence of water. It is possible that once growth has been established and the organisms have become attached to the walls of the fuel tanks, fragments would be easily dislodged and spores produced would recontaminate fuel indefinitely.

As the fuel tanks were shown to contain more than one fungal contaminant, interaction among the different species of micro-organisms become inevitable. A laboratory investigation showed that *C. resinae* may grow profusely, suppressing the other organisms in the Bushnell-Haas mineral salt medium/diesel fuel phases containing the high and balanced nutrient but not in seawater/diesel fuel, tapwater/diesel and distilled water/diesel phases. But *Penicillium corylophilum* survived prolonged immersion (6 weeks) in seawater/diesel and completely suppressed *C. resinae* and *P. variotii* in a mixed inoculum situation. *C. resinae* grew on the same seawater/diesel phases after the growth and removal of *P. corylophilum* but these two species did not grow together.

The ultrastructure of *Cladosporium* spp. spores showed a thick cell wall of several successive layers. The plasma-membrane, underlying the cell wall was resolved to be a trilamellar structure. Spores contained a single nucleus and many unidentified membrane bound bodies and membrane systems. Light and dark electron-dense bodies inside and outside the vacuoles were common to the species. Microbodies were evident in all the species and no difference in the intracellular structures were observed among the *Cladosporium* spp.

Attempts were made to control microbiological growth in N.Z. navy storage tanks at Devonport using biocides where physical methods of control have become impossible. The effects of commercially available (EGME, DEGME, Biobor JF) and experimental biocides (DML-7, Proxel AS, Kathon 886, benomyl and imazalil) on microbiological growth in diesel fuel were studied. All the available products were screened against *C. resinae*, *Penicillium* spp. and *P. variotii* in the laboratory and field.

EGME, DEGME and Biobor JF were neither biostatic nor biocidal to the fungi in the laboratory but in the field they suppressed *C. resinae* but had no effect on the other micro-organisms present. DML-7 and Proxel AS were inhibitory while benomyl, imazalil and Kathon 886 gave total control of the predominant micro-organisms especially *C. resinae*. All the compounds tested passed the engine performance and corrosion tests.

8.

RECOMMENDATIONS

(i) Regular surveillance and monitoring of the stored fuel for the predominant contaminants is a necessity because this study demonstrated the presence of *C. resinae*, *Penicillium* spp. and *Paecilomyces variotii* in diesel fuel.

(ii) 'Good-housekeeping' procedures should be implemented which eliminate free water from the tanks thereby minimizing the likelihood of microbial growth becoming established. The pumping of water from the separating tank into tank 3 should be completely stopped and the fuel passed through filter systems before pumping into storage tanks.

(iii) The concrete underground tanks may no longer be suitable for storing clean fuel and a complete re-design of the fuel system and storage tanks at Devonport should be considered. Upright steel tanks with conical bases would allow water to settle at the bottom from which it could be drained easily.

(iv) Personnel who are handling and managing the fuel should be made aware of the consequences of microbiological contamination of fuel.

(v) If the fuel is kept free from contamination in the storage tanks, there should be little trouble in the shipboard tanks unless these are water-compensated tanks. Intake of tapwater rather than coastal or harbour water would help to keep the problem in check.

(vi) There is an urgent need to treat all fuel entering the TCV with a biocide before it is pumped back into the shipboard tanks.

(vii) If it is impossible to control microbiological contamination by maintaining a water-free system, the effectiveness of surface active biocides for inhibiting microbial growth and reducing sludge generation in storage tanks and water compensated tanks in ships ought to be considered. The water-soluble inhibitors like DML-7 and Proxel AS may migrate rapidly into the aqueous phase and it might be necessary to replenish the fuel system regularly with the compounds. These inhibitors should be used only in clean tank systems. Biocides such as benomyl, imazalil and Kathon 886, which are active at low concentrations may be used to dose the tanks intermittently or even injected along the fuel line, whenever there is a fresh supply of fuel introduced into the tanks.

(viii) If biocides are used, it is important to monitor the fuel regularly in order to maintain an effective concentration at the fuel/water interface.

(ix) The use of DML-7, Proxel AS, benomyl, imazalil and Kathon 886 in fuel would require clearance by engine manufacturers and other fuel authorities before using in the shipboard tanks.

(x) As a precaution, since most of the compounds are capable of producing toxic by-products in the aqueous phase, it may be advisable to neutralise the compounds before draining the water into the wharf or sea. The chemical manufacturers must provide suitable chemical antagonists that will render the biocides safe for the environment.

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APPENDICES

* Usually the bottom samples are analysed for water contents. The samples were not analysed for water regularly but tank 3 bottom usually contained excessive amount of water.

0 No colonies were observed in that sample.

\+ Numbers refer to the numbers of fungal colony forming units per litre of fuel calculated from counts of the fuel filtered aliquots of each sample.

T 0.6 m from the top of the tank ceiling.

M 2 m below the surface of the fuel.

B 6 m below the surface of the fuel.

V.B. Very bottom, approximately 10 mm from the VB to 300 mm.

Appendix 3.1 Fungi isolated from the different levels of tank 2 between 1982 – 1984: Mean number[+] of colonies forming units/litre of fuel per month.

Fungi	May 1982				July 1982				Aug. 1982				Oct. 1982				Nov. 1982				March 1983				April 1983				May 1983			
	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.
C. resinae	10	5	20	-	31	40	47	-	0	159	80	-	0	45	50	27	13	46	30	15	0	0	0	0	4	40	30	10	3	18	5	13
Penicillium spp.	19	23	11	-	18	11	8	-	218	54	36	-	47	68	70	66	27	39	24	15	25	42	25	20	181	221	59	63	85	87	101	190
P. variotii	13	17	6	-	40	0	14	-	0	20	40	-	633	30	0	-	10	0	10	10	0	20	20	0	0	10	10	1000	0	125	3	0
P. lilacinus	0	0	0	0	0	0	0	0	0	0	0			5											0	0	0	20				
Trichoderma spp.									0	0	20	-																				
Fusarium sp.					0	0	4	-	0	0	20						20	0	0	0									3	5	5	5
Aspergillus sp.																					0	20	0	0								
A. fumigatus																													0	0	0	0
Botrytis sp.																																
Gliocladium roseum																	0	0	0	10												
Basidiomycetes																													0	3	0	0
Rhizopus sp.																	0	0	30	0												
Mucor sp.									40	20	20	-					0	10	505	0												
Yeast													20	0	0	0																
Alternaria sp.					0	4	0	-													0	40	0	0								
C. cladosporioides																																

Appendix 3.1(cont.) Fungi isolated from the different levels of tank 2 between 1982 - 1984: Mean number+ of colonies forming units/litre of fuel per month.

Fungi	June 1983				July 1983				Sept. 1983				Oct. 1983				Nov. 1983				Dec. 1983				Jan. 1984				Feb. 1984			
	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.
C. resinae	10	5	12	3	13	20	13	56	15	10	10	20	20	40	29	30	25	30	25	35	20	28	30	61	8	23	37	13	27	30	14	16
Penicillium spp.	217	69	24	39	90	111	48	213	38	28	38	81	23	29	61	58	57	10	18	50	113	54	97	109	83	208	98	79	147	54	28	29
P. variotii	0	0	0	3	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15	0	8	0	0	0	0	0	0
P. lilacinus							6		50	0	0	0													500	128	125	0				
Trichoderma spp.																					0	0	0	10								
Aspergillus niger									45	33	20	70																				
Aspergillus sp.	0	3	0	0													0	10	0	0												
Chaetomium sp.	0	3	0	0	5	0	0	0																								
Basidiomycetes					10	5	0	0	100	0	0	0	0																			
Rhizopus sp.									0	0	100	90	35	0	0	0																
Mucor sp.																													0	0	25	10
Yeast	0	0	3	100																					0	0	0	3				
Aureobasidium sp.													0	10	0	0	0	0	0	10									3	0	0	0

Appendix 3.1(cont.) Fungi isolated from the different levels of tank 2 between 1982 - 1984: Mean number+ of colonies forming units/litre of fuel per month.

Fungi	March 1984				May 1984				June 1984				July 1984				Aug. 1984				Sept. 1984				Oct. 1984				Nov. 1984			
	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.
C. resinae	0	5	25	0	0	35	15	15	5	0	10	0	17	13	19	47	39	17	30	16	16	22	36	40	24	34	38	112	12	11	15	27
Penicillium spp.	74	232	330	307	368	140	113	37	29	34	26	32	93	25	38	142	222	125	79	233	144	198	1000	870	140	96	132	136	22	17	22	12
P. variotii	0	5	7	0	180	563	5	15	41	43	123	0	10	0	0	17	166	3	2	2	150	2	15	102	0	0	60	50	3	16	76	127
P. lilacinus	0	0	0	20	0	100	0	0	350	238	102	707	0	333	0	0					0	8	0	0	0	0	2	0				
Ulocladium sp.																	0	0	0	2					0	0	20	0				
Basidiomycetes					0	0	0	25													400	0	250	0					100	0	150	0
Mucor sp.																	0	0	0	10	0	4	0	0	0	2	0	0				
Yeast																									2	0	0	0				
Alternaria sp.																																
Aureobasidium sp.																																

Appendix 3.2 Fungi isolated from the different levels of tank 3 between 1982 - 1984: Mean number[+] of colonies forming units/litre of fuel per month.

Fungi	May 1982			July 1982			Aug. 1982			Oct. 1982				Nov. 1982				March 1983				April 1983				May 1983				June 1983			
	T	M	B	T	M	B	T	M	B	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.
C. resinae	17	25	90	83	87	83	160	327	633	242	195	999	420	53	141	642	577	35	178	267	917	90	100	90	243	14	10	32	30	10	9	30	100
Penicillium spp.	16	12	13	35	7	20	102	160	110	61	104	30	27	64	63	40	143	67	98	202	238	215	493	70	110	201	262	181	374	314	224	159	60
P. variotii	19	17	5	50	60	11	310	17	20	333	95	20	20	13	10	0	15	0	0	150	10	60	10	0	10	0	0	0	0	10	9	30	100
Trichoderma spp.							0	20	0																								
Fusarium sp.				0	0	24	0	220	0					15	10	15	0					0	0	0	40					0	0	0	70
Aspergillus sp.							0	120	0	0	20	0	0	45	20	0	0									3	0	0	4				
Chaetomium sp.							0	20	20																					0	10	0	0
Botrytis sp.										0	0	0	40	10	0	0	0																
Gliocladium roseum														0	1000	0	0	0	50	0	0												
Rhizopus sp.										0	20	0	20	20	0	0	50	10	0	0	0												
Mucor sp.																										0	0	0	3	0	0	0	163
Yeast														0	30	30	70	30	0	0	0					0	0	0	3				
Alternaria sp.	0	0	8																											0	0	0	60
C. cladosporioides																																	
Phomopsis sp.																																	
	* 147 ppm			* 147 ppm			* 128 ppm																										

Appendix 3.2(cont.) Fungi isolated from the different levels of tank 3 between 1982 - 1984: Mean number+ of colonies forming units/litre of fuel per month.

Fungi	July 1983				Sept. 1983				Oct. 1983				Nov. 1983				Dec. 1983				Jan. 1984				Feb. 1984				March 1984			
	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.
C. resinae	25	15	7	0	30	0	10	0	20	0	0	0	20	10	30	10	455	238	538	467	261	713	75	740	232	269	407	514	64	118	0	132
Penicillium spp.	250	209	178	259	75	60	85	100	121	155	166	82	142	73	98	125	351	344	294	382	275	319	194	657	108	118	126	220	402	356	138	288
P. variotii					0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	13	3	0	10	30	0	0	500	0
Trichoderma spp.																	0	125	0	0	0	0	25	0					0	0	375	250
Fusarium sp.																	0	0	10	43	3	0	8	28								
Ulocladium sp.																													0	5	0	0
Aspergillus niger																																
Aspergillus sp.	5	20	0	0	50	35	0	0	20	15	0	0																	0	5	0	0
Rhizopus sp.	0	0	3	0																												
Mucor sp.	0	5	0	0	0	0	100	0																								
Yeast					0	0	0	90	0	0	0	100					5	0	0	0					13	0	0	0	3	0	0	0
Alternaria sp.																					0	0	3	0								
Aureobasidium sp.																	25	0	0	0												

Appendix 3.2(cont.) Fungi isolated from the different levels of tank 3 between 1982 - 1984: Mean number[+] of colonies forming units/litre of fuel per month.

Fungi	May 1984				June 1984				July 1984				Aug. 1984				Sept. 1984				Oct. 1984				Nov. 1984			
	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.	T	M	B	V.B.
C. resinae	0	0	0	10	0	0	0	0	0	0	0	0	19	78	8	198	4	10	14	30	44	18	14	0	19	16	12	64
Penicillium spp.	347	210	238	300	47	274	165	203	266	77	260	284	232	284	225	152	684	1000	1000	500	900	530	202	808	144	201	193	186
P. variotii	15	10	150	0	3	10	75	0	10	0	3	3	12	2	100	6	2	2	20	130	2	0	6	112	5	19	171	88
Trichoderma spp.					75	0	282	232	417	0	0	0																
Aspergillus niger																					0	0	0	2				
Aspergillus sp.	5	0	5	10					0	3	0	0	0	0	2	0												
Botrytis sp.																									0	0	0	1
Mucor sp.	0	0	0	15													150	2	4	4					0	0	100	50
Yeast													0	2	0	0					10	0	4	0	0	0	0	50
Alternaria sp.													0	0	0	2												
C. cladosporioides																									0	1	0	0
Epicoccum sp.																									2	0	1	2

Appendix 3.3 Fungi and Bacteria isolated from the separating tank and wharf samples (1982-1984). Mean number of colony forming units/litre of fuel per month.

	Separating Tank																										Wharf Sample		
	Jul.1982		Apr.1983		May 1983		June 1983		July 1983		Sept.1983		Oct.1983		Nov.1983		Dec.1983		Jan.1984		Feb.1984		Mar.1984						
	Top	Bot.+	Top	Bot.	Top	Bot.	Top	Bot.	Top	Bot.	Top	Bot.	Top	Bot.	Top	Bot.	Top	Bot.	Top	Bot.	Top	Bot.	Top	Bot.	Jul.82	Aug.82	Oct.83
Fungi																													
C. resinae	1000	1000	300	338	50	275	150	500	171	96	253	477	40	71	0	0	28	0	0	0	0	0	0	0	93	239	334
Penicillium spp.	450	600	320	290	478	227	1000	1000	1000	1000	325	100	200	0	500	0	81	0	1000	1000	100	0	290	0	65	322	558
P. variotii	0	0	400	300	0	0	0	0	0	0	205	0	0	0	0	0	0	0	0	0	0	0	10	0	24	20	452
Mucor sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	40
Yeast	30	67	0	0	0	0	0	0	0	0	0	100	0	0	55	0	1000	0	0	0	0	0	0	0	8	20	0
Aspergillus spp.	0	0	0	0	0	0	0	0	0	0	100	90	0	30	0	0	0	0	0	0	0	0	40	0	0	0	0
Fusarium sp.	0	75	0	0	0	5	0	0	0	0	0	0	0	30	0	0	0	0	0	0	0	0	0	0	54	0	0
Alternaria sp.	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichoderma sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0
Bacteria																													
Bacillus sp.	100	0	0	0	0	0	0	80	0	0	0	0	0	30	0	0	100	0	0	0	0	0	0	0	19	110	80
Pseudomonas spp.	70	150	0	700	0	0	0	706	0	250	0	275	0	20	178	300	0	0	0	0	0	0	100	0	0	10	0
Micrococcus sp.	0	0	10	0	0	0	0	0	45	90	0	150	70	208	10	0	661	0	172	500	0	0	25	0	0	0	0
Desulfovibrio desulfuricans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1000	0	1000	0	0	1000	1000	0	1000	0	0	0

+ Bot. - Bottom

Appendix 5.1 Partition coefficients of imazalil in the octanol-water system.

Results from the physico-chemical department (Dr J. Peiters, Janssen Pharmaceutica N.V., Belgium).

Buffer System	R23979 (A 09/1)	
	pH	log R
Citrate-phosphate	2.15	0.42
	4.05	1.43
	6.18	3.27
	8.02	3.90
Borate-NaOH	9.82	3.87

R23979 : imazalil (base)

$$R = (m_t - m_w)\, v_w / m_w . v_o$$

m_t : total weight of R23979
m_w : weight in aqueous phase
v_w : volume of aqueous phase
v_o : volume of octanol phase

Remark : the solubility of imazalil decreases at lower pH.

Appendix 6.1 The effect of the additives DEGME and Biobor JF on microbiological growth on Diesel fuel at various levels of the tanks in the field trial at Devonport (April 1984 - August 1984).

Fungi	The number of colony forming units per litre of fuel																										
	17 Apr 84			9 May 84			25 May 84			16 Jun 84			28 Jun 84			17 Jul 84			27 Jul 84			10 Aug 84			28 Aug 84		
	T*	M*	B*	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B
															Tank 4 (untreated check)												
Cladosporium resinae	36	144	22	92	-	60	0	528	498	0	115	203	14	51	23	67	86	80	16	11	57	2	1	50	12	11	645
Paecilomyces variotii	0	0	0	0	-	0	0	20	55	69	9	0	337	57	6	30	0	0	801	675	525	0	0	3	0	305	780
Penicillium spp.	70	46	20	566	-	216	0	160	75	48	5	26	38	33	41	105	129	87	47	176	32	2	3	50	16	47	134
Alternaria sp.	4	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aspergillus sp.	4	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium sp.	0	0	0	0	-	0	0	0	0	0	0	0	2	0	0	0	0	16	0	0	0	0	0	0	0	0	0
Mucor sp.	4	0	0	0	-	0	0	0	0	0	0	0	0	11	11	0	0	0	0	0	0	0	0	0	0	0	0
Trichoderma sp.	0	0	0	0	-	0	1000	616	732	305	416	166	137	97	124	0	0	500	0	0	0	0	0	0	0	0	15

* T - Top M - Middle B - Bottom

Appendix 6.1(cont.) The effect of the additives DEGME and Biobor JF on microbiological growth on Diesel fuel at various levels of the tanks in the field trial at Devonport (April 1984 - August 1984).

Fungi	The number of colony forming units per litre of fuel																										
	17 Apr 84			9 May 84			25 May 84			16 Jun 84			28 Jun 84			17 Jul 84			27 Jul 84			10 Aug 84			28 Aug 84		
	T*	M*	B*	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B
Tank 5 (DEGME)																											
Cladosporium resinae	13	4	4	0	0	4	4	0	0	20	31	22	0	0	2	0	0	0	4	0	1	0	0	6	0	0	0
Paecilomyces variotii	0	0	0	0	8	28	740	200	204	2	41	0	3	0	1	0	0	0	650	450	715	0	15	0	385	235	50
Penicillium spp.	9	4	12	12	31	26	116	32	124	59	57	6	32	30	32	234	693	446	25	18	71	3	2	2	5	12	1
Fusarium sp.	0	0	0	0	0	0	0	0	0	13	0	127	0	4	0	0	0	333	0	0	0	0	0	0	0	0	0
Trichoderma sp.	0	0	0	0	0	0	572	0	752	55	55	212	33	121	0	0	0	0	0	0	10	0	0	0	0	0	0
Mucor sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	0	0
Tank 6 (Biobor JF)																											
Cladosporium resinae	16	8	40	0	0	0	0	0	0	2	2	7	6	12	0	0	0	0	2	2	37	1	1	1	1	1	5
Paecilomyces variotii	0	4	0	8	0	0	370	3	390	6	11	0	9	2	2	37	100	0	685	845	991	3	0	18	675	635	316
Penicillium spp.	156	15	236	24	17	5	100	7	170	0	2	6	74	54	44	95	183	353	173	35	13	1	1	1	31	35	53
Cladosporium cladosporioides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0	0	0	0	0
Fusarium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	6	0	0	0	0	0	0	0	0	0	0
Mucor sp.	4	0	0	0	26	0	0	0	0	0	300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichoderma sp.	0	0	0	0	0	0	0	250	440	261	72	0	0	65	121	0	0	0	0	2	0	0	0	0	125	100	75

Appendix 6.2 The effects of the biocides IML-7 and Proxel AS on microbiological growth in Diesel fuel at various levels of the tanks in the field trial at Devonport (October 1984 - February 1985).

Fungi	The number of colony forming units per litre of fuel																										
	31 Oct 84			14 Nov 84			29 Nov 84			19 Dec 84			25 Jan 85			28 Feb 85				7 Mar 85				19 Apr 85			
	T*	M*	B*	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B	VB*	T	M	B	VB*	T	M	B	VB*
	Tank 4 (untreated check)																										
Cladosporium resinae	325	475	413	54	74	560	62	35	265	30	160	284	0	20	700	375	115	1000	1000	0	0	1000	1000	3	25	219	1000
Paecilomyces variotii	20	18	13	26	1	80	61	476	770	0	0	0	22	300	200	5	5	5	0	0	0	0	0	0	0	5	0
Penicillium spp.	345	613	125	108	77	625	28	22	89	32	100	212	110	136	700	210	200	875	400	111	266	190	1000	81	125	108	1000
Mucor sp.	0	0	0	150	0	0	0	0	0	409	450	325	500	400	220	250	0	0	0	50	92	50	0	0	0	0	0
Epicoccum sp.	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aspergillus major	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Alternaria sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0

* T - Top
* M - Middle
* B - Bottom
* VB - Very Bottom

Appendix 6.2(cont.) The effect of the biocides DML-7 and Proxel AS on microbiological growth on Diesel fuel at various levels of the tanks in the field trial in Devonport (October 1984 - February 1985).

Fungi	The number of colony forming units per litre of fuel																											
	31 Oct 84			14 Nov 84			29 Nov 84			19 Dec 84			25 Jan 85			28 Feb 85				7 Mar 85				19 Apr 85			
	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B	VB*	T	M	B	VB*	T	M	B	VB*
Tank 2 (DML-7)																												
Cladosporium resinae	0	0	0	0	0	0	40	60	0	4	0	0	8	0	0	425	272	15	19	510	61	26	27	0	0	0	0
Paecilomyces variotii	0	0	0	0	0	0	800	900	800	125	0	50	0	0	18	10	0	0	25	0	0	0	0	0	0	0	0
Penicillium spp.	3	0	0	0	0	1	4	5	0	63	9	8	28	78	0	119	54	5	29	191	61	29	13	53	0	0	10
Mucor sp.	0	0	0	0	0	0	0	0	0	150	300	0	210	500	100	0	0	0	0	0	187	0	100	0	0	0	0
Yeast	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0
Aspergillus sp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0
Tank 3 (Proxel AS)																												
Cladosporium resinae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	5	0	0	0	0	0	0	0	0	0
Paecilomyces variotii	0	0	0	1	0	0	735	375	0	0	250	0	2	0	0	25	0	5	0	25	0	0	0	0	0	0	0
Penicillium spp.	3	0	0	16	8	0	5	4	0	205	63	0	12	4	0	65	45	15	0	10	10	7	0	15	10	5	0
Mucor sp.	0	0	0	0	0	0	0	0	0	375	225	0	0	200	0	0	250	0	0	50	0	0	0	0	0	0	0
Rhizopus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	250	0	0	0	0	0	0	0	0	0	0	0
Aspergillus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0

Appendix 6.3 The effects of the biocides IML-7 and Proxel AS on microbiological growth in Diesel fuel field trial (May 1985). Fuel sample replication study.

Fungi	The number of colony forming units per litre of fuel											
	Top				Middle				Bottom			
	*R1	R2	R3	Mean	R1	R2	R3	Mean	R1	R2	R3	Mean
Tank 4 (untreated check)												
Cladosporium resinae	12	0	0	4	3	1	0	1	130	600	223	318
Penicillium spp.	34	276	6	105	181	318	62	187	130	415	655	400
Trichoderma sp.	5	0	0	2	0	0	0	0	0	0	0	0
Paecilomyces variotii	141	13	0	51	111	14	8	44	0	2	5	2
Alternaria sp.	1	3	0	1	0	0	0	0	0	0	0	0
Epicoccum sp.	1	0	0	1	0	0	0	0	0	0	0	0
Mucor sp.	0	200	0	67	0	0	150	50	0	0	0	0
C. cladosporioides	0	0	1	1	2	0	0	1	0	0	0	0
Aspergillus niger	0	0	1	1	1	1	0	1	0	0	0	0
Fusarium sp.	0	0	0	0	0	2	0	1	0	0	0	0
Botrytis sp.	0	0	0	0	0	0	1	1	0	0	0	0
Aspergillus sp.	0	0	0	0	0	0	0	0	1	0	0	1
Pink Yeast	0	0	0	0	0	0	0	0	0	1	0	1
Tank 2 (IML-7)												
Cladosporium resinae	0	2	0	1	0	0	0	0	0	1	2	2
Penicillium spp.	1	10	7	6	11	3	3	6	0	15	0	5
Paecilomyces variotii	2	0	0	1	0	0	0	0	5	43	6	18
Mucor sp.	0	0	0	0	100	0	0	33	100	100	0	67
Alternaria sp.	0	0	0	0	0	3	0	2	0	0	0	0
Tank 3 (Proxel AS)												
Cladosporium resinae	0	0	2	1	0	0	0	0	0	0	0	0
Penicillium spp.	11	17	11	13	6	6	4	5	8	5	2	5
Paecilomyces variotii	1	0	0	1	0	0	0	1	0	1	1	1
Trichoderma sp.	0	400	0	133	0	0	0	0	200	200	210	203
Mucor sp.	300	100	0	133	400	400	200	333	0	0	0	0
Rhizopus sp.	0	200	300	167	0	0	0	0	0	0	0	0
Aspergillus niger	0	1	0	1	0	0	0	0	0	0	0	0

Appendix 6.4 The effects of the biocides benomyl and imazalil on microbiological growth in Diesel fuel at various levels of the tanks in the field trial at Devonport (July 1985 – November 1985).

Fungi	The number of colony forming units per litre of fuel																																	
	5 Jul 85				30 Jul 85				20 Aug 85				3 Sep 85				17 Sep 85				1 Oct 85				16 Oct 85				31 Oct 85				19 Nov 85			
	T*	M*	B*	VB*	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB
	Tank 4 (untreated check)																																	
Cladosporium resinae	225	425	527	-	221	306	780	878	11	41	285	146	33	96	937	843	1	5	512	617	33	5	447	800	0	1	1000	567	0	9	494	295	0	0	667	1000
Paecilomyces variotii	490	21	43	-	8	2	37	3	95	70	13	0	3	8	0	17	67	108	5	1	2	1	2	2	13	0	1	38	362	279	25	33	101	17	39	3
Penicillium spp.	122	117	318	-	117	89	594	637	29	45	38	52	208	238	122	279	120	392	467	492	933	906	445	628	2	57	300	319	6	294	105	338	11	157	350	492
Zygorhynchus sp.	8	368	225	-	75	23	42	119	350	233	666	825	266	133	0	33	145	67	0	117	86	24	9	142	200	200	67	400	32	272	133	567	33	8	33	5
Arthrinium sp.	0	0	0	-	6	1	1	0	2	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. cladosporioides	0	0	0	-	0	0	0	0	0	0	0	0	6	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Yeast	0	0	0	-	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	2	3	0	0	0	0	0	0	0	0	0	0
Trichoderma sp.	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	67	0	0	0	0	0	0	0	0	0	167	608	666	170	0
Aspergillus niger	0	0	0	-	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	1	0	0	0	0	0	1	0
Alternaria sp.	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0

Appendix 6.4(cont.) The effects of the biocides benomyl and imazalil on microbiological growth in Diesel fuel at various levels of the tanks in the field trial at Devonport (July 1985 - November 1985).

Fungi	The number of colony forming units per litre of fuel																																			
	5 Jul 85				30 Jul 85				20 Aug 85				3 Sep 85				17 Sep 85				1 Oct 85				16 Oct 85				31 Oct 85				19 Nov 85			
	T*	M*	B*	VB*	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB
																								Tank 5 (benomyl)												
Cladosporium resinae	10	21	0	0	3	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Paecilomyces variotii	0	8	0	0	83	83	0	0	108	6	0	0	0	0	0	0	0	0	0	0	0	3	0	0	153	150	67	0	67	0	0	0	8	73	0	3
Penicillium spp.	57	55	1	1	12	22	0	3	3	6	1	0	4	1	1	0	5	5	1	1	7	18	0	0	0	1	1	0	3	3	4	1	19	8	6	1
Zygorhynchus sp.	17	33	217	0	17	150	0	0	0	0	0	0	16	33	33	0	133	67	0	67	67	67	0	0	0	100	0	0	33	0	17	0	3	0	0	0
Alternaria sp.	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aspergillus niger	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arthrinium sp.	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Yeast	0	0	0	0	0	1	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
C. cladosporioides	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trichoderma sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix 6.4(cont.) The effects of the biocides benomyl and imazalil on microbiological growth in Diesel fuel at various levels of the tanks in the field trial at Devonport (July 1985 - November 1985).

Fungi	The number of colony forming units per litre of fuel																																			
	5 Jul 85				30 Jul 85				20 Aug 85				3 Sep 85				17 Sep 85				1 Oct 85				16 Oct 85				31 Oct 85				19 Nov 85		
	T*	M*	B*	VB*	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB	T	M	B	VB
Tank 6 (imazalil)																																				
Cladosporium resinae	20	7	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Paecilomyces variotii	67	0	11	2	1	0	0	0	0	0	0	0	0	8	0	0	0	2	0	0	6	118	0	0	3	1	0	0	1	5	0	0	8	0	0	0
Penicillium spp.	67	148	26	1	27	12	0	0	7	5	0	0	0	0	0	0	3	26	5	0	0	1	0	0	2	1	0	0	1	2	0	0	1	1	0	0
Zygorhynchus sp.	0	0	0	0	0	67	0	0	0	67	0	0	17	50	0	0	158	33	0	0	21	1	83	0	0	0	0	0	0	0	33	33	0	0	0	0
Aspergillus niger	0	0	0	0	0	0	133	0	0	0	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alternaria sp.	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Yeast	0	0	0	0	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Trichoderma sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	133	0	0	0	0	0	0	67	0	0	0	0	0	0	0	0	0	0	0
Arthrinium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

* T - Top M - Middle B - Bottom VB - Very Bottom

Appendix 6.5 The effects of the biocides benomyl and imazalil on microbiological growth in Diesel fuel field trial (July 1985 - November 1985).
Fuel sample replication study.

Fungi	The number of colony forming units per litre of fuel															
	Top				Middle				Bottom				Very Bottom			
	*R1	R2	R3	Mean	R1	R2	R3	Mean	R1	R2	R3	Mean	R1	R2	R3	Mean
Tank 4 (untreated check)																
Cladosporium resinae	58	54	43	52	70	158	21	28	624	667	527	606	634	664	631	643
Penicillium spp.	170	190	157	172	207	230	349	262	250	191	439	293	439	357	375	390
Paecilomyces variotii	73	151	114	113	48	53	31	44	31	9	3	14	20	2	14	12
Zygorhynchus sp.	175	116	112	134	166	34	183	128	56	150	206	137	289	316	228	278
Arthrinium sp.	2	2	1	2	1	1	1	1	1	1	0	1	0	0	0	0
Aspergillus niger	1	1	1	1	0	0	0	0	2	0	0	1	0	1	0	1
Alternaria	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
C. cladosporioides	0	2	0	1	0	2	0	1	0	0	0	0	0	0	0	0
Trichoderma sp.	56	81	67	68	133	89	22	81	0	22	34	19	0	38	25	21
Yeast	0	0	2	1	0	1	0	1	0	0	0	0	0	0	0	0
Tank 5 (Benomyl treated)																
Cladosporium resinae	2	2	1	2	2	2	3	2	0	0	0	0	0	0	0	0
Penicillium spp.	13	14	10	12	13	11	15	4	1	2	1	1	1	1	1	1
Paecilomyces variotii	46	40	54	47	31	53	25	36	0	1	22	8	0	0	0	0
Zygorhynchus sp.	0	50	45	32	45	78	28	50	33	28	28	21	22	1	0	8
Alternaria sp.	2	3	0	2	1	0	0	1	1	0	0	1	0	0	0	0
Aspergillus niger	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
Arthrinium sp.	2	1	2	2	1	1	0	1	0	0	0	0	0	0	0	0
Yeast	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0
C. cladosporioides	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Trichoderma sp.	0	0	11	4	0	0	0	0	0	0	0	0	0	0	0	0
Tank 6 (Imazalil treated)																
Aspergillus niger	0	0	0	0	0	0	11	4	0	0	0	0	0	0	0	0
Cladosporium resinae	7	2	0	3	0	2	0	1	0	0	0	0	0	0	0	0
Penicillium spp.	26	6	4	12	30	33	1	21	2	9	1	4	0	0	3	1
Paecilomyces variotii	3	3	22	8	1	7	37	15	0	4	0	1	0	0	3	1
Zygorhynchus sp.	24	31	11	22	14	22	14	17	22	33	28	28	11	0	6	6
Arthrinium sp.	1	4	0	2	0	1	0	1	0	0	0	0	0	0	0	0
Trichoderma sp.	0	0	22	7	0	44	0	15	0	0	0	0	0	0	0	0
Alternaria sp.	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0
Yeast	1	4	3	3	1	0	0	1	0	0	0	0	0	0	0	0

Table II.1 The effects of the biocides DEGME and Biobor JF on microbiological growth in diesel fuel field trials at Devenport (April 1984 - August 1984): mean number of colony forming units* per litre of diesel fuel.

	UNTREATED CHECK (Tank 4)				DEGME (3000ppm) (Tank 5)				BIOBOR (270ppm) (Tank 6)			
DATE TESTED	*Cladosporium resinae*	*Paecilomyces variotii*	*Penicillium* spp.	*Trichoderma* sp.	*Cladosporium resinae*	*Paecilomyces variotii*	*Penicillium* spp.	*Trichoderma* sp.	*Cladosporium resinae*	*Paecilomyces variotii*	*Penicillium* spp.	*Trichoderma* sp.
17 April 1984	67	0	45	0	7	0	6	0	21	1	136	0
9 May 1984	76	0	391	0	1	12	23	0	0	3	15	0
25 May 1984	342	25	78	783	1	381	91	441	0	254	92	230
16 June 1984	106	26	26	296	24	14	41	107	4	6	11	111
28 June 1984	29	147	37	119	1	1	31	51	6	4	57	62
17 July 1984	78	0	107	166	0	0	458	0	0	46	242	0
27 July 1984	28	667	85	0	1	605	38	3	14	840	74	1
10 August 1984	18	1	18	0	2	5	2	0	1	7	1	0
28 August 1984	223	362	66	5	0	223	6	0	2	542	40	100

* Mean for top, middle and bottom of tanks 4, 5, 6

p = 0.002 X = 9 n = 9

p = 0.500 X = 4 n = 7

0.254 X = 6 n = 9

X = 5 n = 6 p = 0.109

p = 0.002 X = 9 n = 9

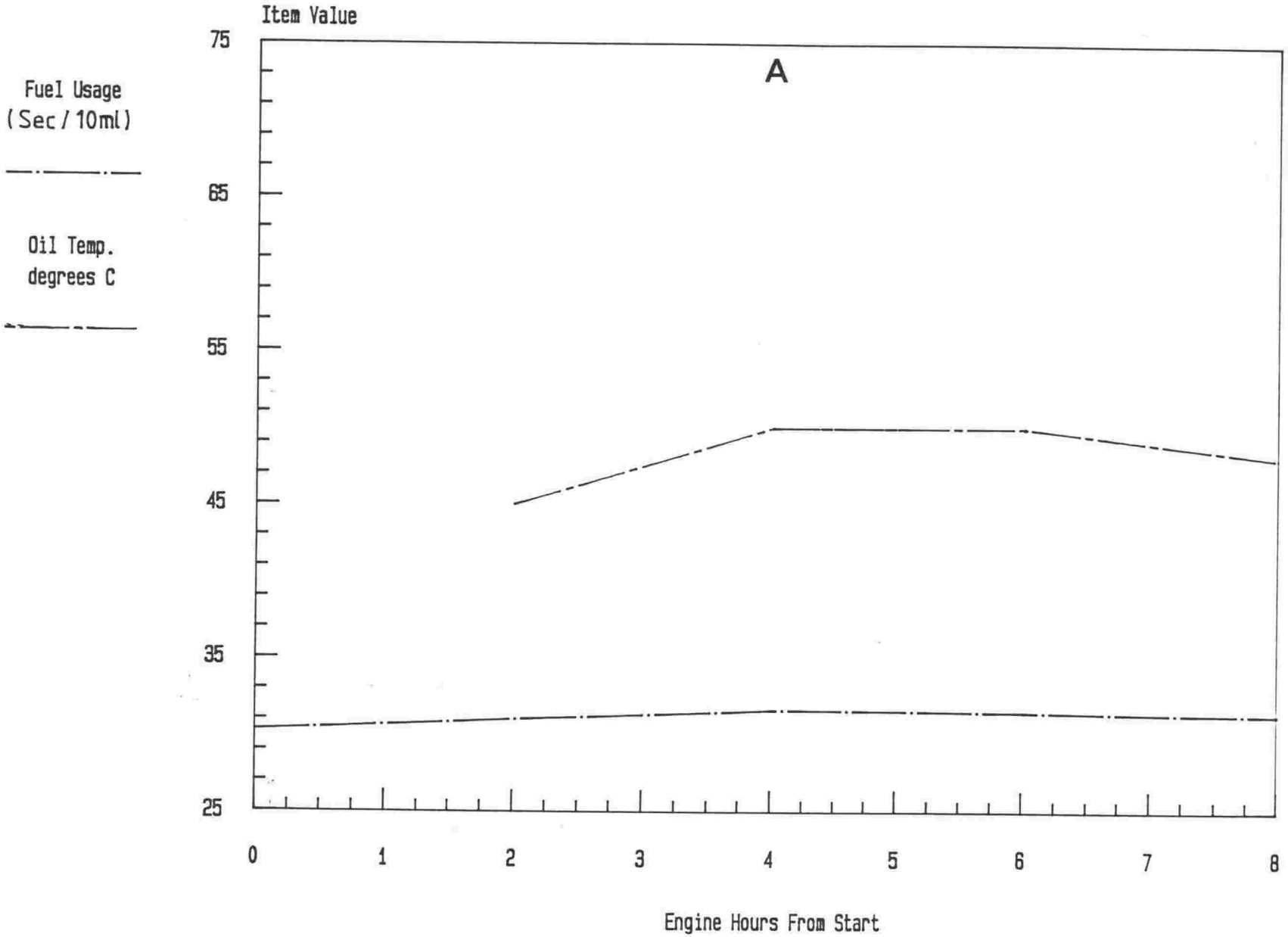
0.980 X = 2 n = 9

0.500 X = 5 n = 9

0.344 X = 4 n = 6

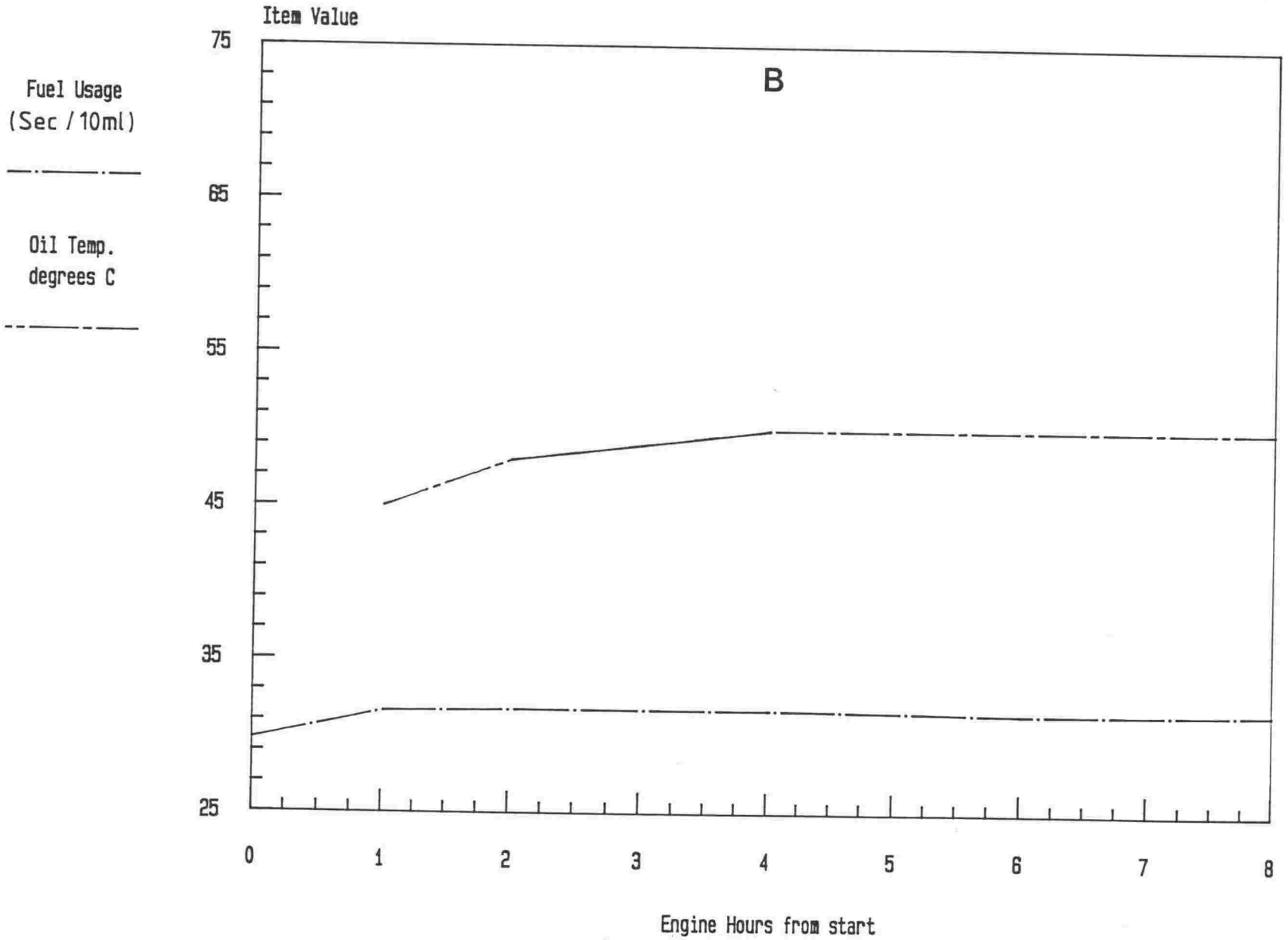
DIESEL ENGINE ADDITIVE TRIALS

Control Diesel (1)



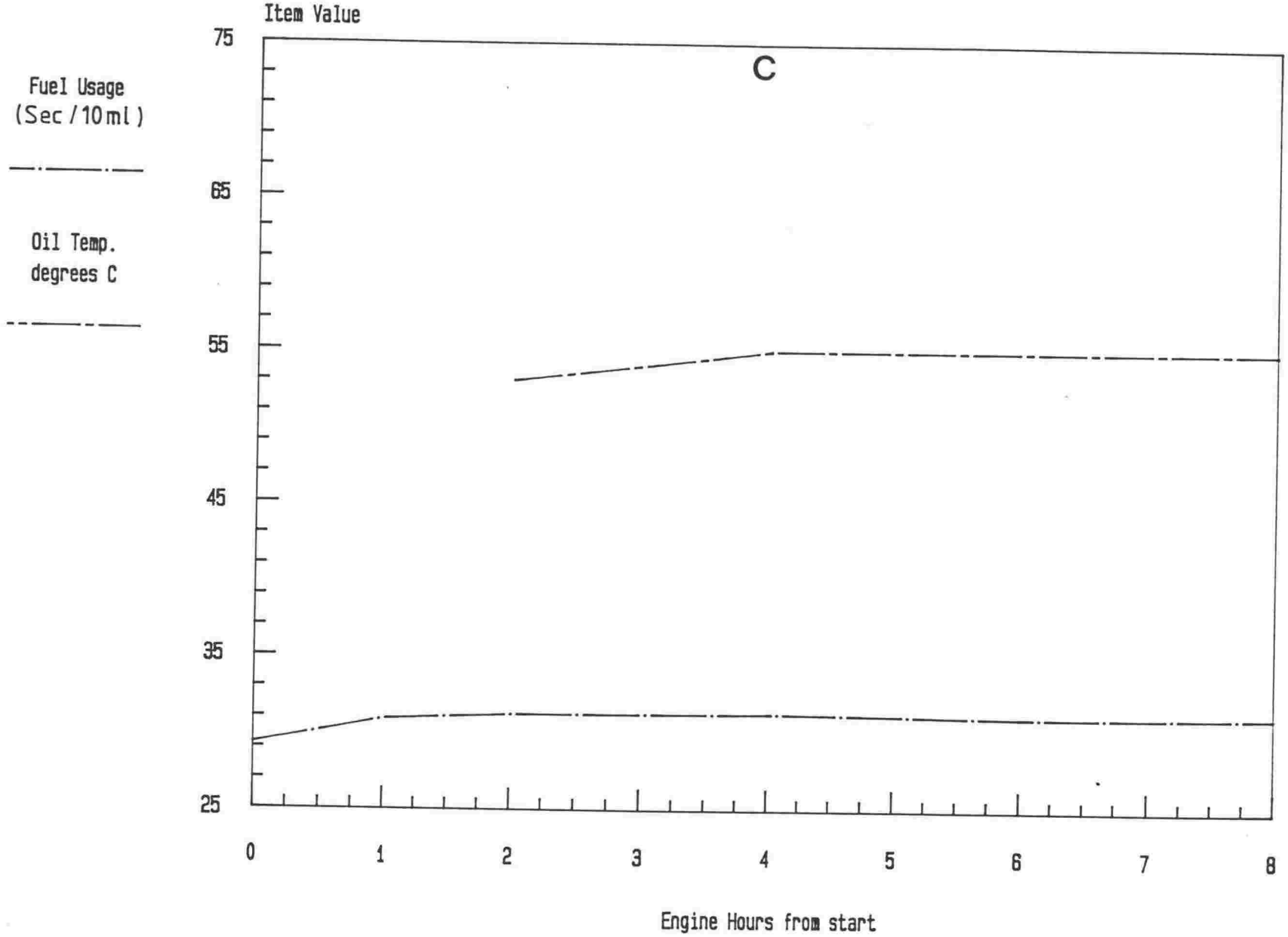
DIESEL ENGINE ADDITIVE TRIALS

Biobor JF 270 ppm



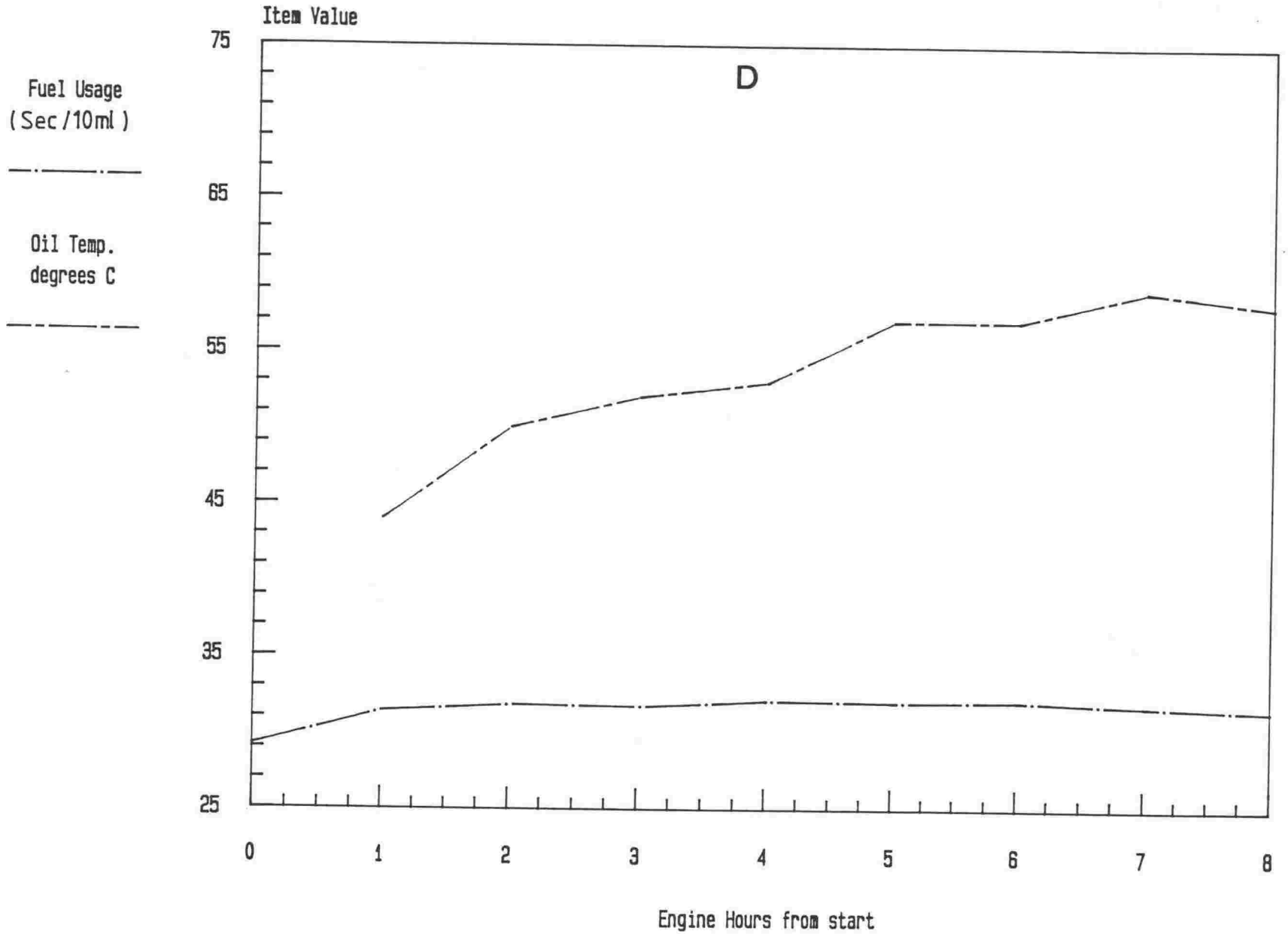
DIESEL ENGINE ADDITIVE TRIALS

DEGME 3000 ppm



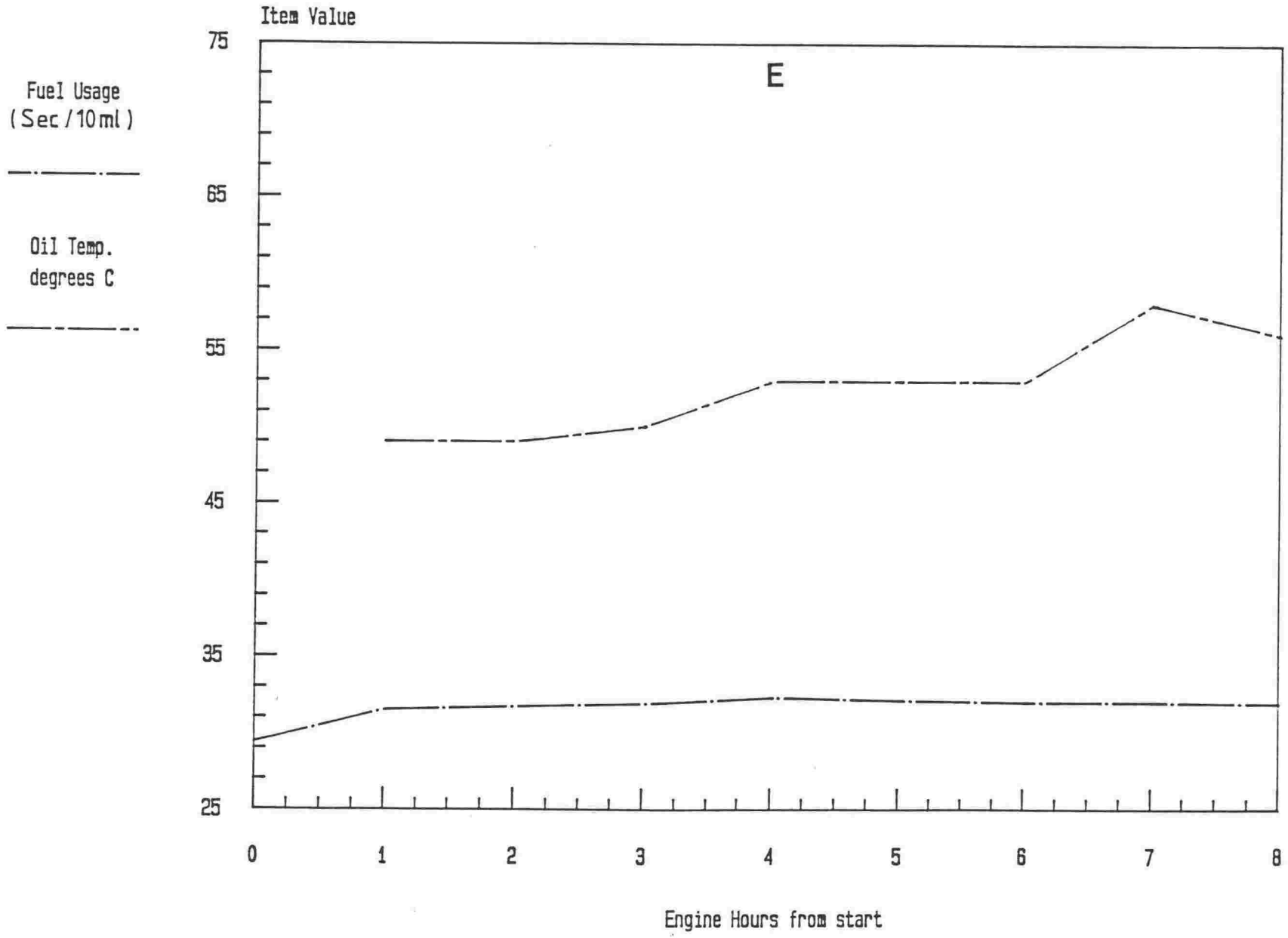
DIESEL ENGINE ADDITIVE TRIALS

Control Diesel (2)



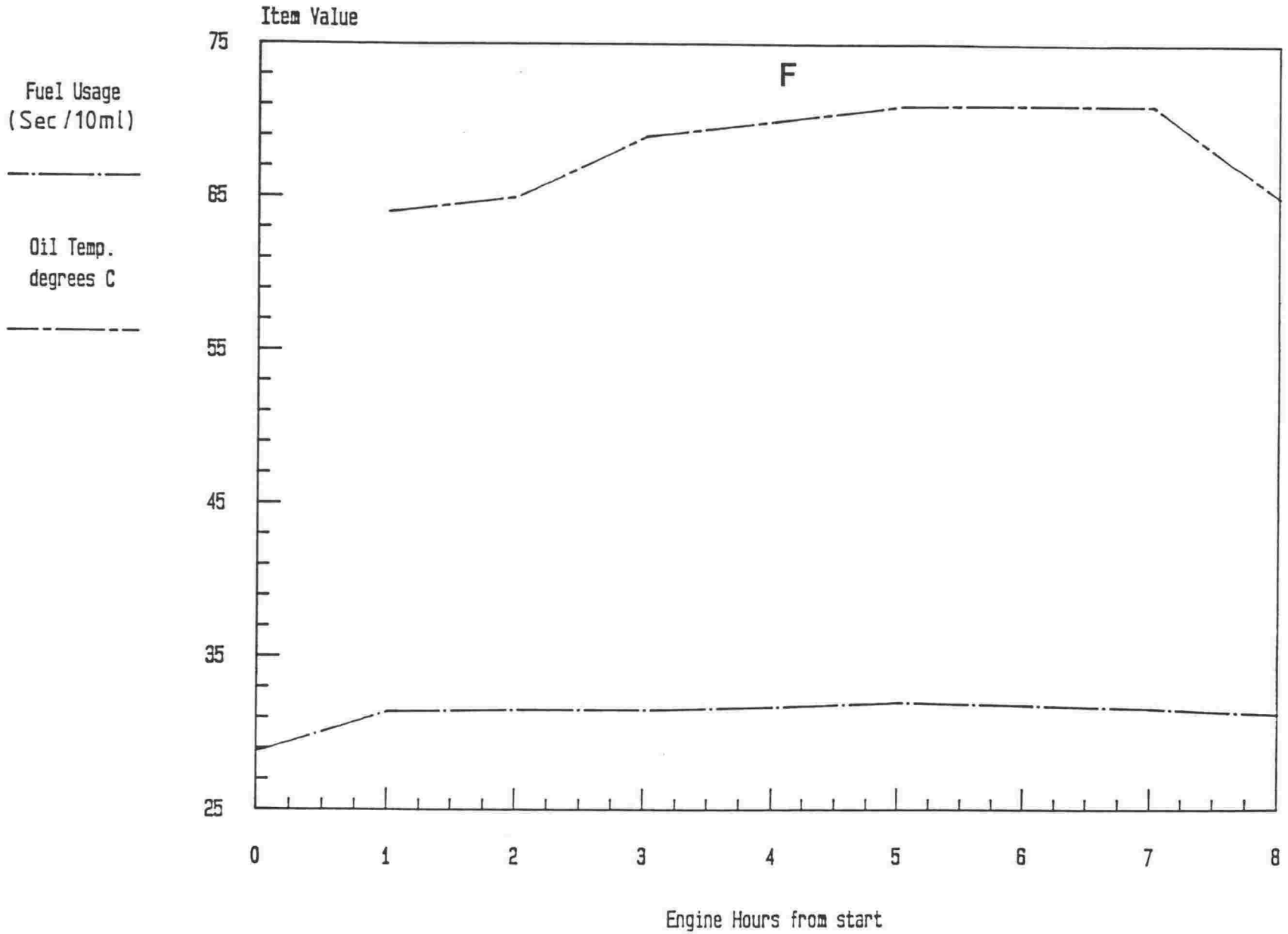
DIESEL ENGINE ADDITIVE TRIALS

DML 7 300 ppm



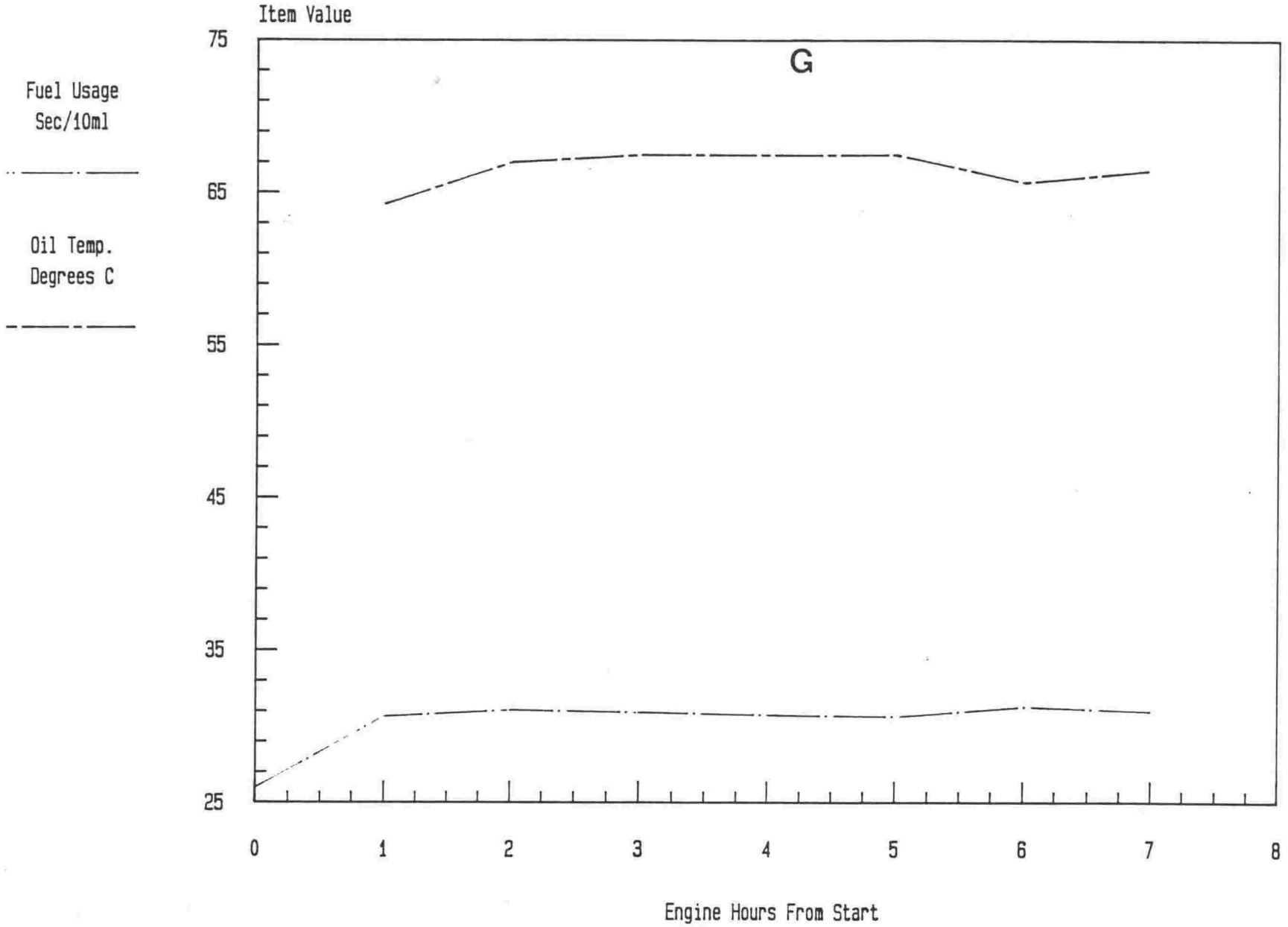
DIESEL ENGINE ADDITIVE TRIALS

Proxel AS 300 ppm



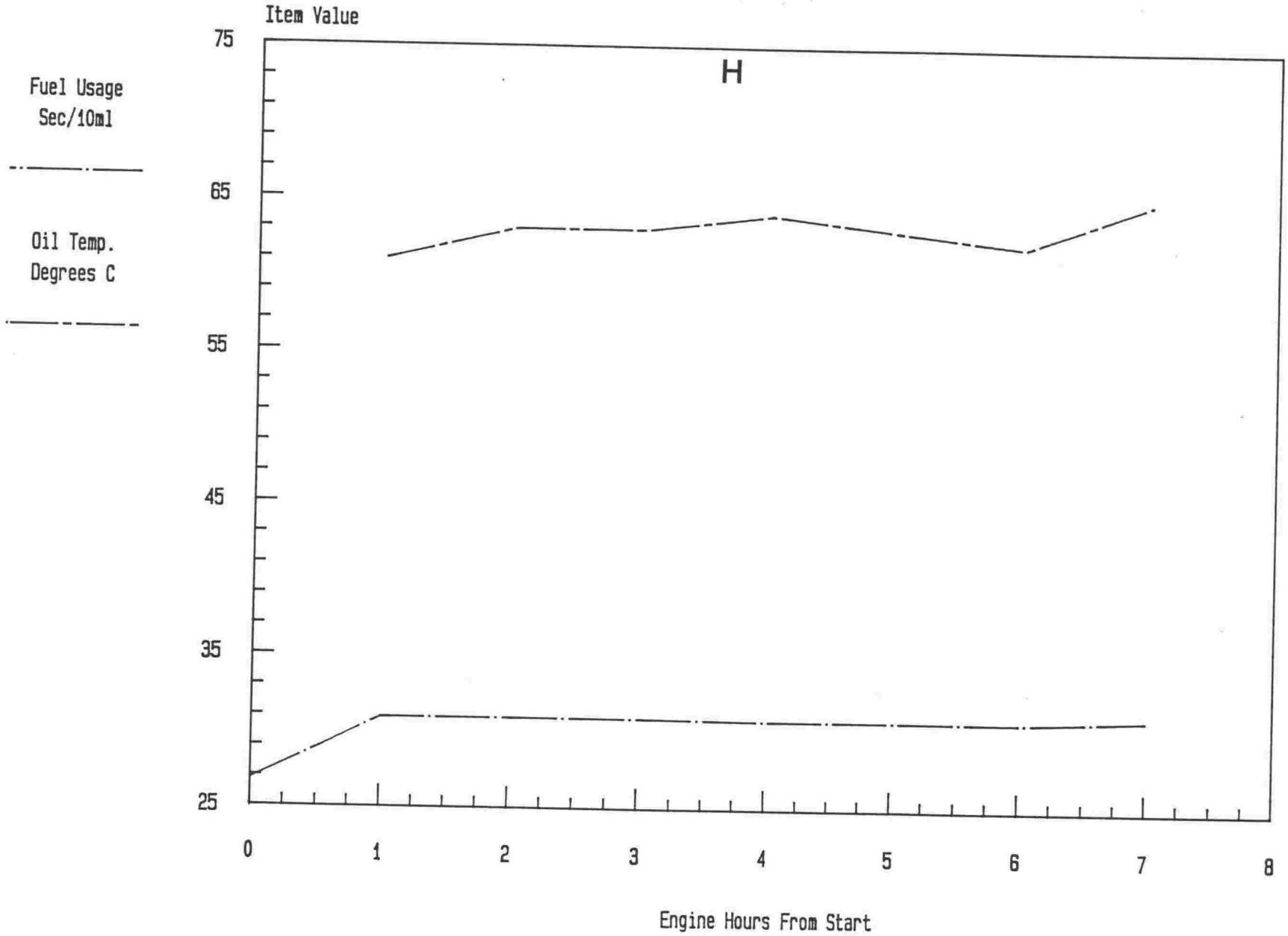
DIESEL ENGINE BIOCIDE ADDITIVE TRIALS

Diesel Control (3)



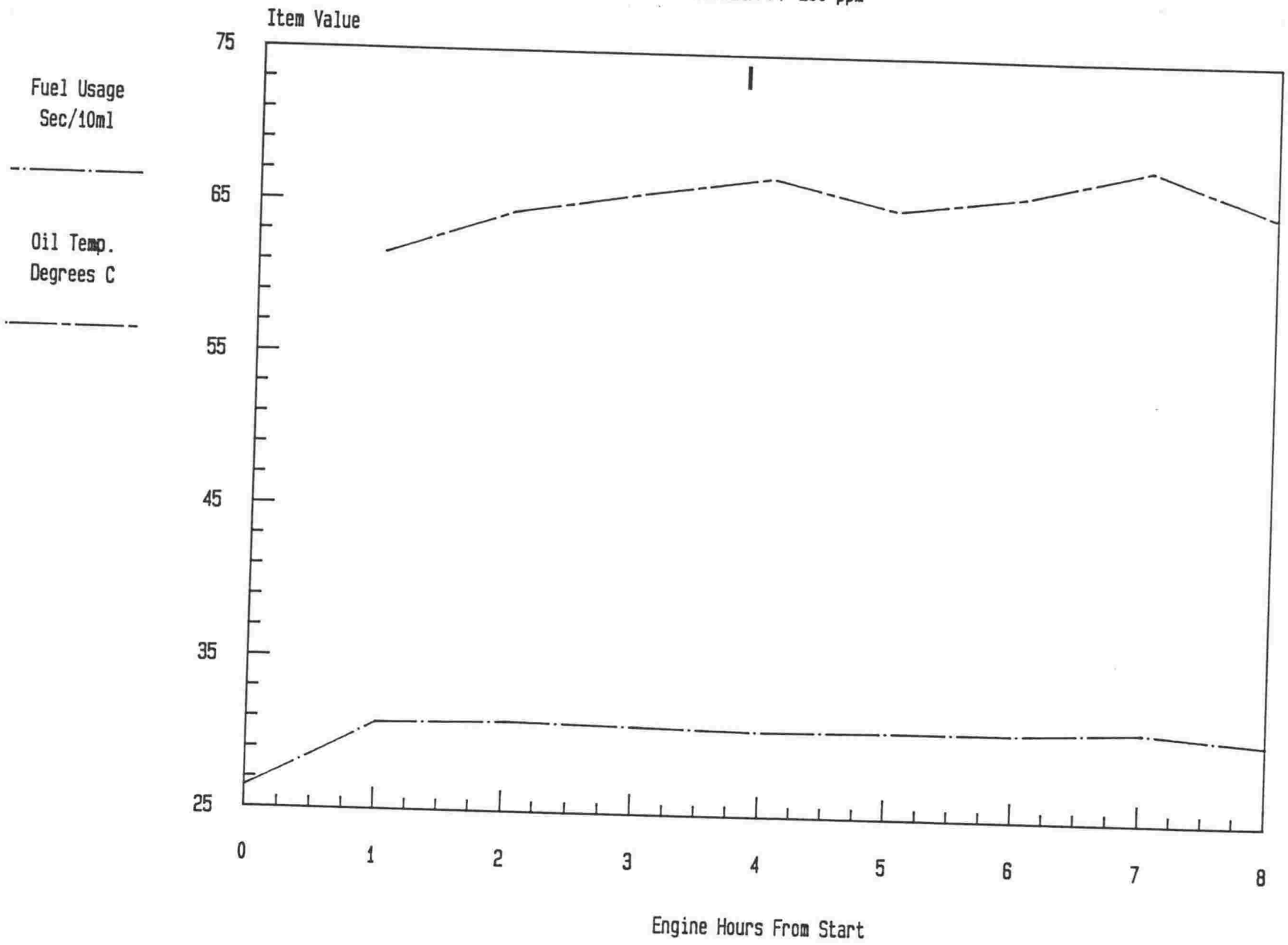
DIESEL ENGINE ADDITIVE TRIALS

Benomyl 50 ppm with DMF



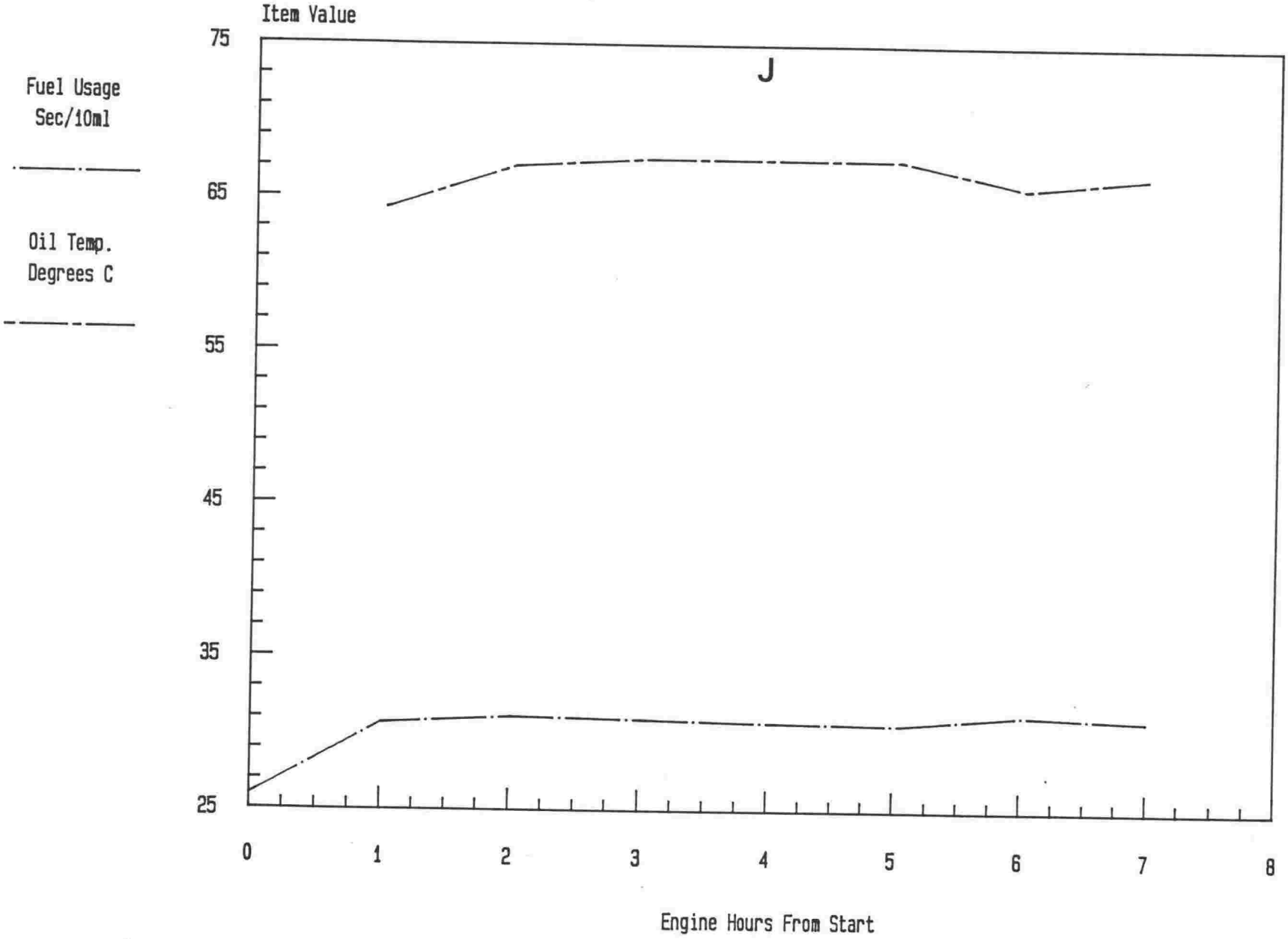
DIESEL ENGINE ADDITIVE TRIALS

Imazalil 200 ppm



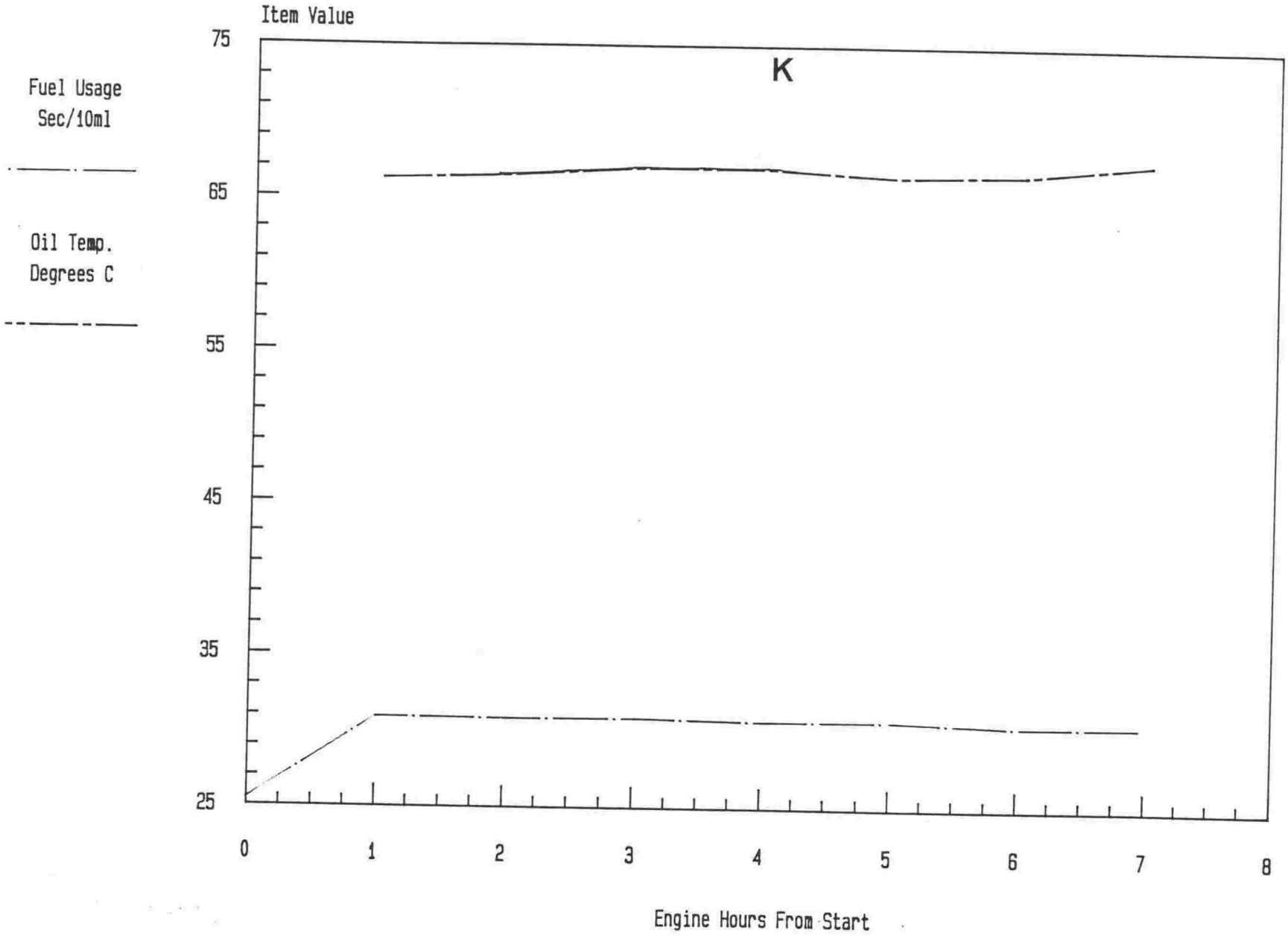
DIESEL ENGINE ADDITIVE TRIALS

Diesel Control 4.



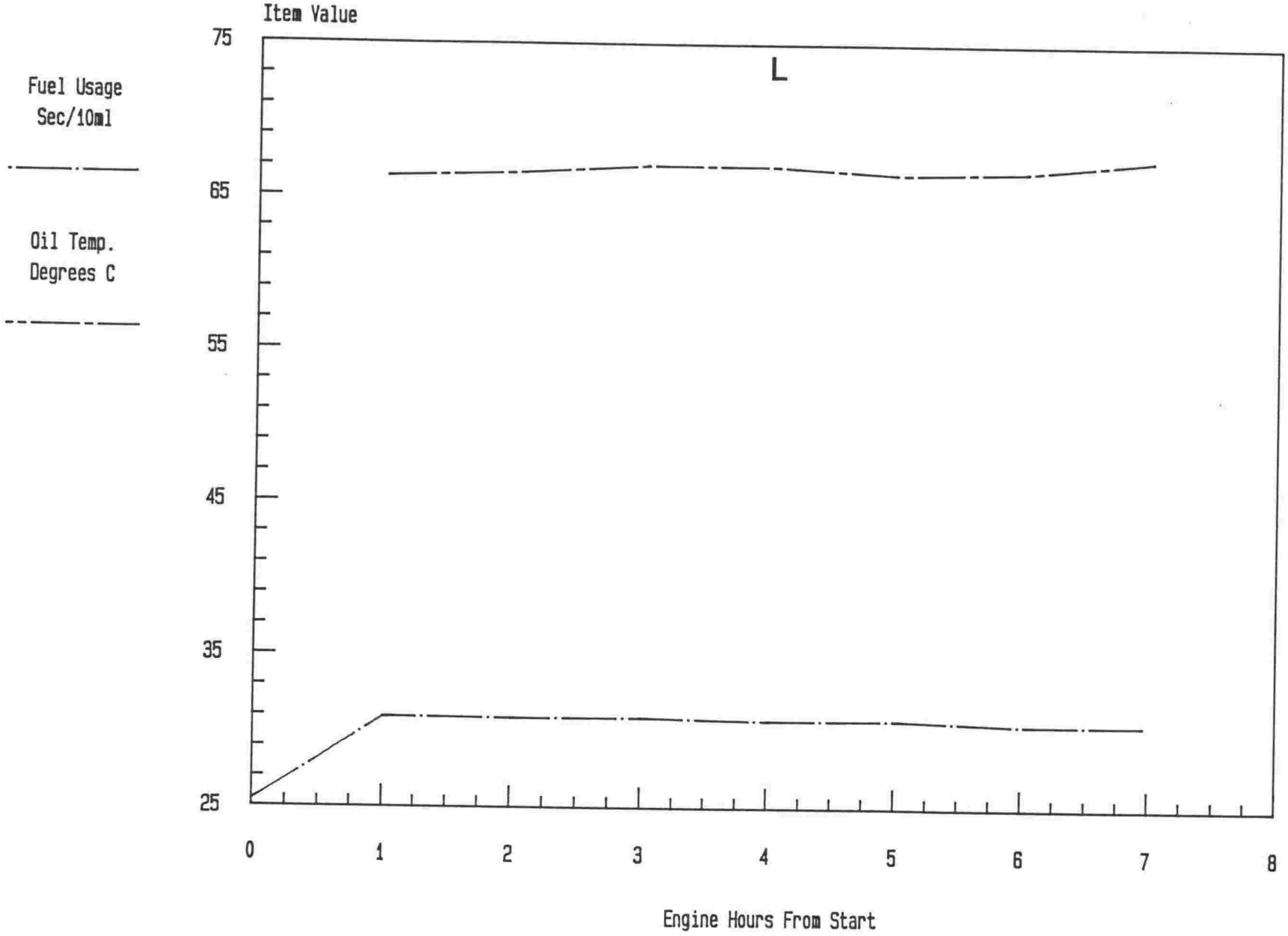
DIESEL ENGINE BIOCIDE ADDITIVE TRIALS

Kathon 100 ppm



DIESEL ENGINE ADDITIVE TRIALS

Kathon 200 ppm



ADDENDUM

1. DESCRIPTION OF THE THREE MAIN CONTAMINATING FUNGI OF DIESEL FUEL ISOLATED IN THIS STUDY

Cladosporium resinae (Lindau) de Vries = *Hormoconis resinae* (Lindau) V. Arx & de Vries comb. nov. Teleomorph: *Amorphotheca resinae* Parbery.

Colonies on malt extract agar (MEA) plates, after 5 days at 25°C, powdery, due to profuse sporulation with little aerial mycelium, hazel to brown in colour with a white margin.

Conidiophores up to 2 mm long, 2.5 - 6 µm thick, smooth. Ramoconidia, when present, clavate or cylindrical, 8 - 20 µm long, 3 - 7 µm thick, smooth. Conidia borne on ramoconidia solitary or in chains of rarely more than 3 conidia, not separated by scars, broadly ellipsoidal or ovoid, 0 septate, brown or olivaceous brown, smooth, commonly 3 - 6x2 - 3.5 µm.

Three other forms have been described (Sheridan, Tan and Nelson, 1972) but only *f. avellaneum* was isolated from diesel fuel in this study.

It has been found that four carboxylic acids were produced by *C. resinae* and identified as dodecanoic, acetic, glycolic and glyoxylic acids (Siporin and Cooney, 1975), which are corrosive to aluminium and its alloy.

Paecilomyces variotii Bainier. Teleomorphs: *Byssochlamys*, *Thermoascus*, *Talaromyces*. Colonies on MEA floccose appearance, usually coloured uniformly brown or olive brown from conidia; reverse pale.

Penicilli borne from aerial hyphae on short stipes of irregular pattern, a cluster of phialides alone or with metulae and phialides; phialides 12 - 20 µm long, tapering gradually. Conidia mostly sub-spheroidal to ellipsoidal, sometimes cylindroidal or pyriform, usually 3.0 - 5.0 µm long, smooth-walled.

P. variotii was said to stimulate the growth of barley seedlings. Contaminated grain proved to be toxic to young ducklings. As the causal agent of paecilomycosis it has been isolated from a generalised infection in a dog, an aborted calf, mycotic stomach ulcer in a calf, kidney infection of a horse, from brain and lung of a turkey and a rat caecum (Domsch, Gams and Anderson, 1980).

Penicillium corylophilum Dierckx.
Colonies on MEA strictly velutinous, mycelium white. Conidiogenesis moderate, dull green, clear exudate occasionally present, reverse pale at the margins, but usually dull green to very dark green centrally. Conidiophores borne from subsurface hyphae, stipes 100 - 250 µm long; rough-walled. Penicilli with two metulae the offset one often longer than the axial, phialides ampulliform, 7 - 11 µm long; conidia spherical to subspheroidal, commonly 2.5 - 3.0 µm diameter, smooth-walled.

P. corylophilum was reported to degrade starch and grows on media with 5% tannin or on substrates with hexadecane, dodecane, octadecane, cyclohexane, toluene, benzene or kerosene as sole C source in the presence of yeast extract. It produces D-xylonic acid, ethylene and viridicatin-related metabolite (Domsch, Gams and Anderson, 1980).

This is the first study to establish that *Paecilomyces variotii* and *Penicillium corylophilum* grow in diesel at the fuel/water interface. Due to the limitation in time, no attempts were made to study the metabolites of *P. variotii* and *Penicillium corylophilum* and this could be a major study by itself. *C. resinae* degrades hydrocarbons and its metabolites has been widely studied.

2. PH DATA ON THE FUNGAL GROWTH STUDIES

C. resinae grew at the seawater/diesel fuel interface after *P. corylophilum* had been grown with an accompanying decrease in pH of about 3 pH units (8.3 to 8). It is clear from these results that the normal pH of seawater (8.3) was too high to allow growth of *C. resinae*. *P. corylophilum*, however, was able to grow in seawater/diesel fuel medium.

The reduction in pH accompanying the growth of *P. corylophilum*, appeared to be the major factor in promoting the growth of *C. resinae*.

The pH of BH/diesel fuel, distilled water/diesel fuel, tapwater/diesel fuel remained neutral (pH 7) during and after the growth of the single and mixed cultures. There was no marked change in pH.

3. STATISTICAL ANALYSES

Some of the problems encountered were:-

a) Impossibility of getting large number of multiple fuel samples from Auckland.

b) Irregularity in the fuel transport from Auckland to Wellington.

c) Fluctuation in the number of colony forming units even when continuous replicate samples were taken from each level at the same time.

Considering the above factors, statistical analysis may have distorted the raw data.

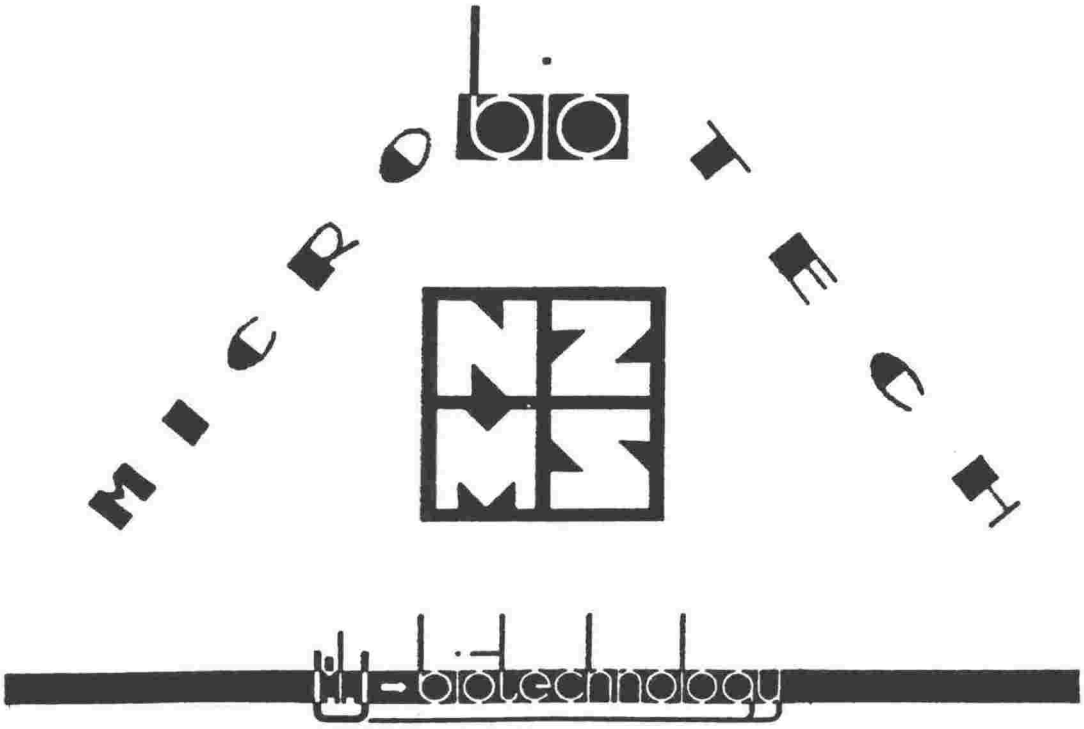
According to Mr. R.M. Renner (Statistician), statistical modelling was not used in this study because the distributions of the colony-forming units controlling for concentration and other explanatory variables, were not known and too erratically skewed to suggest plausible transformations. Further, certain main effects that were observed appeared to be non-linear and convex, precluding monotonic transformations. Certain non-parametric tests were appropriate and consequently employed (see page 190).

Reference

Domsch, K.H., Gams, W., and Anderson, Traute-Heidi. 1980. Compendium of soil fungi. Volume I. Academic Press, London.

Sheridan, J.E., Tan, Y.L., and Nelson, J. 1972. Studies on the 'kerosene fungus' *Cladosporium resinae* (Lindau) De Vries. Tuatara 19:130-165.

Siporin, C., and Cooney, J.J. 1975. Extracellular lipids of *Cladosporium (Amorphotheca) resinae* grown on glucose or on n-alkanes. Applied Microbiology 39(5):604-609.



PROCEEDINGS

OF THE COMBINED
29th N.Z. MICROBIOLOGICAL SOCIETY CONFERENCE
and
17th N.Z. BIOTECHNOLOGY CONFERENCE
at
MASSEY UNIVERSITY, PALMERSTON NORTH
May 27 - 30, 1985

EDITOR
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Department of Microbiology & Genetics
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THE EFFECT OF BIOCIDES ON MICROBIOLOGICAL GROWTHS IN MIDDLE DISTILLATE FUEL

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Problems involving blockages and fuel gauge failures have occurred in diesel fuelled New Zealand Navy ships, "The Bounty" (pers. comm.), and in Auckland Regional Authority buses [1]. In a survey carried out during 1982-83 the contaminating fungi in N.Z. navy storage tanks were identified and documented [3]. The predominant contaminant was Cladosporium resinae as in kerosene type jet fuel [8]. Penicillium spp. and Paecilomyces variotii were also frequently isolated. Bacteria occurred infrequently.

Cladosporium resinae is generally considered to be the most troublesome contaminant of jet and diesel fuel [2]. Laboratory growth studies, however, indicate that P. variotii and Penicillium spp. could produce mycelial growths similar to C. resinae at the fuel/water interface. Efforts should, therefore, be made to control these in addition to C. resinae.

There are several lines of approach to preventing microbiological growths in fuel systems: Good housekeeping, addition of biocides and use of tank linings. A combination of all three is probably the best approach. Good housekeeping and biocides can be used to minimize growth, and tank linings can minimize corrosion should contamination occur. Good housekeeping primarily involves removal of free water from ground storage tanks and on-board ship tanks thereby minimizing the likelihood of microbial growths becoming established. However, in underground fuel installations such as those of N.Z. navy at Devonport, free water cannot be entirely removed because of the design of the system. Inevitably, water is present and the use of biocides must be considered. Corrosion is not a problem because the wall of the underground storage tanks are made of concrete. It could be a problem, however, further downstream particularly in tank ships.

The addition of biocides to jet fuel has been widely studied [5,7] but little work has been done on diesel fuel. The choice of a fuel biocide is restricted due to stringent specifications enforced by engine manufacturers. According to Hill [4] the desirable properties of a biocide are as follows: It must (1) be combustible, with no residual ash, (2) not interfere with the fuel properties on the combustion process, or any other aspect of engine performance, (3) be soluble (or very miscible) in fuel but preferentially soluble in water, (4) present no health hazard during handling or combustion, (5) not be corrosive, (6) preferrably achieve a total 'kill' of micro-organisms at minimum dose. To this list could be added (7) it must be environmentally acceptable (where sea-water is used for displacement). No such biocide has been developed for commercial use. However, a few biocides have achieved market penetration, although deficient in some of the listed characteristics. These are Biobor JF, EGME and DEGME widely used in jet fuel.

The present study involves attempts to control microbiological growth in N.Z. navy underground tanks at Devonport where physical methods of control have become impossible. The effects of commercially available (EGME, DEGME, Biobor JF) and experimental biocides (Proxel AS, DML-7, Kathon 886, benomyl and imazalil) on microbiological growths in diesel fuel were studied in both laboratory and field. All the available products were screened against C. resinae, Paecilomyces variotii and Penicillium spp. So far only DEGME, Biobor, Proxel AS, and DML-7 have been studied in the field.

METHODS

1. Laboratory Tests

(a) Poison plate test:- The biocides were incorporated into 2% molten malt agar (MEA) medium at 50°C at concentrations of 0, 10, 50, 100, 300 ppm (0.001-0.03%), but EGME and DEGME at 0, 1000, 2000, 3000, 4000, 5000 ppm (0.1-0.5%). The units of measurement commonly used are ppm and %, as in the literature and not the metric unit µg/ml. 5 mm diameter plugs of the fungal growth were obtained from two week old cultures on MEA media and were placed centrally in each plate. The diameters of the colonies were measured after 24 hrs and subsequently at daily intervals upto 7 days. Five replicates were made for each concentration.

(b) Liquid culture test:- The activities of the biocides were tested against the mixed inoculum of the predominant fungi in varying concentrations of Bushnell-Haas mineral medium (B/H) and sterile diesel fuel.

B/H medium was prepared according to the method of Parbery and Thistlethwaite [6]. The diesel fuel was sterilized by millipore filtration (0.22 µm) and layered over the B/H medium. The ratio of B/H fuel and fuel:B/H were 5:0, 5:1, 5:2, 5:3, 5:4 and 5:5. The concentrations of benomyl, DML-7, imazalil, Kathon 886, Proxel AS used were 0, 50, 200, 300 ppm. Biobor JF concentrations were 0, 125, 170 and 270 ppm (commercially recommended) DEGME and EGME were 0, 1000, 2000, and 3000 ppm.

The conidial suspensions were prepared from two week old cultures grown on V-8 juice agar at 25°C. B/H medium (20 cm^3 containing 0.1% Tween 80 to aid dispersion of spores) was shaken over the V-8 juice agar culture and the resulting suspension centrifuged and resuspended in B/H. 0.1 cm^3 of the spore suspension was inoculated into B/H:fuel containing the biocides.

The tests were done in 200 cm^3 medicine flat bottles (sterilized in a hot oven at 200°C for 3 hrs). The bottles were incubated in the laboratory for six weeks. At the end of the growth period, the growth was evaluated visually.

2. Field Test

Biobor JF and DEGME were added at concentrations of 270 ppm and 3000 ppm respectively DML-7 and Proxel AS at 300 ppm to small uncoated steel tanks at Devonport containing 25,500 litres of diesel fuel. An untreated check tank was also used. Samples were collected into sterile preserving jars and tested fortnightly for over 3 months.

RESULTS

1. Laboratory Tests

Imazalil prevented growth of C. resinae and Penicillium spp. at 50 ppm but not Paecilomyces variotii. Kathon 886 was effective on all contaminating fungal growth at 50 ppm and benomyl at 10 ppm. DML-7 and Proxel AS was completely biostatic on the predominant fungi at 300 ppm. They inhibited the formation of mycelial mats but viable spores were present. Biobor JF, EGME and DEGME had little effect on mycelial growths regardless of the concentration (table 1).

2. Field Test

Sign test showed that DEGME and Biobor JF significantly reduced C. resinae ($p < 0.01$), but had little effect on P. variotii and Penicillium spp. (table 2). Proxel AS and DML-7 controlled C. resinae and Penicillium spp. but had little effect on P. variotii, as in the laboratory. Isolates of C. resinae from DEGME and Biobor JF treated tanks grew vigorously at all concentrations in the laboratory.

Table 1. Growth of *Cladosporium resinae*, *Paecilomyces variotii* on agar plates containing different concentrations of biocides (at 8 days) (laboratory tests).

Concentration of biocides (ppm)	*Cladosporium resinae*						*Paecilomyces variotii*						*Penicillium* spp.					
	0	10	50	100	200	300	0	10	50	100	200	300	0	10	50	100	200	300
Benomyl	3	0	0	0	0	0	3	0	0	0	0	0	3	0	0	0	0	0
Biobor JF	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
DEGME*	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
DML-7	3	3	3	2	1	1	3	3	3	3	3	3	3	3	3	2	1	1
EGME*	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Imazalil	3	2	1	0	0	0	3	3	2	2	2	2	3	1	0	0	0	0
Kathon 886	3	1	0	0	0	0	3	1	0	0	0	0	3	2	1	0	0	0
Proxel AS	3	3	3	2	2	1	3	3	3	3	3	3	3	3	3	2	2	1

0 - no growth; 1 - a little growth (6-10 mm); 2 - moderate growth (11-30 mm); 3 - profuse growth (31-80 mm); * - 1000, 2000, 3000, 4000 and 5000 ppm.

Table 2. The effect of the biocides DEGME and Biobor JF on microbiological growth in diesel fuel field trials at Devonport (April 1984-August 1984). Mean number of colony forming units per litre diesel fuel.

Date tested	Untreated check (Tank 4)				DEGME (3000 ppm) (Tank 5)				Biobor (270 ppm) (Tank 6)			
	*Cladosporium resinae**1	*Paecilomyces variotii*	*Penicillium* spp.	*Trichoderma* sp.	*Cladosporium resinae**2	*Paecilomyces variotii*	*Penicillium* spp.	*Trichoderma* sp.	*Cladosporium resinae**3	*Paecilomyces variotii*	*Penicillium* spp.	*Trichoderma* sp.
17 April 1984	67	0	45	0	7	0	6	0	21	1	136	0
9 May 1984	76	0	391	0	1	12	23	0	0	3	15	0
25 May 1984	342	25	78	780	1	381	91	441	0	254	92	230
16 June 1984	106	26	26	296	24	14	41	107	4	6	11	111
23 June 1984	29	133	37	119	1	1	31	51	6	4	57	62
17 July 1984	78	10	107	166	0	0	458	0	0	46	242	0
27 July 1984	28	667	85	0	1	605	38	3	14	840	74	1
10 August 1984	18	1	18	0	2	5	2	0	1	7	1	0
28 August 1984	223	362	66	5	0	223	6	0	2	542	40	100

Using sign test *2 and 3 show a significant reduction in relationship to *1 ($p < 0.01$).

Engine tests were carried out on all the field tested biocides at Auckland, before incorporating the products into bulk fuel. The engine tests have shown Biobor JF, DEGME, Proxel AS and DML-7 to be acceptable (D.S.E. pers. comm.).

DISCUSSION

Benomyl, Imazalil and Kathon 886 were the most effective of the biocides tested in the laboratory. Imazalil, however, had no effect against P. variotii. These have not yet been tested in the field. DEGME and Biobor JF performed poorly in the laboratory tests but inhibited C. resinae temporarily in the field. The reason for this is unknown. However, the C. resinae isolates from DEGME and Biobor JF tanks grew vigorously when tested in the laboratory. DML-7 and Proxel AS performed well against C. resinae and Penicillium spp. in both the laboratory and field tests, but were less effective against P. variotii. Laboratory studies showed that P. variotii and Penicillium spp. are capable of forming thick mycelial mats at the fuel/water interface. Hence it is important to control these growths in the storage tanks.

There are large fluctuations in the numbers of colonies recovered from the three storage tanks. This could be due to a number of factors:- clumping of fungal mycelium and spores, settling effect, interaction among the dominant fungi, seasonal and biocide effects. Studies are currently underway to determine the cause of the large fluctuation encountered.

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MYCOFLORA OF STORED DIESEL FUEL IN NEW ZEALAND

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Abstract: Twelve monthly samples of diesel fuel from two bulk storage tanks were examined for the presence of fungal contamination during 1982-1983. Over 25 fungal species were isolated. *Cladosporium resinae* was the predominant fungus occurring in 98% of the samples, followed by *Penicillium* spp. (93%). A list of fungi identified and frequency of isolation is given.

Introduction

Microbial contamination of diesel fuel in storage tanks, shipboard fuel tanks and locomotives has been studied by various workers: Stormont (1962), Zeitlow & Hostetler (1962), Flippin, Smith & Mickelson (1964), Wright & Hostetler (1963), Hendey (1964), Neihof (1980), May & Neihof (1981), Turner (1981) and Bruce (1982), but not as widely as for jet fuel.

Cladosporium resinae has been reported as the major contaminant of diesel although recently Bruce (1982) observed *Penicillium* spp. in the fuel/water system and suggested that they might be as important colonizers as *C. resinae*. Whether *Penicillium* spp. are as harmful as *C. resinae* is not yet established.

The corrosive activity of *C. resinae* on aluminium-copper alloy and stainless steel in the diesel/water system was studied by Hansen, Tighe-Ford and George (1981). They found that stainless steel was not attacked but significant corrosion occurred on aluminium-copper alloy.

In New Zealand, major problems with diesel fuel blockages and fuel gauge failures have occurred in navy ships, "The Bounty" (pers. comm.), and in buses (news reports – see Anon 1981) but little work has been carried out to establish the main cause of the problem.

Cladosporium resinae is known to be widespread in aircraft fuel, in soils and airspora in New Zealand (Sheridan 1972) but a survey of microbial contamination in diesel fuel systems in this country has not been made. No publication has actually documented the fungi found in stored diesel fuel.

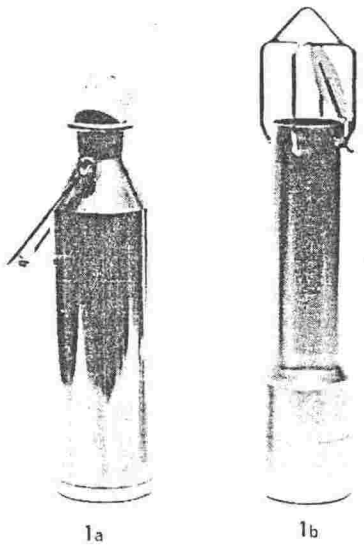
In the studies reported here fungi were identified in monthly samples of diesel fuel collected from two concrete storage tanks sited in Auckland during 1982-83. A list of fungi identified is given together with the numbers and frequency of isolation.

Materials and methods

Sampling techniques

Fuel from the top, middle and bottom of the storage tanks was sampled by a weighted sampling can, shown in Fig. 1a. This is a brass bottle with a fuel holding capacity of one litre, which fills with fuel after being lowered to the appropriate level when the stopper is removed by the captive rope.

Fig. 1a. Samplers



1a 1b

The 'very bottom' of the tank was sampled by a 'zone sampler' also sometimes called the 'thief' as in Fig. 1b. The sampler allows the fuel to pass through as it descends through the fuel in the tank. This is made possible by the top and bottom valves opening due to the pressure of the fuel against the descending sampler. When the sampler becomes stationary, the valves close and the sample is retained as the sampler is returned to the surface.

The fuel samples were immediately transferred into clean, sterile, glass preserving jars in such a manner as to minimize contamination from the environment and transported to Wellington where the microbiological examination was carried out. The sampling bottles and the glass preserving jars were washed in detergent, rinsed with 95% alcohol and sterilized at 200°C in a hot oven for three hours before collection of the diesel fuel. During 1982-83 twelve monthly samples of diesel fuel were collected from each of two concrete storage tanks using the sterile samplers described above.

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Microbiological procedures

Fungal assays were carried out on the samples both immediately and 24 hours after sampling. Later this was extended to 4-5 days to observe any changes in the composition and relative abundance of the mycoflora with time. Because the time of testing of samples after collection did not appear to be critical, immediate testing in Auckland was discontinued.

100 ml of each sample, after thorough shaking, was filtered through a 0.45 μm millipore membrane filter. V-8 juice agar at ≈ 45°C was poured over the filter membrane in a sterile Petri dish. The plate was incubated at 25°C and examined after 5 days. 100 ml of each sample was filtered separately and the membrane filter examined directly under phase contrast to obtain some idea of the spore types present and compare with viable cultures. All tests were carried out in duplicate.

In growth experiments, *Cladosporium resinae*, *Penicillium* spp. and *Paecilomyces variotii* were grown separately and as mixed fungal inoculum in sterile diesel fuel/Bushnell-Haas mineral salts medium (B/H). The ratio of fuel:B/H used was 1:1. The diesel fuel was sterilized by millipore filtration (0.22 μm millipore membrane filter). The conidial suspensions were prepared from two week old cultures grown on V-8 juice agar at 25°C. 20 ml of B/H medium was shaken over the V-8 juice agar culture and the resulting suspension centrifuged in a 15 ml sterile glass centrifuge tube at 1520 x g for 20 mins. 0.1 ml of the above fungal spore suspensions were inoculated into the fuel:B/H (approximately 500,000 spores/ml). The tests were set up in 200 ml medicine flat bottles (sterilized in an oven at 200°C for 3 hours). The aluminium caps with the rubber liner removed, were screwed on loosely and the bottles incubated at 25°C for six weeks. At the end of the growth period, growth was assessed visually. Where there appeared to be little or no growth the contents of the bottles were filtered through 0.45 μm membrane filters, partially wet in sterile water and diesel fuel. Lactophenol cotton blue was applied to the filters which were then observed microscopically.

The airspora in the immediate vicinity of the storage tank was monitored by a Kramer-Collins 7-day spore sampler (Kramer *et al.* 1976).

Culture Media

The V-8 juice agar was prepared as follows. 20 grams of Davis agar (other brands should also be suitable) was added to 800 ml tap water in a glass vessel which was steamed until the agar melted. To this was added 200 ml of V-8 juice (Campbell's Soups Ltd.). The medium was mixed gently, distributed into medicine flats and autoclaved at 103 kPa (15 p.s.i.) for 10 minutes. Bushnell-Haas mineral salts medium was prepared according to the method of Parbery & Thistlethwaite (1973).

Fungal Identifications

Fungi were examined microscopically and identified using available keys. The Commonwealth Mycological Institute, Kew, England, identified or confirmed identification of a number of species; these are indicated by an asterisk in Table 1.

Table 1. *Fungi isolated from New Zealand stored diesel fuel 1982-83.*

Name of Fungus	Mean number of colony forming units/litre				Percentage frequency of isolation	
	Top	Middle	Bottom	Very Bottom		
Cladosporium resinae (Lindau) de Vries	40	160	972	998		98
*Penicillium corylophilum** Dierckx	50	43	473	550	40	
*Penicillium digitatum** (Pers. ex Fr.) Sacc	1	3	55	200	10	
*Penicillium echinulatum** Raper & Thom ex Fassatiova	3	8	28	70	10	93
*Penicillium frequentans** Westling	7	12	15	25	23	
*Penicillium spinulosum** Thom	10	9	10	13	10	
Paecilomyces variotii Bainier	3	8	300	425		50
Fusarium spp.	4	8	17	24		30
Mucor sp.	0	10	23	11		26
*Paecilomyces lilacinus** (Thom) Samson	0	10	13	7		24
Alternaria alternata (Fr.) Keissler	4	9	18	0		23
*Trichoderma harzianum** Rifai	12	3	1	0		18
Cladosporium cladosporioides (Fresen.) de Vries	5	9	2	0		17
Rhizopus sp.	5	6	0	0		15
Chaetomium globosum Kunze ex Fr.	7	5	0	0		14
Alternaria sp.	4	8	0	0		13
Aureobasidium pullulans (de Bary) Arnaud	6	1	2	1		12
*Aspergillus sejunctus** Bainier and Sartory	0	13	5	0		11
Yeast	0	1	10	17		10
Unidentified	2	5	4	1		9
*Aspergillus versicolor** (Vuill.) Tiraboschi	0	9	0	0		8
Phomopsis sp.	3	8	0	0		7
Aspergillus niger Van Tiegh	0	23	0	18		5
Epicoccum purpurascens Ehrenb. ex Schlecht	5	3	0	0		4
Gliocladium roseum Corda	4	2	0	0		3
Ulocladium sp.	4	1	0	0		3

*Identified or identification confirmed by the Commonwealth Mycological Institute, Kew, England.

Quantitative estimation of the level of contamination in the tanks was made by recording the number of samples in which a particular fungus occurred and the number of colony forming units of the fungus.

Results

A list of fungi showing numbers and frequency of isolation is given in Table 1. All of the monthly samples tested were contaminated with microorganisms. The predominant fungus was *C. resinae* isolated from 98% of the samples. *Penicillium* spp. were isolated from 93% and *Paecilomyces variotii* from 50% of the samples. The highest number of colony forming units (cfu) in the samples was obtained for *C. resinae* where almost 1000 cfu/litre occurred in the bottom and 'very bottom' samples. All the above fungi grew vigorously in diesel fuel/Bushnell-Haas mineral salts medium at 25°C, forming a thick mycelial mat at the interface.

Among the five *Penicillium* spp., *Penicillium corylophilum* had the highest number of colony forming units in the diesel fuel. The *Penicillium* spp. produced separate distinctive and individual colonies on the media.

It appeared from microscopic observations and growth experiments in diesel fuel/B/H that *Penicillium* spp. remain as spores in the presence of *C. resinae*.

During the period when *Penicillium* spp. were observed in high numbers in the atmosphere, there was a corresponding increase in numbers in the fuel. *Alternaria alternata* occurred in abundance on filters observed directly but was infrequently isolated as colonies from the filters on agar plates.

Discussion

Over 25 fungal species were isolated from stored New Zealand diesel fuel during a twelve month period in 1982-83. The most frequently isolated fungi were *Cladosporium resinae*, *Penicillium* spp. and *Paecilomyces variotii*, as in jet fuel (Sheridan & Soteros 1974). There was a gradient in contamination levels of *C. resinae*, *Penicillium* spp. and *Paecilomyces variotii* with the highest concentration at the bottom and 'very bottom'. This was probably due to the greater proportion of water present at these levels.

A 24 hour delay in testing samples after collection and in transportation had little effect on the recovery of the above fungi.

Penicillium spp. generally accompanied *C. resinae* but only the latter formed tough mats of mycelium. Growth experiments indicated some form of interaction between *Penicillium* spp. and *C. resinae* possibly similar to that suggested by Neihoff (1980) for *Candida albicans* and *C. resinae* in seawater systems. *Aspergillus fumigatus* was not isolated from diesel fuel in these studies, although it occurred in New Zealand jet fuel (Sheridan & Soteros 1974).

Microbial interactions on the culture media plates could result in antagonism or masking of some fungi. Direct observations of filters revealed a wide variety of fungal spore types in stored diesel fuel. Airspora studies suggest that many such as *Alternaria, Epicoccum, Penicillium, Ulocladium* and others could have entered the tanks from the atmosphere.

The mycoflora of New Zealand diesel fuel cannot be compared with that of other countries because of unavailability of published data. To our knowledge this is the first report to document fungi found in diesel fuel.

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