INHIBITION OF MICROBIAL COLONIZATION OF SHIPBOARD FUEL SYSTEMS

by

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A thesis submitted to the

UNIVERSITY OF ASTON IN BIRMINGHAM

for the degree of

DOCTOR OF PHILOSOPHY

JANUARY 1982

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Summary

The aim of the investigation was to study the problem of colonization of shipboard fuel systems and to examine the effect of a number of environmental factors on microbial growth and survival in order to find potential preservative treatments.

A variety of microbial species were isolated from samples taken from fuel storage tanks. Bacteria were more numerous than yeasts or fungi and most microorganisms were found at the fuel/water interface. The salinity, pH and phosphate concentration of some water bottoms were characteristic of sea water. Others were brackish, acidic and varied in phosphate content.

Microorganisms were cultured under a number of environmental conditions. After prolonged incubation, the inoculum size had no effect on the final biomass of Cladosporium resinae but the time required to achieve the final mass decreased with increasing spore number. Undecane supported better growth of the fungus than diesel fuel and of four types of diesel fuel, two allowed more profuse growth. With sea water as the aqueous phase, a number of isolates were inhibited but the addition of nutrients allowed the development of many of the organisms. Agitation increased the growth of C. resinae on glucose but inhibited it on hydrocarbons.

The optimum temperature for growth of C. resinae on surface culture lay between 25°C and 30°C and growth was evident at 5°C but not at 45°C. In aqueous suspension, 90% of spores were inactivated in around 60 hours at 45°C and the same proportion of spores of C. resinae and Penicillium corylophilum were destroyed after about 30 seconds at 65°C. The majority of bacteria and all yeasts in a water bottom sample were killed within 10 seconds at this temperature. An increase in the concentration of an organo-boron compound caused more rapid inactivation of C. resinae spores and raising the temperature from 25°C to 45°C significantly enhanced the potency of the biocide.

FUNGI, FUEL SYSTEMS, SEA WATER, FILTRATION, GROWTH

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ACKNOWLEDGEMENTS

I wish to thank Dr D. Allsopp for his supervision and guidance and to acknowledge the help and encouragement given to me by Dr H.O.W. Eggins and Mr D.R. Houghton. Also, many thanks to the staff at the Biodeterioration Centre and at the Central Dockyard Laboratory, especially at Eastney, for their assistance. Thanks go to Mrs S. Lloyd for typing the thesis.

This work has been carried out with the support of the Procurement Executive, Ministry of Defence.

To my wife, Patricia

CHAPTER 1

INTRODUCTION

1.1 The problem of microbial growth in shipboard fuel systems.

1.2 History of microbial growth in fuel systems.

1.2.1 Introduction

1.2.2 Aviation fuel systems

1.2.3 Shipboard fuel systems

1.3 Formation of biomass and exocellular products in shipboard fuel systems.

1.3.1 Introduction

1.3.2 Biomass formation

1.3.3 Formation of exocellular products

1.4 Control of microbial growth in shipboard fuel systems.

1.5 Aims.

INTRODUCTION

1.1 The problem of microbial growth in shipboard fuel systems

A number of ships are propelled by gas turbine engines. According to Trenary (1975), particulate matter in the fuel is removed by filtration to prevent blocking of the fine orifices of the engine control system. Water in the fuel (see section 2.1) may cause problems with combustion or contain sodium - usually in the form of sea water - which when combined with sulphur from the fuel can cause corrosion of the engine turbine blades (Ritchie, 1971). Water is therefore removed from the fuel by gravity settling, centrifugation and water droplet coalescence equipment (see Figs. 1, 2 and 3) fitted in the fuel supply system of ships with gas turbine propulsion.

The filters have a mean pore size of five microns and the coalescers contain a layer of glass-fibre with a mean pore size of one micron (Trenary, 1975; see Fig. 4). Hence, particulate matter and biomass, resulting from the growth of microorganisms in fuel tanks, eventually accumulates in the filters and coalescers and impedes the flow of fuel to the engines. Ultimately, the engines become starved and shut down. Gage (1979, 1980) has reported incidents of gas turbine ships experiencing engine failure which was attributed to filter blockage by microbial biomass.

Normally, loss of power due to fuel starvation by filter blockage is prevented by continuously monitoring the pressure difference across the filters and coalescers. At a critical pressure difference for a given flow rate

(see Fig. 5) fuel is supplied to the engines by alternative systems and the filters and coalescers are replaced. However, frequent replacement of these components is undesirable because of high costs and the need to keep a large store of filters and coalescers aboard the ship.

1.2 History of microbial growth in fuel systems

1.2.1 Introduction

The first account of microbial attack on a hydrocarbon seems to have been a report by Miyoshi (1895) of the penetration of thin layers of paraffin wax by hyphae of the fungus Botrytis cinerea. Störmer (1908) and Söhngen (1913) showed that degradation of aromatic hydrocarbons, petrol paraffin, crude oil and paraffin wax took place in the presence of oxygen, but it was later demonstrated that hydrocarbons could be attacked by bacteria under anaerobic conditions (Beckmann, 1926). In the same year, Bastin (1926) reported the presence of sulphate-reducing bacteria in oil-field waters, and these organisms were confirmed as the major hydrocarbon degraders under anaerobic conditions (Tausson and Aleshina, 1932). It was found that the ability to utilize hydrocarbons was not restricted to a few species only, but was widespread among microorganisms (Bushnell and Haas, 1941; Zobell, 1946). The use of hydrocarbons as engine fuels has meant that hydrocarbon-utilizing micro-organisms have caused problems in the fuel systems of aircraft (Prince, 1961), locomotives (Wright and Hostetler, 1963) and ships (Houghton and Gage, 1979).

1.2.2 Aviation fuel systems

The problem of microbial growth in fuel systems was first encountered by the aviation industry. According to Lansdown (1965) there were unpublished reports of microbial attack on aviation gasoline as early as 1937, but the first published report was not until eight years later (Zobell, 1945). In 1952, a number of fuel pump failures in aircraft operating from the Suez Canal zone were attributed to sulphate-reducing bacteria in the water bottom of an aviation gasoline storage tank (Anon, 1953). A similar cause was identified for severe fuel pump corrosion in aircraft operating from Butterworth in Malaya (Anon, 1957).

With the change from gasoline-driven piston engines to kerosene-powered turbo-prop and turbo-jet engines for military and the commercial aircraft, the anaerobic sulphate -reducing bacteria no longer predominated in aircraft fuel systems. Biomass formed by aerobic microorganisms tended to block apertures and cause fuel pump malfunctions and was widely believed to be the cause of unexplained crashes by early jet aircraft in tropical areas (Parbery, 1971a).

Fuel filter clogging in Boeing B-47 and KC-97 aircraft in 1958 was attributed to the presence of bacterial slimes at the water/fuel interface in fuel tanks (Bakanauskas, 1958). Prince (1961) and Churchill and Leathen (1962) isolated fungi from United States Air Force fuel systems. Leathen and Kinsel (1963) described 109 bacterial and 75 fungal isolates obtained from fuel and

and water samples from United States Air Force bases. Because of their relatively large size compared to bacteria and the ramifying nature of the hyphae, fungi were thought to contribute mainly to the problem of filter blockage (Lansdown, 1965). According to Hendey (1964), Darby et al. (1968), Rubidge (1974) and Park (1975), the fungus Cladosporium resinae was considered to be the most important deteriogen in aircraft fuel systems. The name Cladosporium resinae (C. resinae) has been used throughout the thesis since that is the common usage in the context of colonization of fuel systems. The perfect state of the fungus is Amorphotheca resinae (Parbery, 1969a; see Appendix I).

In addition to fouling of filters, fuel screens and fuel quantity capacitance probes, microbial growth was also thought to cause severe corrosion of aircraft fuel tanks (Ward, 1963; Hendey, 1964).

The environmental conditions in supersonic aircraft are different from those in sub-sonic aircraft. "During supersonic flight, fuel is heated because the fuel acts as a heat sink for the dissipation of heat created by friction due to the high speeds of the aircraft" (Thomas and Hill, 1976). The elevated temperature of the fuel may provide a suitable environment for the growth of thermophilous fungi. Scott and Forsyth (1974) used an enrichment technique to isolate the thermophilous fungus Aspergillus fumigatus from a number of samples and Thomas and Hill (1976) showed that the fungus had the potential to grow in some of the fuel tanks of supersonic aircraft. Colonization by Aspergillus fumigatus would be particularly undesirable because of its association with broncho-pulmonary infections in Man (Cruickshank, 1965).

1.2.3 Shipboard fuel systems

The problem of microbial growth in shipboard fuel systems became apparent with the introduction of marine gas turbine engines as propulsion units for warships and the requirement for filters and coalescers to remove particulate matter and water from the fuel. Gas turbine engines have a number of significant advantages over the conventional steam plant as propulsion units for major warships, which include:

a) Rapid start-up from cold

b) Low maintenance requirements

c) Low manning requirement

d) Precise and rapid response to controls (Vallis, 1973)

A number of navies have gas turbine warships, and in the Royal Navy, four classes of major warship have this type of propulsion as sole power source (Moore, 1980):

a) Type 21 (Amazon) class frigates (Fig. 6)

b) Type 42 (Sheffield) class destroyers (Fig. 7)

c) Type 22 (Broadsword) class frigates (Fig. 8)

d) 'Invincible' class lightweight aircraft carriers (Fig. 9)

In addition, a number of warships use gas turbines in conjunction with conventional steam propulsion systems; 'County' class destroyers (Fig. 10), Type 81 (Tribal) class frigates (Fig. 11) and the single Type 82 class guided-missile destroyer, HMS Bristol (Fig. 12).

Filter blockage by microbial biomass in Type 21 frigates has been reported by Gage (1979). HMS Sheffield, a Type 42 destroyer, nearly came to a stop in a force-eight gale (Gage, 1979) and Niteroi, a British-designed, all gas turbine warship built for the Brazilian Navy, experienced engine failure during speed trials (Gage, 1980; pers. comm.). According to

Gage (1980), a Type 21 frigate lost three out of four gas turbine engines through over heating. These incidents were attributed to filter blockage by microorganisms.

The detection of large amounts of microbial biomass in fuel systems may require that fuel tanks have to be drained and cleaned in order to avoid filter blockage problems (Houghton and Gage, 1979). However, tank cleaning can take a ship out of service or delay construction, leading to high costs. The Type 42 destroyer, HMS Glasgow, took six weeks longer to commission because the tanks required cleaning (Gage, 1980; pers. comm.).

1.3 Formation of biomass and exocellular products in shipboard fuel systems

1.3.1 Introduction

Given suitable conditions, microbial growth may take place at any stage during the transportation and storage of fuel. Royal Fleet Auxiliary (RFA) tankers (see Fig. 13) are the main means of transporting fuel between refineries, land storage tanks and warships. Approximately 90% of warship refuelling occurs at sea (Wettern, 1979).

Land storage tanks (see Fig. 14) retain diesel fuel up to a maximum period of two years. They may hold either 8,000 or 5,700 tons. In some cases, fuel is removed by means of a suction device which can be positioned just below the level required to refuel a waiting tanker. This procedure avoids contamination by water bottoms which are in any case routinely tapped off every four weeks.

Warships have several kinds of fuel tanks. Fig. 15

shows a simplified layout of a Type 42 destroyer fuel system. The storage tanks may be displaced or undisplaced and are integral with the hull of the ship (see Fig. 16). The physical shape of an integral fuel tank is therefore complex and the presence of internal structural components and pipework makes cleaning a difficult and invariably manual operation.

Displaced tanks are shown in Fig. 17. As fuel is removed, sea water from header tanks is used as compensation, the purpose of which is to maintain the trim and stability of the ship. Fuel is transferred to undisplaced storage tanks by centrifuges.

Service tanks receive fuel from the undisplaced tanks and supply it to the gas turbine engines via the filtration and water removal equipment. Ready-use tanks contain previously filtered fuel and can supply fuel directly to the engines in the event of a failure in the normal supply system.

1.3.2 Biomass formation

The most obvious product of microbial growth in fuel systems is the biomass which is formed by the development of microorganisms in water associated with the fuel. Many hydrocarbon-utilizing organisms have a high affinity for the oil phase and therefore tend to grow into a layer of biomass at the fuel/water interface (see Fig. 18).

The water also becomes turbid due to the growth of microorganisms and detachment from the interfacial layer. Fig. 19 shows the deposition of biomass on the walls and supporting structures of a shipboard fuel tank.

1.3.3 Formation of exocellular products

Growth of *Cladosporium resinae* on hydrocarbons is

accompanied by an increase in the hydrogen ion concentration of the aqueous phase (Cofone et al., 1973; de Mele et al., 1979), due mainly to the production of organic acids (Lin et al., 1971; McKenzie et al., 1976). These can cause corrosion of aluminium alloys (McKenzie et al., 1976) and may impair the water removal properties of coalescers because, acting as surfactants, the acids prevent the aggregation of tiny water droplets into large drops which separate from the fuel under the action of gravity (Trenary, 1975).

1.4 Control of microbial growth in shipboard fuel systems

The requisite conditions for microbial growth are as follows:

a) a viable inoculum

b) an energy source

c) nutrients to provide the essential materials from which biomass is synthesized.

d) suitable physico-chemical conditions

e) absence of inhibitors which prevent growth.

A change in one of these requirements may alter the growth of one or all of the microbial species present in fuel systems. A deliberate alteration in a growth requirement which decreases or arrests microbial growth - or inactivates microorganisms - constitutes a control measure.

1.5 Aims

The aims of this study are:

a) To discover some of the microbial species and their numbers in samples taken from fuel systems (Chapter 2).

b) To determine the salinity, pH and concentration

of phosphate and dissolved oxygen in some water bottoms (Chapter 3).

c) To examine the effect of inoculum size (Chapter 4).

d) To investigate growth on hydrocarbons (Chapter 5).

e) To examine the effect of water bottom composition on microbial growth. (Chapter 6)

f) To elucidate the effect of hydrocarbon concentration, culture volume and agitation (Chapter 7).

g) To investigate the effect of temperature and an organo-boron compound on the growth and survival of microorganisms (Chapter 8).

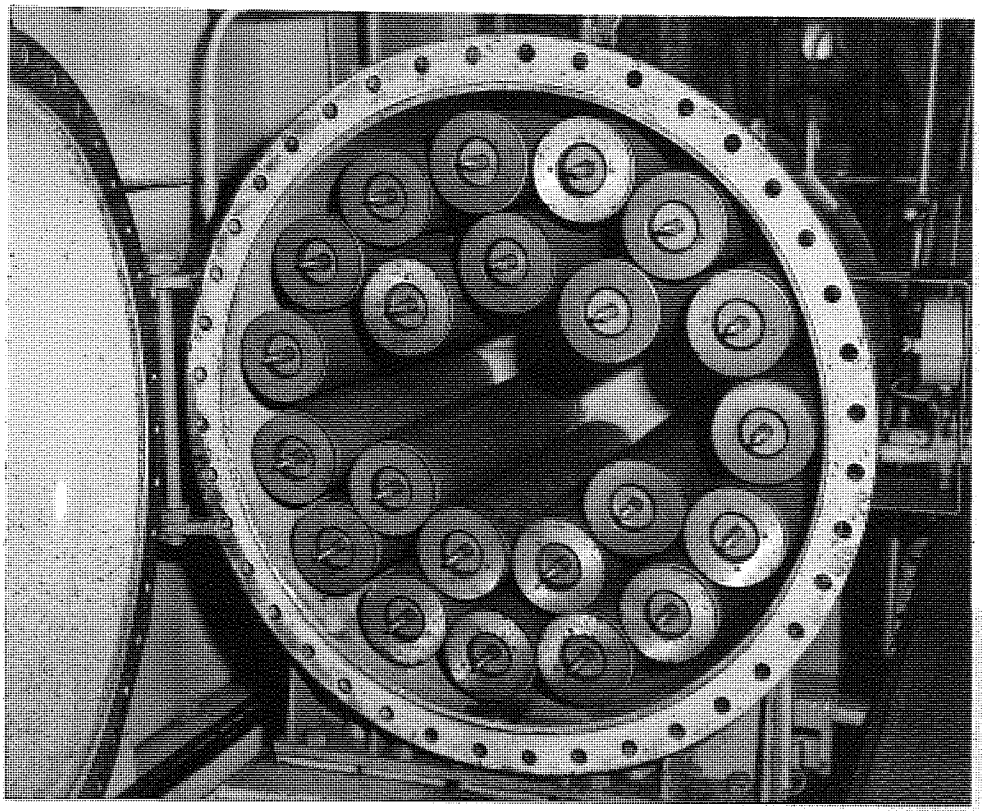

Fig. 1 Water droplet coalescence equipment showing twelve coalescer elements in the lower half of the chamber and ten separator elements in the upper half.

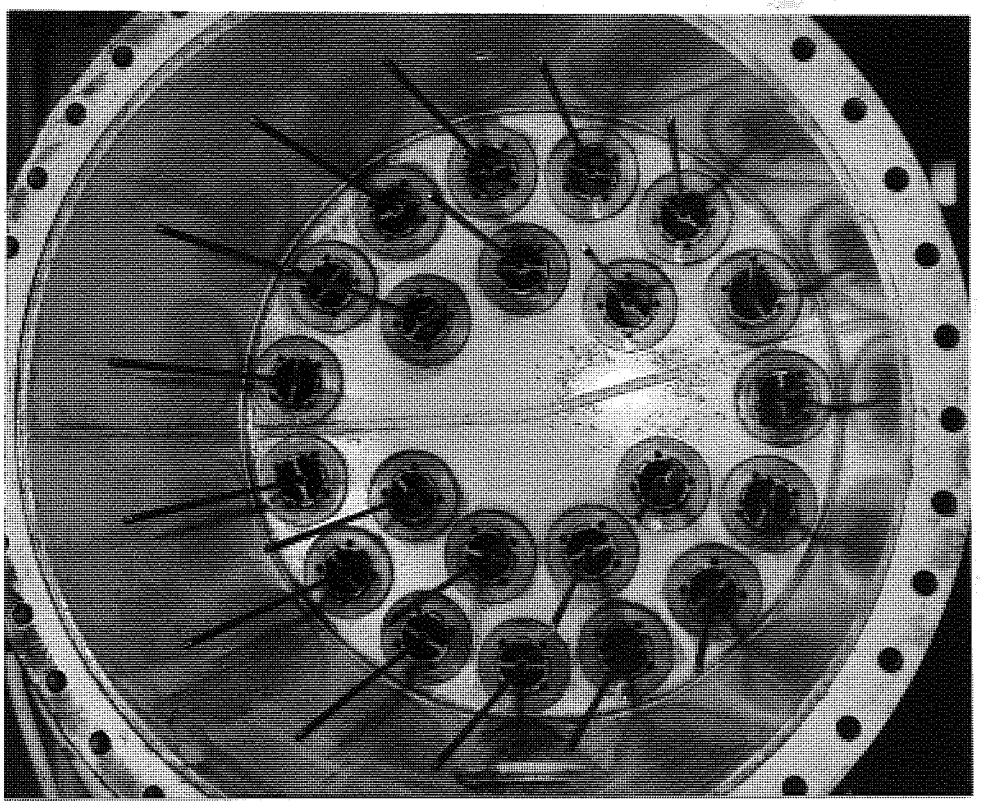

Fig. 2 Water droplet coalescence equipment with coalescer and separator elements removed.

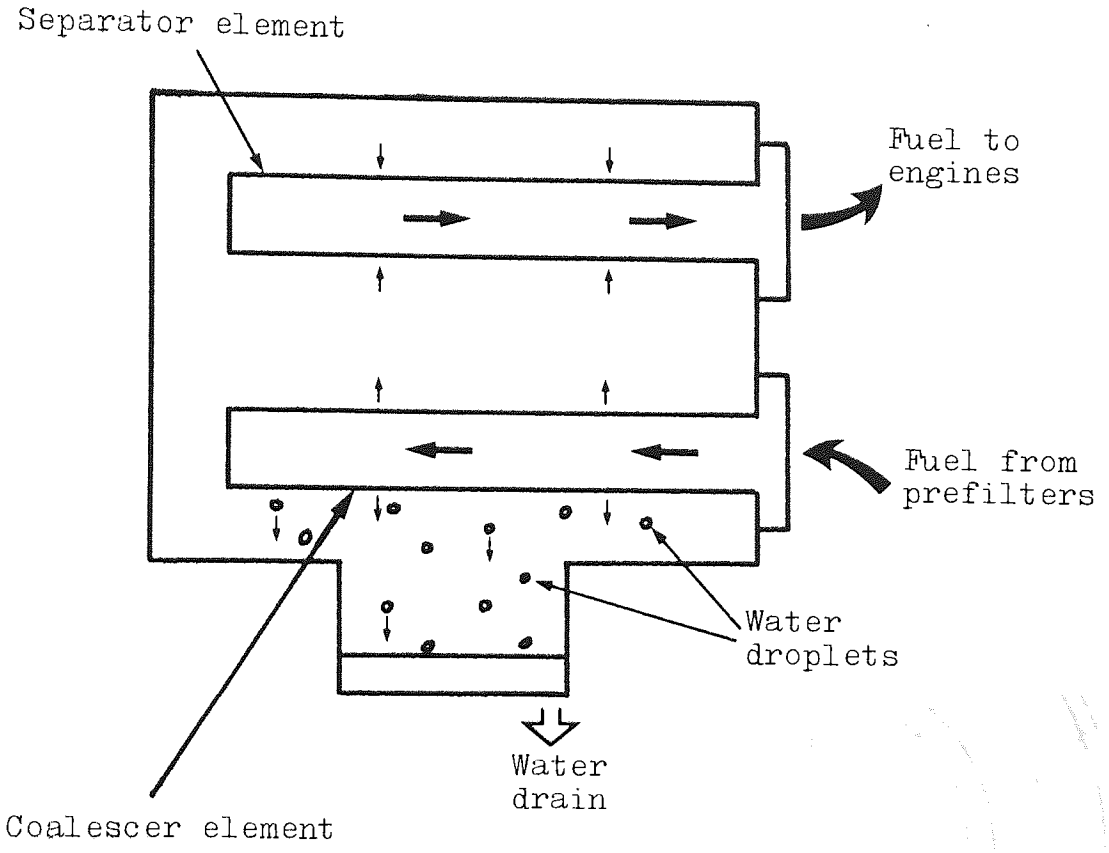

Fig. 3 Water coalescence equipment - diagram of operation.

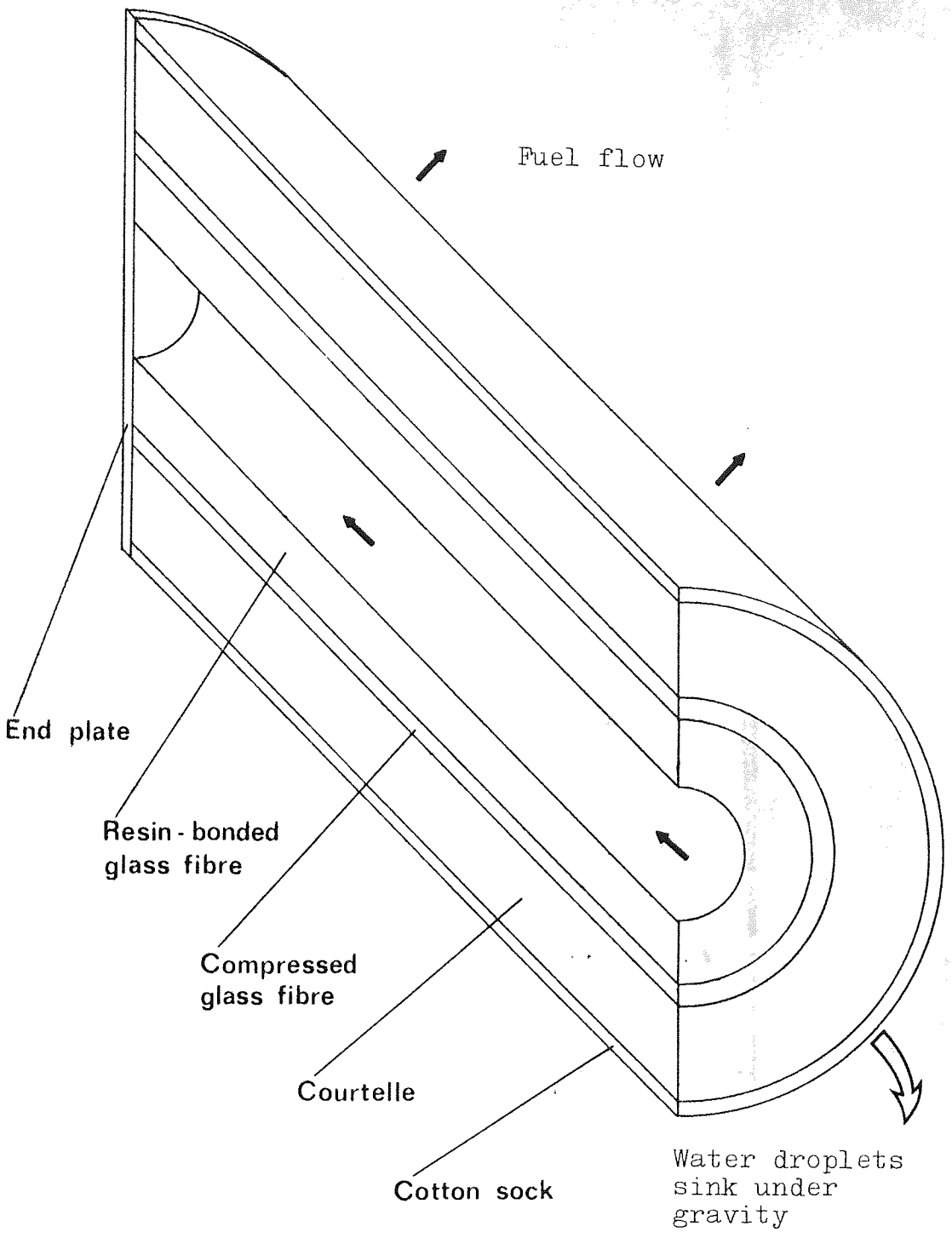

Fig. 4 Diagram of coalescer element composition
(ca. 1 metre in length)

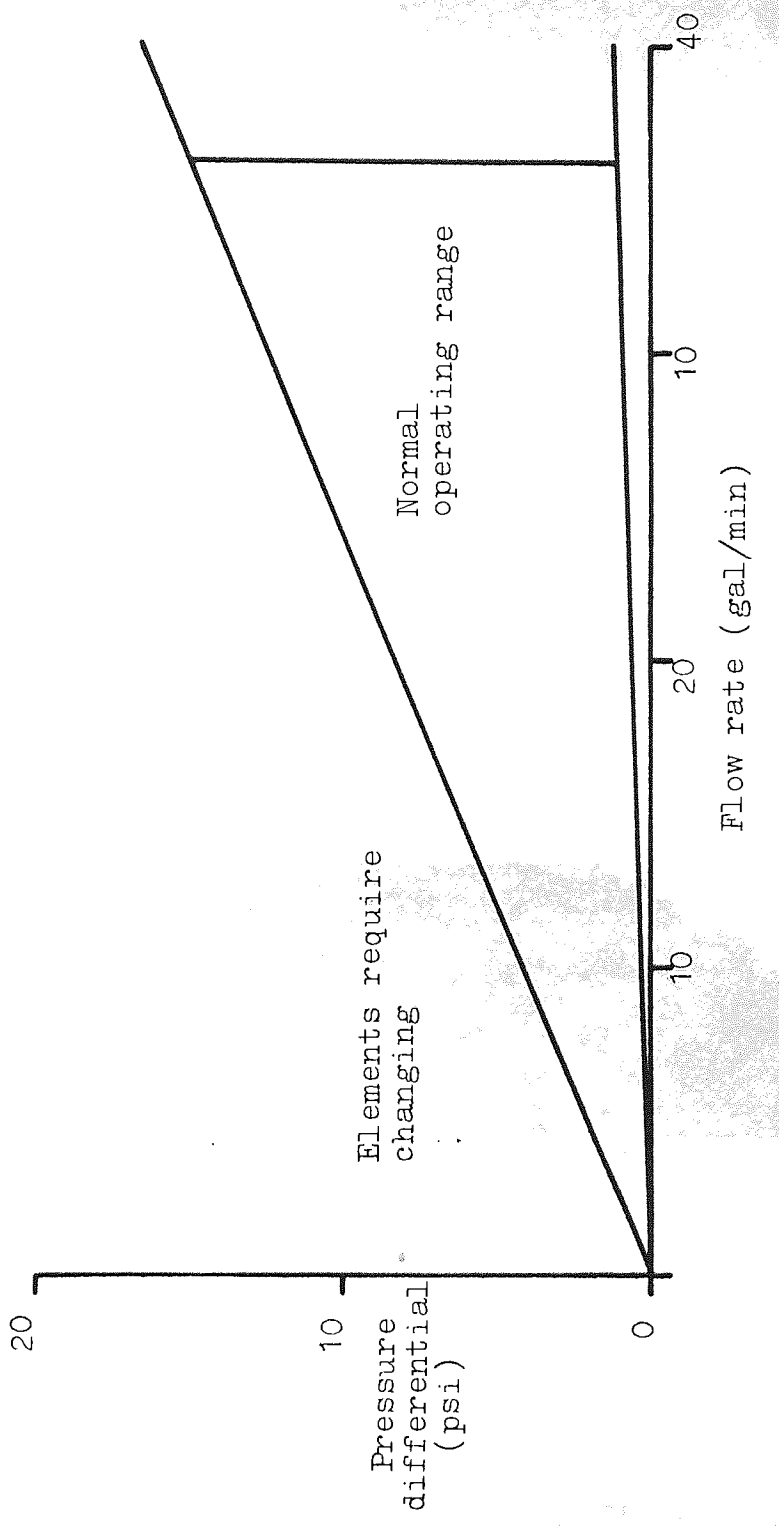

Fig. 5 Pressure/flow characteristics of a typical coalescer/separator system.

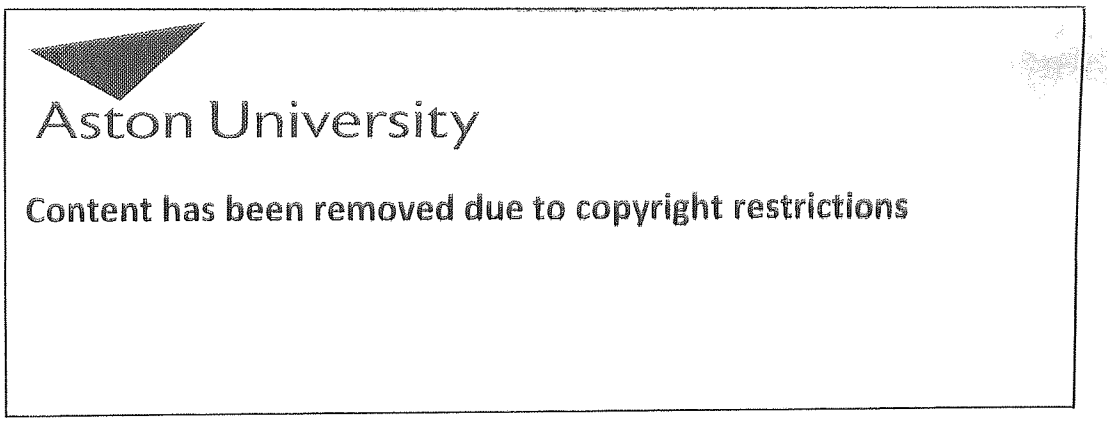

Fig. 6 The Type 21 (Amazon) class frigate, HMS Alacrity.

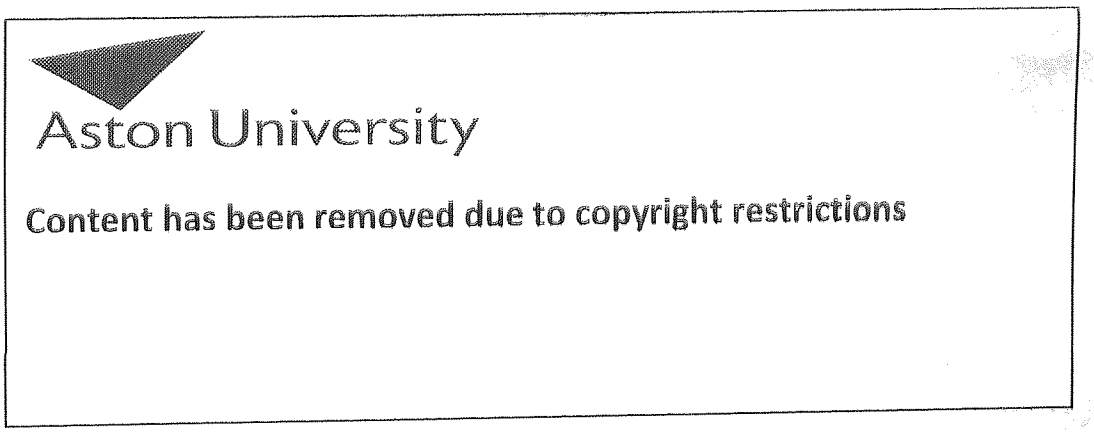

Fig. 7 The Type 42 (Sheffield) class destroyer, HMS Birmingham

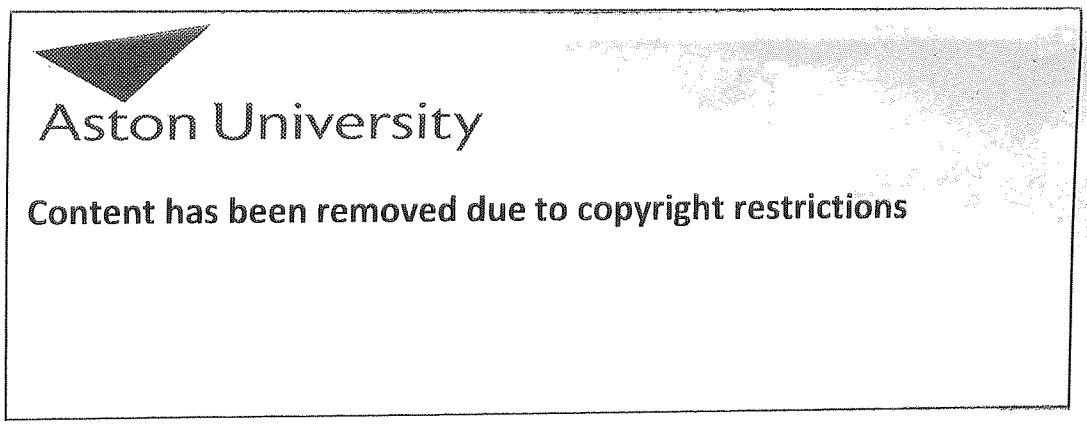

Fig. 8 The Type 22 (Broadsword) class frigate, HMS Broadsword.

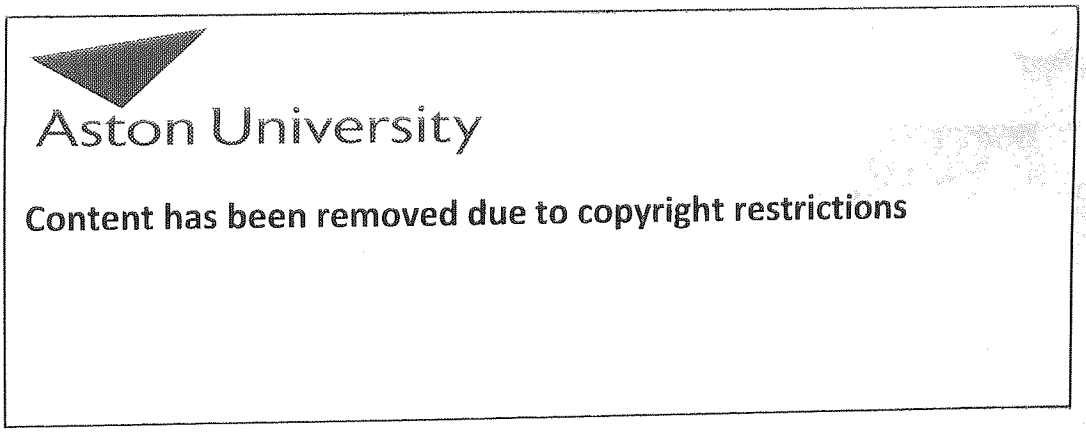

Fig. 9 The 'Invincible' class lightweight aircraft carrier, HMS Invincible.

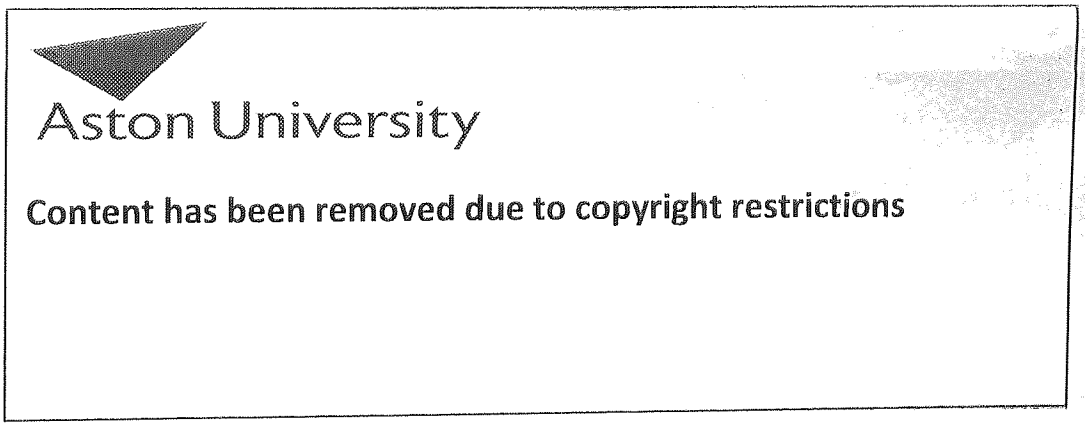

Fig. 10 The 'County' class destroyer, HMS London.

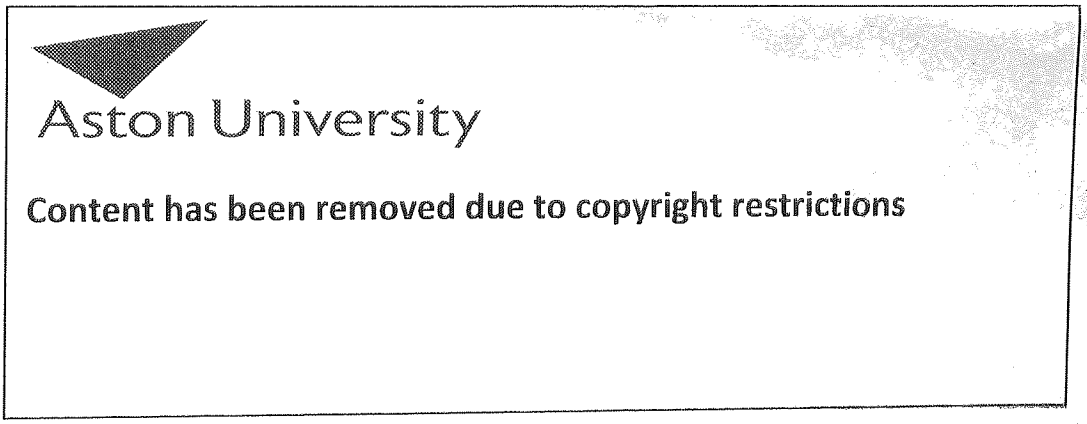

Fig. 11 The Type 81 (Tribal) class frigate, HMS Ashanti.

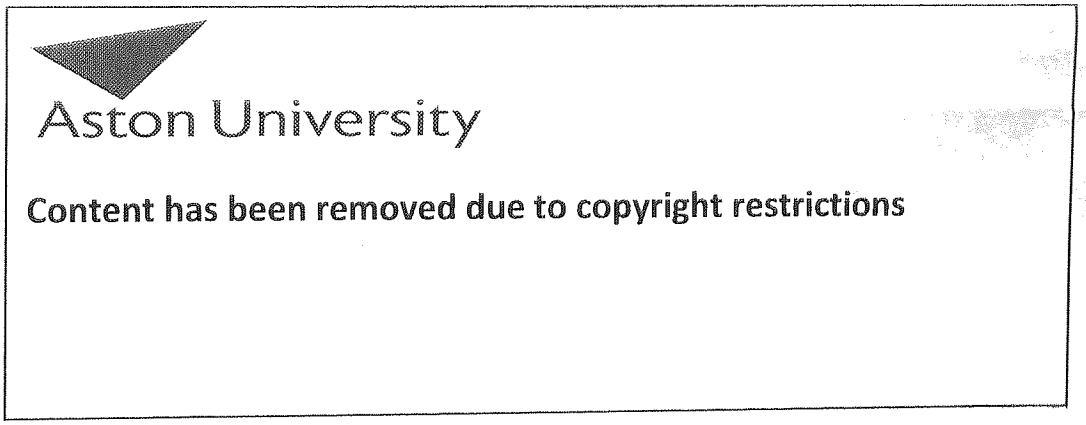

Fig. 12 The Type 82 class guided-missile destroyer, HMS Bristol.

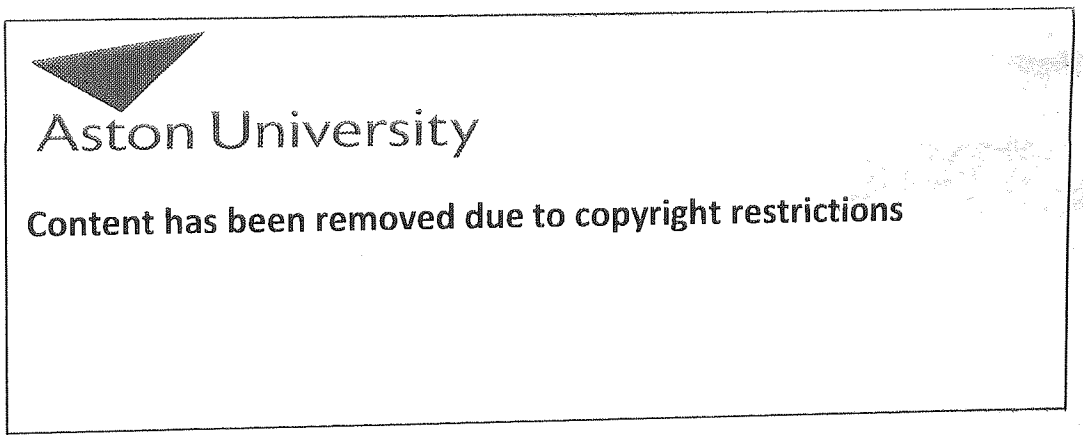

Fig. 13 The Royal Fleet Auxiliary tanker, Olwen.

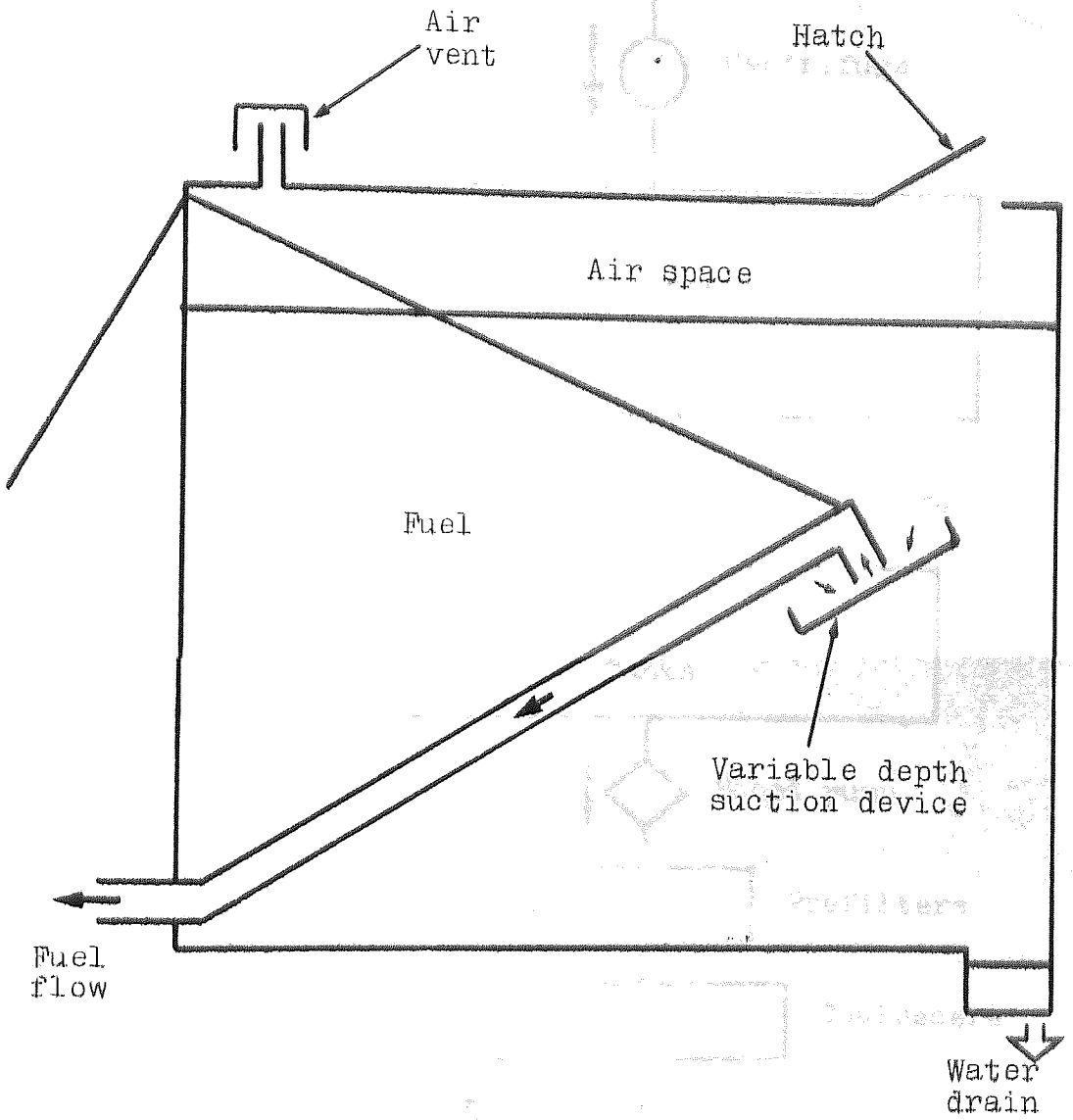

Fig. 14 Diagram of a diesel fuel land storage tank

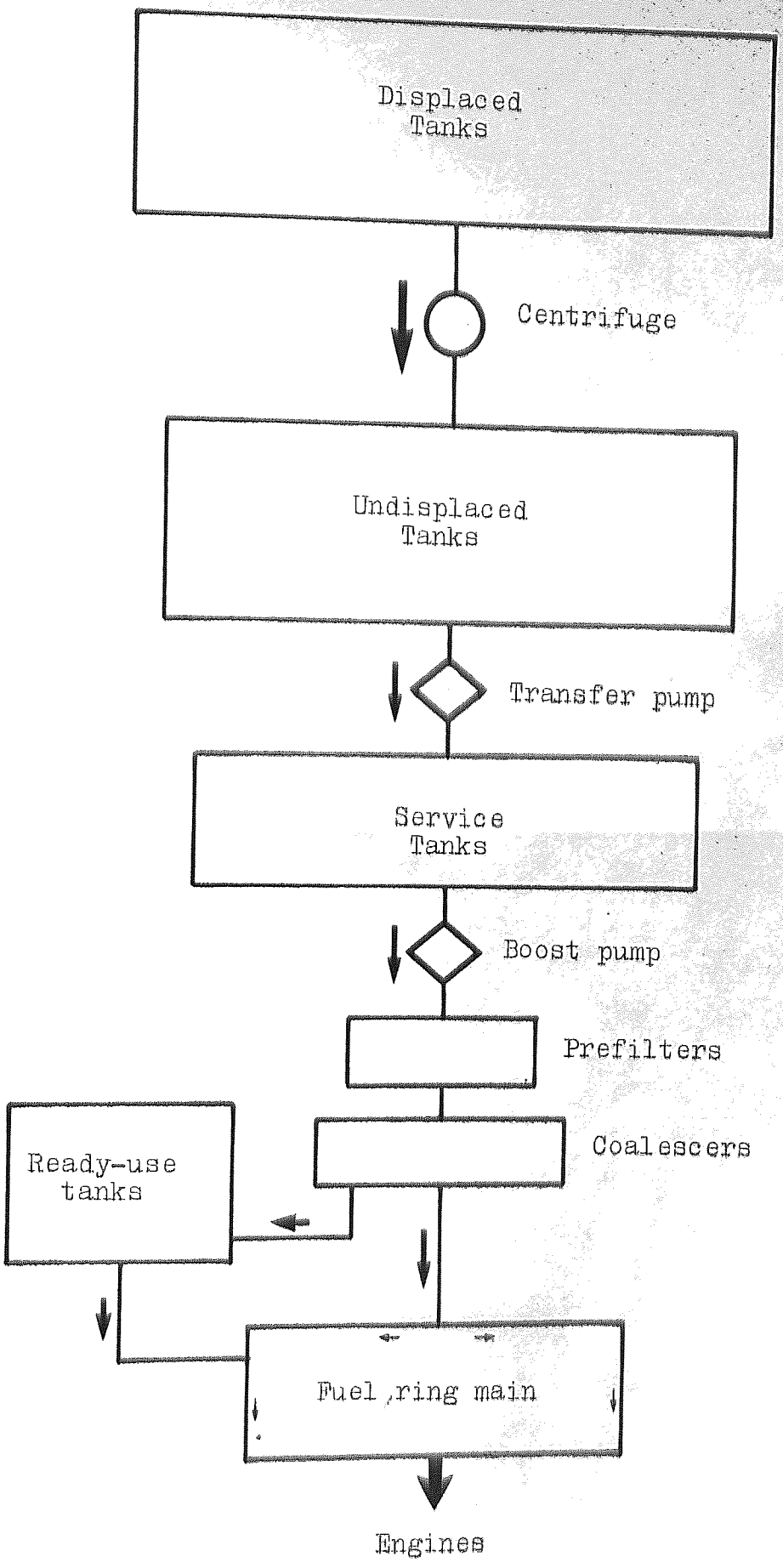

Fig. 15 Diagram of a Type 42 destroyer fuel system.

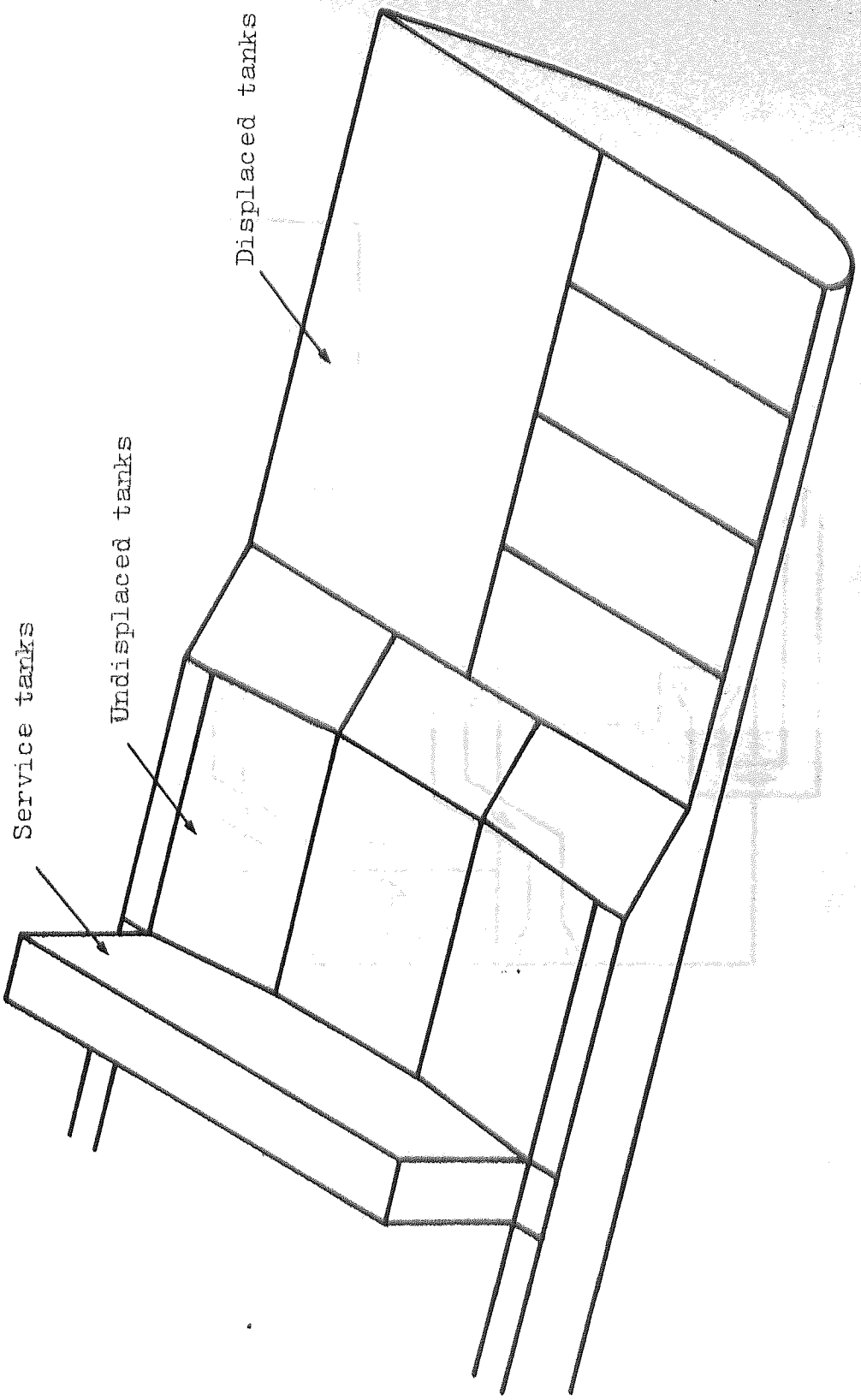

Fig. 16 Typical fuel tank layout in a Type 42 destroyer

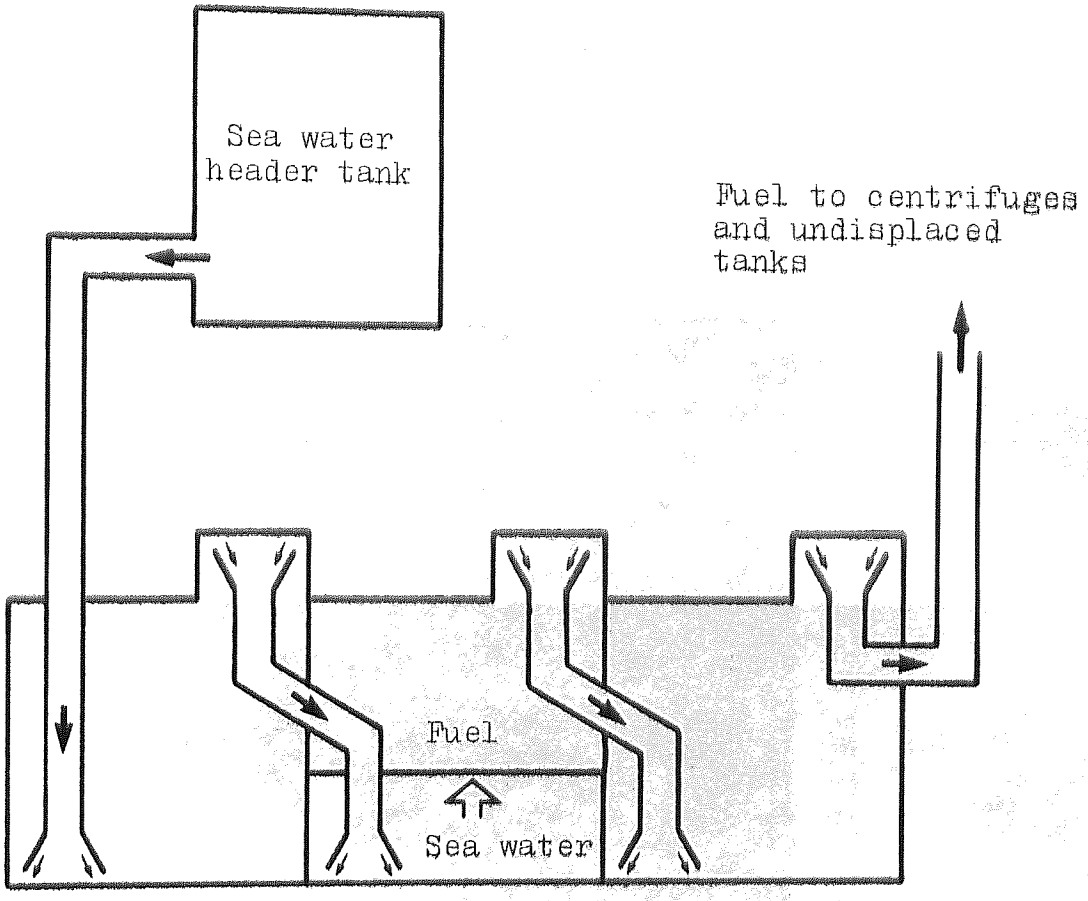

Fig. 17 Diagram of the sea water displacement system in Type 42 destroyers.

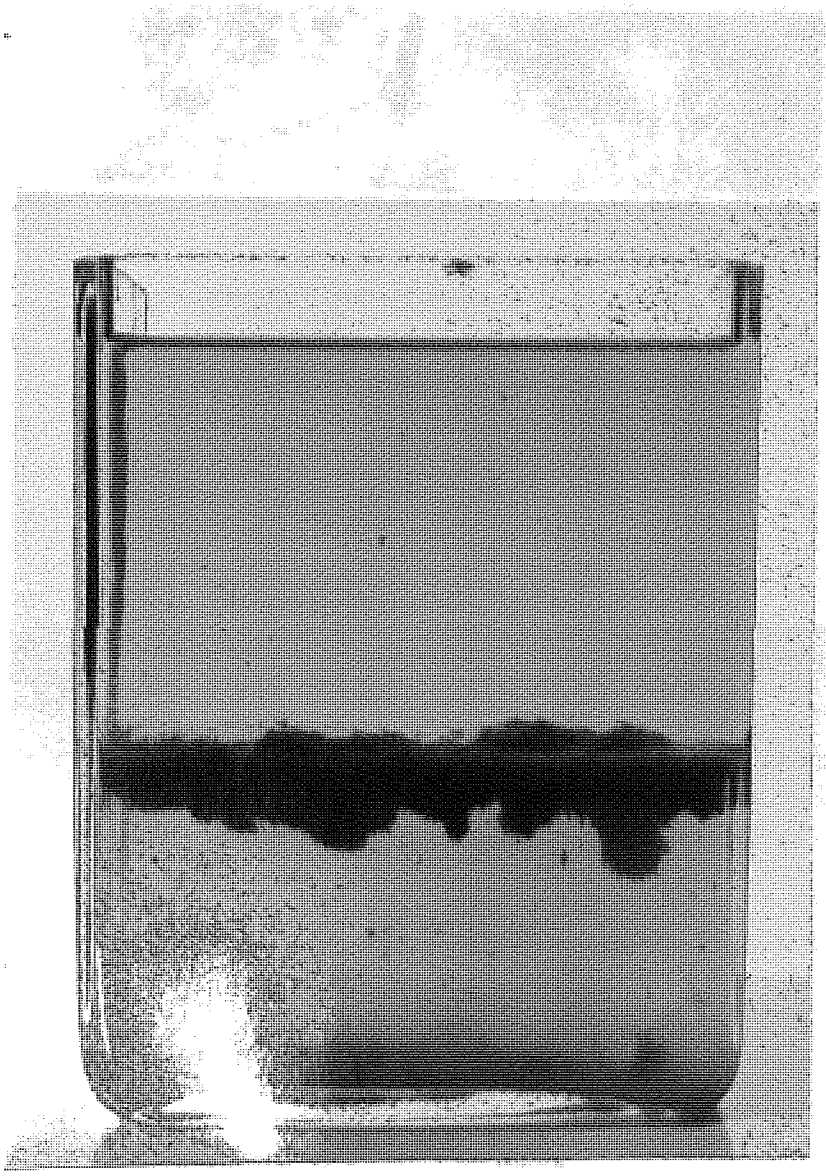

Fig. 18 Fuel/water sample showing microbial growth at the interface.

Fig. 19 Interior view of a shipboard integral fuel storage tank.

CHAPTER 2

ISOLATION AND ENUMERATION OF MICROORGANISMS FROM FUEL/WATER SAMPLES

2.1 Introduction

2.2 Methods

- 2.2.1 Collection of samples
- 2.2.2 Isolation of fungi and yeasts for use in further studies.
- 2.2.3 Enumeration of microorganisms in a sample from HMS Broadsword.
- 2.2.4 Enumeration of microorganisms in a sample from HMS Cardiff.
- 2.2.5 Treatment of results

2.3 Results

2.4 Conclusions

2.5 Discussion

ISOLATION AND ENUMERATION OF MICROORGANISMS FROM FUEL/WATER SAMPLES

2.1 Introduction

The diversity of the microbial flora in shipboard fuel systems is likely to be determined by the number of species in the inoculum and the growth conditions which will select for particular species. Lonsane et al. (1975) have listed a large number of microorganisms which are capable of utilizing hydrocarbons. A number of workers (Leathen and Kinsel, 1963; Edmonds and Cooney, 1967; Rubidge, 1974; Scott and Forsyth, 1974) have isolated many microbial species from aircraft, a similar environment to that found aboard ships. The fungus, Cladosporium resinae has been considered to be the dominant organism in aircraft (see sub-section 1.2.2) and Houghton and Gage (1979) reported that the same species is an important organism involved in the colonization of shipboard fuel systems.

Microorganisms can enter tanks from a number of sources, which include:-

a) the atmosphere

b) soil

c) sea water

d) previously colonized fuel

Fuel tanks are vented and this is the probable means of entry of airborne microorganisms. Cladosporium resinae has been isolated frequently from the air (Harvey, 1967; Chabert and Nicot, 1968; Sheridan and Nelson, 1971).

Soil can come into contact with fuel during handling near land storage tanks, for instance by dragging hose-pipes on the ground. Soil harbours large numbers of microorganisms (Berkeley, 1971) and a number of reports

have shown that Cladosporium resinae is an inhabitant of soil (Parbery, 1967, 1969a, 1969b; Sheridan et al., 1971). The role of marine hydrocarbon-utilizing microorganisms in the degradation of oil at sea has been well documented (Gibbs, 1975; Ahearn and Meyers, 1976; Gutnick and Rosenberg, 1977; Atlas, 1978). Sea water is introduced deliberately into some fuel systems (see sub-section 1.3.1) and is often an accidental contaminant by spray or leakage.

Houghton and Gage (1979) reported that the fuel in land storage tanks and Royal Fleet Auxiliary (RFA) tankers may contain high microbial numbers. A warship which embarks fuel from these sources can therefore become infected with a large microbial inoculum and suffer problems of filter clogging.

The extent of microbial colonization in shipboard fuel systems will depend on the following factors:

a) inoculum size

b) age of the fuel-water system (or incubation time)

c) concentration of nutrients

d) the physico-chemical conditions

e) concentration of inhibitors

The effects of these factors on growth are discussed in subsequent chapters. However, measurement of the extent of microbial development is examined in this chapter.

Rogers and Kaplan (1965) estimated the number of microorganisms in fuel samples by passing 500ml through a 0.2 micron filter which was placed on an agar medium and incubated. A similar technique was used by Houghton and Gage (1979) except that the number of hyphal fragments retained on the filter was counted under a microscope.

The microbial numbers in fuel/water samples have also been determined by dilution and plating of the water bottom on suitable media (Rogers and Kaplan, 1965; Engel and Swatek, 1966).

Microbial numbers have been found to be considerably higher in water bottoms than in fuel. Rogers and Kaplan (1965) reported counts of 4 to 55 bacteria per 500ml of aviation jet fuel and 50,000 to 75,000/ml of aqueous phase. Fungal counts were 2 to 18 per 500ml of fuel and 2 to 114/ml of water bottom. Similar findings were reported by Engel and Swatek (1966).

2.2 Methods

2.2.1 Collection of samples

Samples were obtained from shipboard fuel systems by the following methods:

a) Tanks were emptied and entered to obtain samples from residual pools and deposits on the walls and floor.

b) They were taken from drain cocks situated at low points of tanks.

c) The stripping system of Type 42 destroyers (see section 4.5 and Fig. 28) was used to obtain them from tank bottoms.

2.2.2 Isolation of fungi and yeasts for use in further studies

Aliquots were taken from fuel/water samples, diluted in physiological saline (see Appendix III) and samples

(0.1ml) from the dilutions were spread on malt extract agar (Oxoid) plates. The plates were incubated at 25°C for five days. Pure cultures of fungi and yeasts were obtained by repeated sub-culture on malt extract agar at 25°C and maintained on slants of the same medium at 4°C.

Fungi were identified at the Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, TW9 3AF and yeasts at the National Collection of Yeast Cultures, Food Research Institute, Colney Lane, Norwich, Norfolk, NR4 7UA.

2.2.3 Enumeration of microorganisms in a sample from HMS Broadsword

An aliquot of aqueous phase was taken from a fuel/water sample from HMS Broadsword, diluted in physiological saline and aliquots (0.1ml) from the dilutions were spread on malt extract and nutrient agar (Oxoid) plates. After incubation at 22 and 37°C for up to seven days, the numbers of bacterial yeast and fungal colonies on each plate were counted.

2.2.4 Enumeration of microorganisms in a sample from HMS Cardiff

Aliquots were taken from the aqueous phase and interface of a fuel/water sample from HMS Cardiff, diluted in physiological saline and aliquots (0.1ml) were spread on malt extract and plate count agar (Oxoid) plates. Malt extract agar plates were incubated at 25°C and plate count agar dishes at 22 and 37°C. After incubation for up to seven days, the numbers of bacterial, yeast and

fungal colonies were counted. Bacterial colonies were sub-cultured and distinguished by Gram reaction and shape. Fungal colonies of Cladosporium resinae and species of Penicillium and Cylindrocarpon (Fig. 20) were counted separately from other fungi.

2.2.5 Treatment of results

The mean colony counts of replicate plates and standard deviations were calculated using the formulae in Appendix VI.

2.3 Results

Table 1 lists the fungi and yeasts isolated from fuel/water samples for use in further studies. The results of the enumeration of microorganisms in the water bottom of a sample from HMS Broadsword are shown in Tables 2 and 3 and Fig. 21. The enumeration of microorganisms in the water bottom of a sample taken from HMS Cardiff is shown in Tables 4 and 5 and at the fuel/water interface in Tables 6 and 7. Fig. 22 depicts the HMS Cardiff results.

2.4 Conclusions

The total microbial count in the water bottom of a sample from HMS Broadsword was considerably higher than that obtained from the HMS Cardiff sample. Higher counts were obtained from the fuel/water interface than from the water bottom of the HMS Cardiff sample.

In all cases, bacteria were the most numerous oganisms. Gram negative bacteria greatly outnumbered the Gram positive bacteria in the HMS Cardiff sample. A Cylindrocarpon sp.

was the dominant fungus, Cladosporium resinae and species of Penicillium forming only a minority of the fungi detected.

2.5 Discussion

It is clear from these studies that the microbial population in shipboard fuel systems can be formed from a large number of species. This diversity is an important factor when considering potential control measures. Many inhibitors or treatments are selective for one or a group of microorganisms and hence may allow the development of species which are either resistant to or which relish the altered conditions. It may therefore be necessary to use a range of control methods.

The higher concentration of microorganisms in the sample from HMS Broadsword may be due to one or a combination of the factors mentioned in section 2.1, for example, the age of the fuel and water, concentration of phosphate and the volume of water. These effects are discussed in later chapters.

The higher microbial counts at the fuel/water interface than in the water bottom of the HMS Cardiff sample suggests that the organisms have a high affinity for the oil phase. Mimura et al. (1971) reported that hydrocarbon-utilizing yeasts had a higher affinity for the oil phase than those which were unable to assimilate hydrocarbons. This provides further evidence to the reports of Rogers and Kaplan (1965) and Engel and Swatek (1966) that the distribution of microorganisms in fuel/water systems is not homogenous. These findings are important when attempting to estimate the extent of microbial colonization of a fuel

system or tank. It is likely that the microbial count in a single sample may not be truly representative of the situation. Houghton and Gage (1979) have appreciated this problem and suggest that "information supplied with the sample becomes invaluable" when trying to determine the true level of microbial contamination.

Bacteria were the most numerous organisms in the samples investigated. It is not known whether they can contribute significantly to fuel filter blockage.

The dominant fungus in the HMS Cardiff sample was a Cylindrocarpon sp.. Cladosporium resinae, the fungus most commonly identified with aircraft fuel tank colonization (Hendey, 1964; Darby et al., 1968; Rubidge, 1974) was also isolated but was not present in large numbers.

Table 1 List of microorganisms isolated from fuel/water samples

SPECIMEN CODE	MICROORGANISM	COLLECTION NUMBER	ORIGIN
EW1	*Cladosporium resinae* (Lindau) de Vries	CMI 249456	HMS ALACRITY
EW2	*Cladosporium resinae* (Lindau) de Vries	NOT ACCESSED	GOSPORT LAND STORAGE TANK
EW3	*Cladosporium resinae* (Lindau) de Vries	NOT ACCESSED	RFA BLUE ROVER
EW4	*Penicillium corylophilum* Dierckx	CMI 249457	RFA BLUE ROVER
EW5	*Candida guilliermondii* var. *guilliermondii* (Castellani) Langeron et Guerra	NCYC 973	RFA BLUE ROVER
EW6	*Rhodotorula glutinis* var. *glutinis* (Fres.) Harrison	NCYC 974	RFA BLUE ROVER
EW74	*Cylindrocarpon* sp.	CMI 252757	HMS CARDIFF

Table 2 Counts obtained from a dilution of water bottom from a fuel/water sample from HMS Broadsword

ORGANISM	MEAN COLONY COUNT OF FIVE REPLICATE PLATES (STANDARD DEVIATION)			
	RECOVERY TEMPERATURE (^{o}C) AND MEDIUM			
	22 MEA	22 NA	37 MEA	37 NA
Total	243.6(11.3)	152.0(18.1)	26.4(16.8)	11.2(2.6)
Bacteria	220.4(14.8)	145.6(20.0)	26.4(16.8)	0(0)
Yeasts	21.1(9.4)	6.4(2.9)	0(0)	10.2(3.1)
Fungi	2.0(3.1)	0(0)	0(0)	1.0(1.7)

MEA - Malt extract agar

NA - Nutrient agar

Table 3 Enumeration of organisms in the water bottom of a fuel/water sample from HMS Broadsword

ORGANISM	NUMBER OF ORGANISMS/ML OF WATER BOTTOM			
	RECOVERY TEMPERATURE (^{o}C) AND MEDIUM			
	22 MEA	22 NA	37 MEA	37 NA
Total	2.4×10^{8}	1.5×10^{8}	2.6×10^{7}	1.1×10^{7}
Bacteria	2.2×10^{8}	1.4×10^{8}	2.6×10^{7}	ND
Yeasts	2.1×10^{7}	6.4×10^{6}	ND	1.0×10^{7}
Fungi	2.0×10^{6}	ND	ND	1.0×10^{6}

ND - Not detected

Table 4 Counts obtained from the water bottom of a fuel/water sample from HMS Cardiff

ORGANISM	MEAN COLONY COUNT OF FIVE REPLICATE PLATES (STANDARD DEVIATION)		
	RECOVERY TEMPERATURE (^{o}C) AND MEDIUM (SAMPLE DILUTION)		
	22 PCA (10^{-1})	25 MEA (NEAT) (a)	37 PCA (NEAT)
Total	55.6(6.9)	29.2(14.8)	68.0(10.7)
Bacteria (total)	52.4(7.1)	9.5(4.5)	67.6(10.9)
G-ve rods	52.4(7.1)	9.5(4.5)	67.2(11.6)
G+ve rods	0(0)	0(0)	0.4(0.9)
G+ve cocci	0(0)	0(0)	0(0)
Yeasts	0.2(0.4)	2.8(2.1)	0.2(0.4)
Fungi (total)	3.0(1.9)	17.0(8.8)	0.2(0.4)
C. resinae	0(0)	1.0(1.4)	0.2(0.4)
Penicillium sp.	0(0)	0.5(1.0)	0(0)
Cylindrocarpon sp.	2.6(2.3	13.0(8.3)	0(0)
Unidentified fungi	0.4(0.9)	2.5(3.3)	0(0)

PCA - Plate count agar

(a) - Mean colony counts of four replicate plates

Table 5 Enumeration of microorganisms in the water bottom of a fuel/water sample from HMS Cardiff

ORGANISM	NUMBER OF ORGANISMS/ML OF WATER BOTTOM		
	RECOVERY TEMPERATURE (°C) AND MEDIUM		
	22 PCA	25 MEA	37 PCA
Total	5600	290	680
Bacteria (total)	5200	95	680
G-ve rods	5200	95	670
G+ve rods	ND	ND	4
G+ve cocci	ND	ND	ND
Yeasts	20	28	2
Fungi (total)	300	170	2
C. resinae	ND	10	2
Pencillium spp.	ND	5	ND
Cylindrocarpon sp.	260	130	ND
Unidentified fungi	40	20	ND

Table 6 Counts obtained from the fuel/water interface of a sample from HMS Cardiff

ORGANISM	MEAN COLONY COUNT OF FIVE REPLICATE (PLATES (STANDARD DEVIATION)		
	RECOVERY TEMPERATURE (^{o}C) AND MEDIUM (SAMPLE DILUTION)		
	22 PCA (10^{-1})	25 MEA (NEAT)	37 PCA (10^{-1})
Total	97.6(18.4)	42.2(9.5)	15.2(10.9)
Bacteria (total)	92.4(20.2)	1.6(1.5)	15.2(10.9)
G-ve rods	92.4(20.2)	1.6(1.5)	11.6(8.0)
G+ve rods	0(0)	0(0)	3.2(6.6)
G+ve cocci	0(0)	0(0)	0.4(0.9)
Yeasts	0.2(0.4)	4.0(2.5)	0(0)
Fungi (total)	5.0(3.4)	36.6(11.6)	0(0)
C. resinae	0(0)	1.6(0.5)	0(0)
Penicillium spp	0(0)	0.4(0.5)	0(0)
Cylindrocarpon sp.	4.0(2.4)	24.8(14.4)	0(0)
Unidentified fungi	1.0(1.2)	9.8(5.0)	0(0)

Table 7 Enumeration of microorganisms at the fuel/water interface of a sample from HMS Cardiff

ORGANISM	NUMBER OF ORGANISMS/ML		
	RECOVERY TEMPERATURE (^{o}C) AND MEDIUM		
	22 PCA	25 MEA	37 PCA
Total	9800	420	1500
Bacteria (total)	9200	16	1500
G-ve rods	9200	16	1200
G+ve rods	ND	ND	320
G+ve cocci	ND	ND	40
Yeasts	20	40	ND
Fungi (total)	500	370	ND
C. resinae	ND	16	ND
Penicillium spp.	ND	4	ND
Cylindrocarpon Sp.	400	250	ND
Unidentified fungi	100	100	ND

Fig. 20 Spores of Cylindrocarpon sp.

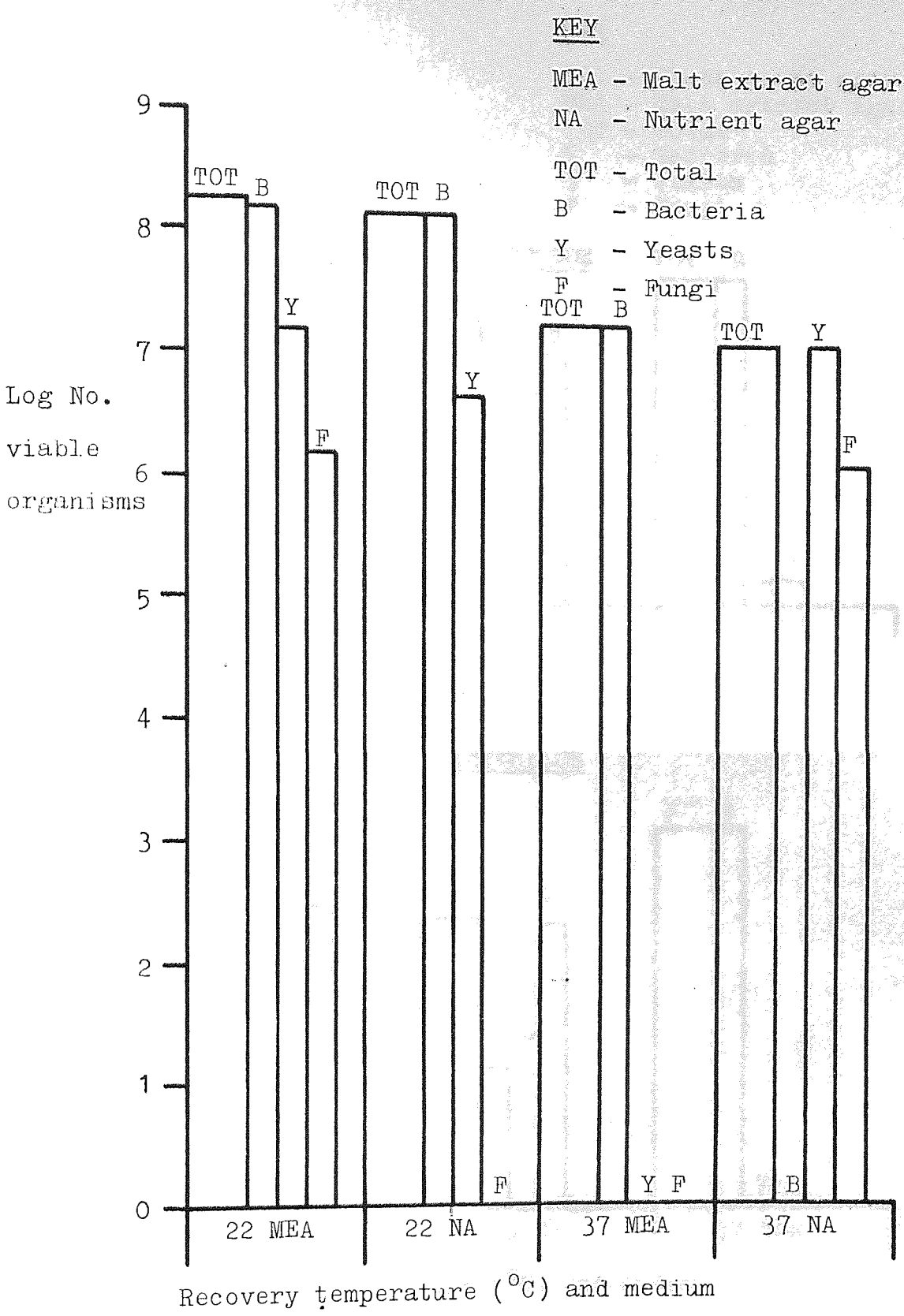

Fig. 21 Enumeration of organisms in the water bottom of a fuel/water sample from HMS Broadsword

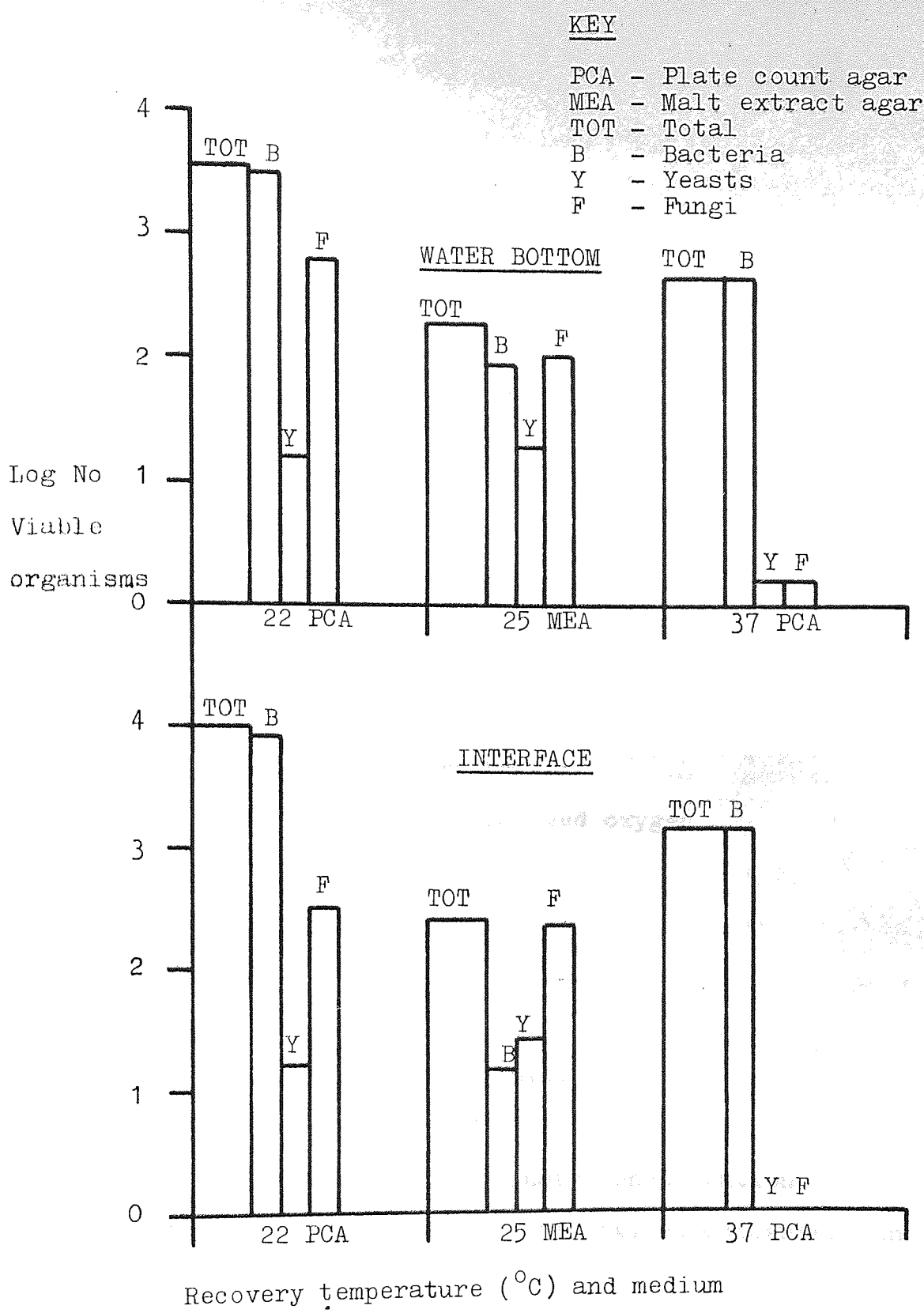

Fig. 22 Enumeration of organisms in a fuel/water sample from HMS Cardiff

CHAPTER 3

MEASUREMENT OF SALINITY, pH AND CONCENTRATION OF PHOSPHATE AND DISSOLVED OXYGEN IN WATER BOTTOMS

3.1 Introduction

3.2 Methods

- 3.2.1 Collection of samples
- 3.2.2 Measurement of salinity, pH and phosphate concentration
- 3.2.3 Measurement of dissolved oxygen concentration

3.3 Results

3.4 Conclusions

3.5 Discussion

- 3.5.1 Measurement of salinity
- 3.5.2 Measurement of pH
- 3.5.3 Measurement of phosphate concentration
- 3.5.4 Measurement of dissolved oxygen concentration

MEASUREMENT OF SALINITY, pH AND CONCENTRATION OF PHOSPHATE AND DISSOLVED OXYGEN IN WATER BOTTOMS

3.1 Introduction

The salinity, pH and concentration of phosphate and dissolved oxygen in the water bottoms of fuel tanks are parameters which are likely to affect microbial growth. These effects are considered in depth in subsequent chapters. However, measurement of the above parameters is examined in this chapter.

Hendey (1964) showed that Cladosporium resinae could not grow in kerosene in the absence of water. Fuel can become contaminated by fresh or salt water. The former type can be present due to condensation which will occur in any vented fuel tank subject to temperature variation. Also, land storage tanks have top hatches (see Fig. 14) which might be left open, allowing the ingress of rain water. Sea water may have been added deliberately in some fuel systems (see sub-section 1.3.1) or might be there as a result of spray or accidental leakage. Thus, the water bottoms are likely to vary in salinity because of mixing of fresh and salt water.

The production of exocellular organic acids by micro-organisms (see sub-section 1.3.3) and the presence of sea water will affect the hydrogen ion concentration of the water bottoms. Acid secretion will lower the pH whereas contamination by sea water, which has a value of around 8 (Sverdrup et al., 1955), will tend to increase it. Sea water also contains phosphate (Sverdrup et al., 1955) therefore its presence in fuel systems can contribute to

the phosphate levels in water bottoms. Uptake of phosphate by microorganisms is also likely which would tend to decrease its concentration.

The concentration of oxygen in water bottoms will depend on the rate of consumption by respiring microorganisms and the rate of replenishment from the atmosphere.

3.2 Methods

3.2.1 Collection of samples

Samples were obtained from fuel tanks by the methods described in sub-section 2.2.1.

3.2.2 Measurement of salinity, pH and phosphate concentration

Water samples were passed through a 0.45 micron filter (Millipore). The filtrate was measured for salinity by titration with silver nitrate solution (Harvey, 1957) and the results were reported in parts per thousand (‰). pH was measured with a portable pH meter (Electronic Instruments, Model 3030). Phosphate concentration was determined by the modified molybdenum blue method of Wooster and Rakestraw (1951).

3.2.3 Measurement of dissolved oxygen concentration

The concentration of dissolved oxygen in water samples was measured with a portable dissolved oxygen meter (Electronic Instruments, Model 1520). Readings of % saturation were converted to mg l^{-1} using the tables of Truesdale et al. (1955).

3.3 Results

The results of the measurement of salinity, pH and phosphate concentration in water bottom samples are shown in Tables 8, 9 and 10 and Figs. 23, 24 and 25 respectively. Dissolved oxygen readings are shown in Table 11.

3.4 Conclusions

The salinity of water samples taken from sea water displaced fuel tanks ranged from 32.8 to 33.6‰, which compares with a typical salinity range of 33 to 37‰ for the ocean (Davies, 1972). For undisplaced and service tanks of Type 42 destroyers, the readings were 30.2, 16.6 and 28.2‰. Samples taken from service tanks of ships with no displacement system had salinities of 11.7, 16.8 and 21.0‰. The salinity of water bottoms taken from Gosport land storage tanks ranged from 5.1 to 24.6‰.

The pH of water samples taken from sea water displaced fuel tanks ranged from 5.8 to 7.5 and averaged 6.8. Samples taken from undisplaced, service and land storage tanks ranged in pH from 4.6 to 6.4 and averaged 5.4.

The phosphate concentration in water samples taken from displaced and undisplaced tanks of Type 42 destroyers ranged from 12.4 to 37.2 ug P l^{-1}. The phosphate concentration in a water bottom sample taken from Gosport land storage tank No. 8 was 6.2 ug P l^{-1}.

The oxygen concentration in water samples taken from Gosport land storage tanks ranged from 1.4 to 3.0 ug l^{-1}.

The dissolved oxygen meter was calibrated from 0 -

100% saturation. However, after the oxygen levels in the water bottom samples were measured, a calibration check showed that the instrument measured from 0 - 160% saturation, which was probably caused by diesel fuel affecting the membrane of the electrode. This finding casts doubts on the readings obtained (see sub-section 3.5.4).

3.5 Discussion

3.5.1 Measurement of salinity

Sea water was present to some extent in all of the water bottoms, the salinity ranging from 5.1 to 33.6. However, the readings varied according to the type of fuel tank sampled.

According to Davies (1972), salinity varies between about 33 and 37‰ in the open ocean. The salinity of sea water obtained from the mouth of Langstone Harbour, Hants. on a rising tide was 33.8‰ (see Table 22 in Chapter 6). The water bottoms taken from sea water displaced fuel tanks were almost fully saline, showing that normal procedures of using salt water as compensation were carried out. Undisplaced and service tanks in ships with displacement systems also contained sea water, possibly due to inefficient centrifugation of the fuel.

Salt water was also detected in the service tanks of HMS Active and HMS Bristol, which do not possess ballasting systems. The brackish conditions were probably formed by mixing of salt and fresh water. Brackish water bottoms were also found in land storage tanks. They receive most of their fuel from ocean-going tankers, therefore the fuel is likely to contain salt water bottoms, which are also transferred to the land tanks.

3.5.2 Measurement of pH

The water bottoms taken from displaced fuel tanks were almost fully saline and therefore could be expected to have similar hydrogen ion concentrations to that of sea water. According to Sverdrup et al. (1955), the pH of sea water varies between about 7.5 and 8.4. The pH of a sample taken from the mouth of Langstone Harbour was 7.8 (see Table 22 in Chapter 6). The results show that the displaced tank water bottoms were more acidic than sea water, which is probably due to the production of organic acids by microbial activity (see sub-section 1.3.3).

The water bottoms taken from undisplaced, service and land storage tanks were more acidic than those from displaced fuel tanks and is probably due to greater microbial activity. Hydrogen ion concentration might therefore be a useful indicator of the extent of colonisation of a fuel system, acid water indicating extensive microbial development and neutral or alkaline conditions suggesting low numbers of microorganisms.

3.5.3 Measurement of phosphate concentration

The water bottoms in the fuel tanks have been shown to contain varying amounts of sea water, therefore their phosphate levels may be compared with recorded concentrations for the ocean. Armstrong et al. (1970) found that the winter maximum for phosphate in the English Channel was 13.95 ug P l^{-1} and Bradfield et al. (1976) found similar levels in five Cornish bays. The measurements of phosphate concentration in water bottoms taken from shipboard fuel tanks show that three samples contained similar

amounts to those obtained by Armstrong et al. (1970) and Bradfield et al. (1976) for sea water. The remaining samples - especially those from the service tanks of HMS Active - contained higher levels.

High phosphate concentrations may be due to the source of the sea water. Estuarine and coastal waters near populated areas tend to have higher levels. In Southampton water, 80.6 ug P l^{-1} was recorded by Raymont (1972) and high concentrations were also recorded in a survey of Liverpool Bay by Jones and Folkard (1971). It is possible that the high levels of phosphate measured in some water bottoms may be due to the ingression of polluted water. The low phosphate concentration found in the sample taken from the land storage tank may be due to uptake by microorganisms.

The aqueous concentration of nitrogen is also likely to have an important effect on the growth of fuel-utilizing microorganisms. However, phosphate was measured to give a general indication of the nutrient levels in water bottoms and was easier to assay than nitrogen.

3.5.5 Measurement of dissolved oxygen concentration

Unfortunately the readings obtained by the dissolved oxygen meter cannot be considered reliable and therefore alternative techniques are required in order to obtain quantitative measurements of oxygen levels in water bottoms. Alternative methods were not investigated due to lack of time.

Table 8 Salinity of water bottom samples

SAMPLE SOURCE	SALINITY (‰)
Type 42 Displaced Tank (HMS Sheffield)	33.6
Type 42 Displaced Tank (HMS Sheffield)	32.9
Type 42 Displaced Tank (HMS Sheffield)	33.1
Type 42 Displaced Tank (HMS Sheffield)	32.8
Type 42 Displaced Tank (HMS Glasgow)	33.6
Type 42 Undisplaced Tank (HMS Sheffield)	30.2
Type 42 Undisplaced Tank (HMS Glasgow)	16.6
Type 42 Service Tank (HMS Sheffield)	28.2
Type 21 Service Tank (HMS Active)	11.7
Type 21 Service Tank (HMS Active)	16.8
Type 82 Service Tank (HMS Bristol)	21.0
Gosport Land Storage Tank No. 8	5.1
Gosport Land Storage Tank No. 5	10.7
Gosport Land Storage Tank No. 13	19.8
Gosport Land Storage Tank No. 23	22.6
Gosport Land Storage Tank No. 35	24.6

Table 9 pH of water bottom samples

SAMPLE SOURCE	pH
Type 42 Displaced Tank (HMS Sheffield)	7.5
Type 42 Displaced Tank (HMS Sheffield)	6.6
Type 42 Displaced Tank (HMS Sheffield)	7.0
Type 42 Displaced Tank (HMS Sheffield)	5.8
Type 42 Displaced Tank (HMS Sheffield)	7.4
Type 42 Displaced Tank (HMS Sheffield)	6.1
Type 42 Displaced Tank (HMS Sheffield)	6.9
Type 42 Displaced Tank (HMS Sheffield)	6.3
Type 42 Displaced Tank (HMS Glasgow)	7.5
Type 42 Undisplaced Tank (HMS Sheffield)	4.7
Type 42 Undisplaced Tank (HMS Glasgow)	5.5
Type 42 Service Tank (HMS Sheffield)	4.6
Type 21 Service Tank (HMS Active)	5.3
Type 82 Service Tank (HMS Bristol)	6.4
(Gosport) Land Storage Tank No. 8	6.1

Table 10 Phosphate concentration in water bottom samples

SAMPLE SOURCE	PHOSPHATE CONCENTRATION ($\mu g\ P\ l^{-1}$)
Type 42 Displaced Tank (HMS Sheffield)	12.4
Type 42 Displaced Tank (HMS Sheffield)	24.8
Type 42 Displaced Tank (HMS Sheffield)	15.5
Type 42 Displaced Tank (HMS Sheffield)	24.8
Type 42 Displaced Tank (HMS Glasgow)	21.7
Type 42 Undisplaced Tank (HMS Sheffield)	15.5
Type 42 Undisplaced Tank (HMS Glasgow)	37.2
Type 21 Service Tank (HMS Active)	46.5
Type 21 Service Tank (HMS Active)	55.8
(Gosport) Land Storage Tank No. 8	6.2

Table 11 Dissolved oxygen concentration in water bottom samples

SAMPLE SOURCE	DISSOLVED OXYGEN CONCENTRATION (% SATURATION)	SAMPLE TEMPERATURE (^{o}C)	SAMPLE SALINITY (‰)	DISSOLVED OXYGEN CONCENTRATION ($mg\ l^{-1}$)
Gosport Land Storage Tank No. 5	33	16	10.7	3.0
Gosport Land Storage Tank No. 13	30	16	19.8	2.6
Gosport Land Storage Tank No. 23	16	15	22.6	1.4
Gosport Land Storage Tank No. 35	34	16	24.6	2.8

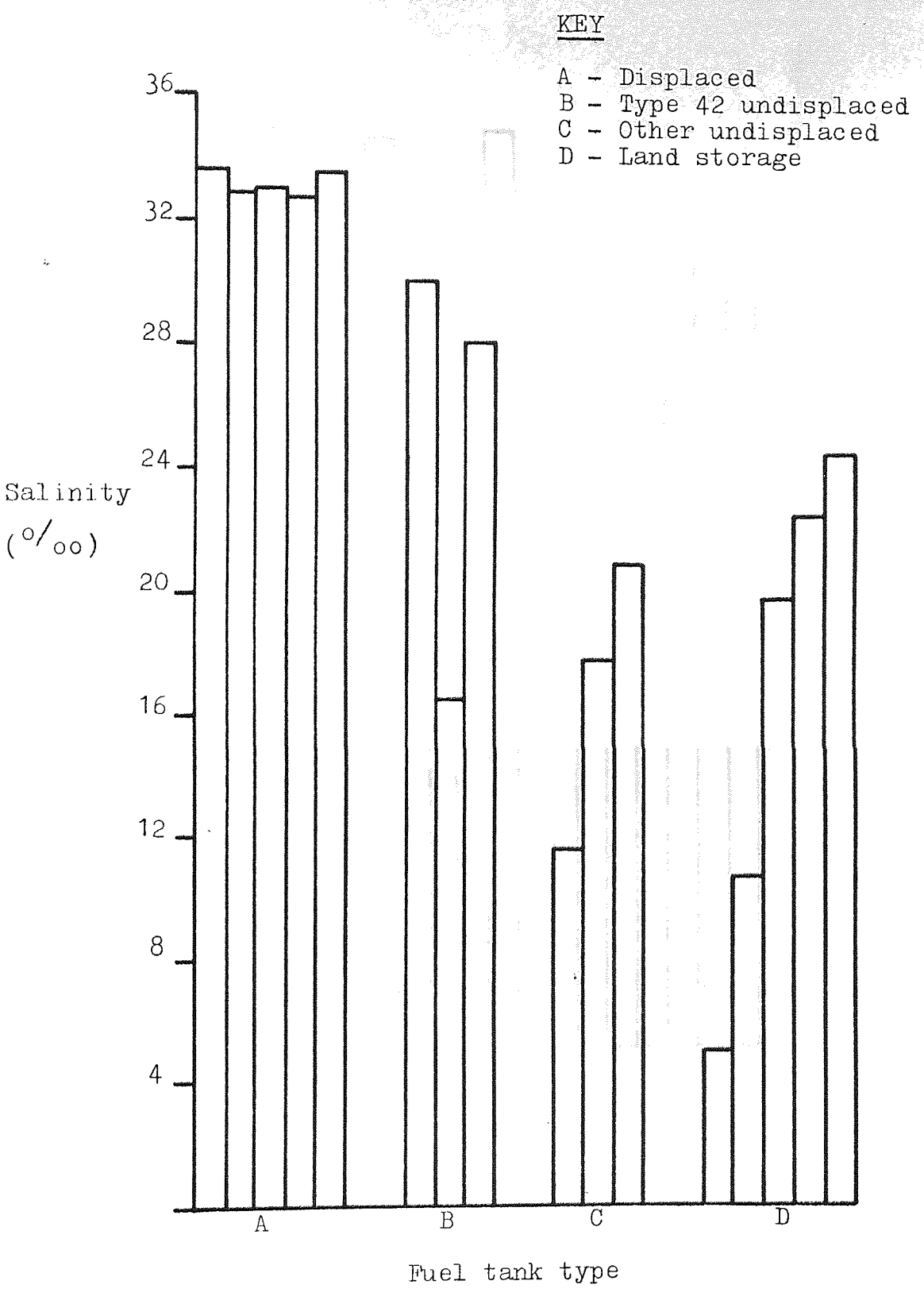

Fig. 23 Salinity of water bottom samples

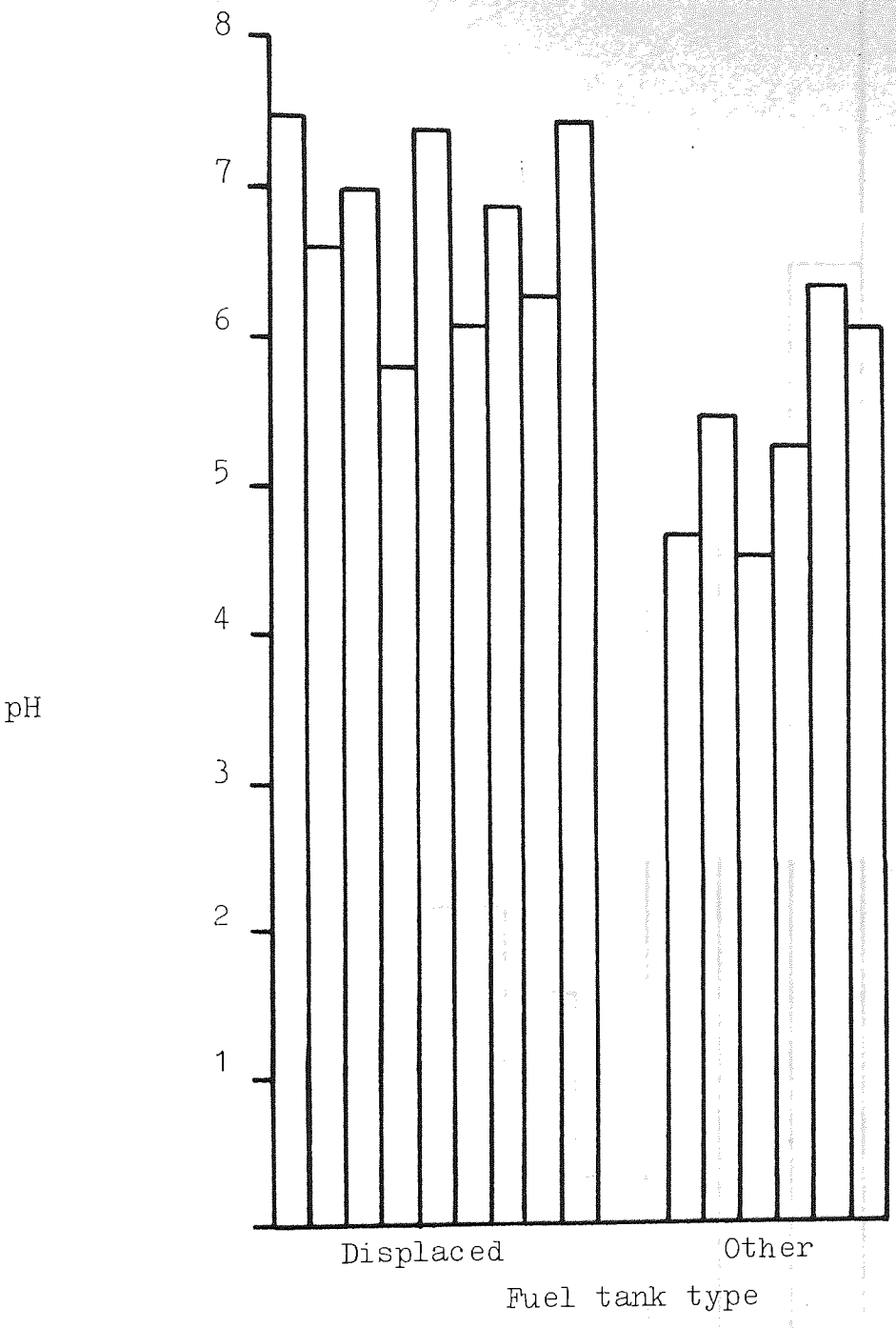

Fig. 24 pH of water bottom samples

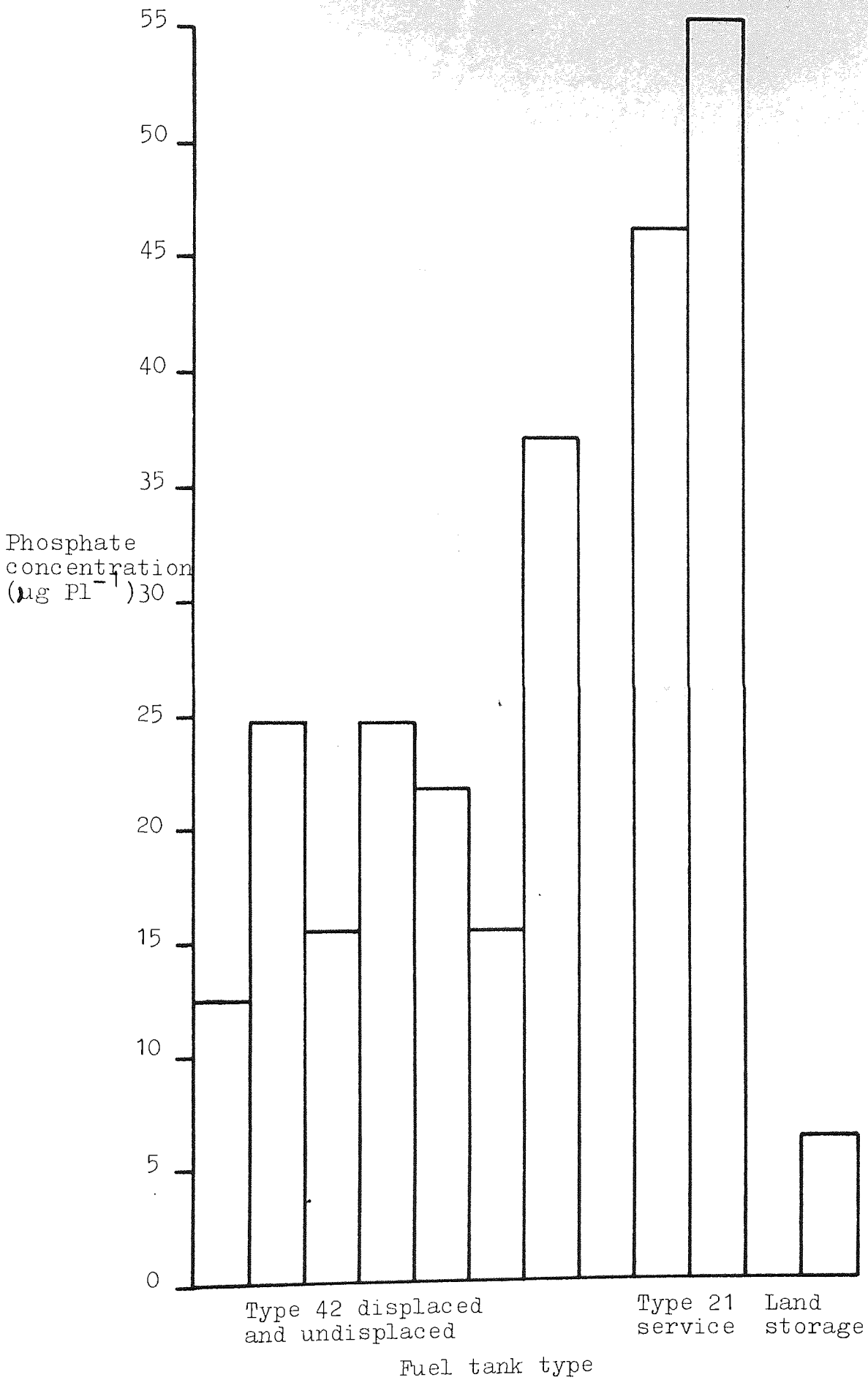

Fig. 25 Phosphate concentration in water bottom samples

CHAPTER 4

EFFECT OF INOCULUM SIZE ON THE GROWTH OF *Cladosporium resinae*

4.1 Introduction

4.2 Methods

4.2.1 Organisms

4.2.2 Media

4.2.3 Effect of inoculum size

4.3 Results

4.4 Conclusions

4.5 Discussion

EFFECT OF INOCULUM SIZE ON THE GROWTH OF *Cladosporium resinae*

4.1 Introduction

The inoculum size (the number of microorganisms) which can infect fuel systems may vary from a few airborne spores to gross contamination by previously colonized fuel. The size of the infection may be an important factor in determing the rate of biomass formation in fuel tanks.

After an initial lag period, the growth of micro-organisms in a culture (or in a fuel tank) can be expressed as

$$x = x_o \, e^{\mu t} \qquad \text{(Pirt, 1975)}$$

where x = final biomass

x_o = initial biomass (inoculum)

μ = specific growth rate

t = time.

Hence, the final biomass obtained is proportional to the inoculum size. The growth equation might be complicated by the diversity of species, variable specific growth rates caused by changes in the environment, lag phase differences, etc. Nevertheless, the overall situation probably remains the same, that in a given time period, the extent of microbial colonisation will be greater if the system is infected with a larger number of organisms. Hence, the time taken to achieve an unacceptable level of biomass formation is also inversely proportional to the inoculum size. It is therefore in the interests of fuel operators to prevent contamination of fuel by large numbers of microorganisms.

4.2 Methods

4.2.1 Organisms

Two strains of C. resinae were used in this study. EW1 was originally isolated from a diesel fuel/water sample from HMS Alacrity (see Table 1). ATCC 22711 was obtained from the American Type Culture Collection. The strain was originally isolated from United States Air Force jet aviation fuel (JP4). Spore suspensions were prepared as described in Appendix II.

4.2.2 Media

The composition of the mineral salts solution used is described in Appendix III. The hydrocarbons used were undecane and Reference Diesel Fuel, High Sulphur (see Appendix III).

4.2.3 Effect of inoculum size

General growth conditions are described in Appendix IV. In the first experiment, conical flasks containing mineral salts solution (25ml) and undecane (0.5ml) were inoculated with spore suspensions (1ml) of C. resinae ATCC 22711, ranging in concentration from 10^4 to 4×10^7 spores ml^{-1}. The flasks were agitated at 200 r.p.m. for 30 days after which growth was estimated by dry weight determination (see Appendix V).

In the second experiment, boiling tubes containing $^1/_{10}$ strength mineral salts solution (50ml) and diesel fuel (50ml) were inoculated with spore suspensions (0.1ml) of EW1, ranging in concentration from 50 to 5×10^6 spores

ml^{-1}. Dry weights were determined after 60 days incubation.

The mean dry weights of replicate flasks or tubes and standard deviations were calculated using the formulae in Appendix VI.

4.3 Results

The results of the effect of inoculum size on the growth of *C. resinae* ATCC 22711 in shake-flask culture are shown in Table 12 and Fig. 26.

The results for strain EW1 under static growth conditions are shown in Table 13 and Fig. 27.

4.4 Conclusions

The results of varying the inoculum size on the growth of ATCC 22711 under shaken conditions (see Table 12 and Fig. 26) show that a significant difference - as estimated by dry weight determination after 30 days incubation - was obtained between inocula of 10^4 and 10^6 spores per culture. No significant difference was obtained between 10^6, 10^7 and 4×10^7 spores per flask.

Variation of the inoculum size under static conditions (see Table 13 and Fig. 27) resulted in a significant growth difference between 5 and 50 spores per culture. No significant difference was obtained between the higher inoculum sizes - 50, 5×10^3 and 5×10^5 spores per tube.

4.5 Discussion

Better growth of *C. resinae* was obtained with larger spore inocula than with relatively small inocula. This is

probably due to the relationship between final and initial biomass according to the growth equation, $x = x_o e^{\mu t}$ (see section 4.1). The results show that more biomass is obtained with larger inocula, as specified by the equation, and points to the need to reduce the number of microorganisms infecting fuel systems.

Little difference in final biomass was obtained with the larger spore inocula. It is possible that at the time of biomass estimation, these cultures had attained maximum population density (x max). Substitution of the constant x max into the growth equation shows that the time taken to reach x max varies inversely with the initial biomass. Hence, lower inoculum sizes do not affect the total biomass concentration which can be obtained but require progressively longer incubation times to attain the maximum population density.

Precautions should therefore be taken to reduce the numbers of microorganisms infecting the fuel. Although sea water is introduced deliberately into some fuel systems (see sub-section 1.3.1) thus providing a source of hydrocarbon-utilizing microorganisms (see section 2.1), it is nevertheless very important to prevent the accidental ingression of salt water spray and leaks. The fuel should also be kept free of dirt and soil, perhaps by more frequent filtration and care whilst handling. Highly contaminated stocks should not be used for refuelling ships with fine filtration equipment. It may be possible to earmark fuels containing low microbial numbers for use in gas turbine ships.

Microorganisms are normally associated with an

aqueous phase, therefore the size of the inoculum can be reduced by the removal of water from the fuel. Three techniques are in current use:

a) gravity settling

b) centrifugation

c) coalescence of water droplets

Service and ready-use tanks are not integral with the ship's hull (see Fig. 16), therefore water can be removed by means of a tap situated at the lowest point of the tank. Storage tanks are integral with the hull, therefore in some warship classes (Type 42 and 'County' class destroyers; Types 22 and 81 class frigates), water bottoms are removed by means of a suction device situated close to the lowest point of the tank floor (see Fig. 28). Unfortunately, removal can never be complete. Water tends to remain behind strengthening girders, ledges and in the area of the suction device.

Centrifuges are fitted in Type 42 destroyers (see Fig. 15), Type 22 frigates and 'Invincible' class aircraft carriers and are designed to remove water, particulate matter and microorganisms from the fuel. However, according to Gage (1980), they are not always 100% efficient.

Removal of water by coalescence is carried out along with filtration prior to the combustion of the fuel (see Fig. 15). It may be possible to use filters and coalescers during refuelling (see Fig. 29) in order to keep the fuel clean and dry, but there is a disadvantage in that refuelling would take longer.

Table 12 Effect of inoculum size on the growth of *C. resinae* in undecane under shaken conditions

NUMBER OF SPORES/ FLASK	MEAN DRY WEIGHT OF TWO REPLICATE FLASKS (STANDARD DEVIATION) (mg)
10^4	12.5 (0.7)
10^5	14.0 (-) (a)
10^6	19.5 (2.1)
10^7	24.0 (2.8)
4×10^7	21.2 (6.0)

(a) - Result of one flask only

Table 13 Effect of inoculum size on the growth of *C. resinae* in diesel fuel under static conditions

NUMBER OF SPORES/ TUBE	MEAN DRY WEIGHT OF THREE REPLICATE TUBES (STANDARD DEVIATION) (mg)
5	13.7 (2.1)
50	17.3 (0.6)
5×10^3	18.3 (0.6)
5×10^5	17.7 (0.6)

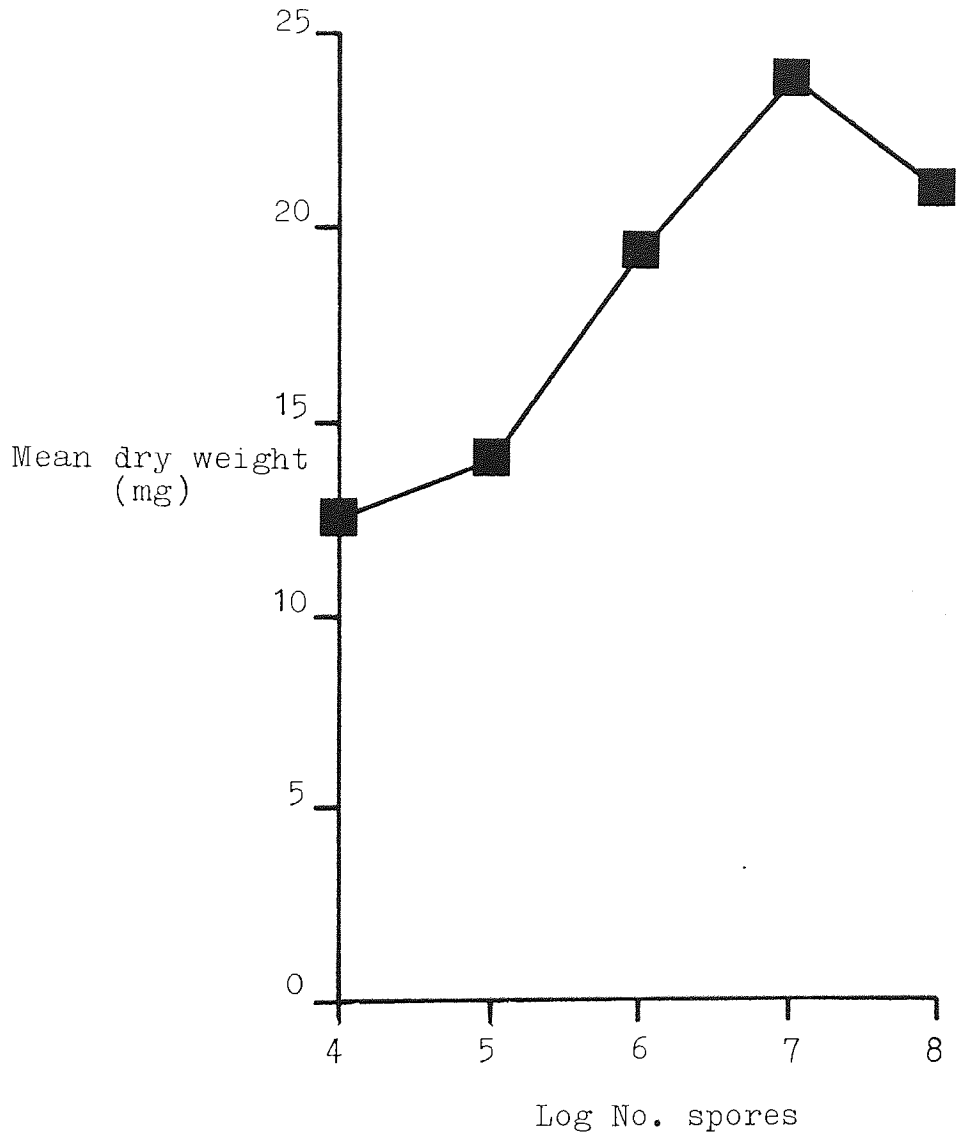

Fig. 26 Effect of inoculum size on the growth of *C. resinae* on undecane under shaken conditions

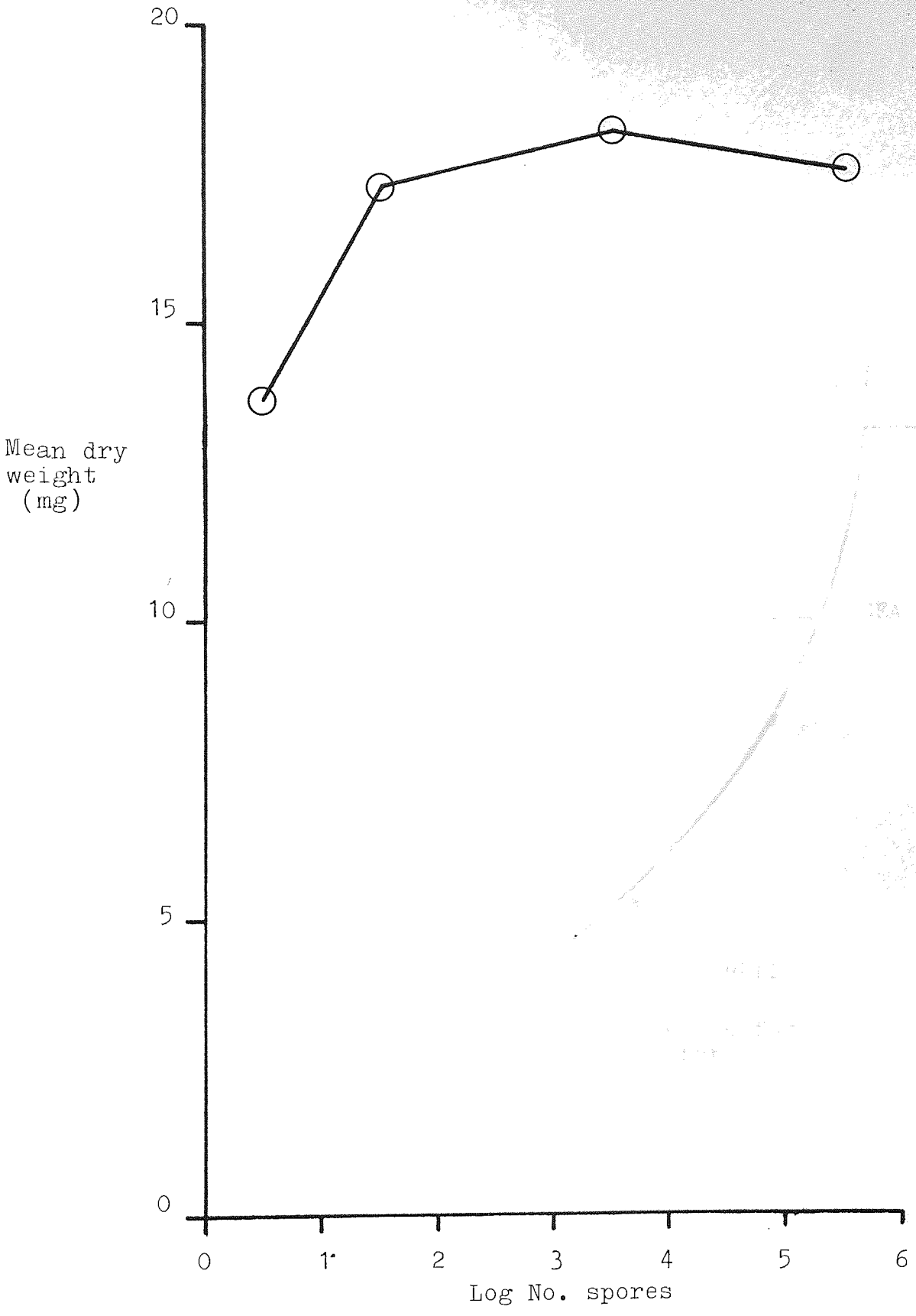

Fig. 27 Effect of inoculum size on the growth of C. resinae on diesel fuel under static conditions

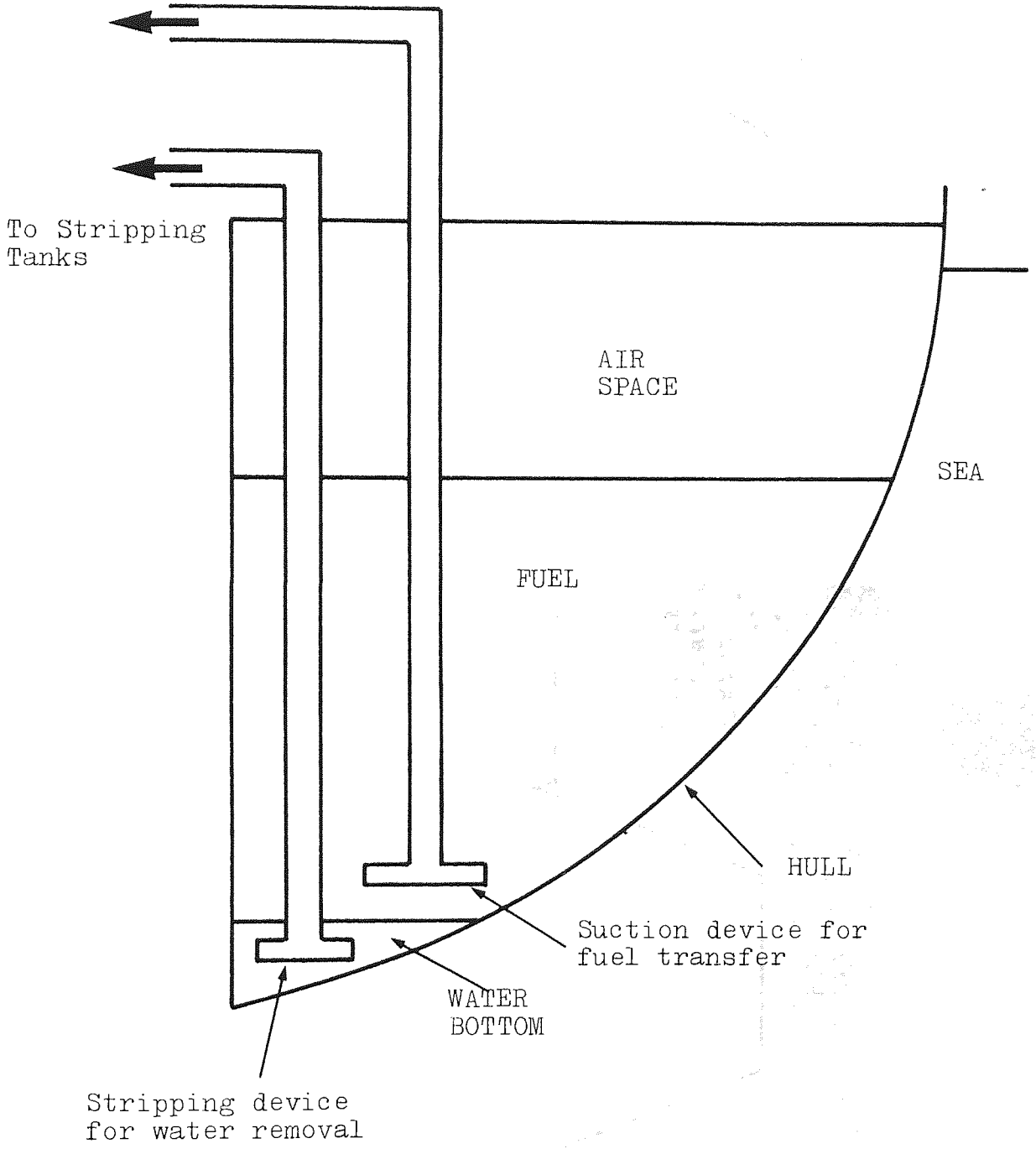

Fig 28 Diagram of a typical shipboard integral fuel storage tank

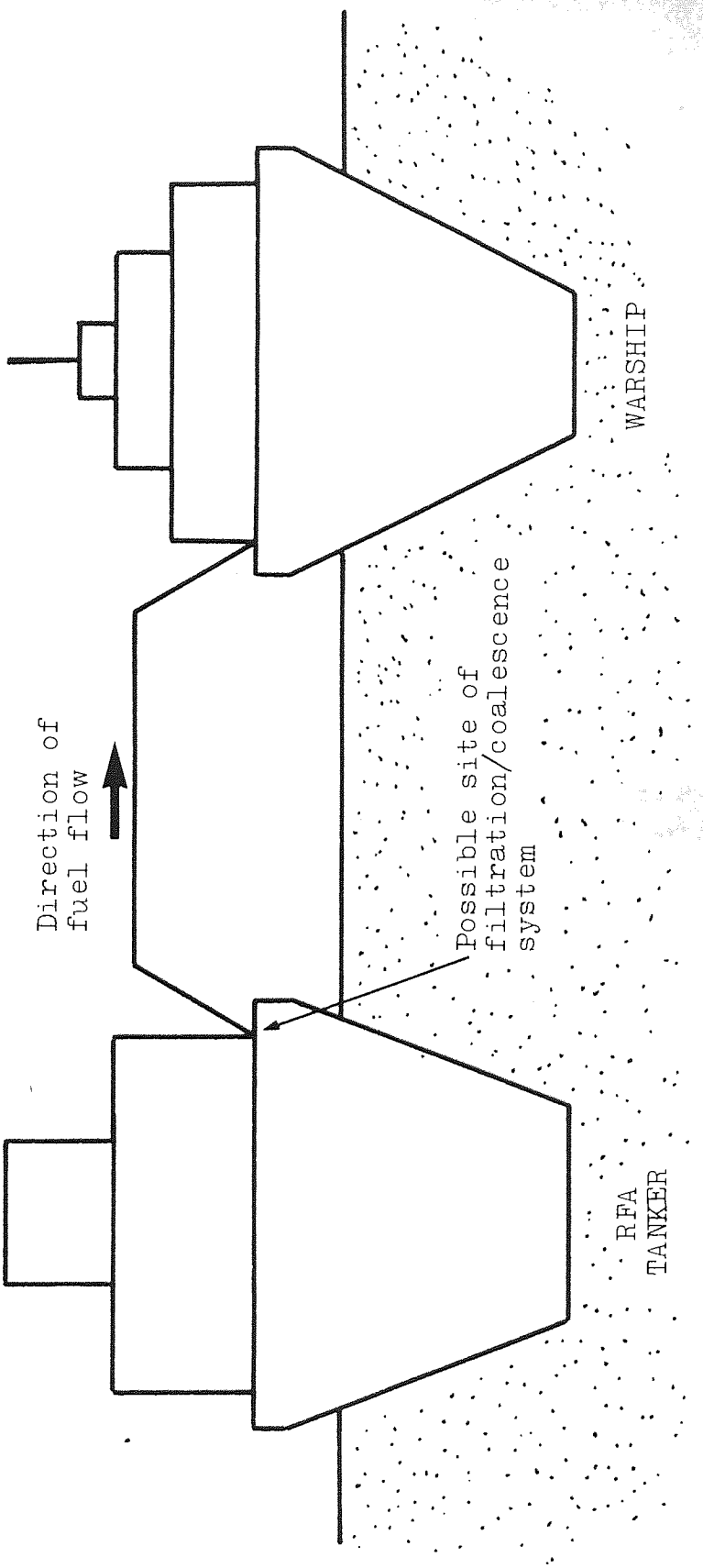

Fig. 29 Diagram of refuelling at sea

CHAPTER 5

GROWTH OF *Cladosporium resinae* ON HYDROCARBONS

5.1 Introduction

5.2 Methods

5.2.1 Organisms

5.2.2 Media

5.2.3 Growth in undecane and diesel fuel

5.2.4 Growth in four diesel fuels

5.2.5 Treatment of results

5.3 Results

5.4 Conclusions

5.5 Discussion

GROWTH OF *Cladosporium resinae* ON HYDROCARBONS

5.1 Introduction

In fuel systems, the energy which organisms require to synthesize cell material and for other functions such as motility is obtained from the catabolism of hydrocarbons in the fuel. Cofone et al. (1973) tested the ability of *C. resinae* to grow on a number of pure hydrocarbons. Growth was obtained on n-alkanes from hexane to nonadecane with best development on undecane. Gas chromatographic studies by McKenzie et al. (1976) showed that the n-alkanes from undecane to tetradecane in aviation kerosene were utilized by *C. resinae*.

Cofone et al. (1973) also reported growth on some alkenes, branched alkanes, cyclic hydrocarbons, benzene, toluene, isopropyl-benzene, ortho- and meta-xylene. Development on these substrates was generally slower and less extensive than on the n-alkanes. It is likely that the n-alkanes in fuel are preferentially utilized and are therefore the main substrates for microbial growth.

The fuels used by the Royal Navy for combustion in gas turbine engines are diesel fuels, which have a distillation range of approximately 180 - 380°C. They are a complex mixture of alkanes, alkenes and aromatic compounds, the relative proportions of which vary with the original crude oil source.

For normal operations, NAVAL 20 POUR (47/20 DIESO) is used. GENERAL PURPOSE (UK (MT) DIESO) which contains a red indicator dye and other additives to show it bears revenue duty, is used by commercial shipbuilders before warships are commissioned into service. REFERENCE DIESEL

FUEL, HIGH SULPHUR is used in the development testing of gas turbine engines.

5.2 Methods

5.2.1 Organisms

The following strains of *C. resinae* were used in this study:

a) ATCC 22711 (see sub-section 4.2.1)

b) EW1 (see Table 1)

c) EW2 (see Table 1)

d) KJIB1 obtained from Dr R.N. Smith, Hatfield Polytechnic, Herts.

The strains were maintained on malt extract agar slants at 4°C. Cultures were inoculated either by the transfer of spores with an inoculating loop or by the addition of spore suspensions (see Appendix II for preparation).

5.2.2 Media

The composition of the mineral salts solution used is described in Appendix III. The hydrocarbons used were undecane and the four diesel fuels described in Appendix III.

5.2.3 Growth in undecane and diesel fuel

General growth conditions are described in Appendix IV. In the first experiment test tubes containing mineral salts solution (5ml) and undecane (1ml) or diesel fuel (1ml) were inoculated with spores of *C. resinae* ATCC 22711, EW1, EW2 and KJIB1. The cultures were incubated for 26

days after which growth or otherwise was determined by visual observation (see Appendix V).

In the second experiment, conical flasks containing mineral salts solution (50ml) and undecane (5ml) or diesel fuel (5ml) were inoculated with spore suspensions of ATCC 22711 and EW1 (1ml, 10^7 spores ml^{-1}). The cultures were shaken at 100 r.p.m. for 74 days after which growth was estimated by dry weight determination (Appendix V).

5.2.4 Growth in four diesel fuels

Boiling tubes containing mineral salts solution (50ml) and one of the four diesel fuels described in Appendix III (50ml) were inoculated with spore suspensions of ATCC 22711 and EW1 (0.1ml; 10^7 spores ml^{-1}). After incubation for 60 days, dry weights of the cultures were measured.

5.2.5 Treatment of results

The mean dry weights of replicate flasks or tubes and standard deviations were calculated using the formulae in Appendix VI.

5.3 Results

The results of the growth of four strains of *C. resinae* in undecane and diesel fuel in test tube cultures are shown in Table 14. ATCC 22711 and EW1 were also grown in shake-flask culture and the results are shown in Table 15 and Fig. 30. The dry weights obtained from the growth of ATCC 22711 and EW1 on four diesel fuels are shown in Table 16 and Fig. 31. The appearance of the cultures can be seen in Figs. 32 to 35.

5.4 Conclusions

All four strains of C. resinae were capable of growth on diesel fuel. Three of the strains developed on undecane but no growth of EW2 was observed within the incubation period of 26 days (see Table 14). Under shaken conditions, meagre growth of ATCC 22711 and EW1 was obtained on diesel fuel. However, growth on undecane under these conditions was good. ATCC 22711 grew much more profusely than EW1 (see Table 15 and Fig. 30).

The same two strains were also cultured on four diesel fuels in boiling tubes. EW1 grew better than ATCC 22711. The dry weights of EW1 were similar for each fuel whereas ATCC 22711 grew better on NAVAL 20 POUR (47/20 DIESO) from Curacao and GENERAL PURPOSE (UK (MT) DIESO).

5.5 Discussion

All four strains of C. resinae grew in diesel fuel in the absence of an alternative carbon and energy source, confirming that the fungus is capable of utilizing constituents in the fuel (see section 5.1). Undecane did not support the growth of one of the strains, namely EW2. However, variation in growth response by different isolates of C. resinae to a number of alkanes was also noted by Teh and Lee (1973) which may be explained by the considerable natural variability of the fungus (Parbery, 1971a).

Undecane allowed better growth of C. resinae than diesel fuel in agitated culture. This finding is supported by the results of the agitation experiments in Chapter 7

and by the report of Cooney and Proby (1971) who used a stirred batch fermenter. Conversely, Teh and Lee (1973) reported little difference in growth on alkane and jet aviation fuel under static culture conditions. However, the results in Chapter 7 show that the fungus developed more profusely in undecane than in diesel fuel, whether cultured in a shaken or undisturbed environment.

It is unlikely that growth of the fungus is limited by the amount of substrate it can utilize in diesel fuel since alkanes account for about 50% of the total composition. The fuel probably contains compounds, such as certain aromatics, which inhibit growth.

C. resinae ATCC 22711 grew more profusely than EW1 on undecane and is further evidence of the natural variability of the fungus, described by Parbery (1971a). The reason is probably due to the nature of the habitat from whence each strain was isolated. ATCC 22711 was originally isolated from jet aviation fuel, which contains a high proportion of short chain hydrocarbons like undecane. EW1 was found in diesel fuel which contains less undecane than jet fuel. Hence, ATCC 22711 is more suited to growth on undecane than EW1 and this difference is reflected in their growth response to the alkane.

The finding that EW1 grows more vigorously than ATCC 22711 on diesel fuels supports this conclusion. Cultures of the two strains on various diesel fuels are shown in Figs. 32 to 35. The fungus grows as a mat at the oil-water interface. White, vegetative development of ATCC 22711 and brown, sporulating growth of EW1 can be seen.

ATCC 22711 grew less well in two of the diesel fuels examined which is probably due to their different chemical

compositions. Fuels which are known to inhibit microbial growth could possibly be earmarked for use in gas turbine ships.

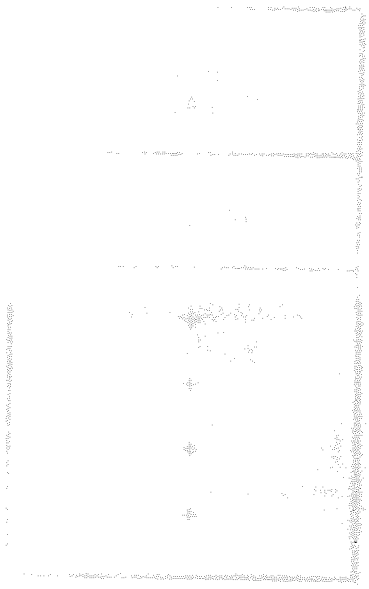

Table 14 Growth of C. resinae on undecane and and diesel fuel under static conditions

ORGANISM	ESTIMATION OF GROWTH AFTER 26 DAYS INCUBTAION	
	UNDECANE	DIESEL FUEL
C. resinae ATCC 22711	+	+
C. resinae EW1	+	+
C. resinae KJIB1	+	+
C. resinae EW2	-	+

+ - Visible growth observed

- - No visible growth observed

Table 15 Growth of C. resinae on undecane and diesel fuel under shaken conditions

ORGANISM	MEAN DRY WEIGHT OF THREE REPLICATE FLASKS (STANDARD DEVIATION)	
	UNDECANE	DIESEL FUEL
C. resinae ATCC 22711	946.3(34.4)	1.3(1.2)
C. resinae EW1	39.3(5.0)	0(0)

Table 16 Growth of C. resinae in four diesel fuels

ORGANISM	MEAN DRY WEIGHT OF TWO REPLICATE TUBES (STANDARD DEVIATION) (mg)			
	NAVAL 20 POUR (47/20 DIESO) (CURACAO)	NAVAL 20 POUR (47/20 DIESO) (BAHRAIN)	REFERENCE DIESEL FUEL HIGH SULPHUR	GENERAL PURPOSE (UK (MT) DIESO)
C. resinae ATCC 22711	20.5(0.7)	(12.0(1.4)	14.5(0.7)	21.5(3.5)
C. resinae EW1	37.0(7.1)	37.5(2.1)	31.5(3.5)	33.5(2.1)

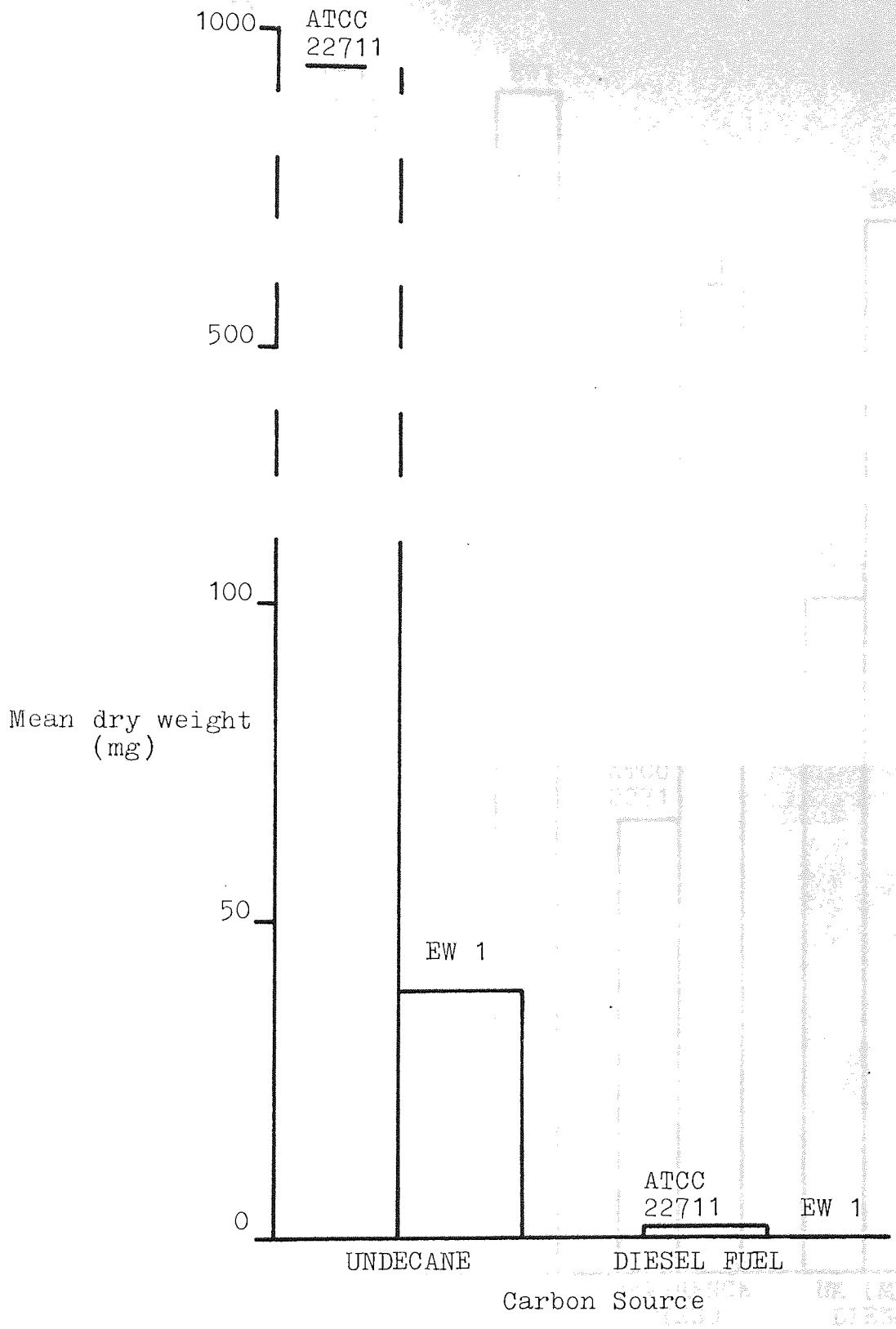

Fig. 30 Growth of two strains of C. resinae on undecane and diesel fuel under shaken conditions

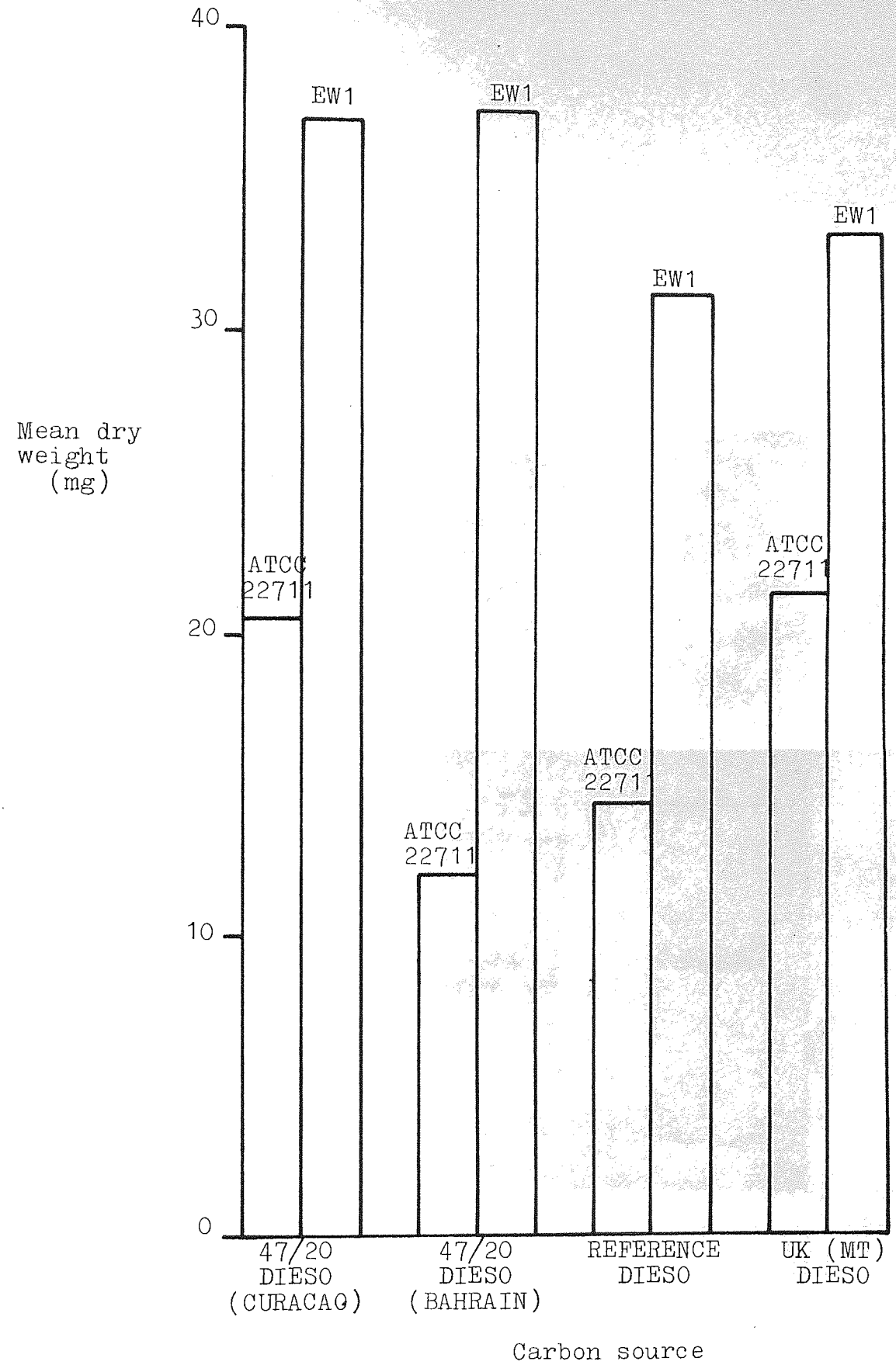

Fig. 31 Growth of two strains of C. resinae on four diesel fuels.

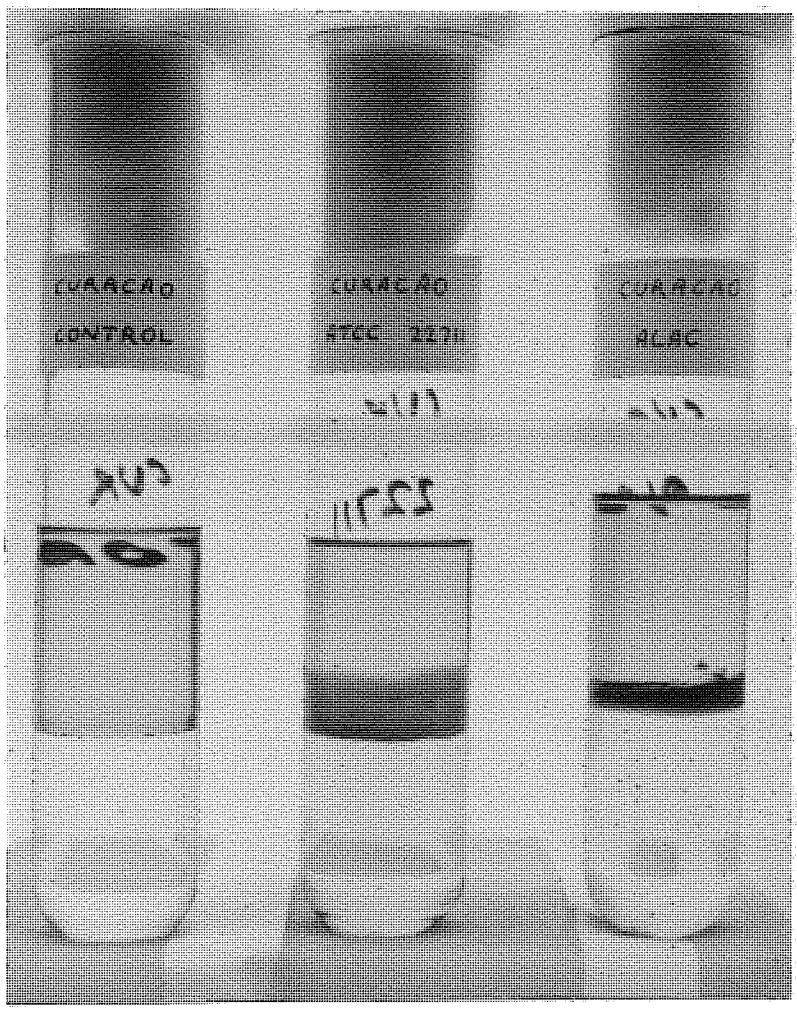

Fig. 32 Growth of C. resinae on NAVAL 20 POUR (47/20 DIESO) from Curacao.

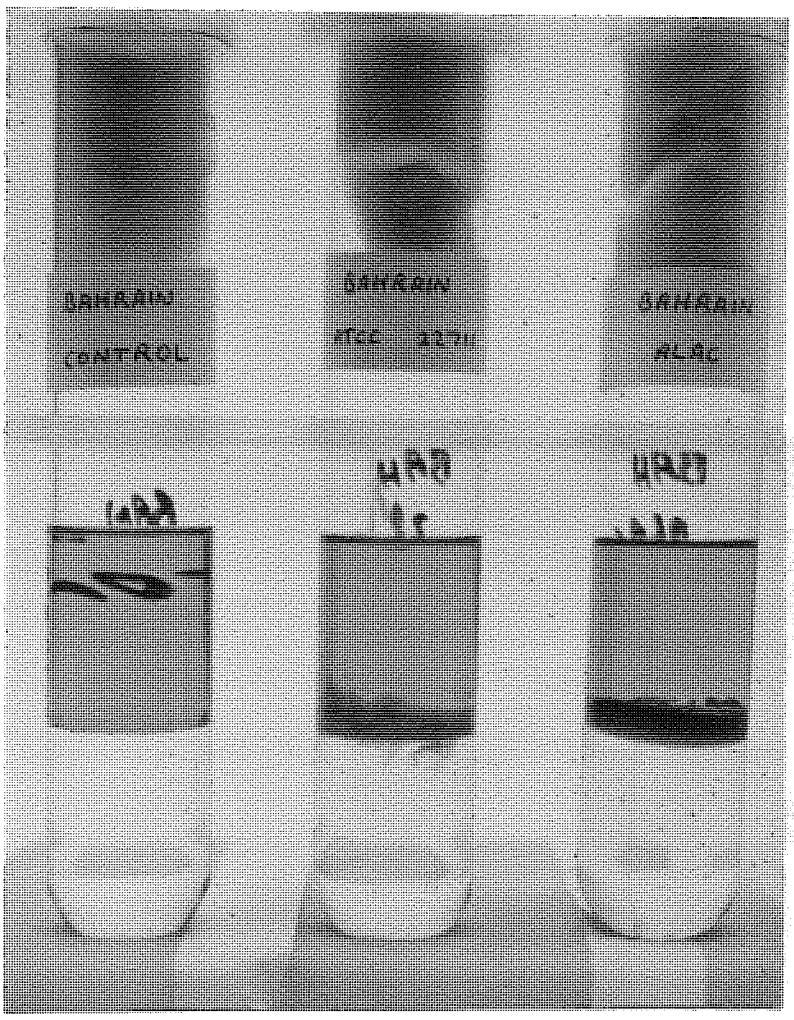

Fig. 33 Growth of C. resinae on NAVAL 20 POUR (47/20 DIESO) from Bahrain.

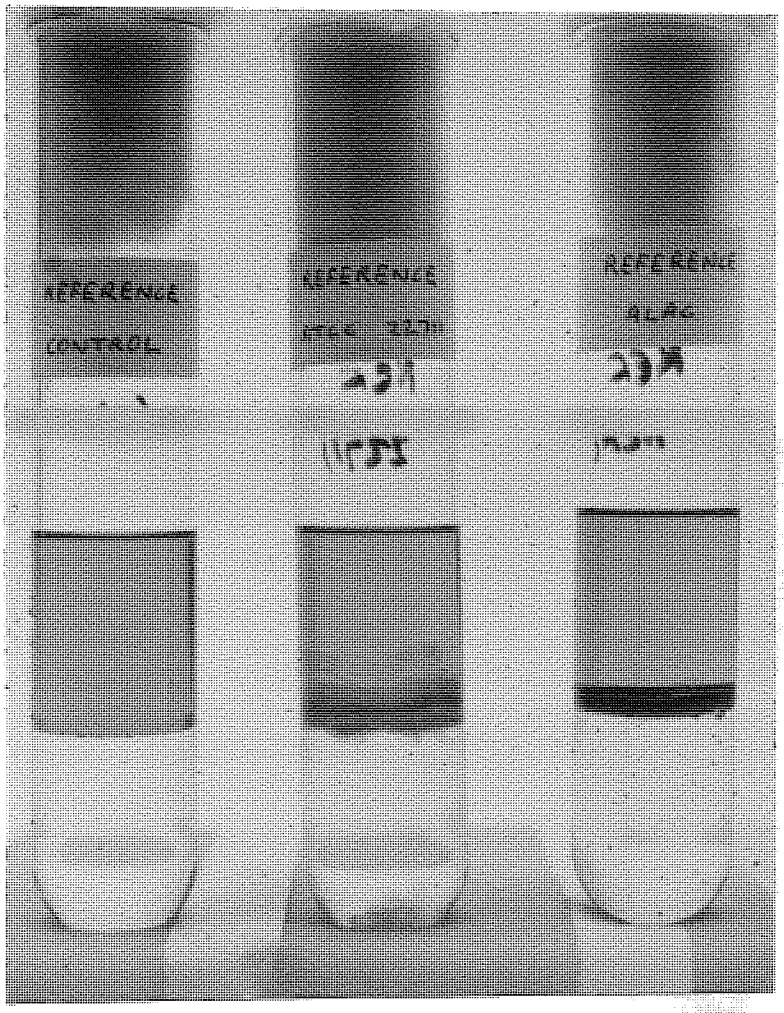

Fig. 34 Growth of C. resinae on REFERENCE DIESEL FUEL, HIGH SULPHUR.

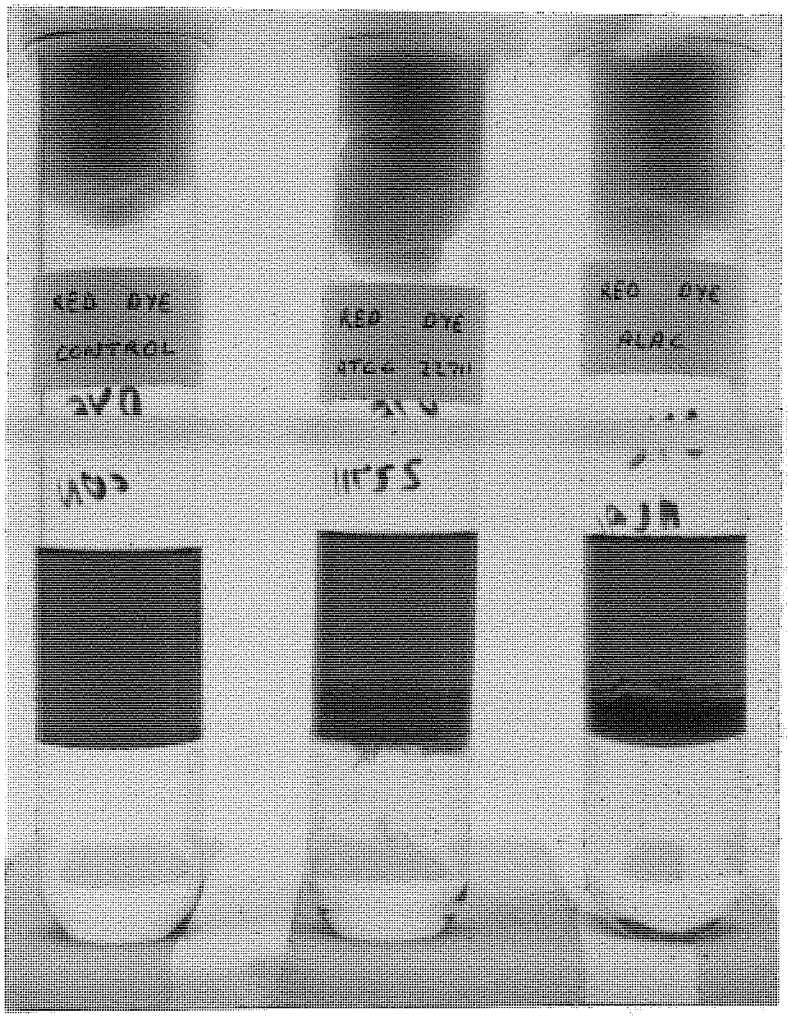

Fig. 35 Growth of C. resinae on GENERAL PURPOSE (UK (MT) DIESO).

CHAPTER 6

EFFECT OF AQUEOUS PHASE COMPOSITION ON MICROBIAL GROWTH

6.1 Introduction

6.2 Methods

6.2.1 Organisms

6.2.2 Media

6.2.3 Growth in mineral salts solution

6.2.4 Comparison of growth in sea water and mineral salts solution

6.2.5 Growth in a range of salinites

6.2.6 Growth of microorganisms in various aqueous media

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6.3 Results

6.4 Conclusions

6.4.1 Growth in mineral salts solution

6.4.2 Growth in sea water

6.4.3 Growth in various aqueous media

6.5 Discussion

EFFECT OF AQUEOUS PHASE COMPOSITION ON MICROBIAL GROWTH

6.1 Introduction

Hendey (1964) showed that C. resinae could not grow in kerosene in the absence of water. The composition of the aqueous phase is therefore likely to affect the growth of C. resinae and other microorganisms found in fuel systems. A change in the concentration of one or more water-soluble compounds can affect microbial developments by:

a) altering the nutrient levels

b) changing the concentration of inhibitors

The nutrients required for microbial growth are obtained from the diesel fuel and the aqueous phase. Carbon and hydrogen are usually provided by the energy source (Pirt, 1975), which are the hydrocarbons in the fuel.

The oxygen required for growth is obtained from the aqueous phase. The solubility of oxygen in air-saturated water is a few mg l^{-1} which is slight compared to the amount which can be consumed by respiring microorganisms. Hence, conditions may become anaerobic unless the oxygen solution rate is high. Diesel fuel appears to facilitate the transfer of oxygen from the atmosphere to the aqueous phase. Addition of kerosene to a shake flask has been reported to increase the transfer dramatically (Mimura et al., 1969). The reason may lie in the high solubility of oxygen in oil - about 100-fold more soluble than in water - which could endow the fuel with an oxygen carrier property (Pirt, 1975).

Nitrogen and sulphur compounds in the fuel probably

dissolve in the aqueous phase where they may be catabolized to provide microorganisms with nitrogen and sulphur. An alternative source of these elements, as well as phosphorus potassium, magnesium and 'trace' elements is from inorganic salts in the aqueous phase.

Sea water is the most likely source of these nutrients. The occurrence of phosphate in the ocean and hence in fuel systems has been described in Chapter 3. It appears that inorganic nutrient levels can vary considerably in the marine environment, especially high concentrations being found in polluted waters near populated areas (Jones and Folkard, 1971; Raymont, 1972). The sea water used to displace fuel in Type 42 destroyers (see Fig. 16) is often taken on while the ship is in harbour, therefore the water bottoms in the tanks are likely to contain high nutrient levels. Consequently, the growth of hydrocarbon-utilizing microorganisms is encouraged, leading to the problems described in Section 1.1.

Although higher nutrient concentrations tend to encourage microbial growth, Parbery and Thistlethwaite (1973) found that C. resinae grew better on aviation fuel when the concentration of Bushnell and Haas (1941) mineral salts solution was reduced to 1/10 strength. They suggested that an aqueous phase of high ionic strength could inhibit hydrocarbon transport into the aqueous phase. Presumably the effect would be to decrease uptake of the substrate by the organism with consequent reduction in growth. This phenomenon is investigated further in this chapter.

The presence of certain compounds in the aqueous phase can inhibit microbial growth. The possible toxic effects of

diesel fuel compounds has been discussed in Chapter 5 and the effect of deliberately added biocides in Chapter 8.

However, the effect of salinity on growth is examined in this chapter.

Byrne and Jones (1975) have summarized the effect of salinity on the vegetative growth, reproduction and germination of a range of fungi. As expected, high concentrations of sea water were favoured by marine species and low salinities by terrestrial fungi. It is likely therefore that salt water will discourage the development of C. resinae and other microorganisms which are considered to be terrestrial. The effect of sea water on growth is therefore also investigated in this chapter.

6.2 Methods

6.2.1 Organisms

The microorganisms studied in this chapter are listed in Table 17.

Spore suspensions were prepared as described in Appendix II.

6.2.2 Media

The preparation of mineral salts solution and hydrocarbons is described in Appendix III. Sea water was obtained from the mouth of Langstone Harbour, Hants., on a rising tide and sterilized by passage through a 0.22 micron filter. Yeast extract and bacteriological peptone were obtained from Oxoid and glucose from British Drug Houses.

6.2.3 Growth in mineral salts solution

General culture conditions are described in Appendix IV. In the first experiment, boiling tubes containing a range of concentrations of mineral salts solution from 0 to 100% (50ml) and diesel fuel (10ml) were inoculated with spore suspensions of *C. resinae* ATCC 22711 (0.1ml, 10^7 spores ml^{-1}). The cultures were incubated for 60 days after which growth was estimated by dry weight determination (see Appendix V).

In the second experiment, conical flasks containing full or $^1/_{10}$ strength mineral salts solution (50ml) and glucose (1%), diesel fuel (10ml) or a combination of glucose (1%) and diesel fuel (10ml) were inoculated with spore suspensions of EW1 (0.1ml; 10^7 spores ml^{-1}). The flasks were either shaken at 100 r.p.m. or left undisturbed. Growth was estimated by dry weight measurement after seven and fourteen days for cultures containing glucose and after 30 and 60 days for those containing diesel fuel only.

6.2.4 Comparison of growth in sea water and mineral salts solution

Conical flasks containing sea water (25ml) or mineral salts solution (25ml) and undecane (0.5ml) were inoculated with spore suspensions of ATCC 22711 (1ml; 10^7 spores ml^{-1}). The cultures were shaken at 200 r.p.m. and dry weights were determined after 30 days incubation.

6.2.5 Growth in a range of salinities

Test tubes containing a range of concentration of sea

water in distilled water from 0 to 100% (5ml) and diesel fuel (1ml) were inoculated with spores of ATCC 22711, EW1, EW2 and KJIB1. The cultures were incubated for 40 days and examined by visual observation for growth (see Appendix V).

6.2.6 Growth of microorganisms in various aqueous media

Test tubes containing the following aqueous media (5ml):

a) distilled water
b) $^{1}/_{10}$ strength mineral salts solution
c) sea water
d) $^{1}/_{10}$ strength mineral salts solution in sea water
e) sea water containing yeast extract (0.5%) and bacteriological peptone (0.5%) and diesel fuel (1ml)

were inoculated with spores or yeast cells of the micro-organisms listed in Table 17, except KJIB1. The cultures were incubated in the dark at 25 $\pm$1°C and the time taken to observe visible growth was reported.

6.2.7 Treatment of results

The mean dry weights of replicate flasks or tubes and standard deviations were calculated using the formulae in Appendix VI.

6.3 Results

The results of the growth of C. resinae ATCC 22711 in a range of concentrations of mineral salts solution are shown in Table 18 and Fig. 36. The results of the growth of EW1 in full and $^{1}/_{10}$ strength salts solution are shown in Table 19 and Fig. 37 for static cultures and in Table 20 and Fig. 38 for shaken cultures.

The growth of ATCC 22711 in sea water and mineral salts solution is compared in Table 21 and Fig. 39. Four strains were tested in a wide range of concentrations of sea water and the findings are shown in Table 22 and Fig. 40.

The microorganisms listed in Table 17 (with the exception of C. resinae KJIB1) were tested for their ability to grow in various aqueous media, described in sub-section 6.2.6. The results of growth in distilled water and mineral salts solution are shown in Table 23. The results for sea water and enriched sea water are shown in Table 24.

6.4 Conclusions

6.4.1 Growth in mineral salts solution

C. resinae ATCC 22711 was capable of growth in diesel fuel and each concentration of mineral salts solution tested. Slight growth of the fungus was detected in distilled water and increased to a maximum dry weight of 11.3mg per tube at 10% of the original concentration of mineral salts solution. As the concentration increased from 10 to 100%, growth decreased. (See Table 18 and Fig. 36).

EW1 was grown in full and $^{1}/_{10}$ strength mineral salts solution with glucose, diesel fuel and a combination of glucose and fuel as carbon sources. The results for static cultures are shown in Table 19 and Fig. 37. In general, better growth was obtained in full strength solution.

Table 20 and Fig. 38 show the results for agitated

cultures. Those containing glucose yielded more biomass in full strength than in $^{1}/_{10}$ strength mineral salts solution The dry weights obtained for flasks containing diesel fuel only were extremely low and no difference in growth between the two concentrations of salts solution could be realized.

Regardless of the concentration of mineral salts, faster growth was observed on glucose than on diesel fuel. Combining glucose and fuel yielded more growth than glucose only, the difference being more apparent with static than agitated cultures (see also Fig. 43 and 44).

6.4.2 Growth in sea water

C. resinae ATCC 22711 grew much more profusely in mineral salts solution than in sea water (see Table 21 and Fig. 39). The results in Table 22 and Fig. 40 show that all four strains of C. resinae developed in distilled water and in 10% sea water. EW2 was unable to grow above this concentration. ATCC 22711 was the only strain capable of growing in 50% brine. No strains developed in 75 or 100% sea water.

6.4.3 Growth in various aqueous media

The microorganisms were placed in four groups based on their ability to grow in the various aqueous media (see Tables 23 and 24). Groups 1 and 2 developed in distilled water and diesel fuel. With the addition of mineral salts, group 3 isolates were also able to grow.

No microorganisms developed in sea water and diesel fuel. However, the addition of mineral salts allowed

the growth of groups 1 and 3. When the sea water was supplemented with yeast extract and bacteriological peptone, each species tested was able to grow.

Group 4 isolates were unable to grow in any of the aqueous media when diesel fuel was supplied as the only carbon and energy source.

6.5 Discussion

"As a general rule, the growth rate of microorganisms remains unaffected by the concentrations of its nutrients until these have fallen to very low values" (Stanier et al., 1971). The results obtained in this chapter show that C. resinae amd some species of Penicillium were capable of some development in only distilled water and diesel fuel. Parbery and Thistlethwaite (1973) also reported the meagre growth of two strains of C. resinae in distilled water and aviation fuel. Hence, a few microorganisms would be capable of surviving and even developing to some degree in fuel systems where nutrients were reduced in some way to extremely low values.

Nevertheless, growth in distilled water was limited by the concentration of inorganic nutrients because biomass production in mineral salts solution was far more profuse.

Microbial colonization of fuel systems can therefore be discouraged by minimizing the concentration of mineral salts in water bottoms. Levels could be reduced by preventing the accidental ingress of sea water and other sources which are rich in nutrients, such as sewage. Therefore, the use of harbour water to displace fuel is undesirable. Ideally, the

water used should be unpolluted. It may also be possible to actively remove certain nutrients. For example, aerobic microorganisms could be inhibited through lack of dissolved oxygen by sparging the fuel with an inert gas.

Maximum growth of C. resinae ATCC 22711 on diesel fuel was obtained in 1/10 strength mineral salts solution which is in agreement with the findings of Parbery and Thistlethwaite (1973). However EW1 generally grew more profusely in full strength than in 1/10 strength mineral salts solution; whether cultured using glucose or diesel fuel. The results for EW1 therefore conflict with those for ATCC 22711 and those of Parbery and Thistlethwaite (1973). The reason may be due to strain differences.

Faster growth was obtained on glucose than on diesel fuel, which is consistent with the findings of a number of reports (Cooney and Proby, 1971; Cofone et al., 1973; Walker and Cooney, 1973a, b). Combining diesel fuel and glucose yielded more biomass than growth on glucose only. This effect was more marked in static cultures and is therefore probably due to the fuel enhancing oxygen transfer into the aqueous phase (see section 6.1). Thus, growth limitation through lack of dissolved oxygen in static cultures is reduced and higher dry weights are obtained. In agitated culture, oxygen transfer is already high and the extra effect of the fuel is probably insignificant.

Sea water supported sparse or no detectable microbial growth and may be due only to the low levels of inorganic nutrients in the medium. However, many of the organisms

grew to some extent in distilled water and diesel fuel and retardation of the growth of four strains of C. resinae was observed with increasing salinity. Hendey (1964) also reported the retardation of growth of C. resinae above 30% sea water. The findings suggest that high salinities are inhibitory to the microorganisms studied.

Byrne and Jones (1975) have summarized the effect of salinity on the vegetative growth, reproduction and germination of a range of fungi. The vegetative growth of terrestrial fungi exhibited a broad tolerance to increasing salinities, whereas sexual reprcduction and spore germination were markedly affected by high salinities. The lack of growth of the hydrocarbon utilizing organisms in sea water may have been due to the inhibition of germination of the spore inoculum.

The addition of inorganic nutrients to the sea water allowed the growth of four species of Penicillium and the two strains of Candida guilliermondii indicating that growth was limited only by the absence of nutrients in sea water and not by inhibition due to high salinity. Also, the addition of yeast extract and bacteriological peptone to the sea water allowed the growth of all the microorganisms studied. Borut and Johnson (1962) reported that glucose or peptone could overcome the stress induced in terrestrial fungi by high salinity.

These findings stress the need to use only unpolluted sea water for fuel displacement in Type 42 destroyers. Microbial colonization is likely to be discouraged in this environment. The presence of high microbial numbers in sea water/fuel systems may be due to previous develop-

ment in a less hazardous environment.

Some of the microorganisms isolated from fuel/water samples were unable to grow in any of the media with diesel fuel as sole carbon and energy source. These organisms may have lost the ability to utilize hydrocarbons because of repeated sub-culture on malt extract agar. Alternatively, the fuel/aqueous phase media may have been lacking in certain nutrients, vitamins or metals essential to growth of these microorganisms.

Table 17 List of microorganisms studied in Chapter 6

ORGANISM	STRAIN CODE	ORIGIN
Cladosporium resinae	ATCC 22711	(see sub-section 4.2.1)
" "	EW1	(see Table 1)
" "	EW2	" " "
" "	EW3	" " "
" "	KJIB1	(see sub-section 5.2.1)
Penicillium corylophilum	EW4	(see Table 1)
P. janthinellum	PJB1	HMS Sheffield
P. citrinum	PJB2	HMS Lincoln
P. corylophilum	PJB3	HMS Glasgow
P. chrysogenum	PJB7	HMS Ardent
P. brevicompactum	PJB8	HMS Lynx
P. cyclopium	PJB9	Reference diesel fuel
Paecilomyces varioti	PJB5	HMS Birmingham
Fusarium solani	PJB6	HMS Alacrity
Candida guilliermondii	PJB4	HMS Sheffield
" "	EW5	(see Table 1)
Rhodotorula glutinis	EW6	" " "

Note Strains coded PJB were obtained from Miss P.J. Burrows, Portsmouth Polytechnic, Hants.

Table 18 Growth of C. resinae in a range of concentrations of mineral salts solution

CONCENTRATION OF MINERAL SALTS SOLUTION (%)	MEAN DRY WEIGHT OF THREE REPLICATE TUBES (STANDARD DEVIATION) (mg)
0	0.7 (0.6)
1	6.0 (1.0)
10	11.3 (2.1)
50	9.0 (2.0)
100	5.7 (0.6)

Table 19 Growth of C. resinae in 10 and 100% mineral salts solution under static conditions

CARBON SOURCE	INCUBATION PERIOD (DAYS)	MEAN DRY WEIGHT OF TWO REPLICATE FLASKS (STANDARD DEVIATION) (mg)	
		10% SOLUTION	100% SOLUTION
GLUCOSE	7	17.0 (1.4)	29.0 (-)a
	14	52.5 (0.7)	68.0 (26.9)
GLUCOSE AND DIESEL FUEL	7	50.0 (1.4)	41.5 (9.2)
	14	95.0 (-)a	120.0 (-)a
DIESEL FUEL	30	47.5 (20.5)	36.5 (9.2)
	60	81.5 (7.8)	171.0 (2.8)

a - Result of one flask only

Table 20 Growth of *C. resinae* in 10 and 100% mineral salts solution under shaken conditions

CARBON SOURCE	INCUBATION PERIOD (DAYS)	MEAN DRY WEIGHT OF TWO REPLICATE FLASKS (STANDARD DEVIATION) (mg)	
		10% SOLUTION	100% SOLUTION
GLUCOSE	7	40.5 (0.7)	52.5 (4.9)
	14	71.0 (2.8)	99.5 (4.9)
GLUCOSE AND DIESEL FUEL	7	52.0 (1.4)	78.5 (2.1)
	14	70.5 (0.7)	99.0 (11.3)
DIESEL FUEL	30	2.0 (0)	1.5 (0.7)
	60	4.5 (0.7)	2.0 (1.4)

Table 21 Growth of C. resinae in sea water and mineral salts solution

AQUEOUS PHASE	MEAN DRY WEIGHT OF TWO REPLICATE FLASKS (STANDARD DEVIATION) (mg)
MINERAL SALTS SOLUTION	24.0 (2.8)
SEA WATER	1.0 (0)

Table 22 Growth of C. resinae in a range of concentrations of sea water

ORGANISM	DETECTION OF VISIBLE GROWTH					
SEA WATER CONCENTRATION (%)	0	10	25	50	75	100
SALINITY (‰)	0	3.4	8.4	16.9	25.4	33.8
INITIAL pH	5.8	7.6	7.8	7.8	7.8	7.8
C. resinae ATCC 22711	+	+	+	+	–	–
C. resinae EW1	+	+	+	–	–	–
C. resinae KJIB1	+	+	+	–	–	–
C. resinae EW2	+	+	–	–	–	–

Table 23 Growth of deteriogenic organisms on diesel fuel in distilled water and mineral salts solution

ORGANISM	STRAIN CODE	GROUP	TIME TAKEN FOR VISIBLE GROWTH (DAYS)	
			DISTILLED WATER	MINERAL SALTS SOLUTION
Penicillium corylophilum	EW4	1	20	20
P. janthinellum	PJB1		20	20
P. citrinum	PJB2		20	20
P. corylophilum	PJB3		20	20
Cladosporium resinae	ATCC 22711	2	20	20
" "	EW1		26	26
" "	EW2		26	26
" "	EW3		26	26
Candida guilliermondii	EW5	3	NG	4
" "	PJB4		NG	4
Paecilomyces varioti	PJB5	4	NG	NG
Fusarium solani	PJB6		NG	NG
P. chrysogenum	PJB7		NG	NG
P. brevicompactum	PJB8		NG	NG
P. cyclopium	PJB9		NG	NG
Rhodotorula glutinis	EW6		NG	NG

NG = No growth after 60 days incubation

Table 24 Growth of deteriogenic organisms on diesel fuel in sea water and enriched sea water

ORGANISM	STRAIN CODE	GROUP	TIME TAKEN FOR VISIBLE GROWTH (DAYS)		
			SEA WATER	SEA WATER MINERAL SALTS	SEA WATER YEAST EXTRACT BACT. PEPTONE
Penicillium corylophilum	EW4	1	NG	20	4
P. janthinellum	PJB1		NG	26	4
P. citrinum	PJB2		NG	20	4
P. corylophilum	PJB3		NG	20	4
Cladosporium resinae	ATCC 22711	2	NG	NG	10
" "	EW1		NG	NG	10
" "	EW2		NG	NG	10
" "	EW3		NG	NG	10
Candida guilliermondii	EW5	3	NG	4	7
" "	PJB4		NG	5	7
Paecilomyces varioti	PJB5	4	NG	NG	4
Fusarium solani	PJB6		NG	NG	2
P. chrysogenum	PJB7		NG	NG	3
P. brevicompactum	PJB8		NG	NG	3
P. cyclopium	PJB9		NG	NG	2
Rhodotorula glutinis	EW6		NG	NG	7

NG = No growth after 60 days incubation

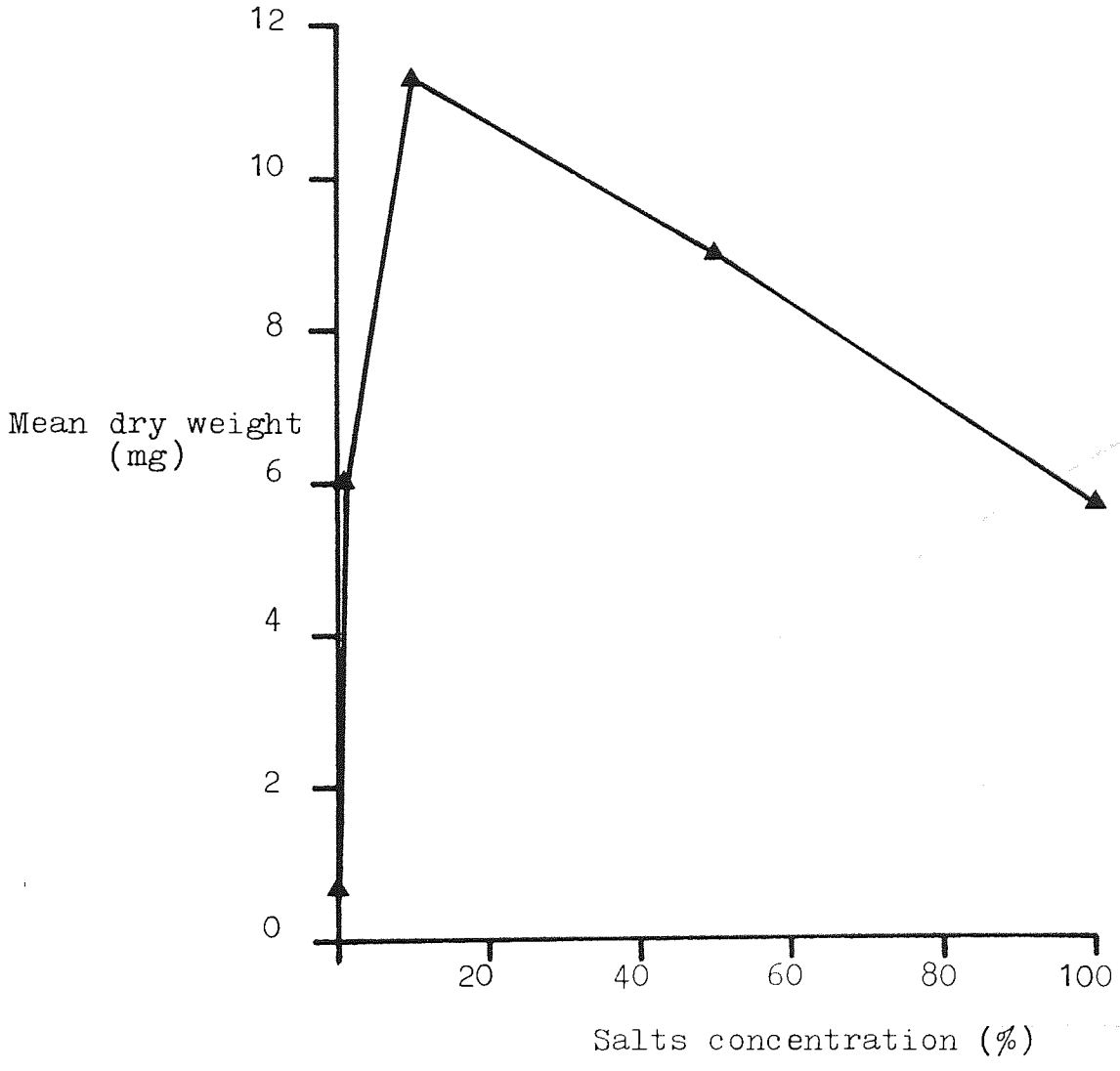

Fig. 36 Growth of C. resinae in varying concentrations of mineral salts solution

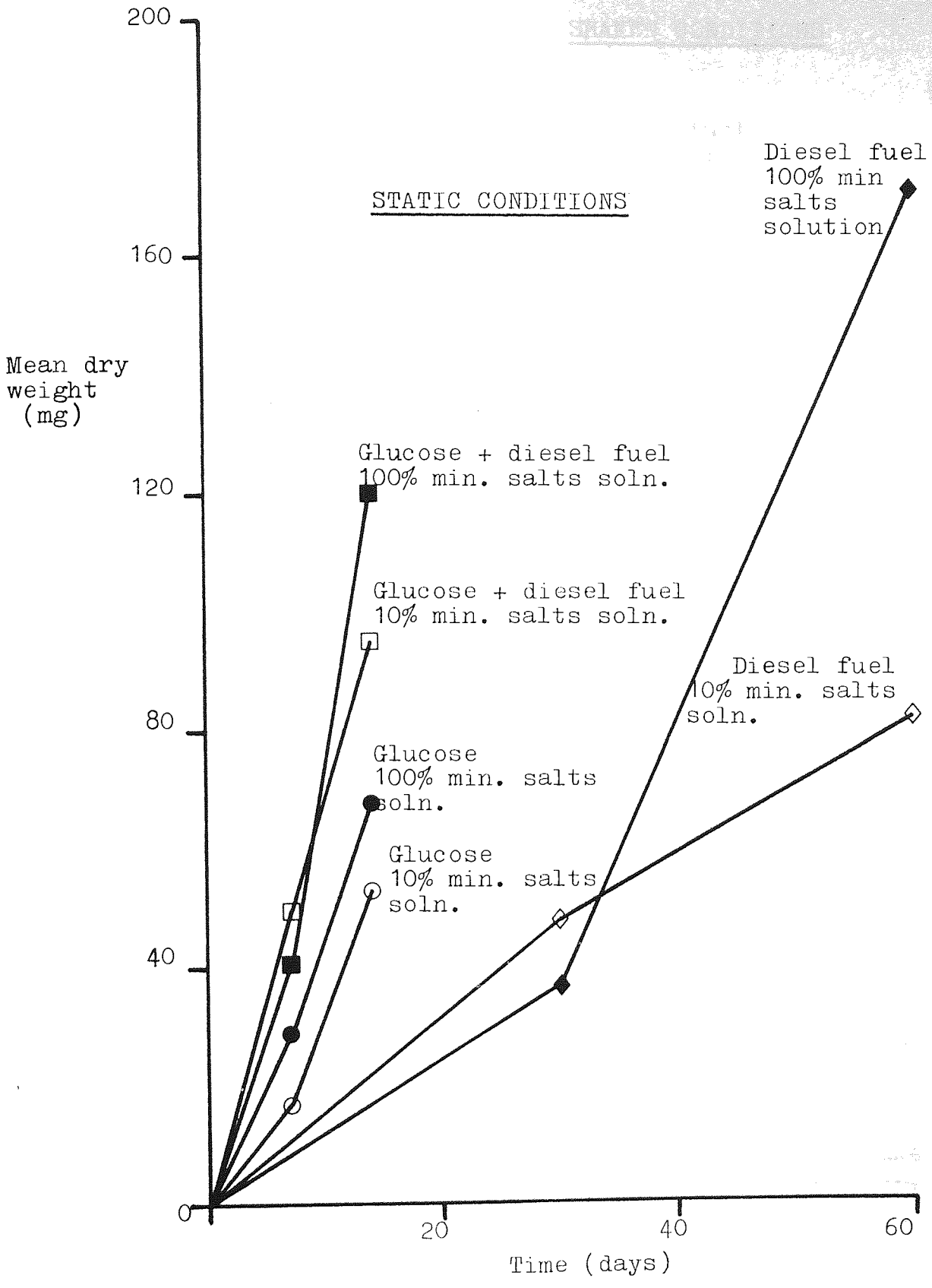

Fig. 37 Growth of C. resinae in various media under static culture conditions.

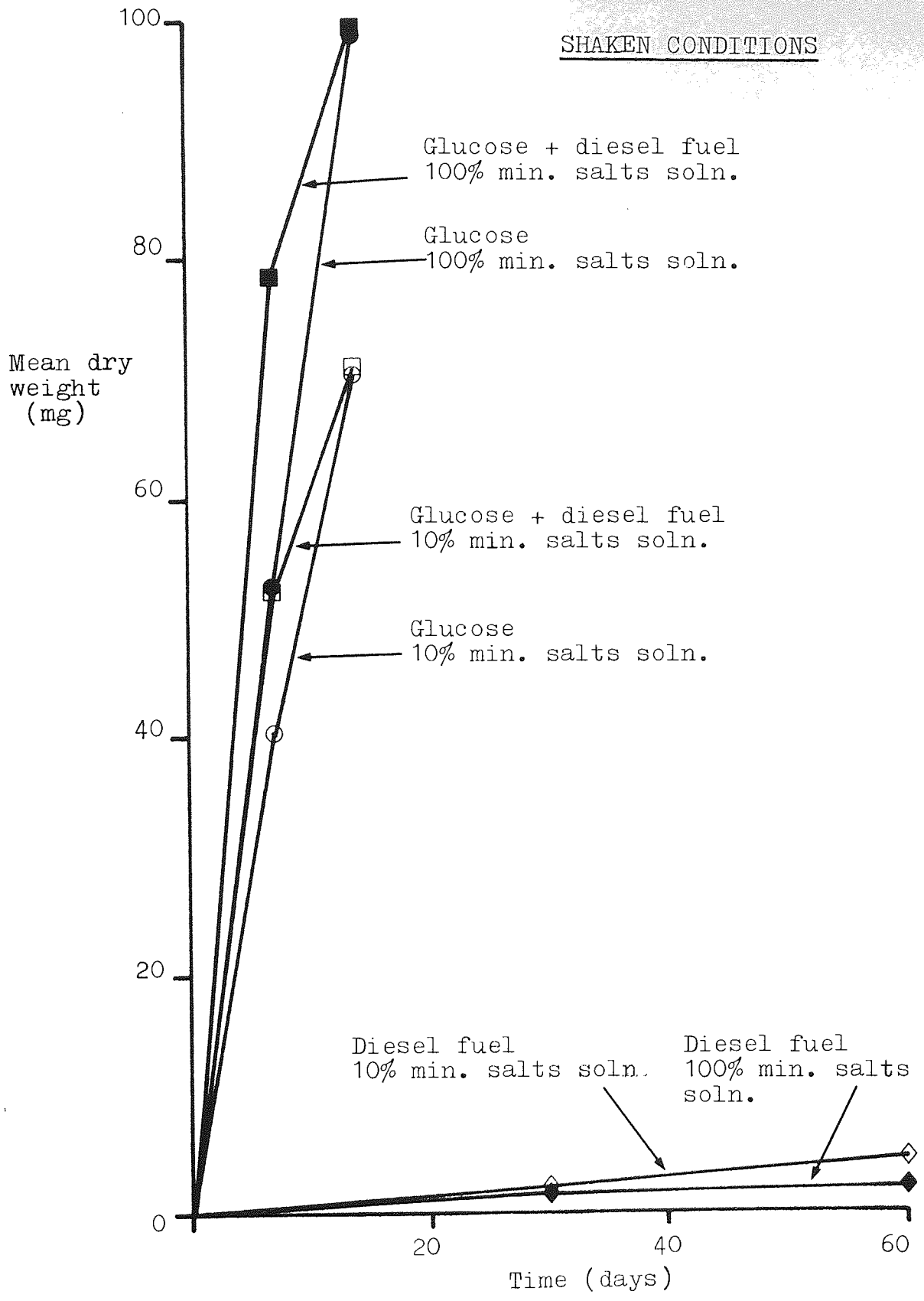

Fig. 38 Growth of C. resinae in various media under shaken culture conditions.

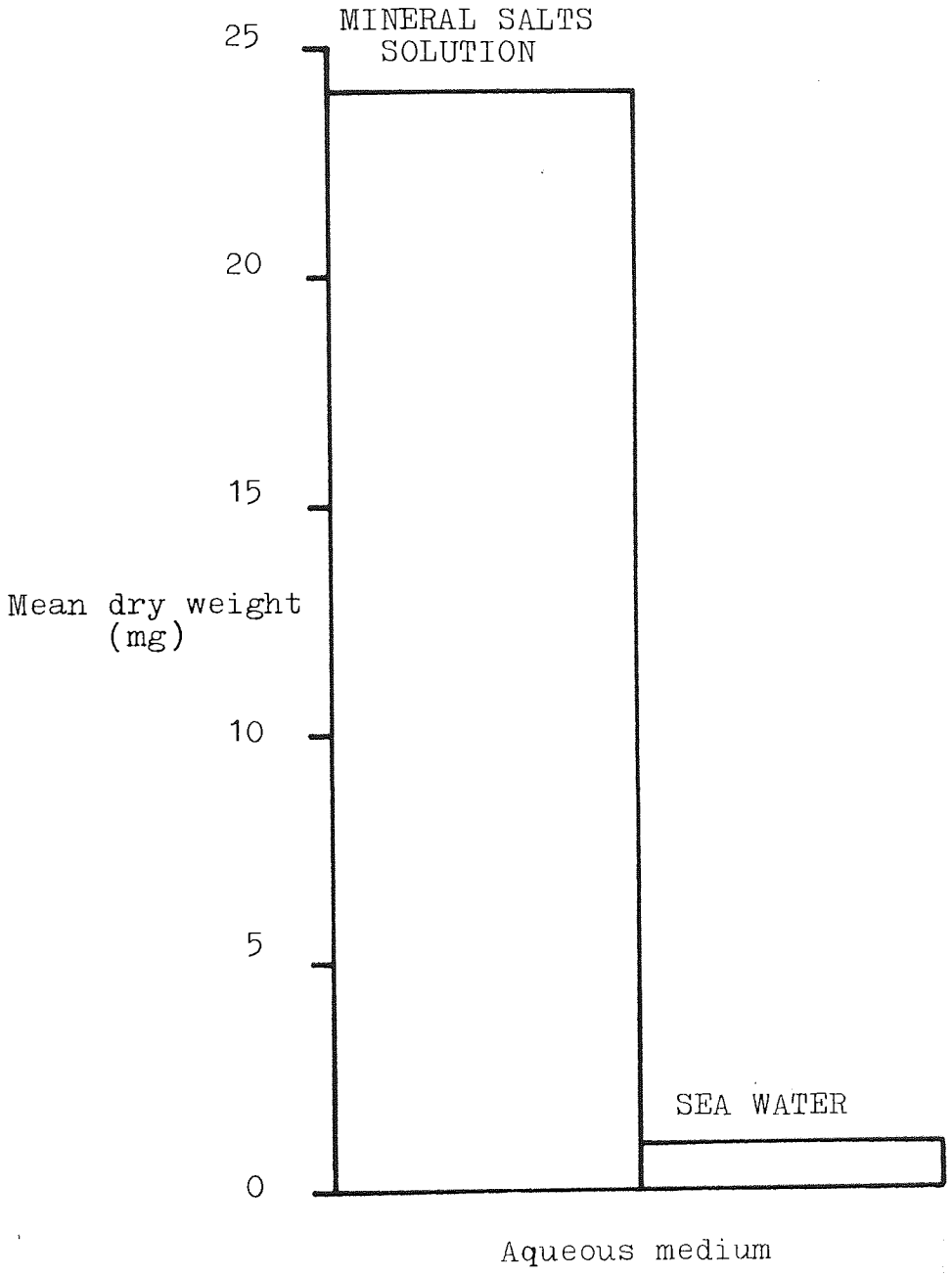

Fig. 39 Growth of C. resinae in mineral salts solution and sea water

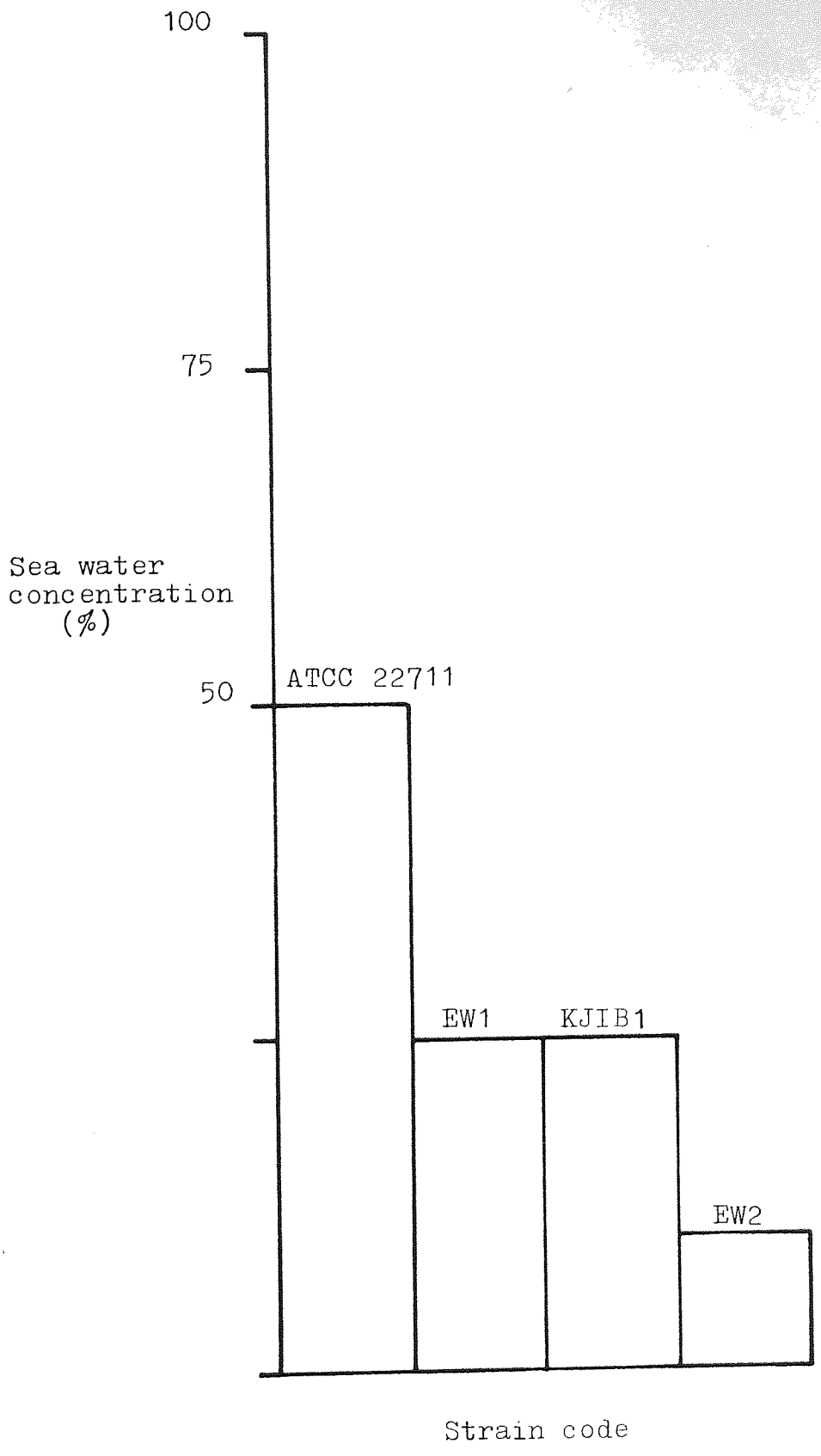

Fig. 40 Growth of four strains of C. resinae in varying concentrations of sea water.

CHAPTER 7

EFFECT OF HYDROCARBON CONCENTRATION, CULTURE VOLUME AND AGITATION ON THE GROWTH OF *Cladosporium resinae*

7.1 Introduction

7.2 Methods

- 7.2.1 Organisms
- 7.2.2 Media
- 7.2.3 Effect of hydrocarbon concentration and culture volume
- 7.2.4 Effect of agitation
- 7.2.5 Treatment of results

7.3 Results

7.4 Conclusions

7.5 Discussion

- 7.5.1 Effect of hydrocarbon concentration
- 7.5.2 Effect of culture volume
- 7.5.3 Effect of agitation

EFFECT OF HYDROCARBON CONCENTRATION, CULTURE VOLUME AND AGITATION ON THE GROWTH OF *Cladosporium resinae*

7.1 Introduction

Some of the environmental conditions which may affect the growth of microorganisms in fuel systems include:

a) amount of water bottom

b) volume of tank contents

c) agitation

Water can accumulate in tanks by the following means (see also section 3.1):

a) condensation due to changes in temperature

b) deliberate and accidental ingress of sea water

c) accidental ingress of rain

The amount in sea water displaced tanks of Type 42 destroyers can vary from 0 - 100% of the total liquid volume (see Fig. 16). The fungus, *C. resinae* is unable to grow on hydrocarbons in the absence of water (Hendey, 1964), hence microbial development in fuel systems could be prevented by the complete removal of water from the fuel. In practice, however, it is extremely difficult to keep the fuel completely dry because of condensation, water ingress and the limitations of the removal equipment, discussed in section 4.5.

Nevertheless, since microorganisms are usually associated with the aqueous phase, large amounts of water could encourage increased biomass formation because of the greater 'biological space' which is available.

The volume of fuel tank contents is likely to vary because of transfer and removal of fuel. Thus, air spaces

may sometimes be created which could assist in aeration of the tank contents. Similarly, sloshing of fuel due to movement of the ship could also improve oxygen transfer. The effect would be to encourage the growth of aerobic microorganisms in the water bottoms, leading to the problems discussed in section 1.1. These effects are examined in this chapter.

7.2 Methods

7.2.1 Organisms

Two strains of C. resinae were used in this study; ATCC 22711 (see sub-section 4.2.1) and EW1 (see Table 1). Spore suspensions were prepared as described in Appendix II.

7.2.2 Media

The preparation of mineral salts solution and hydrocarbons is described in Appendix III.

7.2.3 Effect of hydrocarbon concentration and culture volume

General culture conditions are described in Appendix IV. Conical flasks containing mineral salts solution (25 or 50ml) and undecane (2 or 10% v/v) were inoculated with spore suspensions of C. resinae ATCC 22711 (1ml; 10^7 spores ml^{-1}). The cultures were shaken at 200 r.p.m. for 30 days after which growth was estimated by dry weight determination (see Appendix V).

7.2.4 Effect of agitation

In the first experiment, conical flasks containing mineral salts solution (50ml) and undecane (5ml) or diesel fuel (5ml) were inoculated with spore suspensions of ATCC 22711 (1ml; 10^7 spores ml^{-1}). The cultures were either shaken at 100 r.p.m. or left undisturbed. After an incubation period of 30 days for flasks containing undecane and 60 days for those containing diesel fuel, growth was estimated by dry weight determination.

In the second experiment, conical flasks containing full or $^1/_{10}$ strength mineral salts solution (50ml) and glucose (1%), diesel fuel (10ml) or a combination of glucose (1%) and diesel fuel (10ml) were inoculated with spore suspensions of EW1 (0.1ml; 10^7 spores ml^{-1}). The flasks were either shaken at 100 r.p.m. or left undisturbed. Growth was estimated by dry weight measurement after seven and fourteen days for cultures containing glucose and after 30 and 60 days for those containing diesel fuel only.

7.2.5 Treatment of results

The mean dry weights of replicate flasks and standard deviations were calculated using the formulae in Appendix VI.

7.3 Results

The results of the effect of hydrocarbon concentration and culture volume are shown in Table 25 and Fig. 41. The effect of agitation on ATCC 22711 is shown in Table 26 and Fig. 42. The results of the effect of shaking on EW1 are shown in Tables 27 and 28 and Figs. 43 and 44.

7.4 Conclusions

An increase in undecane concentration from 2 to 10% resulted in increased growth of C. resinae. Doubling the culture volume yielded no significant difference in biomass concentration on 2% undecane and a reduction in growth on 10% hydrocarbon (see Table 25 and Fig. 41).

The results in Table 26 and Fig. 42 show that with either undecane or diesel fuel as sole carbon and energy source, the growth of ATCC 22711 was significantly reduced under shaken conditions. The behaviour of EW1 was also compared under static conditions. The results for cultures in full strength mineral salts solution are shown in Table 27 and Fig. 43. With glucose as sole carbon and energy source, agitation increased fungal growth. The combination of glucose and diesel fuel yielded more biomass in shaken flasks after seven days incubation but less growth after fourteen days. Fungal development was significantly depressed in shaken flasks containing mineral salts solution and diesel fuel only. Similar growth characteristics were obtained for cultures in $^{1}/_{10}$ strength mineral salts solution (see Table 28 and Fig. 44).

7.5 Discussion

7.5.1 Effect of hydrocarbon concentration

An increase in the concentration of undecane from 2 to 10% (v/v) gave an increase in the growth of C. resinae. Ratledge (1968) reported that a Candida sp. grew more rapidly at higher concentrations of n-alkanes than at lower concentrations. These findings suggest that growth

of the organisms is limited by substrate availability at lower hydrocarbon concentrations.

The solubility of n-alkanes above butane decreases with chain length according to the expression

$$\log H = 4.526 - 0.588\ n$$

where H is the solubility in mg l^{-1} and n is the number of carbon atoms in the molecule (Johnson, 1964). The solubility of undecane is therefore 0.0114 mg l^{-1}.

Yoshida et al. (1971) have demonstrated the growth of microorganisms on dissolved hydrocarbons. However, because of the very low solubility of undecane, the microbial growth rate is likely to depend on the rate of undecane solubility. An increase in the concentration of undecane increases the rate of solubility and consequently more growth is obtained.

There is evidence that hydrocarbon-utilizing organism can assimilate hydrocarbons by direct uptake from the oil phase. Johnson (1964) has calculated from diffusion considerations that appreciable growth rates cannot be obtained on the higher alkanes unless a special mechanism for substrate uptake is available. Mimura et al. (1971) demonstrated that hydrocarbon-assimilating yeasts have stronger affinities for oil than yeasts which are unable to utilize hydrocarbons. Wang and Ochoa (1972) found transitions from exponential to linear growth at higher cell concentrations in batch culture which was attributed to hydrocarbon limitation by the available oil-water surface area.

An increase in the oil-water surface area therefore prolongs exponential growth and a greater biomass

concentration is obtained before the onset of linear growth. An increase in the concentration of undecane in a batch culture increases the oil-water interfacial area and consequently more microbial growth is obtained.

In conclusion, the increased growth of C. resinae on higher concentrations of undecane can be explained whether growth is on dissolved undecane, by direct uptake or by a combination of these processes.

7.5.2 Effect of culture volume

Doubling the media volume in the flasks did not affect the biomass concentration obtained on 2% undecane. However, the biomass concentration was reduced significantly on 10% undecane with increase in media volume, indicating limitation of the growth rate.

The limiting factor is likely to be oxygen. Smith and Johnson (1954) have shown that the oxygen solution rate falls rapidly as the liquid volume in flasks is increased. The higher growth rate, and hence greater oxygen demand, on 10% undecane is possibly not satisfied by the slower oxygen solution rate in larger media volumes and hence growth is oxygen-limited. On 2% undecane the oxygen solution rate in the larger volume of media is possibly sufficient to meet the lower oxygen demand of the micro-organisms. Hence, growth on 2% undecane is not oxygen-limited and no reduction in biomass concentration is obtained with the larger media volume.

Ideally, therefore, fuel tanks should be kept as full as possible in order to reduce air spaces which could encourage the growth of hydrocarbon-utilizing microorganisms.

7.5.3 Effect of agitation

An important effect of agitation on a culture is that the transfer of oxygen from the gas to the liquid phase is increased (Pirt, 1975). As discussed in sub-section 7.5.2, an increase in oxygen solution rate increases the growth of microorganisms. This was found to be the case with C. resinae EW1 growing on glucose as sole carbon and energy source.

However, with undecane or diesel fuel as sole carbon and energy source, the growth of C. resinae was inhibited by shaking. Therefore other factors caused by the agitation of two phase cultures are more important than the increase in oxygen solution rate.

The rate of solution of hydrocarbons into the aqueous phase and the oil-water interfacial area are increased with agitation. These effects are thought to increase microbial growth (see sub-section 7.5.1), therefore, are probably not involved in the observation that shaking inhibits the growth.

The most likely reason for inhibition is that agitation increases the homogeneity of the culture. In static conditions, the biomass develops as a mat at the oil-water interface (see Figs. 32 to 35). In shaken cultures, the biomass, nutrients and metabolic products are dispersed throughout the culture. It is possible that dispersion interferes with the mechanism for direct uptake of hydrocarbon from the oil phase (see sub-section 7.5.1) which results in a reduction of growth.

Table 25 Effect of hydrocarbon concentration and culture volume on the growth of C. resinae

AQUEOUS PHASE VOLUME (ml)	UNDECANE CONCENTRATION (% v/v)	MEAN DRY WEIGHT OF TWO REPLICATE FLASKS (STANDARD DEVIATION)	
		(mg)	(gl^{-1} of aqueous phase)
25	2	24.0 (2.8)	0.96
25	10	82.0 (5.6)	3.28
50	2	47.0 (2.8)	0.94
50	10	66.0 (5.6)	1.32

Table 26 Effect of agitation on the growth of *C. resinae* ATCC 22711

GROWTH CONDITIONS	MEAN DRY WEIGHT OF REPLICATE FLASKS (STANDARD DEVIATION) (mg)	
	UNDECANE (2 FLASKS)	DIESEL FUEL (3 FLASKS)
Static	193.5 (30.4)	14.0 (3.6)
Agitated	78.0 (8.5)	Not Detectable

Table 27 Effect of agitation on the growth of C. resinae EW1 in full strength mineral salts solution

CARBON SOURCE	INCUBATION PERIOD (DAYS)	MEAN DRY WEIGHT OF TWO REPLICATE FLASKS (STANDARD DEVIATION) (mg)	
		STATIC	SHAKEN
GLUCOSE	7	29.0 (-)a	52.5 (4.9)
	14	68.0 (26.9)	99.5 (4.9)
GLUCOSE AND DIESEL FUEL	7	41.5 (9.2)	78.5 (2.1)
	14	120.0 (-)a	99.0 (11.3)
DIESEL FUEL	30	36.5 (9.2)	1.5 (0.7)
	60	171.0 (2.8)	2.0 (1.4)

a = result of one flask only

Table 28 Effect of agitation on the growth of C. resinae EW1 in 1/10 strength mineral salts solution.

CARBON SOURCE	INCUBATION PERIOD (DAYS)	MEAN DRY WEIGHT OF TWO REPLICATE FLASKS (STANDARD DEVIATION) (mg)	
		STATIC	SHAKEN
GLUCOSE	7	17.0 (1.4)	40.5 (0.7)
	14	52.5 (0.7)	71.0)(2.8)
GLUCOSE AND DIESEL FUEL	7	50.0 (1.4)	52.0 (1.4)
	14	95.0 (-)a	70.5 (0.7)
DIESEL FUEL	30	47.5 (20.5)	2.0 (0)
	60	81.5 (7.8)	4.5 (0.7)

a = result of one flask only

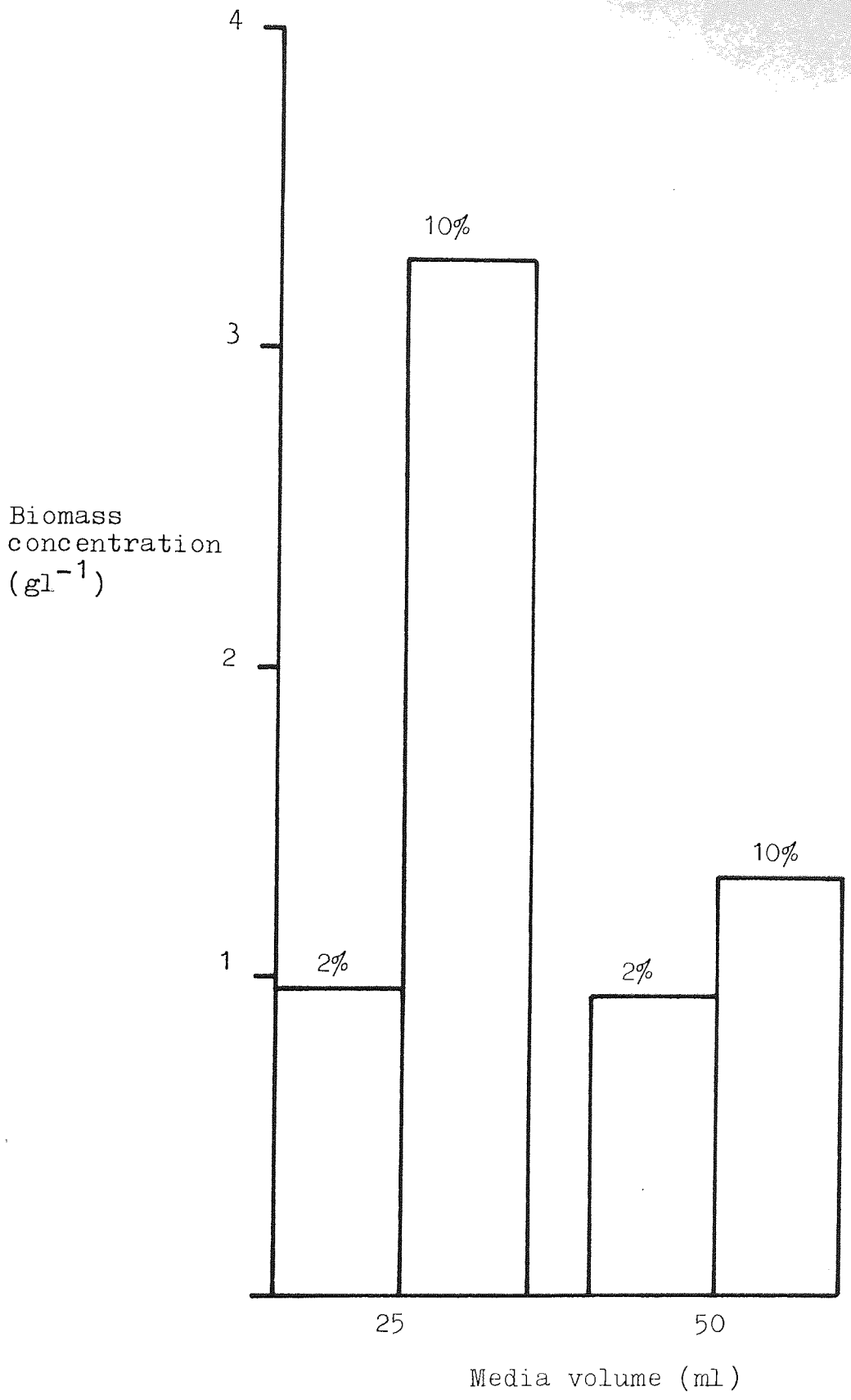

Fig. 41 Growth of C. resinae on 2 and 10% undecane in 25 and 50 ml volumes of media.

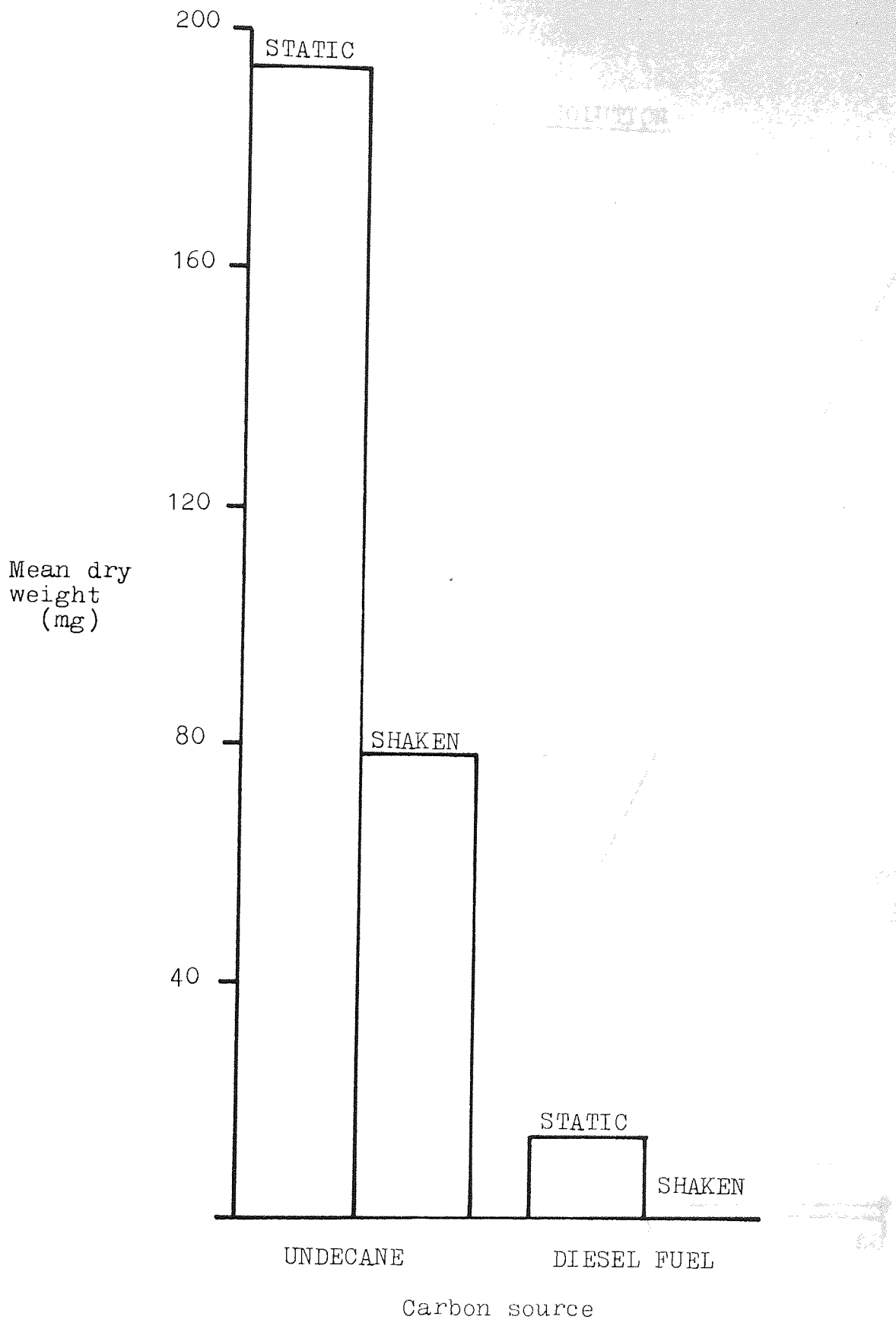

Fig. 42 Growth of C. resinae on undecane and diesel fuel under static and shaken conditions.

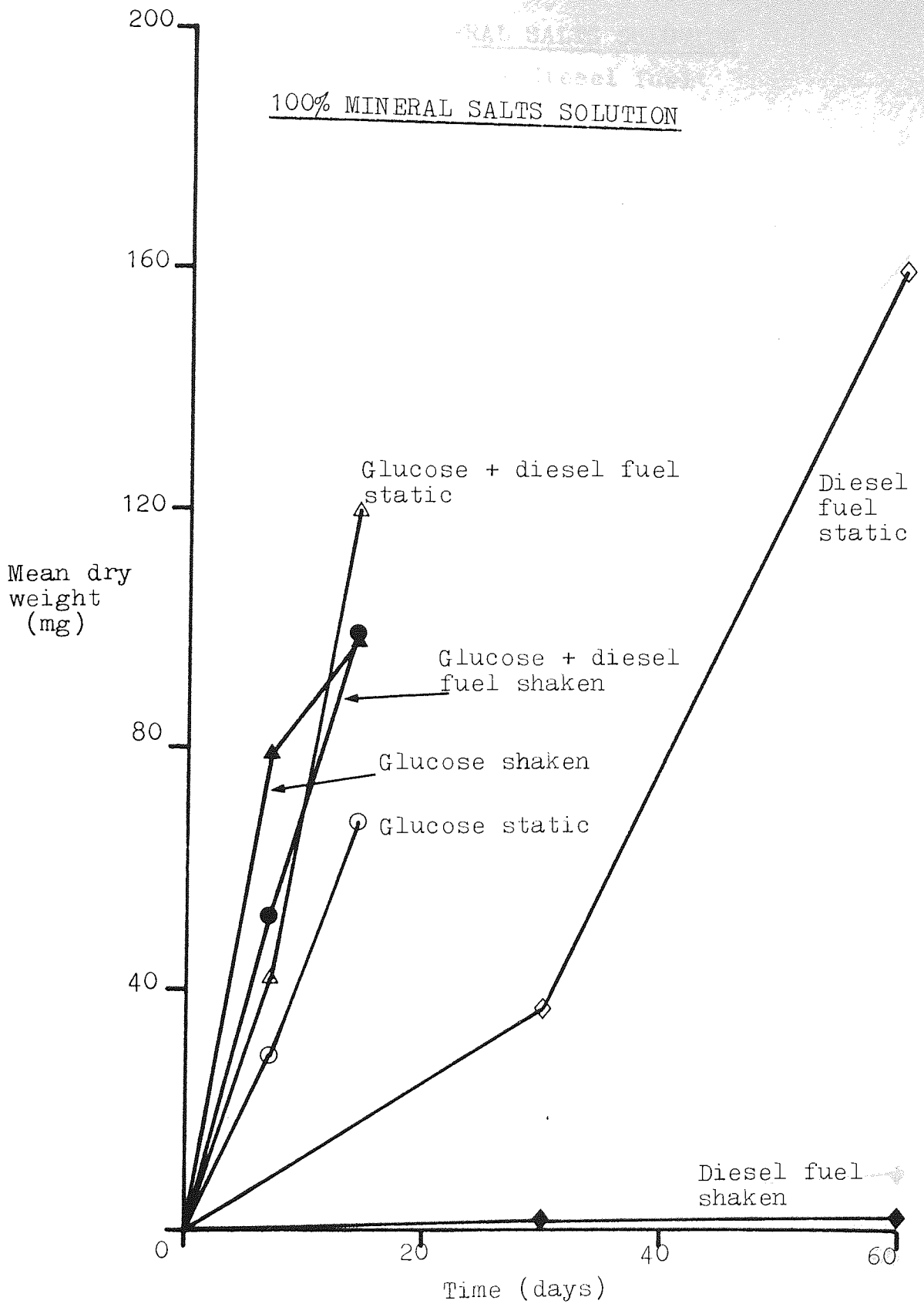

Fig. 43 Growth of C. resinae in 100% mineral salts solution and on various carbon sources under static and shaken conditions.

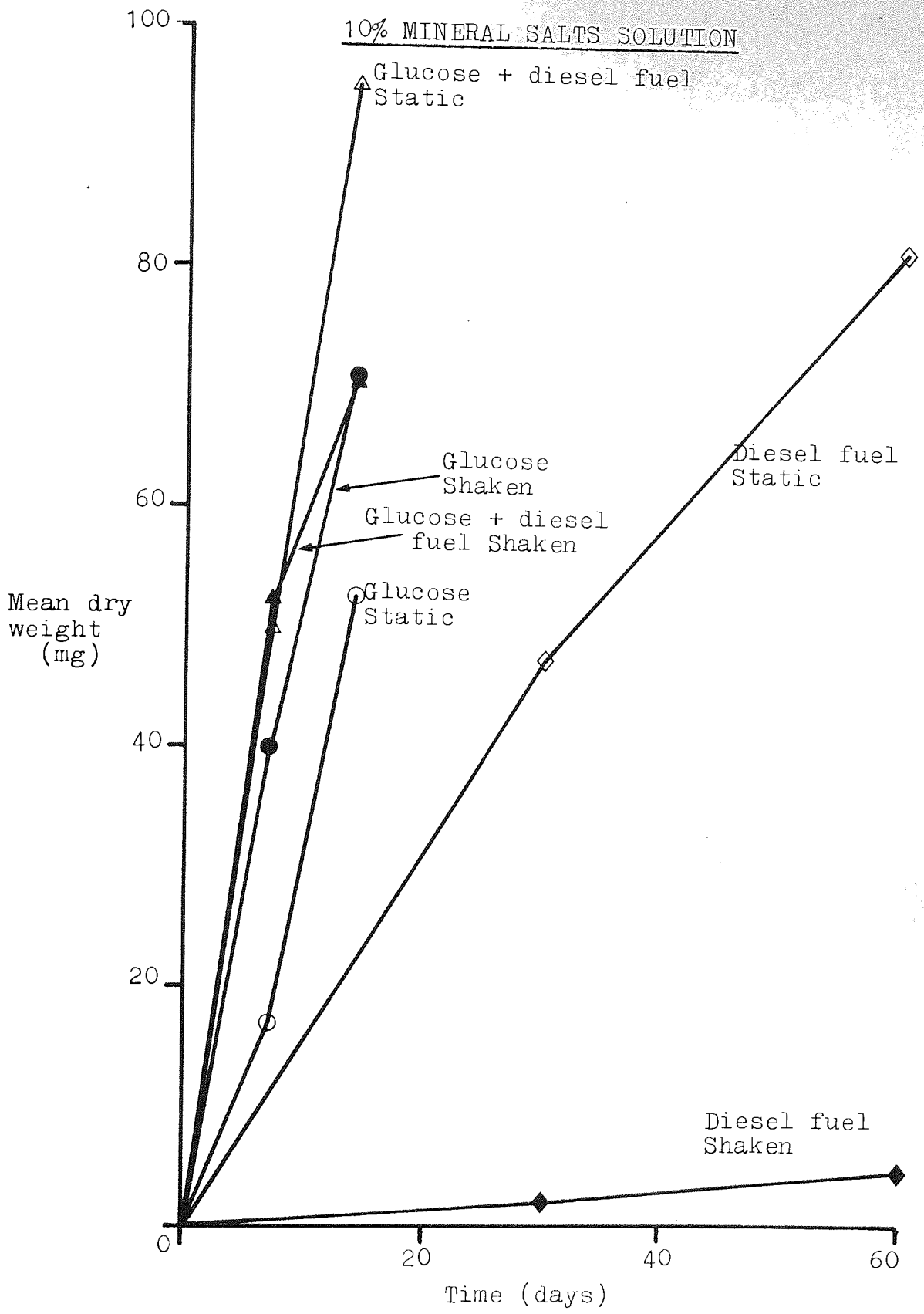

Fig. 44 Growth of C. resinae in 10% mineral salts solution and on various carbon sources under static and shaken conditions.

CHAPTER 8

EFFECT OF TEMPERATURE AND AN ORGANO-BORON COMPOUND ON THE GROWTH AND SURVIVAL OF MICROORGANISMS

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EFFECT OF TEMPERATURE AND AN ORGANO-BORON COMPOUND ON THE GROWTH AND SURVIVAL OF MICROORGANISMS

8.1 Introduction

8.1.1 Effect of temperature

The temperature of diesel fuel will tend to vary with the climate. In land storage tanks, it will be influenced by the action of the weather on the walls and roof. Shipboard storage tanks are integral with the hull (see Fig. 15) therefore the fuel will tend to assume the temperature of the surrounding waters which can vary from around 0°C to about 30°C. Parbery (1971) and Sheridan et al. (1971) found maximum growth of C. resinae around 30°C, therefore, development of the fungus in fuel systems is likely to be fast in summer or tropical waters and slow in winter or the Arctic.

The application of heat above the optimum temperature can bring about the inactivation of micro-organisms. Their death does not take place instantaneously but over a time period, due to individual differences in resistance in the population.

Inactivation by heat gives the following situation:

Viable cells → Dead cells

The inactivation rate at a given temperature therefore depends only on the concentration of viable cells. This condition can be expressed mathematically by

$$-\frac{dV}{dt} = KV \qquad (1)$$

where V is the concentration of viable cells at time t, and K is the rate constant.

For practical use, equation (1) is rearranged to

$$-\frac{dV}{V} = Kdt \qquad (2)$$

and integrated to give

$$-\int_{V_1}^{V_2} \frac{dV}{V} = K \int_{t_1}^{t_2} dt \qquad (3)$$

or

$$\ln \frac{V_1}{V_2} = K (t_2 - t_1) \qquad (4)$$

where V_1 and V_2 are the concentrations of viable cells at times t_1 and t_2 respectively

Equation (4) is rearranged to give

$$(t_2 - t_1) = \frac{1}{K} \ln \frac{V_1}{V_2} \qquad (5)$$

$$(t_2 - t_1) = \frac{2.303}{K} \log \frac{V_1}{V_2} \qquad (6)$$

Let $t_1 = 0$, $V_1 = 100$ and $V_2 = 10$

$$t_2 = \frac{2.303}{K} \qquad (7)$$ (Meynell and Meynell, 1970)

t_2 is known as the Decimal Reduction Time (DRT) or D-value and is the time taken to reduce viable numbers by 90%. The effect can therefore be used as a measurement of the effectiveness of a heat treatment towards a microbial population.

The effect of increasing the temperature is shown by the Arrhenius equation

$$K = Ae^{-E/RT} \qquad (8)$$

or

$$\log K = \log A - \frac{E}{2.303RT} \qquad (9)$$

where A, E and R are constants, T is the absolute

temperature and K the rate constant. The Arrhenius equation shows that the rate constant is directly proportional to the temperature. Since the D-value is inversely proportional to the rate constant (see equation (7)), it follows that the D-value decreases with increase in temperature. Hence, at higher temperatures, the destruction of a microbial population occurs after progressively shorter time periods.

The effect of temperature on the growth and rate of destruction of microorganisms is examined in this chapter.

8.1.2 Effect of an organo-boron compound

Microbial growth in aviation fuel systems has been controlled by the use of biostatic and biocidal compounds. 2- ethoxyethanol (see Fig. 45), which was adopted by the United States Air Force as an anti-icing inhibitor, was also found to deter microbial development (Hitzman, 1965). Biobor J.F., a proprietary mixture of organo-boron compounds (see Fig. 46) has been used to prevent microbial growth by a number of commercial airlines (Hill, 1970).

Both 2- ethoxyethanol and Biobor J.F. have been used on a few occasions to disinfect shipboard fuel systems (Houghton and Gage, 1979). The compounds are normally added to the fuel phase and they pass into any aqueous phase to exert an inhibitory or biocidal concentration.

In the simplest model of microbial inactivation by a biocidal compound, death is assumed to result from the reaction of x molecules of biocide, B, with y molecules of a substrate, V, in the organism:

$$xB + yV \rightarrow BxVy$$

$$\text{BIOCIDE} + \text{VIABLE CELLS} \rightarrow \text{DEAD CELLS}$$

In sterilization, the molar concentration of B is far in excess of V and therefore may be assumed to remain constant. Also, when the reaction between the disinfectant and a single molecule in the organism brings about death, y = 1. The reaction rate for a given biocide concentration is then, approximately,

$$-\frac{dV}{dt} = kV \qquad (10)$$

Thus, a constant proportion of viable organisms die in each interval of time and the survival curve is exponential. This type of reaction is termed "pseudo first order" for although it results from the union of two reactants, B and V, its kinetics resemble those of a true first order reaction in which only one reactant is involved (Meynell and Meynell, 1970).

However, this model does not hold true, since everyday experience tells one that higher concentrations of disinfectant usually act in progressively shorter time periods. In practice, biocide concentration, C, and the time, t_F, corresponding to the survival of a given fraction of cells are found to obey the following relationship:

$$C^n t_F = a \qquad (11)$$

or

$$\log t_F = \log a - n \log C \qquad (12)$$

where n is the concentration coefficient and a is a constant. According to Meynell and Meynell (1970), the concentration coefficient, n, reflects only the properties of the disinfectant.

Provided two survival curves have the same general shape and when the curves are exponential, the inactivation rates can be compared using D-values as described previously.

The Arrhenius equation (see equations 8 and 9) also applies, therefore the inactivation rate for a given biocide concentration should increase with increasing temperature.

The effect of Biobor J.F. on the destruction rate of spores of C. resinae is investigated in this chapter.

8.2 Methods

8.2.1 Organisms

The following strains were used in this study:

a) *C. resinae* ATCC 22711 (see sub-section 4.2.1)

b) *C. resinae* EW1 (see Table 1)

c) *C. resinae* EW3 (see Table 1)

d) *Penicillium corylophilum* EW4 (see Table 1)

Spore suspensions were prepared as described in Appendix II.

8.2.2 Media

The following suspending media were used (see Appendix III for details):

a) distilled water

b) physiological saline

c) reference diesel fuel, high sulphur

d) mineral salts solution containing a trace of diesel fuel.

Malt extract and nutrient agar were obtained from Oxoid. Biobor J.F. was obtained from the United States Borax and Chemical Corporation.

8.2.3 Effect of temperature on growth

Malt extract agar plates were inoculated centrally with spores of *C. resinae* ATCC 22711 and incubated at 25°C for three days. The minimum and maximum diameters of each colony were measured and the mean diameter calculated. The plates were transferred to incubators with pre-determined temperatures from 5 to 45°C and incubated for 168 hours. The mean colony diameters were determined as before and the radial growth rate for each temperature of incubation was

calculated.

8.2.4 Heat inactivation of microorganisms

A sample (1ml) of water taken from a fuel tank of HMS Broadsword was diluted in physiological saline (9ml). Samples (0.1ml) from the dilution were added to physiological saline (9.9ml) or diesel fuel (9.9ml) in test tubes agitated in a water bath at 65°C. After time intervals ranging from 10 to 100 seconds, the test tubes were removed and cooled by immersion in melting ice water. Samples (0.1ml) were diluted in physiological saline (9.9ml) and aliquots (0.1ml) were spread on malt extract and nutrient agar plates. After incubation at 22 or 37°C for up to 14 days, the number of colonies of bacteria, yeasts and fungi on each plate were counted and the mean colony of replicate plates was calculated. The initial microbial numbers were determined by plating after 100 seconds at 25°C.

8.2.5 Heat inactivation of fungal spores

Spore suspensions (0.1ml; 5×10^4 spores ml^{-1}) were added to distilled water (9.9ml) or diesel fuel (9.9ml) in test tubes agitated in a water bath at 55, 60 or 65°C. After time intervals ranging from 10 to 100 seconds, the test tubes were removed and cooled by immersion in melting ice water. Samples (0.1ml) were spread on malt extract agar plates and incubated at 25°C for up to 14 days. The number of colonies on each plate were counted and the mean colony count of replicate plates was calculated. The initial spore numbers were determined by plating after 100 seconds at 25°C.

8.2.6 Effect of an organo-boron compound

Spore suspensions (0.1ml; 5 x 10^6 spores ml^{-1}) were added to mineral salts solution (9.9ml) containing a trace of diesel fuel and Biobor J.F. (0-10%, v/v). The cultures were incubated at 25 and 45°C. At time intervals ranging from 0.5 to 192 hours, samples (0.1ml) were removed and diluted in distilled water (9.9ml). Aliquots (0.1ml) were spread on malt extract agar plates and incubated at 25°C for up to 14 days. The number of colonies on each plate were counted and the mean colony count of replicate plates was calculated.

8.3 Results

8.3.1 Effect of temperature on growth

The results of the effect of temperature from 5 to 45°C on the growth of C. resinae ATCC 22711 are shown in Table 29 and Fig. 47.

8.3.2 Heat inactivation of microorganisms

The results of the effect of heat at 65°C on the survival of microorganisms in a water bottom sample from HMS Broadsword are shown in Tables 30 to 33 for suspension in physiological saline and in Tables 34-37 for diesel fuel. Tables 30 and 34 show the total colony counts obtained, 31 and 35 for bacteria, 32 and 36 for yeasts and 33 and 37 for fungi.

The mean colony counts for each of the recovery conditions were combined and plotted against exposure time, as shown in Fig. 48 for microorganisms suspended in

physiological saline and in Fig. 49 for suspension in diesel fuel.

8.3.3 Heat inactivation of fungal spores

The results of the effect of heat on the inactivation of spores of C. resinae ATCC 22711 at 55, 60 and 65°C are shown in Tables 38, 39 and 40 respectively, and similarly for EW1 in Tables 41 to 43. The effect of suspension in distilled water or diesel fuel on the thermal death of spores of EW3 at 65°C is shown in Table 44. The inactivation of spores of Penicillium corylophilum EW4 at 60 and 65°C is shown in Tables 45 and 46 respectively.

The mean colony counts for each time interval of up to three trials for each fungal strain were combined. The logarithmic values were calculated and plotted against exposure time, as shown in Figs. 50, 51, 52 and 53 for ATCC 22711, EW1, EW3 and EW4 respectively.

The slope of each line determined by the points was calculated by linear regression analysis from which the D-value was found (see Appendix VI). Initial spore counts - obtained by plating after 100 seconds at 25°C - were plotted as at time zero, but were excluded from the calculations. Mean colony counts of zero were also excluded. The D-values for each strain at each temperature are shown in Table 47.

8.3.4 Effect of an organo-boron compound

The results of the effect of Biobor J.F. on the death of spores of C. resinae ATCC 22711 at 25°C are shown in Table 48. The inactivation of spores of EW1 by Biobor J.F.

at 25 and 45°C are shown in Tables 49 and 50 respectively.

The logarithmic values of the mean colony counts were calculated and plotted against exposure time, as shown in Fig. 54 for ATCC 22711 and in Figs. 55 and 56 for EW1. Fig. 55 shows inactivation of spores of EW1 at 25°C in Biobor J.F. concentrations of 0, 1, 4 and 7%. Fig. 56 compares the effect of 0 and 1% Biobor J.F. at 25 and 45°C.

The slope of each line was determined by linear regression analysis from which the D-value was calculated. Mean colony counts of zero were excluded from the calculation. The D-values for each concentration of Biobor J.F. at 25 and 45°C are shown in Table 51.

8.4 Conclusions

8.4.1 Effect of temperature on growth

The results in Table 29 and Fig. 47 show that *C. resinae* ATCC 22711 was capable of growth in the temperature range 5 to 45°C. The optimum temperature lay between 25 and 30°C, maximum growth being obtained at 28°C.

8.4.2 Heat inactivation of microorganisms

The number of yeasts, bacteria and fungi in a sample of water from a fuel tank of HMS Broadsword were obtained after incubation at 25°C for 100 seconds and after heating at 65°C for up to 100 seconds. Microbial numbers were determined by plating on malt extract and nutrient agar at 22 and 37°C. Greatest colony counts of the unheated controls were obtained on malt agar and at 22°C. Bacteria

were more numerous than yeasts which were more numerous than fungi. The application of heat at 65°C significantly reduced the microbial numbers within 10 seconds. A few bacteria and fungi survived up to 100 seconds at 65°C, but no yeasts survived at any stage of the heat treatment. Survival of the microorganisms at 65°C was not affected by suspension in diesel fuel than in physiological saline, although the microbial counts of the unheated controls were reduced by approximately 50% with suspension in diesel fuel.

8.4.3 Heat inactivation of fungal spores

In most cases, the application of heat at 55, 60 and 65°C reduced the numbers of viable spores with time and as expected higher temperatures required shorter exposure times for the same degree of destruction. Spores of Penicillium corylophilum were more resistant than those of C. resinae. The resistance of spores of C. resinae EW3 at 65°C increased slightly with suspension in 1 part distilled water to 100 parts diesel fuel than in distilled water only.

8.4.4 Inactivation of spores by Biobor J.F.

The application of Biobor J.F. reduced the number of viable spores of C. resinae with time, higher concentrations requiring shorter exposure times. The resistance of the spores decreased when the temperature was increased from 25 to 45°C. Without Biobor J.F. no reduction in viable numbers was observed at the lower temperature but at 45°C a D-value of 63.2 hours was obtained. In 1% Biobor J.F. a D-value of 1016.6 hours was obtained at 25°C but at 45°C, no viable spores were obtained after 42 hours.

8.5 Discussion

8.5.1 Effect of temperature on growth

The optimum temperature for the growth of C. resinae ATCC 22711 on malt extract agar lay in the range 25 to 30°C. Maximum growth was obtained at 28°C. Parbery (1971) and Sheridan et al. (1971) measured the growth of C. resinae at 25, 30 and 35°C and found maximum growth at 30°C, indicating temperature optima in the range 25 to 35°C.

Growth of C. resinae was obtained at 5°C, therefore the lower temperature limit for growth is less than 5°C. Christensen et al. (1942) also observed growth of C. resinae at 5°C, but Hendey (1964) detected no growth of the fungus at 4°C. Parbery (1971) found no growth of C. resinae at 0°C on a mineral salts-glucose agar but detected slight growth at this temperature in liquid culture.

The upper temperature limit for growth seems to be around 45°C. Parbery (1971) reported no growth of C. resinae at 50°C on surface culture but detected slight growth in liquid culture at this temperature.

8.5.2 Heat inactivation studies

The application of heat at 65°C rapidly reduced the number of viable microorganisms in a sample taken from HMS Broadsword. A few bacteria survived up to 100 seconds at this temperature and probably arose from spores which are considerably more resistant to heat than vegetative bacteria (Sykes, 1965). A few fungi also survived the treatment. However, the initial fungal count was also extremely low therefore no definite conclusions can be

drawn concerning the destruction of these organisms from this experiment.

The environment in which an organism finds itself either prior to, or during, a heat treatment can influence its resistance (Sykes, 1965). The most important factor in this respect is the presence or absence of water. Resistance to heat is greatly increased in anhydrous conditions and has been attributed by Hawker (1950) to a decrease in the water content of the cells. Bomar (1970) investigated the survival of Aspergillus niger at 60°C in various relative humidity conditions and reported a decrease in resistance with increase in relative humidity. Upsher (1976) found that spores of C. resinae, Paecilomyces varioti and Aspergillus niger were more resistant to heat in completely dry fuel than in wet fuel.

Suspension of the microorganisms from the HMS Broadsword sample in 1 part aqueous phase to 100 parts diesel fuel did not affect their resistance to heat, presumably because the microorganisms are mostly retained in the small volume of aqueous phase. The fuel, therefore, confers no resistance. However, suspension in diesel fuel reduced the number of microorganimms recovered in the unheated controls. Some of them may lose viability when in close contact with diesel fuel (which contains toxic compounds) either during suspension or during incubation with a thin film of fuel on the agar surface.

At 65°C, the destruction of spores of C. resinae and Pencillium corylophilum was rapid. Wet diesel fuel increased the resistance of C. resinae slightly but not

signficantly.

These findings suggest that heating the fuel in shipboard systems to 65°C for short periods of time can kill the majority of the microorganisms and control microbial colonization. Processes have been developed for the continuous in-line heat treatment of fluids and are widely used in the preparation of beverages and dairy products. It may be possible to fit a heat exchanger somewhere in the fuel supply system. It could be sited near land storage tanks or on RFA supply tankers to treat the fuel during refuelling, or in the warship itself to pasteurize the fuel as it is transferred between tanks.

As an alternative to rapid in-line heating at 65°C, the fuel could possibly be maintained at a suitable temperature to discourage the growth of C. resinae. At 45°C, the fungus is either inhibited or gradually inactivated and the practical difficulties of holding the fuel in land storage tanks at this temperature would not be too great. However, the growth of thermophilous organisms such as Aspergillus fumigatus could be encouraged under these conditions which could lead to more serious problems (see sub-section 1.2.2). In order to control microbial colonization a combination of heat and biocide could be applied.

8.5.3 Inactivation of spores by Biobor J.F.

The use of Biobor J.F. to control microbial colonisation in shipboard fuel systems has several drawbacks:

1. It is required at high concentrations

2. It is expensive compared to other biocides
3. It is ineffective when large volumes of water are associated with the fuel because its concentration in the aqueous phase is reduced.
4. It requires a period of days for effective elimination of microorganisms.

Hence research is being carried out to find more active cheaper biocides which do not cause damage to gas turbine machinery or to turbine blades (Houghton and Gage, 1979).

However, the results obtained in this chapter may allow the more efficient use of Biobor J.F.. By raising the temperature of the fuel to 45°C, the destructive effect of the biocide increases significantly. Thus lower concentrations can be used at the higher temperatures which could give considerable cost savings. Heating the fuel to 45°C is a practicable proposition. Furnace fuel oil, which was used to power steam driven ships, had to be heated to reduce its viscosity and heating coils still exist in some shipboard and land storage tanks.

Table 29 Effect of temperature on the growth of *C. resinae*

TEMPERATURE (°C)	MEAN RADIAL GROWTH RATE OF THREE REPLICATES (STANDARD DEVIATION) (μ hr^{-1})
5	13.4 (2.1) (a)
18 ±4	62.1 (9.5)
20	94.2 (2.0)
25	124.6 (0.7)
28	128.0 (5.3)
30	111.3 (4.2) (a)
37	75.4 (4.6)
41	52.5 (13.8)
45	1.0 (1.7)

(a) Mean radial growth rate of two replicates

Table 30 Heat inactivation of HMS BROADSWORD micro organisms in physiological saline at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN TOTAL COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)				
		Recovery temperature (°C) and medium				COMBINED COUNTS
		22 Malt	22 Nutrient	37 Malt	37 Nutrient	
25	100	243.6 (11.3)	152.0 (18.1)	26.4 (15.0)	11.2 (2.6)	433.2
65	10	0.6 (0.8)	0.8 (1.3)	0.4 (0.5)	1.4 (1.0)	3.2
"	20	0.4 (0.5)	0.2 (0.4)	0.2 (0.4)	0.2 (0.4)	1.0
"	40	0.2 (0.4)	0.8 (1.8)	0.4 (0.8)	1.0 (1.5)	2.4
"	60	3.8 (6.1)	0.2 (0.4)	0 (0)	0.4 (0.5)	4.4
"	80	0.2 (0.4)	0.4 (0.8)	0 (0)	0.2 (0.4)	0.8
"	100	3.0 (6.0)	1.0 (1.7)	0 (0)	0.2 (0.4)	4.2

Table 31 Heat inactivation of HMS BROADSWORD bacteria in physiological saline at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN BACTERIAL COLONY COUNTS OF FIVE REPLICATE PLATE PLATES (STANDARD DEVIATION)				
		Recovery temperature (°C) and medium				COMBINED COUNTS
		22 Malt	22 Nutrient	37 Malt	37 Nutrient	
25	100	220.4 (14.8)	145.6 (20.0)	26.4 (16.8)	0 (0)	392.4
65	10	0 (0)	0.6 (0.9)	0 (0)	1.4 (1.1)	2.0
"	20	0 (0)	0.2 (0.4)	0 (0)	0.2 (0.4)	0.4
"	40	0 (0)	0.2 (0.4)	0 (0)	1.0 (1.7)	1.2
"	60	0 (0)	0 (0)	0 (0)	0.4 (0.5)	0.4
"	80	0 (0)	0 (0)	0 (0)	0.2 (0.4)	0.2
"	100	0 (0)	0.6 (0.9)	0 (0)	0.2 (0.4)	0.8

Table 32 Heat inactivation of HMS BROADSWORD yeasts in physiological saline at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN YEAST COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)				
		Recovery temperature (°C) and medium				COMBINED COUNTS
		22 Malt	22 Nutrient	37 Malt	37 Nutrient	
25		21.2 (9.4)	6.4 (2.9)	0 (0)	10.2 (3.1)	37.8
65	10	NO SURVIVORS AT ANY STAGE OF THE HEAT TREATMENT				0
"	20					0
"	40					0
"	60					0
"	80					0
"	100					0

Table 33 Heat inactivation of HMS BROADSWORD fungi in physiological saline at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN FUNGAL COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)				
		Recovery temperature (°C) and medium				COMBINED COUNTS
		22 Malt	22 Nutrient	37 Malt	37 Nutrient	
25	100	2.0 (3.1)	0 (0)	0 (0)	1 0 (0)	3.0
65	10	0.6 (0.9)	0.2 (0.4)	0.4 (0.5)	0 (0)	1.2
"	20	0.4 (0.5)	0 (0)	0.2 (0.4)	0 (0)	0.6
"	40	0.2 (0.4)	0.6 (1.3)	0.4 (0.9)	0 (0)	1.2
"	60	3.8 (6.9)	0.2 (0.4)	0 (0)	0 (0)	4.0
"	80	0.2 (0.4)	0.4 (0.9)	0 (0)	0 (0)	0.6
"	100	3.0 (6.7)	0.4 (0.9)	0 (0)	0 (0)	3.4

Table 34 Heat inactivation of HMS BROADSWORD micro organisms in diesel fuel at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN TOTAL COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)				
		Recovery temperature (°C) and medium				COMBINED COUNTS
		22 Malt	22 Nutrient	37 Malt	37 Nutrient	
25	100	103.2 (20.5)	79.2 (16.4)	7.0 (7.5)	0.2 (0.4)	189.6
65	10	0.2 (0.4)	0.2 (0.4)	0 (0)	0.2 (0.4)	0.6
"	20	0.4 (0.8)	1.0 (1.2)	0 (0)	2.0 (2.1)	3.4
"	40	0 (0)	0.4 (0.5)	0 (0)	0.2 (0.4)	0.6
"	60	0 (0)	1.2 (0.4)	0 (0)	0 (0)	1.2
"	80	0 (0)	0.8 (1.3)	0 (0)	0.4 (0.5)	1.2
"	100	0.2 (0)	0.8 (0.4)	0.2 (0.4)	0 (0)	1.0

Table 35 Heat inactivation of HMS BROADSWORD bacteria in diesel fuel at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN BACTERIAL COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)				
		Recovery temperature (°C) and medium				COMBINED COUNTS
		22 Malt	22 Nutrient	37 Malt	37 Nutrient	
25	100	89.6 (19.2)	73.4 (18.1)	6.8 (6.8)	0 (0)	169.8
65	10	0 (0)	0 (0)	0 (0)	0.2 (0.4)	0.2
"	20	0 (0)	0.4 (0.9)	0 (0)	2.0 (2.3)	2.4
"	40	0 (0)	0.4 (0.5)	0 (0)	0.2 (0.4)	0.6
"	60	0 (0)	0 (0)	0 (0)	0 (0)	0
"	80	0 (0)	0.6 (0.9)	0 (0)	0.4 (0.5)	1.0
"	100	0 (0)	0.8 (0.4)	0 (0)	0 (0)	0.8

Table 36 Heat inactivation of HMS BROADSWORD yeasts in diesel fuel at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN YEAST COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)				
		Recovery temperature (°C) and medium				COMBINED COUNTS
		22 Malt	22 Nutrient	37 Malt	37 Nutrient	
25	100	12.2 (3.1)	5.4 (4.5)	0.2 (0.4)	0.2 (0.4)	18.0
65	10	NO SURVIVORS AT ANY STAGE OF THE HEAT TREATMENT				0
"	20					0
"	40					0
"	60					0
"	80					0
"	100					0

Table 37 Heat inactivation of HMS BROADSWORD fungi in diesel fuel at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN FUNGAL COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)				
		Recovery temperature (°C) and medium				COMBINED COUNTS
		22 Malt	22 Nutrient	37 Malt	37 Nutrient	
25	100	1.4 (2.2)	0.4 (0.5)	0.2 (0.4)	0 (0)	2.0
"	10	0.2 (0.4)	0.2 (0.4)	0 (0)	0 (0)	0.4
"	20	0.4 (0.9)	0.6 (0.5)	0 (0)	0 (0)	1.0
"	40	0 (0)	0 (0)	0 (0)	0 (0)	0
"	60	0 (0)	1.2 (0.4)	0 (0)	0 (0)	1.2
"	80	0 (0)	0.2 (0.4)	0 (0)	0 (0)	0.2
"	100	0.2 (0.4)	0 (0)	0.2 (0.4)	0 (0)	0.4

Table 38 Heat inactivation of spores of C. resinae ATCC 22711 at 55°C

TEMPERATURE (°C)	TIME (s)	MEAN COLONY COUNTS OF THREE OR FIVE REPLICATE PLATES (STANDARD DEVIATION)		COMBINED MEAN COLONY COUNTS OF TWO TRIALS	LOGARITHMIC VALUE
		TRIAL 1	TRIAL 2		
25	100	25.3 (4.7)	33.2 (9.4)	58.5	1.77
55	10	31.3 (4.7)	28.2 (13.6)	59.5	1.77
"	20	19.7 (9.8)	22.4 (6.6)	42.1	1.62
"	40	26.0 (3.5)	28.2 (2.5)	54.2	1.74
"	60	20.0 (1.7)	24 2 (7.8)	44.2	1.64
"	80	22.7 (3.2)	13 4 (7.1)	36.1	1.56
"	100	19.0 (1.7)	14.8 (2.9)	33.8	1.53

Table 39 Heat inactivation of spores of C. resinae AFCC 22711 at 60°C

TEMPERATURE (°C)	TIME (s)	MEAN COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)			COMBINED MEAN COLONY COUNT OF THREE TRIALS	LOGARITHMIC VALUE
		TRIAL 1	TRIAL 2	TRIAL 3		
25	100	33.2 (9.4)	41.2 (5.2)	77.6 (18.7)	152.0	2.18
60	10	16.4 (5.9)	44.8 (27.0)	43.0 (6.8)	104.2	2.02
"	20	12.4 (5.9)	29.8 (13.7)	37.0 (8.0)	79.2	1.90
"	40	6.4 (1.7)	34.0 (2.2)	25.2 (2.8)	65.6	1.82
"	60	3.4 (2.5)	23.4 (4.8)	20.6 (5.0)	47.4	1.68
"	80	1.8 (0.8)	23.2 (5.3)	15.4 (3.7)	40.4	1.61
"	100	0.4 (0.6)	12.0 (7.1)	9.4 (4.3)	21.8	1.34

Table 40 Heat inactivation of spores of C. resinae ATCC 22711 at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)		COMBINED MEAN COLONY COUNTS OF TWO TRIALS	LOGARITHMIC VALUE
		TRIAL 1	TRIAL 2		
25	100	41.2 (5.2)	77.6 (18.7)	118.8	2.07
65	10	21.8 (5.8)	13.8 (4.2)	35.6	1.55
"	20	7.6 (1.1)	2.2 (1.3)	9.8	0.99
"	40	2.0 (1.6)	0 (0)	2.0	0.30
"	60	0 (0)	0 (0)	0	-
"	80	0 (0)	0 (0)	0	-
"	100	0 (0)	0 (0)	0	-

Table 41 Heat inactivation of spores of C. resinae EW1 at 55°C

TEMPERATURE (°C)	TIME (s)	MEAN COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)		COMBINED MEAN COLONY COUNTS OF TWO TRIALS	LOGARITHMIC VALUE
		TRIAL 1	TRIAL 2		
25	100	45.3 (12.3)	35.6 (6.8)	80.9	1.91
55	10	31.3 (9.1)	28.0 (3.9)	59.3	1.77
"	20	41.3 (11.2)	35.8 (2.6)	77.1	1.89
"	40	33.0 (2.6)	28.0 (6.0)	61.0	1.78
"	60	37.0 (13.7)	33.6 (6.6)	70.6	1.85
"	80	35.0 (7.0)	24.8 (4.2)	59.8	1.78
"	100	30.0 (7.8)	21.8 (5.3)	51.8	1.71

Table 42 Heat inactivation of spores of C. resinae EW1 at 60°C

TEMPERATURE (°C)	TIME (s)	MEAN COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)			COMBINED MEAN COLONY COUNT OF THREE TRIALS	LOGARITHMIC VALUE
		TRIAL 1	TRIAL 2	TRIAL 3		
25	100	35.6 (6.8)	49.8 (6.1)	89.6 (17.7)	175.0	2.24
60	10	25.2 (11.1)	55.4 (12.4)	61.2 (9.3)	141.8	2.15
"	20	22.6 (3.5)	42.4 (13.6)	60.0 (10.6)	125.8	2.07
"	40	17.2 (6.8)	57.8 (16.9)	43.8 (7.6)	118.8	2.07
"	60	7.2 (1.9)	39.4 (5.7)	24.8 (7.2)	71.4	1.85
"	80	1.8 (0.8)	42.4 (16.2)	7.2 (2.5)	51.4	1.71
"	100	1.0 (0.7)	7.8 (3.0)	4.2 (1.3)	13.0	1.11

Table 43 Heat inactivation of spores of C. resinae EW1 at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)		COMBINED MEAN COLONY COUNTS OF TWO TRIALS	LOGARITHMIC VALUE
		TRIAL 1	TRIAL 2		
25	100	49.8 (6.1)	89.6 (17.7)	139.4	2.14
65	10	41.0 (5.6)	37.0 (10.6)	78.0	1.89
"	20	10.6 (4.2)	3.8 (2.3)	14.4	1.16
"	40	0.6 (0.5)	0.2 (0.4)	0.8	-0.10
"	60	0.4 (0.5)	0 (0)	0.4	-0.40
"	80	0 (0)	0 (0)	0	-
"	100	0 (0)	0 (0)	0	-

Table 44 Heat inactivation of spores of C. resinae EW3 in distilled water and diesel fuel at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION) (LOGARITHMIC VALUE)	
		Distilled Water	Diesel fuel
25	100	18.2 (5.1) (1.26)	18.8 (2.9) (1.27)
65	10	7.8 (2.8) (0.89)	16.8 (6.3) (1.22)
"	20	6.2 (3.8) (0.79)	9.2 (4.9) (0.96)
"	40	0.2 (0.4) (-0.70)	4.0 (2.9) 0.60)
"	60	0 (0) -	0.4 (0.5) (-0.40)
"	80	0 (0) -	0.2 (0.4) (-0.70)
"	100	0 (0) -	0.4 (0.5) (-0.40)

Table 45 Heat inactivation of spores of Penicillium corylophilum EW4 at 60°C

TEMPERATURE (°C)	TIME (s)	MEAN COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)	LOGARITHMIC VALUE
25	100	94.5 (48.5)	1.98
60	10	55.2 (11.0)	1.74
"	20	116.2 (62.2)	2.06
"	40	36.0 (13.3)	1.56
"	60	42.8 (4.1)	1.63
"	80	74.2 (41.2)	1.87
"	100	76.2 (50.6)	1.88

Table 46 Heat inactivation of spores of Penicillium corylophilum EW4 at 65°C

TEMPERATURE (°C)	TIME (s)	MEAN COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)	LOGARITHMIC VALUE
25	100	94.5 (48.5)	1.98
65	10	44.0 (31.8)	1.64
"	20	20.8 (2.6)	1.32
"	40	3.5 (1.0)	0.54
"	60	13.5 (9.0)	1.13
"	80	0.5 (1.0)	-0.30
"	100	0.5 (0.6)	-0.30

Table 47 Heat inactivation of fungal spores expressed as D-values

ORGANISM	TEMPERATURE (°C)	D-VALUE (s)
Cladosporium resinae ATCC 22711	55	450
	60	148
	65	25
Cladosporium resinae EW1	55	1037
	60	97
	65	21
Penicillium corylophilum EW4	60	a
	65	47
Cladosporium resinae EW3	65 (distilled water)	18
	65 (diesel fuel)	46

a = no loss in viability observed

Table 48 Inactivation of spores of C. resinae ATCC 22711 by Biobor J.F. at 25°C

TIME (hr)	MEAN COLONY COUNTS OF FIVE REPLICATE PLATES (STANDARD DEVIATION)				
	BIOBOR J.F. CONCENTRATION (%, v/v)				
	0	0.1	4	7	10
0.5	45.6 (5.7)	47.8 (9.6)	31.6 (8.1)	21.2 (4.0)	22.4 (5.9)
3.5	39.6 (7.4)	40.2 (6.4)	24.0 (4.2)	15.0 (4.2)	7.8 (2.8)
21.0	45.6 (9.3)	40.2 (19.0)	13.2 (4.4)	8.2 (2.9)	1.6 (1.3)
27.5	43.8 (12.6)	37.8 (8.3)	12.4 (3.0)	5.4 (2.9)	0 (0)
45.0	53.6 (5.4)	34.4 (5.7)	6.0 (2.2)	0.2 (0.4)	0 (0)
51.5	44.6 (9.4)	37.4 (8.2)	5.4 (1.7)	0 (0)	0 (0)
69.0	41.2 (7.6)	35.2 (10.7)	4.8 (2.3)	0 (0)	0 (0)
75.5	53.4 (9.4)	42.6 (10.2)	6.8 (1.9)	0 (0)	0 (0)

Table 49 Inactivation of spores of C. resinae EW1 by Biobor J.F. at 25°C

TIME (hr)	MEAN COLONY COUNTS OF TEN REPLICATE PLATES (STANDARD DEVIATION)			
	BIOBOR J.F. CONCENTRATION (%, v/v)			
	0	1	4	7
0.5	50.0 (16.5)	51.5 (4.9)	42.9 (9.0)	42.3 (10.2)
18.0	55.3 (10.6)	39.4 (4.7)	34.8 (8.0)	20.9 (5.8)
42.0	58.6 (16.3)	41.2 (14.0)	38.7 (14.5)	2.8 (1.8)
66.0	42.4 (16.3)	35.0 (6.9)	39.4 (7.2)	0.3 (0.5)
90.0	83.2 (36.7)	28.8 (10.4)	28.4 (10.3)	0 (0)
114.0	66.2 (25.2)	35.2 (11.1)	32.0 (7.6)	0 (0)
192.0	94.6 (29.2)	31.1 (5.5)	9.3 (1.9)	NOT DONE

Table 50 Inactivation of spores of C. resinae EW1 by Biobar J.F. at 45°C

TIME (hr)	MEAN COLONY COUNTS OF TEN REPLICATE PLATES (STANDARD DEVIATION)			
	BIOBOR J.F. CONCENTRATION (%, v/v)			
	0	1	4	7
0.5	51.1 (11.7)	43.1 (14.3)	38.7 (9.0)	24.8 (6.0)
18.0	27.3 (5.9)	0.3 (0.7)	0 (0)	0 (0)
42.0	18.0 (10.0)	0 (0)	0 (0)	0 (0)
66.0	9.8 (2.3)	0 (0)	0 (0)	0 (0)
90.0	3.3 (2.3)	0 (0)	0 (0)	0 (0)
114.0	0.6 (0.7)	0 (0)	0 (0)	0 (0)
182.0	6.0 (2.5)a	NOT DONE	NOT DONE	NOT DONE

a = Not diluted 100 fold.

Table 51 Inactivation of fungal spores by heat and Biobor J.F. expressed as D-values

ORGANISM	TEMPERATURE (°C)	BIOBOR J.F. CONCENTRATION (%, v/v)	D-value (hr)
Cladosporium resinae ATCC 22711	25	0	a
		0.1	1359
		4	99
		7	24
		10	20
Cladosporium resinae EW1	25	0	a
		1	1016
		4	323
		7	30
Cladosporium resinae EW1	45	0	63
		1	8b
		4	c
		7	c

a = No loss in viability observed

b = Calculated from the results of two time intervals

c = Not calculated because only one result obtained

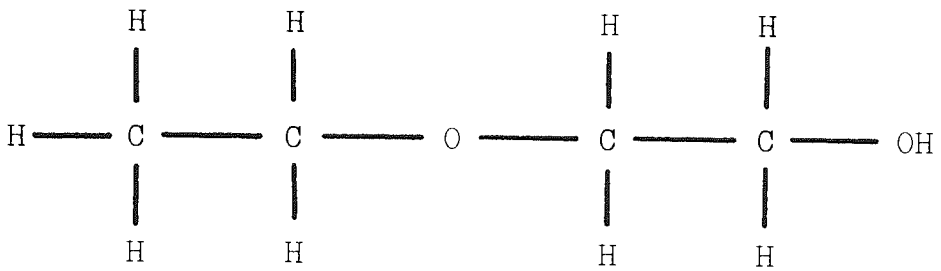
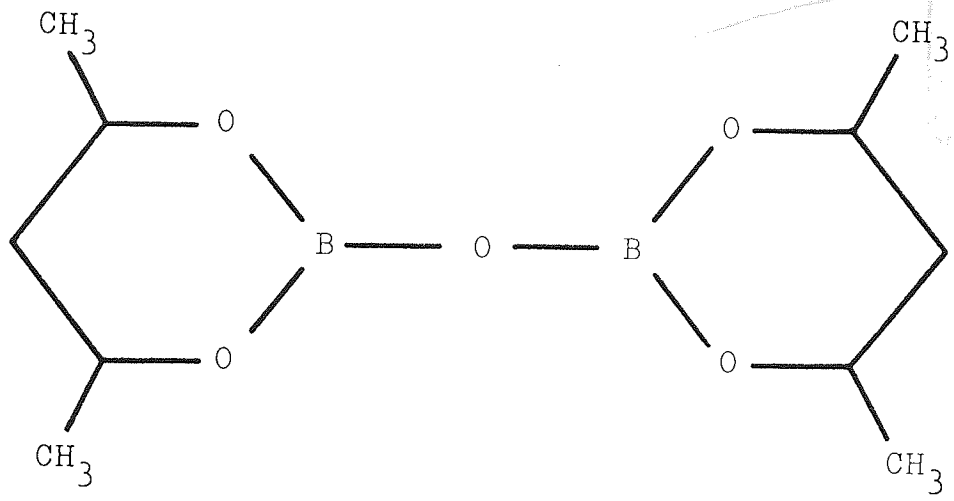


Fig. 45 Structure of 2- ethoxyethanol



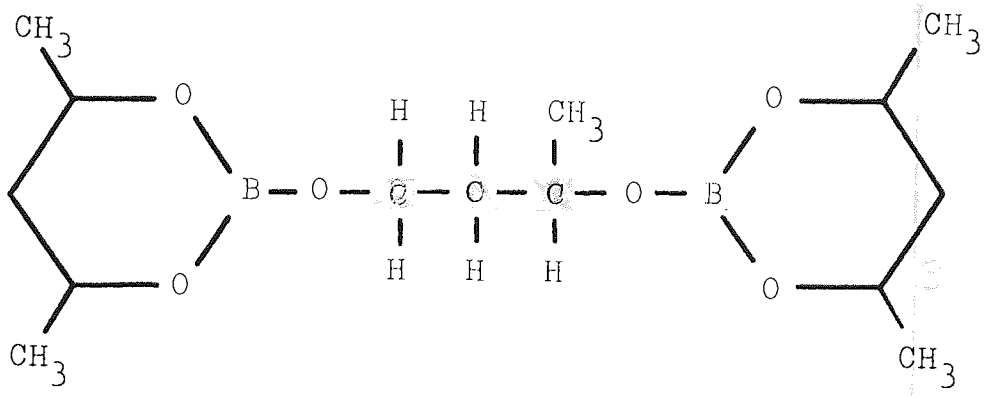


Fig. 46 Structures of the active organo-boron compounds in Biobor J.F.

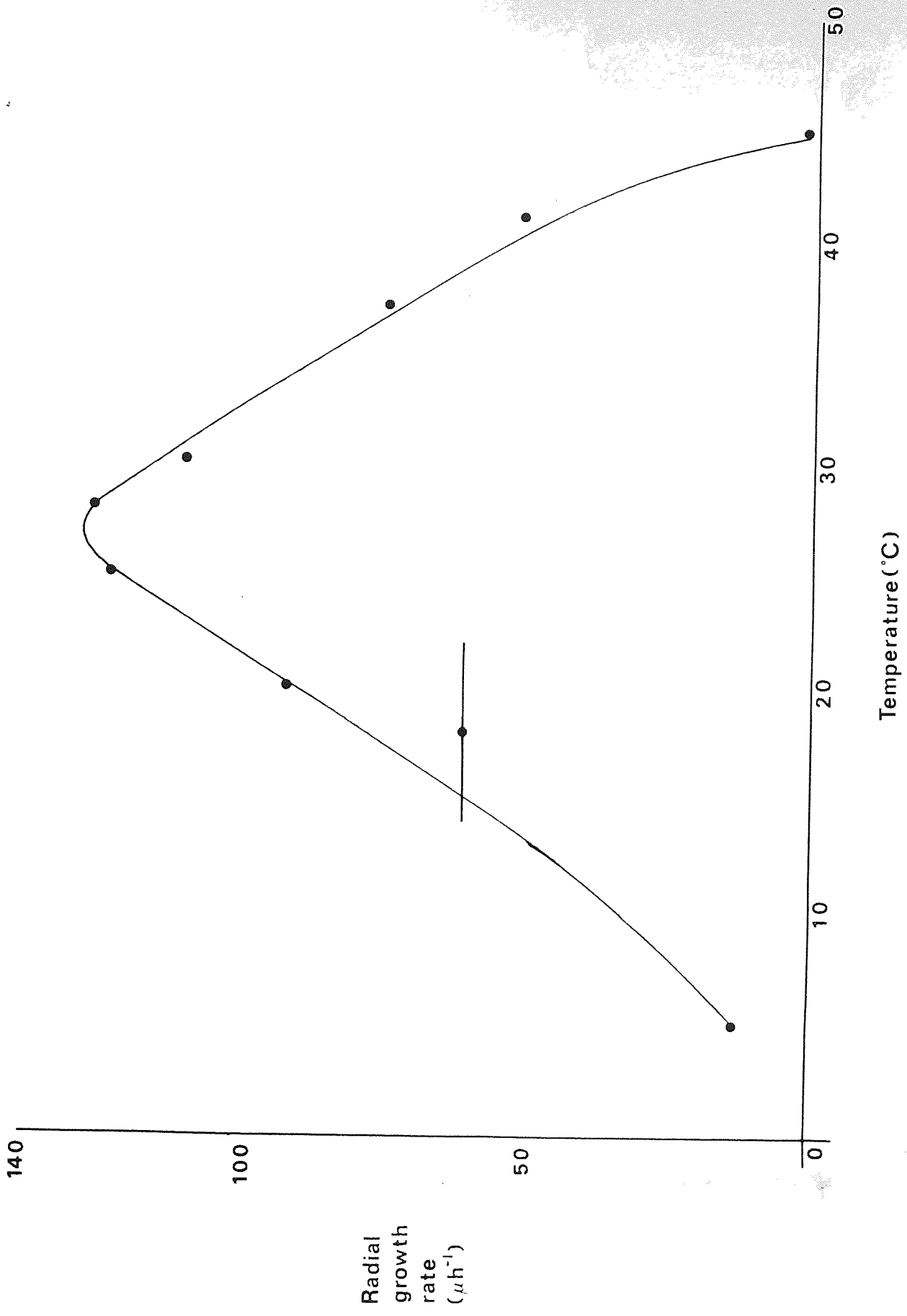


Fig. 47 Effect of temperature on the growth rate of C. resinae

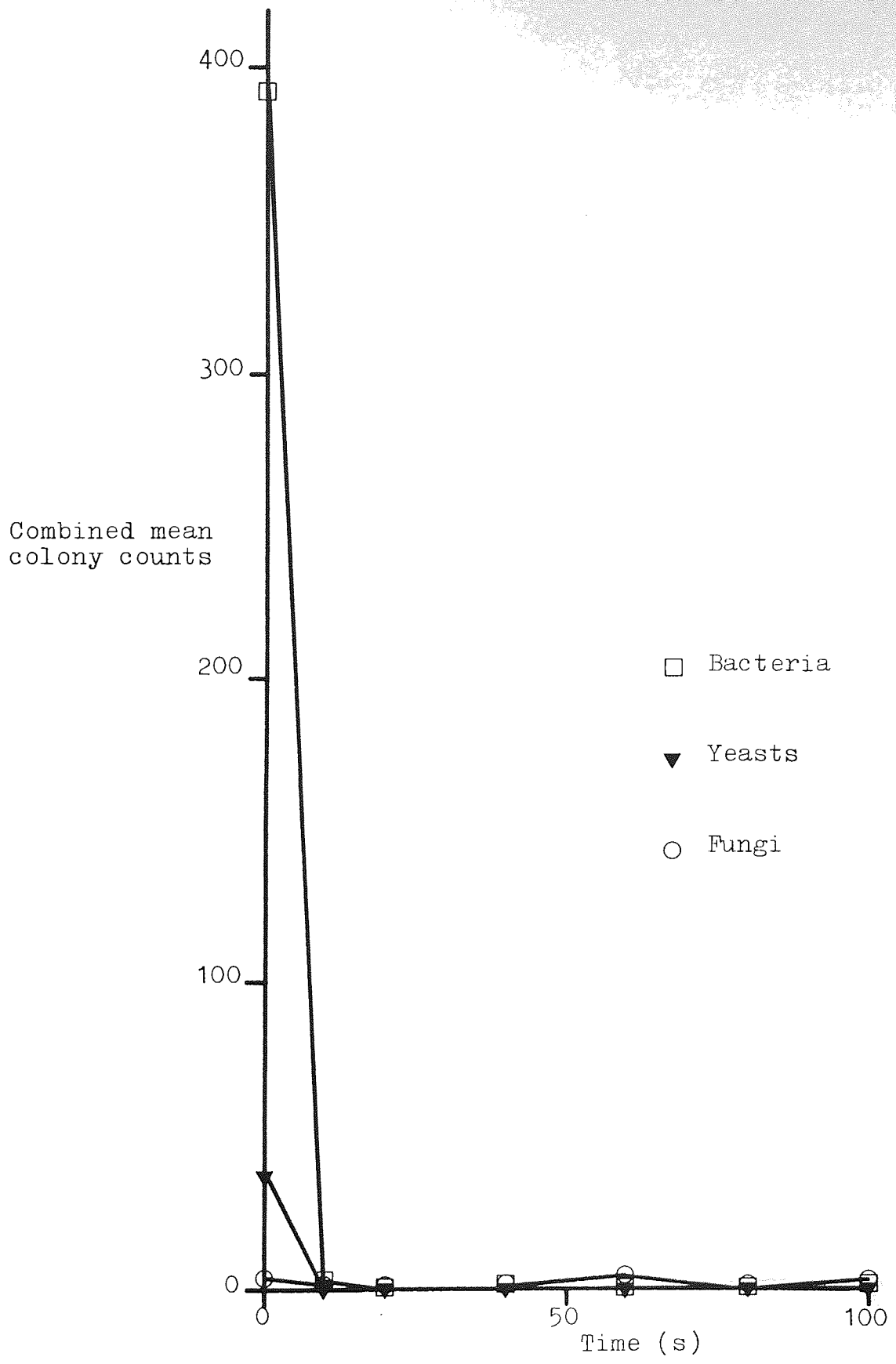


Fig. 48 Heat inactivation of HMS Broadsword micro-organisms in physiological saline at 65°C

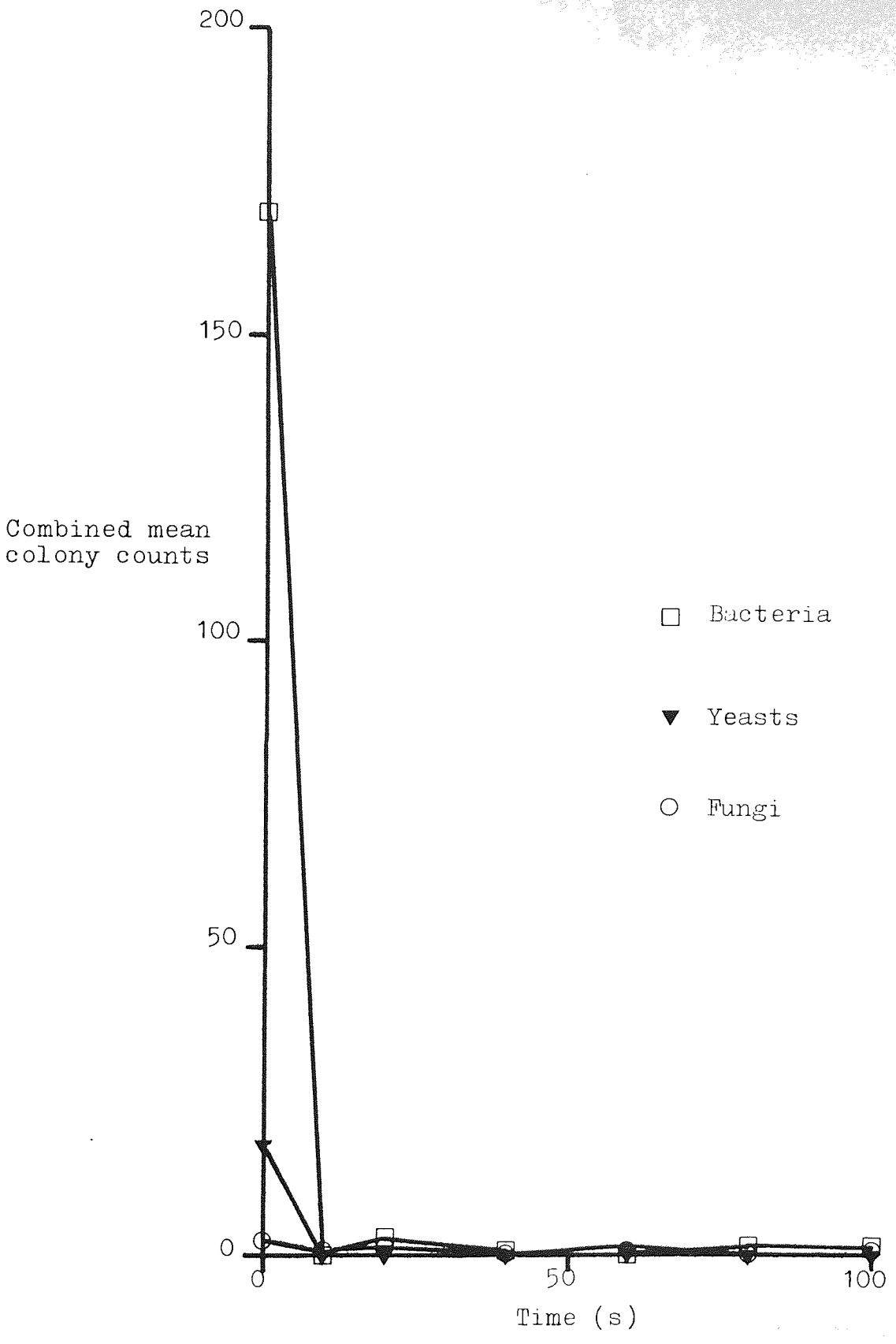


Fig. 49 Heat inactivation of HMS Broadsword micro-organisms in diesel fuel at 65°C

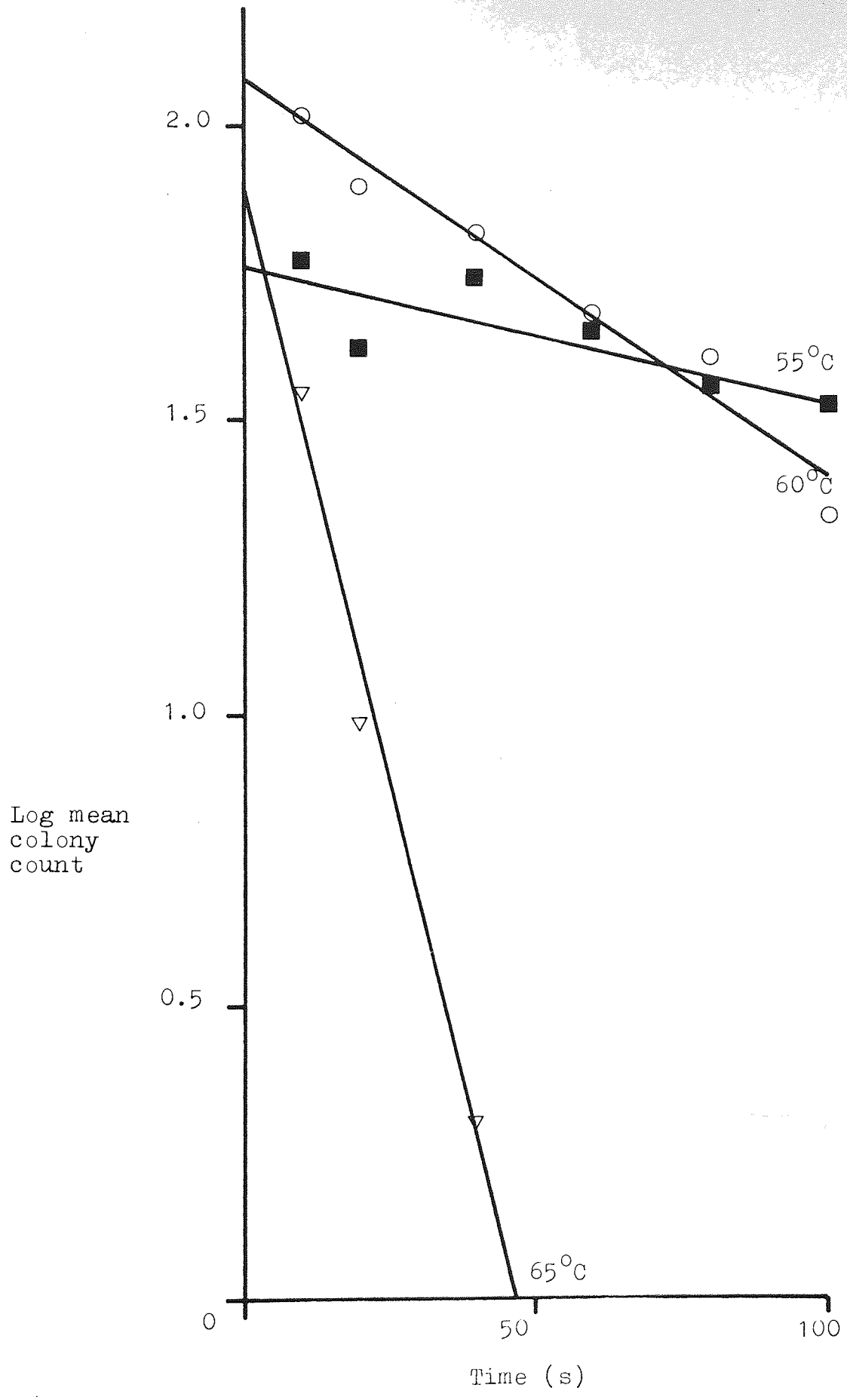


Fig. 50 Heat inactivation of C. resinae ATCC 22711 at 55, 60 and 65°C.

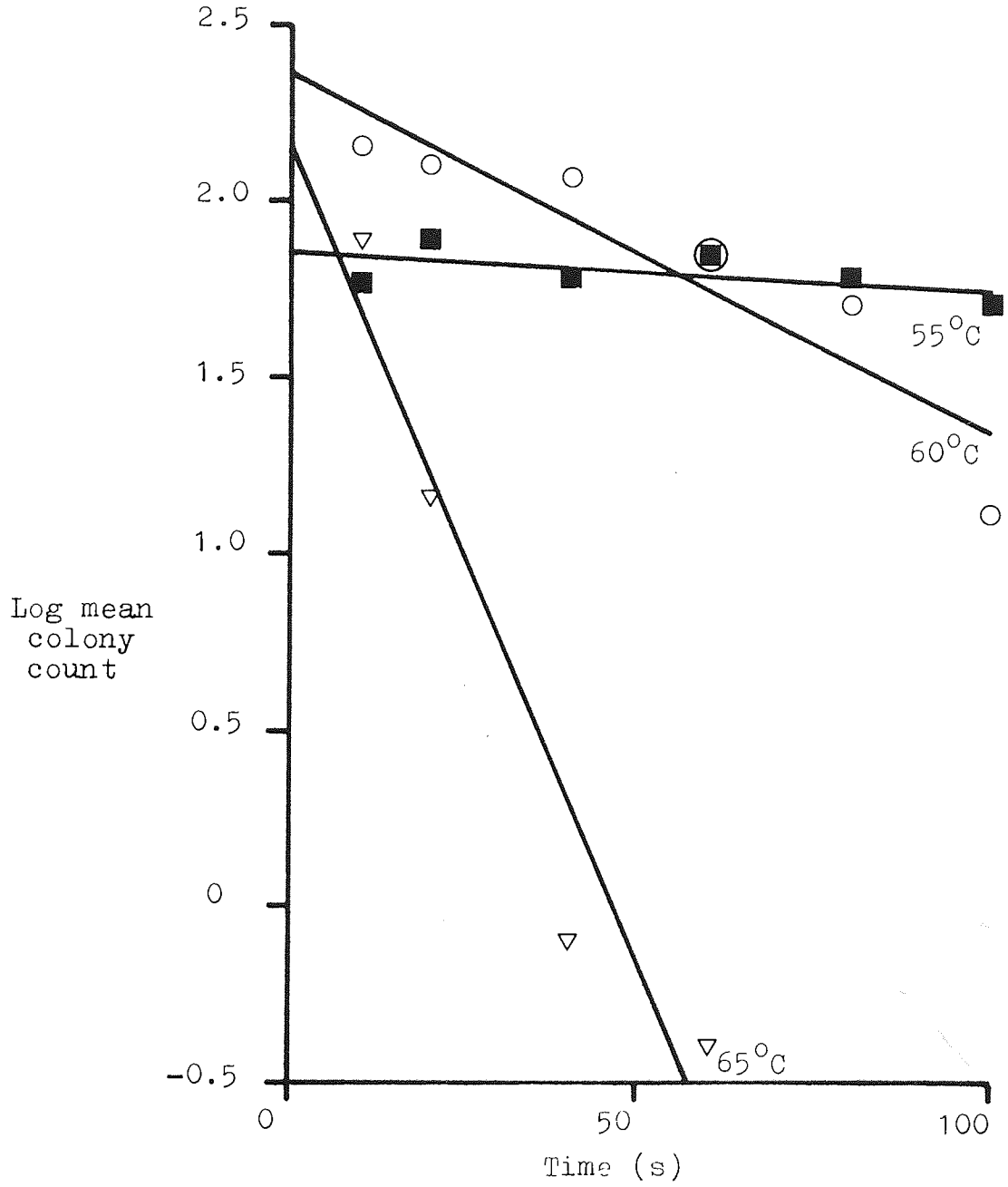


Fig. 51 Heat inactivation of C. resinae EW1 at 55, 60 and 65°C.

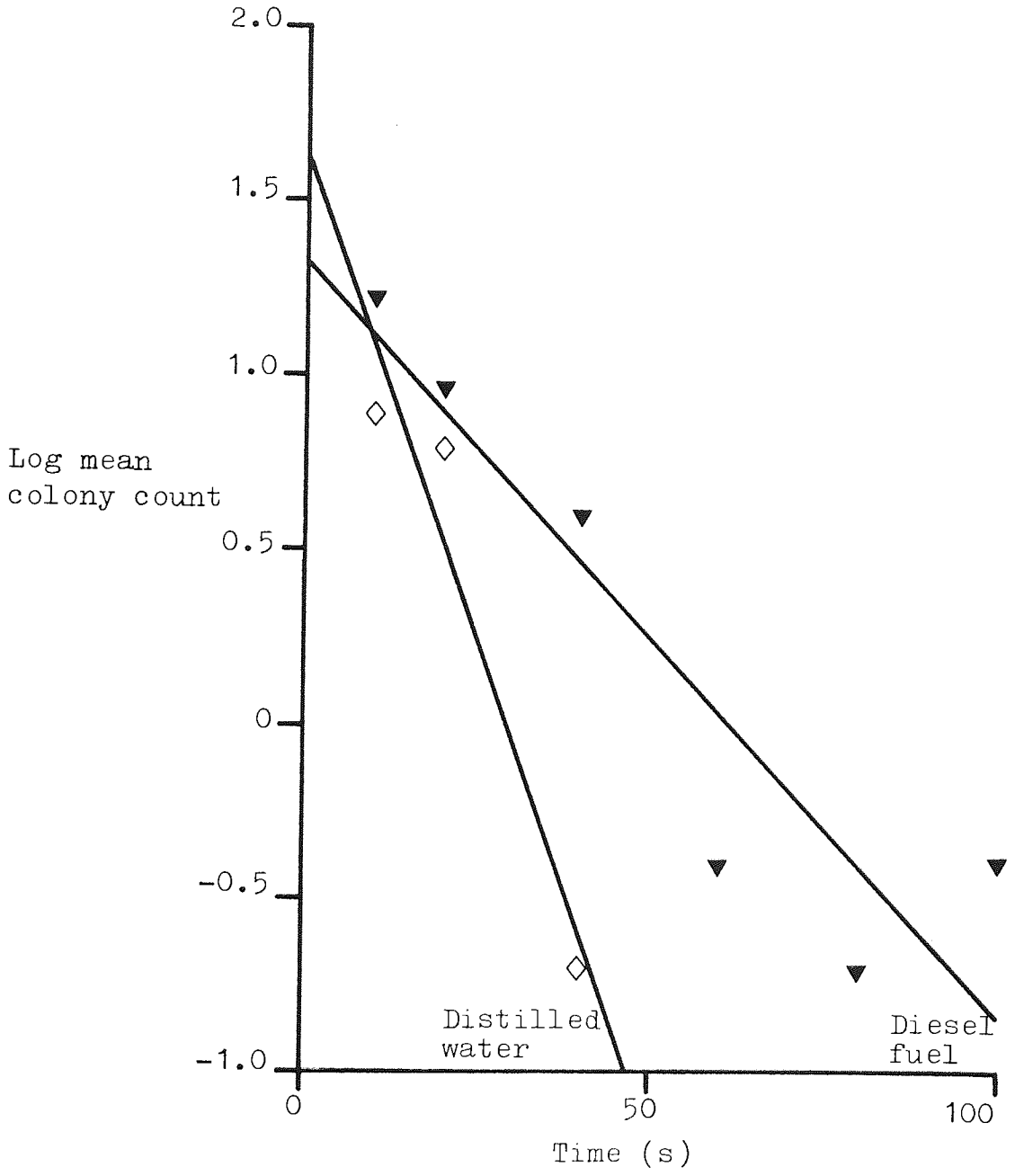


Fig. 52 Heat inactivation of C. resinae EW3 at 65°C in distilled water and diesel fuel.

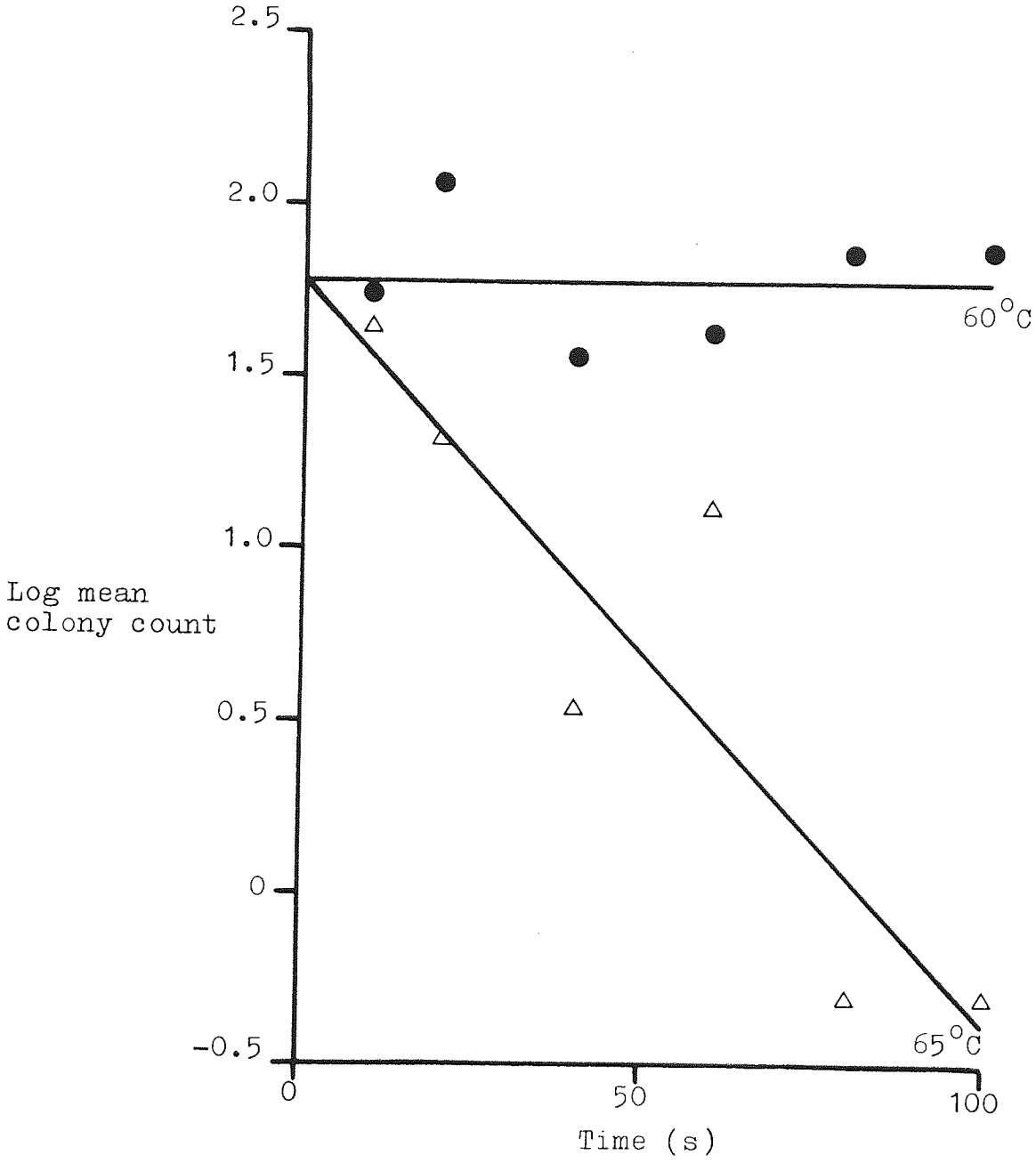


Fig. 53 Heat inactivation of Penicillium corylophilum EW4 at 60 and 65°C

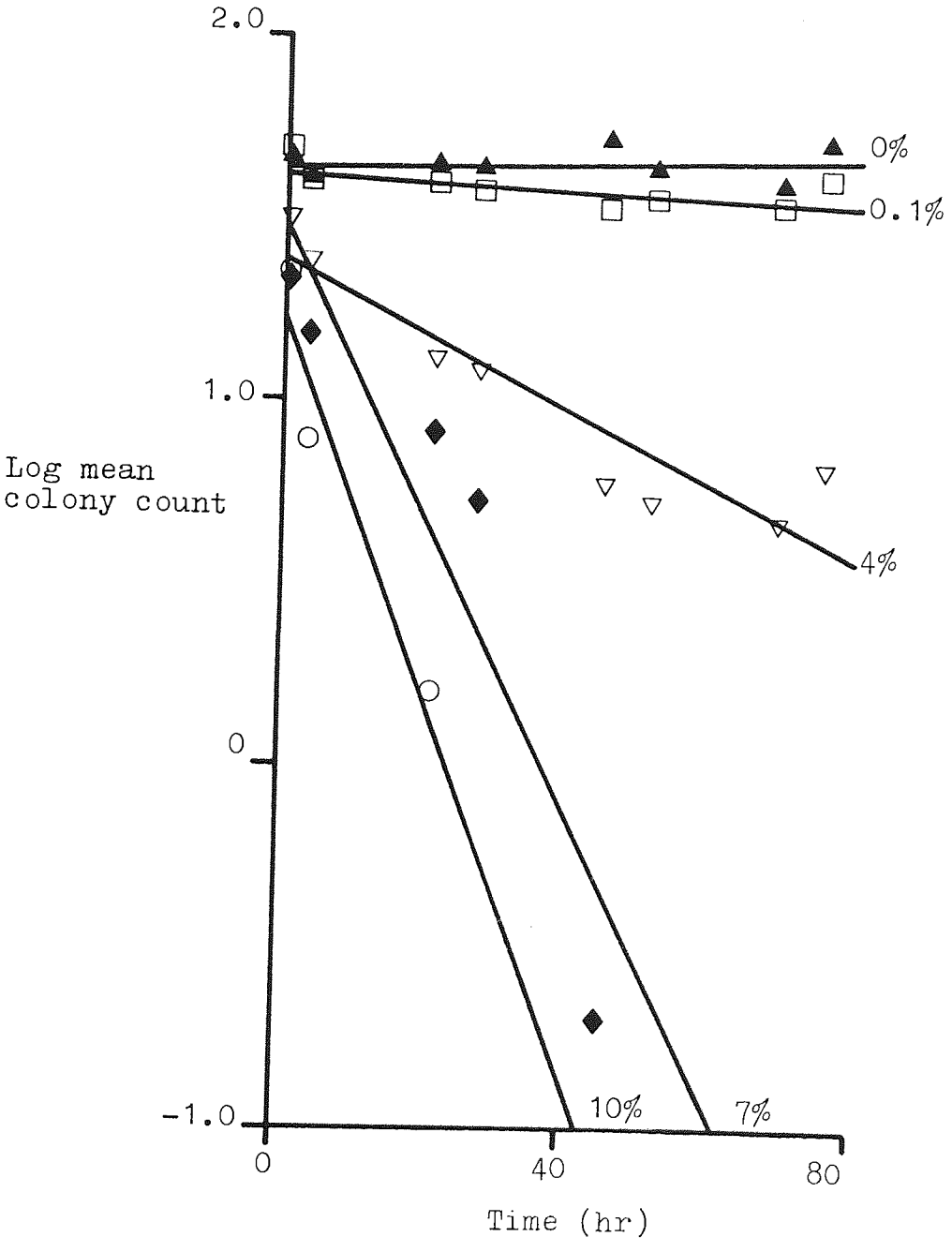


Fig. 54 Inactivation of C. resinae ATCC 22711 by Biobor J.F. at 25°C

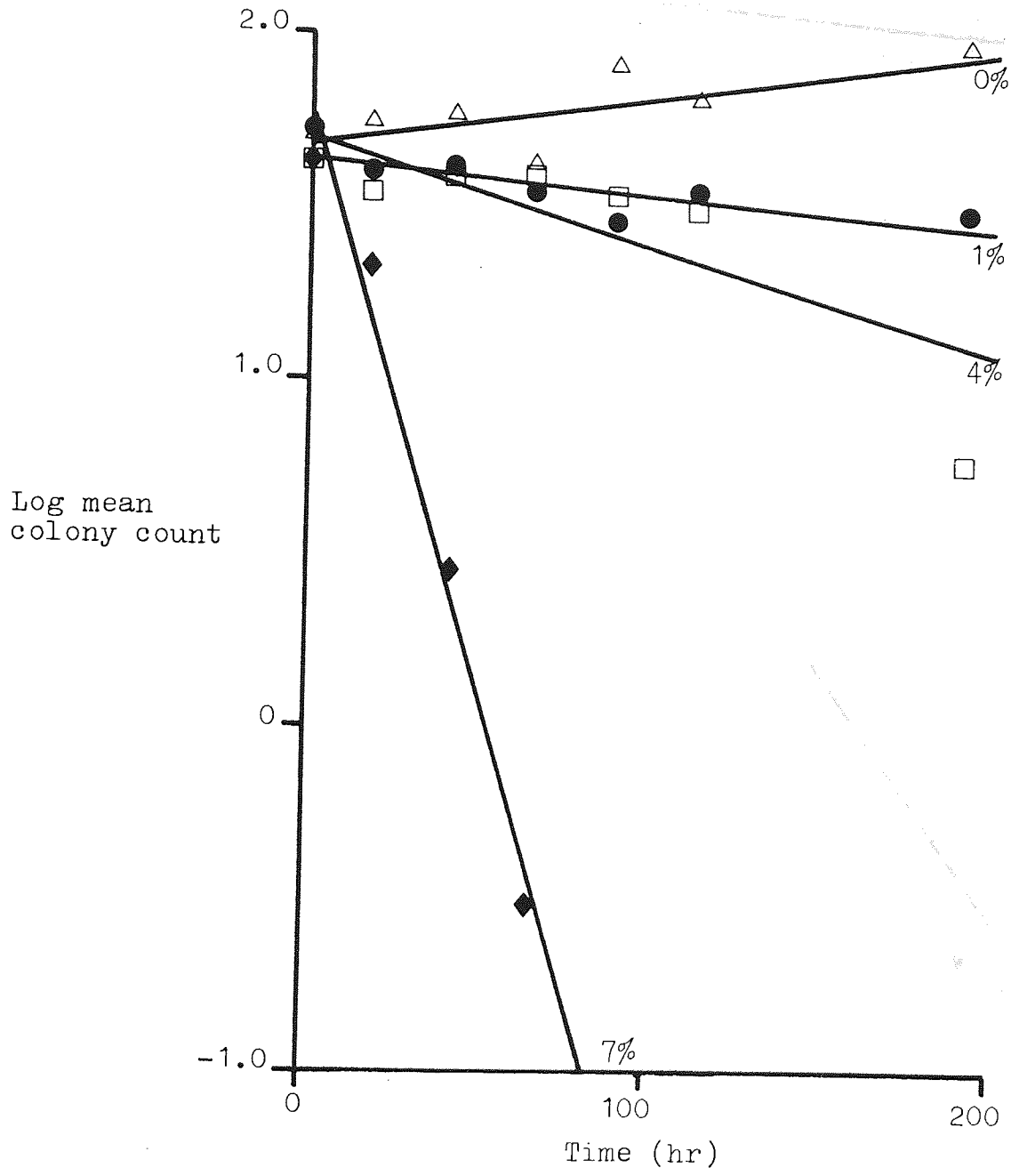


Fig. 55 Inactivation of C. resinae EW1 by Biobor J.F. at 25°C

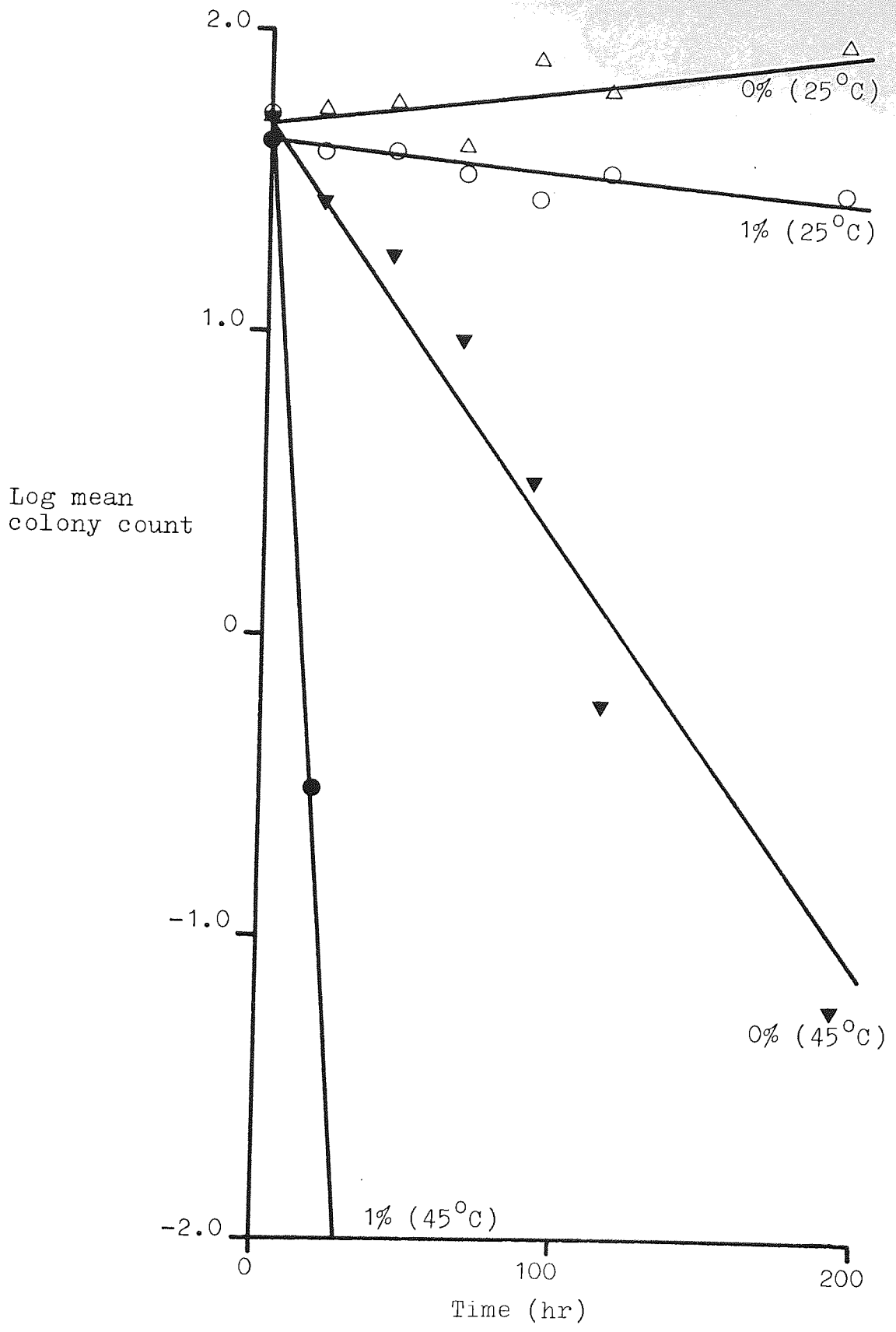


Fig. 56 Inactivation of C. resinae EW1 by Biobor J.F. at 25 and 45°C

CHAPTER 9

FINAL DISCUSSION

FINAL DISCUSSION

Microbial colonization of shipboard fuel systems first posed a serious problem with the introduction of gas turbine propelled warships and the need for clean, dry fuel. Blockage of the fine filtration and water removal equipment occurred due to the build-up of microbial biomass and frequent maintenance and replacement of filters was required. In some cases, engines became starved of fuel and failed to operate.

The biomass is formed by the growth of microorganisms in shipboard and land storage tanks and samples were taken to study the microbial population. Many species of bacteria, yeasts and fungi were found growing on the isolation media and some were obtained in pure culture. Three fungal species were identified as Cladosporium resinae, Penicillium corylophilum and Cylindrocarpon sp. and two yeasts as Candida guilliermondii and Rhodotorula glutinis. The bacteria were mostly Gram-ve rods.

Enumeration studies were carried out on two fuel/water samples and it was found that the distribution of microorganisms in the samples was uneven. The fuel harbours few microbes and a greater concentration was found at the interface than in the water bottom. Thus, hydrocarbon utilizers have a requirement for water and a strong affinity for their carbon and energy source, which is the reason that a layer of biomass forms at the interface (see Figs. 18 and 32-35).

With this knowledge, filter blockage can be alleviated by tapping off only the fuel contained in tanks and not the water bottom or interface. This procedure can be

carried out in land storage tanks which have a variable depth suction device (see Fig. 14). The difference in degree of colonization may be due to a number of factors; age of the environment, nutrient levels and temperature. In both samples, bacteria were more numerous than the yeasts and fungi.

The environmental conditions in fuel systems can affect the ecological balance and the rate of biomass formation. Samples were taken in order to measure salinity, pH and phosphate concentration.

The concentration of sea water in the water bottoms varied considerably. In many cases, its presence is due to leakage and salt spray; however in some warships sea water is added deliberately in order to displace spent fuel and maintain trim and stability. Fresh water can accumulate due to condensation and the brackish conditions in some samples were probably caused by the mixing of marine and condensed water.

Water bottoms of high salinity showed little evidence of microbial development and the pH was around neutral. On the other hand, brackish water bottoms showed signs of biomass formation and had pHs of 4 - 5. The acid conditions were probably caused by the production of organic acids by the microorganisms.

The concentration of phosphate in the water bottoms varied considerably. Many had levels similar to those normally found in the ocean. High concentrations were noted in others, possibly because of contamination by polluted, nutrient-rich coastal water. In one sample, the phosphate concentration was very low, perhaps due to

uptake by the microbial population.

Experiments were carried out to investigate the effect of inoculum size on microbial growth. It was found that after prolonged incubation, the final biomass concentration was unaffected by the number of spores of C. resinae introduced into the culture. However, the time taken to reach the final fungal mass decreased with increasing spore number. Thus, contamination of fuel systems by large numbers of microorganisms can reduce the time taken for the formation of biomass sufficient to cause filter blockage problems. Conversely, if the inoculum size is minimised, fuel can be stored relatively free of colonization for longer periods.

Probably the main source of fuel-utilizers is from previously colonized stocks. Therefore "topping-up" of tanks should be discouraged and only clean fuel be delivered to gas turbine warships. Other potential sources of microorganisms such as polluted harbour water and soil should be excluded from the fuel.

The fungus, C. resinae was cultured on a number of hydrocarbons. Growth was much more profuse on a pure alkane, undecane, than on diesel fuel. Since alkanes account for around 50% of the composition of diesel fuel, the fungus is probably inhibited by the presence of toxic compounds. One strain grew less well in two of four diesel fuels used, indicating that some fuels may contain a higher concentration of toxic components. These could possibly be earmarked for use in gas turbine ships.

It has been established for some time that the presence of water is essential for the growth of

hydrocarbon-utilizing microorganisms. Complete exclusion from fuel systems would therefore prevent colonization. However, because of condensation and the accidental and deliberate addition of water, the fuel is frequently contaminated. As evidence, many of the samples taken from tanks contained water bottoms.

A number of experiments were carried out to determine the effect of aqueous phase composition on microbial growth. Sparse growth of some Penicillium species and C. resinae was observed in diesel fuel containing only distilled water and illustrates the ability of these fungi to develop in an environment of low nutrient levels. The addition of mineral salts to the medium improved growth considerably.

The effect of inorganic salts on growth was examined in more detail. In one case, increasing the concentration yielded more biomass until a critical level was reached after which growth was inhibited. However, a similar experiment using another strain of C. resinae showed that the higher nutrient levels allowed more profuse growth, no critical concentration being apparent. The reason for these conflicting results is possibly due to strain differences.

Sea water generally supported little or no growth which may be due to inhibition or lack of nutrients or a combination of both. Salt water displaced fuel tanks should therefore present a hostile environment for hydrocarbon-utilizing microorganisms. This is apparently not the case, for a number of samples taken from these tanks have shown large deposits of biomass at the

interface. The reason may lie partly in the nutrient levels in the water bottoms. Experiments showed that sea water containing inorganic and organic nutrients supported profuse microbial growth.

It is apparent therefore that nutrients can promote the microbial colonization of fuel systems irrespective of the sea water content of the water bottoms and that it is essential to minimize the contamination of fuel by sources containing high nutrient levels, such as harbour water and decaying organic matter.

The fuel/water ratio, the volume of tank contents and agitation of the environment can affect the growth of microorganisms. More biomass of C. resinae was obtained with higher hydrocarbon concentration, which is probably due to the greater availability of the carbon and energy source to the fungus. An increase in the volume of fuel and water in a flask of fixed size discouraged growth at higher hydrocarbon concentrations which is probably due to limitation of oxygen transfer.

Shaken cultures increased the growth of C. resinae on glucose and is due to the increased transfer of oxygen. However, on hydrocarbons, growth was significantly reduced with agitation, indicating that another factor is outweighing the beneficial effect of increased oxygen transfer. This is likely to be the disruption of direct hydrocarbon uptake by the fungus.

The use of heat to control microbial colonization was investigated and a number of experiments were carried out to determine its effect on the growth and survival of microorganisms. On surface culture, the upper temperature

limit for growth of C. resinae was found to be around 45°C. At this temperature, spores in aqueous suspension were slowly inactivated, 90% of the population dying after about 63 hours. However, heating the fuel to 45°C for prolonged periods could allow the growth of thermophilous microorganisms and is therefore not recommended as a useful preservative treatment. Nevertheless, in conjunction with a biocide, the rate of destruction is increased at this temperature, and could in these circumstances prove to be an extremely effective control measure (see later).

Exposure of fuel-degraders to higher temperatures for short time periods is an alternative means of controlling colonization and systems are available which could carry out such a treatment in ships or land storage installations. Experiments demonstrated that a 90% kill of C. resinae spores occurred after about two minutes at 60°C and around 20 seconds at 65°C. Spores of Penicillium corylophilum were more resistant to heat, a 90% kill occurring after 47 seconds at 65°C.

At this temperature, all of the yeasts and most of the bacteria in a water bottom sample taken from HMS Broadsword were destroyed within 10 seconds of the treatment. Suspension of microorganisms in a medium containing 100 parts diesel fuel to 1 part water (to attempt to simulate shipboard conditions) did not greatly affect the inactivation rate. These studies have shown that the application of heat at 65°C for time periods of less than a minute can destroy the majority of fuel-inhabiting microorganism and thereby prevent colonization of fuel systems.

The organo-boron compound, Biobor J.F., was found to cause the destruction of spores of C. resinae, the rate of inactivation increasing with higher concentrations. At 25°C, 90% of the spores in aqueous suspension were destroyed after 99 hours in 4% and after 20 hours in 10% Biobor J.F. Disinfection of a fuel tank will therefore require a few days exposure to the biocide at normal temperatures.

This is an undesirable situation because a ship will have to be taken out of service for long periods. Another problem with the use of Biobor J.F. can occur. It is normally added to the fuel and partitions into the aqueous phase to set up an inhibitory concentration. However, if the amount of water associated with the fuel is very large, the concentration of biocide may become insufficient to cause the inactivation of the microbial population.

Experiments were carried out with a view to increasing the effectiveness of Biobor J.F. and it was found that raising the temperature significantly increased its potency. In 1% concentration of the compound, a 90% kill was obtained after about 1000 hours at 25°C but after only 8 hours at 45°C. Therefore, the time required to disinfect a tank can be considerably reduced by raising the temperature of the fuel. Also, when large water bottoms are present thereby reducing the biocide concentration, effective control is retained at higher temperatures.

The problem of filter clogging is caused to a large extent by the filtration of biomass which has formed

previously in storage tanks. However, it is possible that growth of microorganisms within the mesh of the filters can contribute to the problem. Scale models of fuel systems are available and these could be used to determine if "in situ" biomass formation takes place and if it plays a significant role in causing blockage. In this event, effective control could be brought about by impregnation of the filters with biocides.

Although the present study has gone some way to determining the nature of the microbial population and the environmental conditions prevailing in fuel tanks, there is scope for a great deal of further work in this area. Bacteria were found in large numbers in the samples examined and their role needs to be resolved. Experiments using pure cultures in scale models could show if, in spite of their small size relative to yeasts and fungi, they can contribute by virtue of their large number to filter clogging. Bacteria may be troublesome in other ways. They may produce surfactants which impair water coalescence or cause the precipitation of insoluble "gums" from the fuel, leading to filter blockage. Bacteria may also alter the environment, allowing the growth of yeasts and fungi which because of their larger size are certainly capable of plugging filters.

A comprehensive programme of sampling, isolation, enumeration and identification followed by laboratory studies of the interactions between various microbial species is required in order to elucidate the ecological basis of fuel system colonization. This study would also indicate the sources of microbial contamination and the

main sites of biomass formation so that corrective measures can be applied. A list of the dominant species involved could be compiled and these used in experiments to find preservative treatments.

More data on the environmental conditions in fuel tanks is needed and could be correlated with information relating to the microbial populations. Thus, conditions which discourage colonization could be identified and maintained. Conversely, environments which enhance microbial development could be highlighted and corrective measures taken to avoid their recurrence.

The measurement of dissolved oxygen in fuel systems was not fully explored and requires further study. Because the catabolism of hydrocarbons requires a great deal of oxygen, it is likely to be a very important environmental parameter. Further measurements of nutrient levels could show where, in the supply chain, these come into contact with the fuel. When the sources are known, action can be taken to prevent further contamination.

The studies on the effect of inoculum size and physico-chemical conditions on microbial growth have shown that colonization can be inhibited by paying careful attention to the quality of the fuel. The following measures could be implemented:

1. Increase the frequency of drainage, centrifugation and coalescence to remove water from the fuel.
2. Seal tanks and ensure hatches and vents are closed in order to prevent ingress of water.
3. Prohibit the use of polluted harbour water in

warships with displacement systems.

4. Prevent contamination by soil and decaying organic matter.
5. Discourage "topping up" of tanks, especially with previously colonized fuel.
6. Reduce storage time where possible.

Before the use of gas turbine engines and the need for fine filtration equipment, ships could burn fuel containing microbial biomass, therefore there was virtually no need to employ many of these measures to keep it clean and dry which would discourage growth. The current situation now demands good fuel handling procedures and training of personnel should be given where necessary.

Biomass can form at many points along the fuel supply chain but its effects are generally only experienced in the warships when the fuel is filtered prior to its combustion. Thus, personnel on supply tankers and land storage depots may be unaware that a problem exists. In many cases, the engineer on board a gas turbine warship can refuse to embark contaminated fuel, but in the middle of the ocean, he may have no choice but to accept the fuel he is offered. This type of situation illustrates the need to train personnel on supply tankers and land storage installations to keep the fuel in good condition.

Frequent monitoring of fuel systems for microbial colonization is important for two reasons. First, if a land storage tank or supply tanker is found to be heavily contaminated with microorganisms, it must not be allowed to transfer fuel and spread the infection. Second, the results can be fed back to fuel handling personnel. If

colonization is serious, it must be pointed out that the fuel is unfit for use and that measures have to be taken to improve its quality. This may mean extensive tank cleaning and re-training.

Further studies on the growth of microorganisms on hydrocarbons are required. The finding that agitation significantly inhibited growth of C. resinae is particularly interesting. It is through further research of this kind that the way in which a species colonizes an environment may be more fully understood and can lead to the application of practical measures designed to inhibit growth and biomass formation.

The use of heat to control microbial colonization is particularly attractive since a large amount of waste heat is generated in ships. The present study has shown that a temperature of 65°C, applied over time periods of less than a minute, can destroy the majority of fuel-inhabiting microorganisms. It should be possible to install heat exchangers in ships, tankers and land storage tank depots and engineering studies are being carried out on a test rig to examine the practical aspects of pasteurization (Houghton, 1981; pers. comm.).

Cheap, effective biocides which are acceptable for use with gas turbine engines are required. However, the present study has shown that the destructive effect of a currently used compound - Biobor J.F. - increased markedly by raising the temperature from 25 to 45°C. Furnace fuel oil, which was used to power steam driven ships, had to be heated to reduce its viscosity and facilities still exist in some land storage and shipboard tanks. The

practice of heating fuel to 45°C in combination with a dose of Biobor J.F. could possibly be introduced to gain signficant savings in loss of operational time and biocide costs.

APPENDICES

APPENDIX I

Cladosporium resinae - TAXONOMY AND DISTRIBUTION

Cladosporium resinae - TAXONOMY AND DISTRIBUTION

Cladosporium resinae was originally isolated as Hormodendron resinae Lindau from the resin of Picea execelsa near Hamburg (Lindau, 1907). It was next reported by Christensen et al. (1942), who found the fungus to be widespread and common on creosoted telephone poles and fence posts, as well as on resinous woods in North America. They recognised it as the same fungus as Lindau had found, but commented that it might be an unknown species of Cladosporium. Marsden (1954) also isolated the fungus from creosoted timber and named it the "creosote fungus". De Vries (1955) compared Cladosporium avellaneum de Vries with the strains of Lindau, Marsden and Christensen and renamed the "creosote fungus" Cladosporium resinae (Lindau) de Vries. The fungus exhibits a number of morphological forms, of which the two extremes are: Cladosporium resinae f. avellaneum and Cladosporium resinae f. resinae. The forma avellaneum is the most common (Parbery, 1969a; see Fig. 57).

The perfect state of Cladosporium resinae was discovered by Parbery and Ford (1968) and the fungus was placed in the genus Amorphotheca which represents a monospecific genus in the Eurotiales (Parbery, 1969a). The ascal state was also isolated directly from soil (Parbery, 1969b).

Cladosporium resinae was first isolated from soil in the vicinity of creosoted matchsticks by Parbery (1967). Subsequent work showed the fungus to be distributed in soils in Australia, Europe (Parbery, 1969c) and New Zealand (Sheridan et al., 1971) and established soil as a natural habitat for C. resinae. The perfect state of the fungus, Amorphotheca resinae, was also isolated directly from soil (Parbery, 1969b).

The ability of Cladosporium resinae to grow in the vicinity of creosote (Christensen et al., 1942; Marsden, 1954; Parbery, 1967) suggests that the fungus belongs to a group of soil organisms which do not actively compete with the vigorous soil saprophytes for substrates. Instead their survival depends on their ability to grow on substrates unavailable to other organisms (Park, 1968). These substrates may be toxic to other organisms or their utilisation requires rarely available enzymes. Cladosporium resinae has been isolated from creosoted wood (Christensen et al., 1942; Marsden, 1954), resinous wood (Lindau, 1907; Christensen et al, 1942; Nicot and Zakartchenko, 1966) and hydrocarbon fuels (Edmonds and Cooney, 1967; Darby et al., 1968; Turner and Ahearn, 1970; Sheridan and Soteros, 1974; Scott and Forsyth, 1974; Houghton and Gage, 1970). The organism has been shown to utilise leaf waxes, vegetable oils, keratin, chitin (Parbery, 1969c), lignin (Marsden, 1954), methyl p-hydroxybenzoate (Sokolski et al., 1965), progesterone (Fonken et al., 1960) fatty acids, fatty alcohols (Teh, 1974), hydrocarbons (Teh and Lee, 1973; Cofone et al., 1973; Walker and Cooney, 1973; Walker et al., 1975) and hydrocarbon fuels (Prince, 1961; Hendey, 1964; Cooney and Kula, 1970; Parbery, 1971b).

Cladosporium resinae has been trapped from the air over Cardiff, Great Britain (Harvey, 1967), Rabat, Morocco (Chabert and Nicot, 1968) and Wellington, New Zealand (Sheridan and Nelson, 1971) which suggests that spore dispersal is airborne.

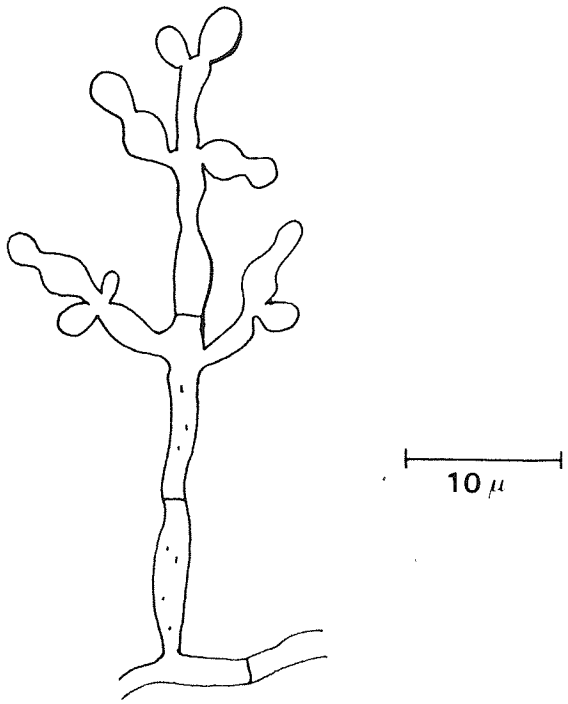


Fig. 57 Conidiophore of Cladosporium resinae f. avellaneum

APPENDIX II

PREPARATION OF SPORE SUSPENSIONS

PREPARATION OF SPORE SUSPENSIONS

A malt extract agar slant was inoculated with fungal spores and incubated at 25°C for 14 days. Distilled water (10ml) was added and the contents were shaken vigorously. The suspension was filtered through eight layers of muslin to remove mycelium and agar and centrifuged at 1300g for five minutes. The supernatant liquid was discarded and the pellet was resuspended in distilled water. After two further washes, the spores were counted in a counting chamber.

APPENDIX III

MEDIA

MEDIA

The mineral salts solution was that of Turner (1977). and had the following composition (%, w/v, except where otherwise stated):

KH_2PO_4	1.25
$Na_2HPO_4 \cdot 12H_2O$	0.2875
NH_4NO_3	0.6
$MgSO_4 \cdot 7H_2O$	0.02
$CaCl_2 \cdot 2H_2O$	0.002
trace element solution	0.1% (v/v)
ferric chloride solution	0.5% (v/v)
pH 5.6	

Trace element solution contained (%, w/v):

$ZnSO_4 \cdot 7H_2O$	0.009
$CaSO_4 \cdot 5H_2O$	0.008
$MnSO_4 \cdot 4H_2O$	0.008
$CoCl_2 \cdot 6H_2O$	0.009
H_3BO_3	0.005
$Na_2MoO_4 \cdot 2H_2O$	0.015

Ferric chloride solution contained (%, w/v):

$FeCl_3 \cdot 6H_2O$	0.167

and was sterilized by passage through a 0.22 micron filter (millipore).

The hydrocarbons used were undecane (British Drug Houses) and diesel fuel, obtained from the National Gas Turbine Establishment, Fairmile, Cobham, Surrey. Four diesel fuels were used;

NAVAL 20 POUR (47/20 DIESO) - source BAHRAIN

NAVAL 20 POUR (47/20 DIESO) - source CURACAO

GENERAL PURPOSE (UK(MT) DIESO)

REFERENCE DIESEL FUEL, HIGH SULPHUR

The hydrocarbons were sterilized by passage through a 0.22 micron filter.

Mineral salts solution containing a trace of diesel fuel was prepared by the following method. Turner's mineral salts solution (10ml) was diluted in distilled water (90ml) and shaken vigorously for 10 minutes with Reference Diesel Fuel, High Sulphur (1ml) in a separating funnel. The contents were allowed to equilibrate in the dark for 48 hours at $4^{o}C$. The aqueous phase was removed, brought to room temperature and sterilized by passage through a 0.22 micron filter.

Physiological saline was prepared by the solution of sodium chloride in distilled water ($8.5g\ l^{-1}$). All salts used were obtained from British Drug Houses.

APPENDIX IV

GENERAL GROWTH CONDITIONS

GENERAL GROWTH CONDITIONS

The microorganisms were grown in test tubes (150 x 19mm), boiling tubes (200 x 38mm) or conical flasks (250ml). The aqueous medium was generally mineral salts solution and the hydrocarbons either undecane or diesel fuel (see Appendix III). Boiling tubes and conical flasks were inoculated with spore suspensions, and test tubes by the transfer of spores (or yeast cells) with an inoculating loop from actively growing colonies on malt extract agar plates.

All cultures were incubated in the dark at $25 \pm 1^{\circ}C$. Conical flasks were either agitated on a rotary shaker (Gallenkamp, 32mm stroke) and shaken by hand daily to minimise the formation of a rind, or left undisturbed. Boiling tube and test tube cultures were not moved during incubation. After a suitable incubation period, growth of the cultures was estimated (see Appendix V).

APPENDIX V

ESTIMATION OF GROWTH

ESTIMATION OF GROWTH

Presence or absence of growth in test tube cultures was estimated by visual observation against uninoculated controls. Aqueous phase turbidity and/or the formation of biomass at the fuel/water interface were acceptable indications of growth.

Conical flask and boiling tube cultures were estimated by dry weight determination. The contents were filtered under vacuum through a pre-weighed crucible (Gallenkamp, Sinta glass 4). The remaining biomass was washed with distilled water and chloroform and the crucible was dried to constant weight at 105°C.

APPENDIX VI

TREATMENT OF RESULTS

TREATMENT OF RESULTS

Means were calculated by the following formula:

$$\bar{x} = \frac{\Sigma x}{n}$$

where $\bar{x}$ = mean

Σx = sum of the replicate values

n = number of replicates

Standard deviations were calculated by the following formula:

$$\sigma = \sqrt{\frac{\sum_{x=i}^{n} (X_i - \bar{x})^2}{n - 1}}$$

D-values were obtained from the inverse of negative gradients of lines of best fit. Gradients were calculated using the following formula:

$$m = \frac{Sxy}{Sxx}$$

where $Sxy = \sum_{i=1}^{n} (X_i - \bar{x})(Y_i - \bar{y})$

$$Sxx = \sum_{i=1}^{n} (X_i - \bar{x})^2$$

(Davies and Goldsmith, 1977).

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