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Genotoxicology of diesel engine exhaust emissions in cultured mammalian cells

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by

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Genotoxicology of diesel engine exhaust emissions in cultured mammalian cells.

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Abstract.

Diesel exhaust emissions were collected from a 2 litre direct injection diesel engine using the Total Exhaust Solvent Scrubbing Apparatus (TESSA). Emission samples were collected from a series of two minute engine runs, at a variety of engine speeds and loads which covered the full operating range of the engine. The exhaust extracts collected were then tested for their cytotoxicity and mutagenic potential in cultured Chinese hamster cells.

Emission samples were found to be extremely toxic, with most causing 100% cytotoxicity at concentrations of less than 100 μg/ml. Chromosome aberration studies indicated that less than 50% of the emission samples collected induced increases in the numbers of cells with aberrations, and less than 10% induced aberrations in cell cultures exposed to emission samples with a supplementary metabolic activation system. Samples tested in *in vitro* sister chromatid exchange assays, induced significant increases in the frequencies of exchanges.

Linear trend statistics calculated from chromosome aberration data, were used to reflect the relative clastogenic potential of individual emission samples. Linear regression of these trend values against physical components of the exhaust emissions, showed a significant correlation between sample mutagenicity in aberration assays and the emission of oxides of nitrogen (NOx) in the exhaust. Mapping of NOx emissions to engine conditions has shown that the mutagenicity of diesel emissions from the test engine tend to be highest under condition of low speed/high load and high speed with increasing load.

Toxicity assays of subfractions of emission samples isolated by column chromatography has shown that the toxicity of the emission samples is associated with the aromatic and polar components of the samples.

The dose responses obtained from mutagenicity assays, in which samples only caused increases in aberrations and chromatid exchanges at the same concentrations at which cytotoxic effects were observed, suggests that the emission may have a limited cytogenetic effects *in vivo*.

Mapping of the clastogenicity of emission samples against engine speed and load, has shown that the most clastogenic samples collected, were emitted under engine conditions that might be expected to occur under urban driving conditions.

Results of epidemiological studies of health effects of diesel emissions, and recent associations between particulate emissions and health effects are discussed.

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List of abbreviations.

ANOVA	Analysis of variance
bpt	boiling point
BrdUrd	5-deoxy-bromouridine
CA	Chromosome aberrations
Ce	Particulate elemental carbon
CHO	Chinese hamster ovary
Co	Particulate organic carbon
CO	Carbon monoxide
COP	Conformity of production
CP	Cyclophosphamide
cs	centisieverts
d.f	Degrees of freedom
DCM	Dichloromethane
DI	Direct injection
DMSO	Dimethyl sulphoxide
DOH	Department of health
EC	Economic community
ECACC	European collection of animal cell cultures
ECE	Economic commission for Europe
EEC	European economic community
EPA	United States Environmental protection agency
ES1-ES19	Emission samples
FAP	Familial adenomatous polyposis
FBS	Foetal bovine serum
FPG	Fluorescence plus giemsa
FTP	Federal test procedure
g/mile	emissions in grammes per mile travelled
g/t	emissions in grammes per test
G-6-P	Glucose 6 phosphate
G6	CHO-K1-PD2-G6 cells

GC	Gas chromatography
GC/MS	Gas chromatography plus mass spectrometry
GLP	Good laboratory practice
HC	Hydrocarbons
HD	Heavy duty diesel engines
HFET	Highway fuel economy test
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
IARC	International agency for research on cancer
IDI	Indirect diesel injection
IPCS	International program on chemical safety
IU	International unit of enzyme activity
KCl	Potassium chloride
kW	kilowatt
LD	light duty diesel engine
LPG	Liquid petroleum gas
MA	Metabolic activation
MMNG	N-methyl-N'-nitro-N-nitrosoguanidine
mpt	melting point
mptc	Mitoses per thousand cells
NADP	Nicotinamide adenine diphosphate monosodium salt
nm	nanometres
Nm	Newton metres
NOx	Oxides of nitrogen
NSCA	National society for clean air
NYCC	New York city cycle
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PCB's	Polychlorinated biphenyls
PHA	Phytohaemagglutinin
ppm	parts per million
ppm/gf	Emissions as parts per million per gramme of fuel consumed

revs/μg	Bacterial revertants per microgramme of exhaust extract
rpm	Revolutions per minute
SCE/chrom	Sister chromatid exchanges per chromosome
SCE/cell	Sister chromatid exchanges per cell
SCE	Sister chromatid exchanges
SI	Spark ignition
SMMT	Society of motor manufactures and traders
SO2	Sulphur dioxide
SOF	Soluble organic fraction of exhaust emissions
SRM	Standard reference material
TA	Type approval
TESSA	Total exhaust solvent scrubbing apparatus
U.N.	United Nations
U.V.	Ultra violet
UKEMS	United Kingdom environmental mutagen society
VOC	Volatile organic compounds
WHO	World Health organisation
Z	Linear Trend Statistic

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AUTHORS DECLARATION.

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Signed S.T. Kingston

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1.0 Introduction.

1.1. Environmental impacts of diesel emissions.

The diesel engine is probably most familiar as the source of clouds of smoke emitted from buses and lorries, particularly in urban areas. However, the diesel in its more refined form as a passenger vehicle engine is gaining in popularity, with an approximately four percent increase annually in new car registrations (Select Committee 1987). With the recent advances in engine technology and performance the image of the diesel car as a slow noisy vehicle, with poor acceleration and low top speed is being overcome. However, the original reason for purchase, that of the relative cheapness of diesel fuel compared to petrol has been reduced, due to government incentives for the "greener" unleaded petrol. Although the fuel economy of diesel engines is superior to that of petrol, extra mileage in the region of 10 to 15,000 miles per annum is required before the benefits in fuel savings can be appreciated. Even so, the market share of the diesel car is increasing particularly in other EC countries (eg. in France diesel cars represent approximately 40% of the vehicle fleet).

Perhaps, the most obvious effect of vehicle emissions, is the soiling of buildings particularly in city centres. Diesel engine emissions are the major contributors to urban soiling. This is due to the higher emissions of particulate matter from diesel engined vehicles. Diesel particulates also have a particularly adhesive nature, due to a coating of adsorbed organic compounds making them "oily" and therefore prone to accumulation on stone surfaces. In the early 1970's the market for building cleaning in the U.K. was estimated £1.5 million (Jones 1970 cited by QUARG 1993). In 1987 the value of this market had increased to an estimated £110 million, although in the 1990's the value has decreased slightly (£79 million), largely as a result of the economic recession (Mansfield 1992). Recommendations from the industry suggest that buildings should be cleaned every eight to ten years.

Although building soiling is probably the most evident form of vehicle pollution, particulate matter in the form of dust and dirt has also been considered as a subjective indicator of the nuisance of road traffic. Two studies (Mackie and Davies 1981 and Clench-Aas *et al.* 1991 cited by QUARG 1993), have highlighted the increased number of reports made concerning disturbance from dust, dirt and traffic volume. A recent report (McCrae and Williams 1992) has indicated that 35% of householders believed vehicle emissions were the

major source of household dust. Up to 50% of these believed road traffic was responsible for the majority of outdoor dirt, and 25% believed that outdoor dirt could be damaging to their health.

1.2. Potential health affects of diesel emission exposure.

The perceived health effects of diesel engine emissions have received much attention since the early 1980's. The initial fears that the emissions themselves had carcinogenic potential, was one factor that resulted in the application of stringent emission legislation in the United States that has virtually eliminated the diesel car from the US. More recently the emphasis has been directed towards the inorganic rather than organic composition of the exhaust. Reports of the increased incidence of asthma attacks and respiratory disorders arising from exposure to elevated concentrations of diesel particulate matter or nitrogen oxide (NOx) emissions have been published (WHO 1990, cited by Royal Commission 1991). In one case there has been a definite link between diesel emission exposure and the onset of reactive airways disease (Wade and Newman 1993). However, the exposure levels were far greater than those encountered in normal traffic conditions.

Apart from the particulate emissions, gaseous emissions have also been shown to play an increasingly important role in the health impacts of diesel emissions. Almost all gaseous components, including sulphur dioxide, carbon monoxide and gaseous hydrocarbons have all been implicated in respiratory disorders particularly in susceptible individuals (Stöber 1992). Another area of recent interest is the effect of chronic exposure on those individuals exposed during work, ie. groups such as lorry drivers garage mechanics, railway workers, etc. Several long term epidemiological studies have been performed, although few have shown any significant contributions of diesel emission exposure to ill health (Guillemin *et al.* 1992, Emmelin *et al.* 1993)

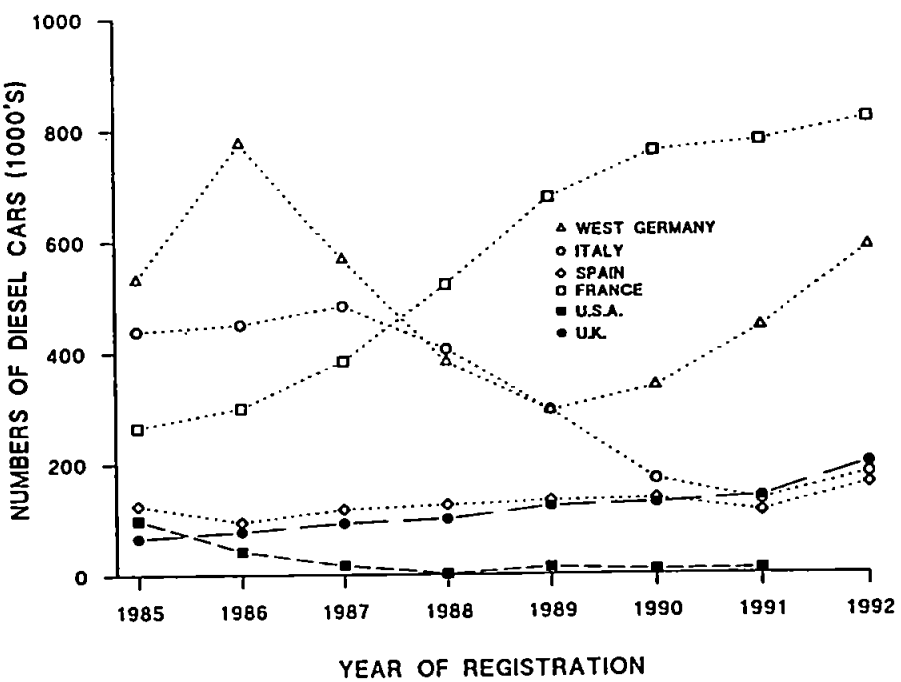
1.3. Trends in fuel consumption, emission burdens and vehicle numbers.

Current trends in diesel car sales for the past decade are shown below (figure 1). The trends in registrations illustrate several points, first and most obviously the almost total lack of diesel cars in the USA. This is almost certainly the result of the emission legislation implemented by the US Environmental

Protection Agency (EPA). Compliance with these limits is at the moment almost beyond the available diesel engine technology and consequently few passenger vehicle manufactures target the US market. One company that still does produce vehicles for the American market are Daimler-Benz. They have proceeded with production models incorporating exhaust particulate traps to keep emissions within the range of standards. The result of this has not been entirely successful, initially the traps became filled much earlier than expected and required either replacement at some cost to the company or regenerating (Select-Commitee 1987). Another, contributory factor to the low number of diesel engines in the US is the relatively low cost of vehicle fuels in the US. Thus, there is no financial incentive to move over to diesel engined vehicles.

In contrast to the US, the picture in the European market suggests that the diesel engine is becoming widely accepted. In France in particular there has been a steady growth in diesel registration since 1986, although the rate of growth has decreased in the 1990's. At this point diesel cars represent up to 40% of the French vehicle fleet.

Figure 1: New diesel car registrations in selected European countries, and the USA.

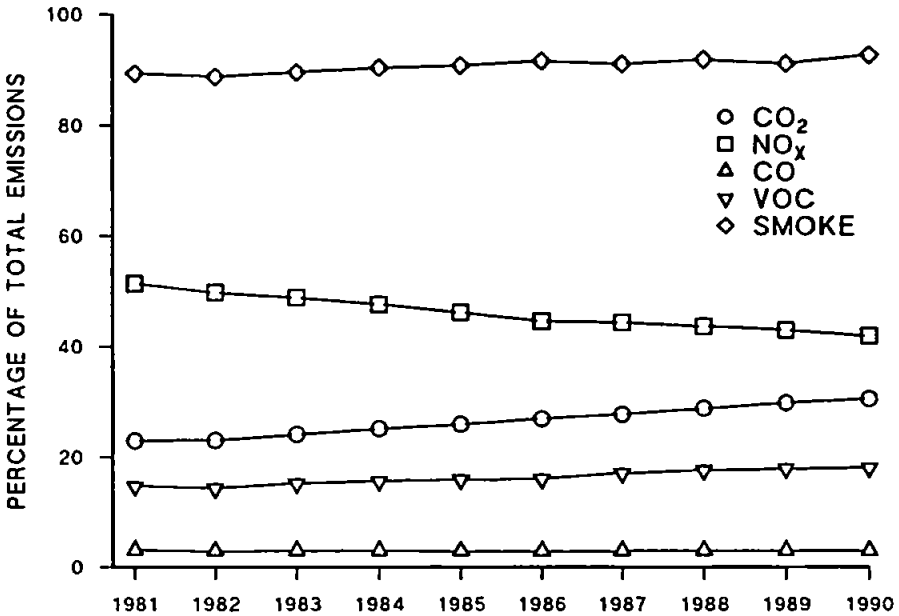


Source SMMT 1993

Another feature of figure 1, is the decline in the German market for diesel vehicles in the late 1980's. This probably also represents the response to tightened emission legislation during this period. There has been a fairly rapid increase in diesel registrations during the 1990's, probably stimulated by advances in engine design by Mercedes-Benz. In most other countries there has been a modest increase in the numbers of diesel registration (ie. the Spanish market increasing by approximately 4% per annum). The most recent data available (SMMT 1993) show that there has been a marked increase in the penetration of the diesel engined passenger vehicles in all European markets during the early 1990's. This may reflect the introduction of the diesel engine option, on several smaller vehicle ranges, coupled with financial incentives. The percentage of diesel vehicles in the UK has risen consistently from 5.3% of the total in 1989, to 8.7% in 1991. The figures for 1992 show a marked increase in purchases, and diesel engined passenger vehicles account for nearly 13% of new registrations (SMMT 1993). Future trends in the UK diesel market are however unpredictable, due to the application of new emission limits and the requirement for electronic emission control devices, which may ultimately lead to an increase in vehicle prices.

With the increasing total number of vehicles on the roads, there is an increase in the total vehicular emission loads in the environment. With the relative increases in the numbers of diesel vehicles, the contribution of their specific emissions may become increasingly important. Figure 2 shows the percentage contribution of diesel engined vehicles to the total UK pollutant emissions of road vehicles over the past decade. The most significant contribution to environmental pollution is by black smoke. The diesel engine is the major cause of smoke in the environment producing approximately 47% of the total UK smoke emissions, with power stations and other industries producing only 19%. In contrast, diesel engined vehicles contribute less than 5% of the total carbon monoxide emissions from road transport. General trends suggest a slight increase in the emissions of carbon dioxide (CO_2) and volatile organic compounds (VOC) with a reduction

Figure 2: Emissions of pollutants from diesel vehicles between 1981-1990 expressed as percentages of the road transport totals in thousands of tonnes.



Source SMMT 1990

in the contribution of Nitrogen oxides (NOx). The decrease in NOx levels is largely attributed to an overall increase in the emissions from spark ignition engines rather than a "real" reduction of diesel emitted NOx.

The latest predictive model of vehicle emission trends in the EC was based on the values for 1988 and covered the intervening years up to 2000 (Samaris *et al.* 1993). Several scenarios were constructed, which included influencing factors such as, the introduction of low sulphur content fuels, concurrent reductions in emission limits, and the underlying economic trends. In all of the cases except one, there was a general reduction in total emission of between 10 and 30 per cent depending on the models employed. The exception was that, where an economic boom period was included in the forecast, in which the emission levels and distance travelled annually were predicted to increase by at least 15%. The consequence of this for the environment particularly in the EC example, is that the emission output is dependent more on the economic climate rather than any introduction of emission legislation or advances in engine technology.

1.4. Vehicle emission legislation.

1.4.1 Legislation of light duty passenger vehicles.

Regulatory legislation for vehicle emissions was first implemented in the United States in the early 1960's in response to urban photochemical smogs produced in Los Angeles. As a result, there are two standards for emission controls, those implemented by the US Environmental protection agency (EPA) nationally, and second, more restrictive controls on vehicles enforced on a state basis. The concern over effects of emissions in the US, prompted the United Nations (UN) in the late 1960's to consider target emission limits for European countries. These regulations were enforced by the Economic Commission for Europe (ECE), a body of the UN. The initial legislation designated as ECE R15, limited only emissions of carbon monoxide (CO) and hydrocarbons (HC), and were applicable only to vehicles of less than 3.5 tons. These regulations were of limited power, being mandatory only in signatory countries. With the formation of the European Economic Community, (EEC), further restrictions on vehicle emission were applied, with the advantage of being compulsory in all member states. The first directive issued by the EEC (70/220/EEC, 1970), had identical constraints to those of the original ECE R15 regulations. Indeed until 1984 amendments to ECE R15 were routinely adopted by the EEC. Subsequent amendments of both ECE R15 and directive 70/220/EEC led to reductions in the permitted emissions of CO and HC during the 1970's. This regulation had the undesirable effect of increasing Nitrogen oxide emissions, which were finally regulated in 1977 by both ECE R15/02 and directive 77/102/EEC. Further restrictions of all emissions were introduced during the 1980's, but it was not until 1984 that light duty, diesel engined vehicles were included in the legislation.

In 1982 the original plan to harmonise emission standards throughout Europe was disrupted when Sweden adopted its own national standard based on the US federal emission standards for 1972 (Neumann and Barthel 1989). Shortly afterwards Austria and Switzerland also adopted independent emission legislation resulting in the division of Europe regarding emission and test procedures. In 1983 discussions on further emission restrictions began and were finally agreed by 1985 in what was known as the " Luxembourg compromise" (Hecq 1989).

This compromise was due to the refusal of Denmark to endorse the proposed limits as tighter restrictions

were already enforced nationally (Select Committee 1987). These limits were finally adopted and published as EEC directive 88/76/EEC. This imposed restrictions of emission based on engine displacement, rather than on vehicle weight as in previous legislation. Passenger vehicles were separated into three categories, those with, (*i*) displacements greater 2l, (*ii*) between 1.4 and 2l, and (*iii*) those with capacities of less than 1.4l. This final category was debated at some length, as smaller engined vehicles form the highest percentage of some European car fleets, *ie;* in Italy 84% of vehicles have capacities less than 1.4l compared to 37% in Western Germany (Neumann and Barthel 1989). In the UK, vehicles of less than 1.4l capacity comprised approximately 43% of the total vehicle fleet, and of these, diesels represented less than 0.15% (SMMT 1990). To overcome the difficulties of replacing a large proportion of the car fleet in some countries, a final directive 89/458/EEC was issued to delay the restriction of emissions from these vehicles until 1996 (Hecq 1989). Finally, directive 88/436/EEC was issued to restrict diesel particulate emissions in passenger cars. The emission limits for several categories of engine and vehicle type with the dates of implementation, are shown in table 1. Two values are set for approval the type approval (TA) refers to results of tests on single representative engines, whereas the later conformity of production approval (COP) refers to testing of engines from the production line.

Table 1: Summary of European passenger vehicle emission limits and dates of implementation.

Engine capacity	Fuel and engine type	Dates of implementation (1st October of each calender year)								
		1988	1989	1990	1991	1992	1993	1994	1995	1996
> 2.0 l	Diesel IDI		TA	COP	1.1g/t----------					
	Diesel DI	Particulates emissions						TA	1.1g/t	COP
	All Diesel + petrol	TA	COP	Diesel (30/8) g/t, Petrol (25/8.5) g/t.----------						
		Gaseous emissions								
>1.4l <2.0l	Diesel IDI		TA	COP	1.1 g/t----------					
	Diesel DI	Particulate emissions						TA	1.1g/t	COP
	Diesel IDI + petrol				TA		COP	(30/8) g/t----------		
	Diesel DI	Gaseous emissions						TA	(30/8) g/t	COP
≤1.4l	Diesel IDI		TA	COP	1.1 g/t ----------					
	Diesel DI	Particulate emissions						TA	1.1g/t-----COP	
	All Diesel			TA	COP		(45/15) g/t----------			
	+ Petrol	Gaseous emissions				TA [1.7.92] COP[31.12.92] (19/5) g/t----------				

N.B: TA Type approval. COP conformity of production.
Particulate emissions in grammes emitted per test = (g/t)
Gaseous emissions: grammes emitted of CO and HC+NOx per test = (CO/HC+NOx)g/t
(Derived from Hecq 1989)

1.4.2. Emission controls of heavy goods vehicles.

In contrast to the regulation of light duty vehicles, heavy duty vehicles (*ie*, those greater that 3.5 tonne's unladen weight) have, until recently been under few controls. Heavy duty diesel engine emissions are currently under legislation from EC directive 88/77/EEC implemented in 1990. Before this limits were based only on smoke emissions described by EC directive 72/306/EEC, which was derived from a British standard set in 1964. This smoke limit was based on observations of smoke emissions from an engine operated at full speed and at varying loads. Emissions were qualitatively assessed by a panel of observers to determine the "acceptability" of the emissions (HMSO 1991). Due to the nature of diesel smoke this is included under diesel particulate regulations as shown below (table 2).

Table 2: Heavy good vehicle emission limits agreed by the European Community.

Legislation	Implementation Date	Limit values in grammes per kilowatt hour				
		Carbon monoxide	Hydrocarbons	Nitrogen oxides	Particulate matter	
					< 85kW	> 85kW
Directive	TA 01/07/88	11.2	2.4	14.4	No Limit	No Limit
88/77/EEC	COP 01/10/90	12.3	2.6	15.8	No Limit	No Limit
Amendment	TA 01/07/92	4.5	1.1	8.0	0.61	0.36
STAGE 1	COP 01/10/93	4.9	1.23	9.0	0.68	0.40
Amendment	TA 01/10/95	4.0	1.1	7.0	0.15	0.15
STAGE 2	COP 01/10/96	4.0	1.1	7.0	0.15	0.15

From Select Committee of the House of Lords on the European Community (1987).

NB: TA = Type approval. COP = conformity of production.

As with light duty engines two dates of implementation are given (TA & COP). Limits on particulate matter in table 2 are separated between two engine classes, those with a maximum rated power above 85 kW, and those below. Smaller engines are allowed to emit more particulate matter under stage 1, this distinction is not recognised for the stage 2 limits (Royal Commission 1989).

The latest legislation agreed by the EEC, was directive 93/59/EEC (1993), which became legislation in the UK on 28/06/93, and came into force on 01/10/93. This directive was specifically designed to reduce emission from light duty vans of less than 3.5 tonnes, in an attempt to close a loophole created in an earlier directive (91/441/EEC). This effectively allowed car derived vans, to have higher emissions than a catalyst equipped car of the same model. The limits imposed by directive 93/59/EEC are shown below (table 3)

Table 3: Emission limits for EEC directive 93/59/EEC, for light duty vans and commercial vehicles of less than 3.5 tonnes.

Vehicle mass (Kg)	Dates of implementation	Permitted CO emissions (g/km)	Permitted HC+NOx emissions	Permitted emission of particulate matter (g/km)
≤1250	TA	2.72	0.97	0.14
	COP	3.16	1.13	0.18
1251-1700	TA	5.17	1.4	0.19
	COP	6.0	1.6	0.22
>1700	TA	6.9	1.7	0.25
	COP	8.0	2.0	0.29

TA = Type approval
COP= Conformity of production
From QUARG 1993

1.4.3. Future trends in vehicle emission legislation.

The limits suggested by the EC directive are currently three years behind those in force in the United States. Reasons for this delay, appear to depend on the diesel fleet and other pertinent emission standards (HMSO 1991). In the US only large vehicles are diesel operated and these are more economical to modify for tighter emission controls than smaller engines. Other factors unique to the European diesel fleet, are controls on fuel economy that are important with respect to NOx emissions. The trends in particulate emissions are expected to increase over the next 20 years, due a rapid increase in the diesel car market in Europe, and limited reductions in emissions of particulate matter proposed by the European commission (Select Committee 1987). Future trends in legislation are the continued tightening of emission limits in passenger vehicles towards the end of the century, which are likely to be similar to those in the united states (QUARG 1993). If such limits are adopted on a worldwide scale this will open the US market to European vehicle manufacturers, possibly generating a resurgence in the popularity of diesel vehicles, if this is so, there is likely to be an increase in the number of diesel vehicles worldwide in the early 21st century.

1.5. The diesel engine, design and production.

1.5.1. Combustion in the diesel engine.

The diesel engine, invented in 1892 by Rudolph Diesel, operates on the compression ignition principle. Fuel is injected into the combustion chamber, which contains air preheated by compression from the piston.

Normal engine compression values are roughly 22.1, where the total volume of the cylinder is compressed into the much smaller volume of the combustion chamber. Within the combustion chamber air is compressed up to pressures of 45 atmospheres and consequently attains temperatures in excess of 500°C. Injection of the fuel under these conditions causes rapid combustion to occur. This is in contrast to spark ignition (SI) engines where, the fuel and air are pre-mixed in the carburettor and are then introduced into the combustion chamber together, combustion is then initiated by a high potential electrical discharge.

The diesel engine is described as "lean burn" *ie.* fuel is combusted with an excess of air, and therefore fuel economy is improved over SI engines. Diesel engines can be classed into two groups, these are direct injection (DI), and indirect injection (IDI). This nomenclature describes the method of fuel introduction into the combustion chamber. DI engines simply contain valves (injectors) which deliver fuel into the combustion chamber as a jet, which is directed into a bowl in the crown of the piston. In IDI engines the fuel is injected into a pre-chamber where air mixing and fuel combustion is initiated. This modification allows wider application of IDI engines, as they are quieter and able to operate over a wider range of speeds than DI engines. However, there is an associated loss of efficiency, which increases fuel costs. As a result DI is widely used in heavy duty vehicles, whereas IDI engines are commonly found in passenger and light duty vehicles. Development of high pressure injectors has enabled the fitting of some direct injection engines to passenger vehicles (QUARG 1993).

1.5.2. Aspiration and turbo-charging.

Diesel engines can be further classified, depending on the method used to induct combustion air into the cylinder (aspiration). Naturally aspirated engines (as used in this study) depend on atmospheric pressure to introduce air into the engine cylinder for combustion. This is obviously inefficient when the engine is operated at high altitude, or more commonly under conditions of high engine speed. At increased engine speed the induction stroke is more rapid and therefore less air is available for combustion. This relative reduction results in a richer combustion mixture, reduced combustion efficiency and therefore higher engine emissions. The most common method employed to overcome this failing, is the use of turbocharging (super charging). Turbochargers are effectively gas turbines operated from the exhaust stream. Typically 40%

of the available energy from fuel combustion is lost through the exhaust stream (Dales & Theissen 1982), and a fraction of the loss can be harnessed to improve fuel combustion. Hot exhaust gases are used to drive a small turbine, the rotational movement generated, is harnessed to compress air to pressures of up to four atmospheres. The compressed air is then either directed into the air intake valves, or passed through a cooling system (usually liquid or air). The compressed air is usually cooled in process known as intercooling, in an attempt to further increase the amount of air available for combustion. Turbocharged air can reach temperatures of up to approximately 150°C, and by intercooling the air density is increased, and therefore the quantity available for combustion is further increased.

Several factors have been shown to affect the emissions of diesel engines. These include timing and duration of fuel injection, cylinder temperature and the amount of available O_2 for combustion. To appreciate the effects of these factors, some basic knowledge of the processes occurring within the combustion chamber is required. Most diesel engines operate on the four stroke combustion cycle, as described below and illustrated in (figure 3). The four stages (strokes) of combustion are described as follows;

a. Induction stroke. During induction, air is drawn into the cylinder through the inlet valve, as the piston reaches the bottom of the stroke the inlet valve is closed.

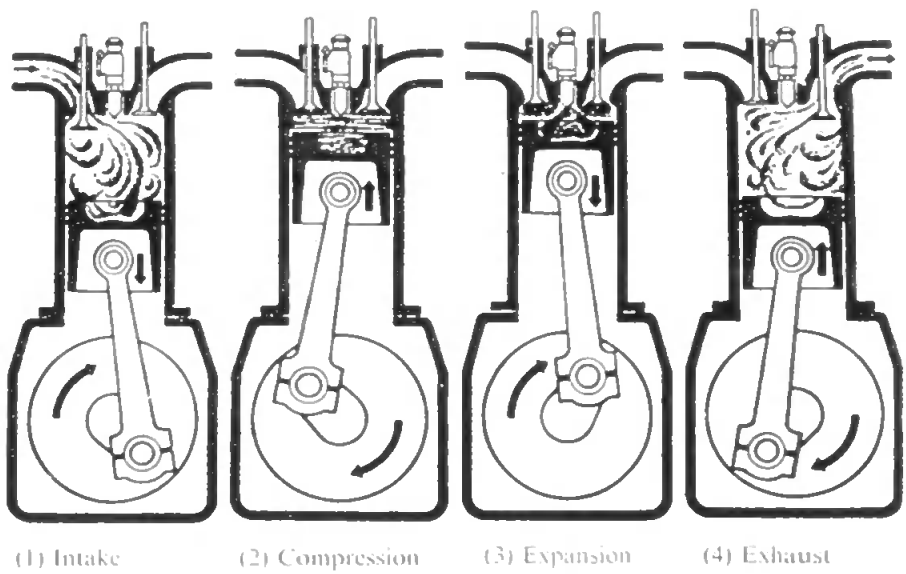
b. Compression stroke. The piston starts to travel back up the cylinder compressing the contained air up to a maximum pressure of approximately 45 Atm, during this compression the air attains temperatures of more than 500°C. As the piston approaches top dead centre of the stroke the fuel is injected as a fine spray into either, the combustion chamber (DI) or into the pre-chamber (IDI). The fuel rapidly mixes with the air and ignites. Combustion causes a further temperature rise and the increasing pressure results in the forcing down of the piston into the expansion stroke.

c. Expansion stroke. This stroke delivers the energy of fuel combustion to the crankshaft. Fuel injection continues after the commencement of the expansion stroke. This results in fuel droplets in oxygen rich regions being fully combusted, while those that fall into oxygen poor regions are only partially combusted.

Fuel injected into regions of no available oxygen become pyrolysed by the intense temperature, causing unburnt hydrocarbons and particulate emissions.

d. Exhaust stroke. As the piston moves upwards from momentum of the crankshaft, the exhaust valve is opened and the combustion gases are exhausted. The exhaust valve closes and the cylinder is set for the induction stroke as the piston again descends.

Figure 3: Combustion sequence in a single cylinder of a four stroke direct injection diesel engine.



From Royal Commission on Environmental Pollution (1991)

1.6. The composition of diesel exhaust emissions.

Raw diesel exhaust can be considered as consisting of three distinct components. These are the gaseous, vapour and particulate phases. The gaseous phase consists mainly of oxides of nitrogen (NOx) either as NO or NO_2. These are formed from reactions occurring within the engine cylinder as a result of the high temperatures pressures, the nitrogen is derived chiefly from the intake air. NOx are dependent on temperature and tend to increase with increasing engine speed and load. Road traffic is responsible for 45% of all NOx emissions in UK (NSCA 1990). These emissions, after leaving the tailpipe, may undergo further reactions with hydrocarbons to produce photochemical pollution. NOx emission may undergo further oxidations to form the major component of acid rain.

The other major components of the gaseous phase are carbon monoxide (CO) and carbon dioxide (CO_2). CO_2 is typically the final gaseous product of hydrocarbon combustion in sufficient oxygen, and is considered harmless from a health aspect. Under extreme conditions of engine speed and loads, when the air:fuel ratio is reduced incomplete combustion may result in the formation of carbon monoxide (CO). CO_2 has a direct health effect, by preferentially binding to haemoglobin. Approximately 85 % of all CO is produced by road traffic (SMMT 1990), although diesel engines produce significantly less than SI engines, due to leaner fuel/air ratio and effects of turbocharging. Minor components of the gaseous phase include, sulphur oxides comprised mainly of sulphur dioxide (SO_2) arising from readily oxidised sulphur components of the diesel fuel. SO_2 may be further oxidised to sulphate (SO_3) but this is usually emitted with the particulate phase.

1.6.1. Vapour phase emissions.

Vapour phase emissions consist of compounds that are liquids at ambient temperature, but are vaporised at the high temperatures of the combustion and exhaust system. The major component of the vapour phase is water derived from complete combustion of hydrocarbons. The presence of water in the exhaust stream also influences the formation of nitric (HNO_3) and sulphuric (H_2SO_4) acids. The reaction of NOx with water results in the formation of both nitrous and nitric acids. These acids may be involved in further modifications of hydrocarbon species. In particular, nitration reactions occur which may enhance the mutagenicity of the emissions considerably. Sulphuric acid is also formed by reaction of sulphur trioxide with water from combustion and is usually emitted from the exhaust as an aerosol. The presence of both nitric and sulphuric acids forms a significant contribution to the problem of acid rain. Sulphuric acid if present at high concentrations can also induce respiratory dysfunction in certain individuals (NSCA).

Other important components of the vapour phase, are volatile, relatively low molecular mass hydrocarbons. These may arise from the pyrolysis of both the fuel and engine oil, or may be the product of pyrosynthesis within the combustion chamber. Although most of the vapour phase organic compounds will be condensed onto particulate matter before reaching the atmosphere, those compounds that remain in the gaseous state may escape detection in conventional exhaust filtration sampling (see section 1.7).

1.6.2. Particulate emissions.

Particulate emissions are defined by the US EPA as any components of the emission excluding water, which are retained on filters placed in the exhaust stream maintained at a temperature not exceeding 52°C. The output of particulates from diesel vehicles is typically between two and ten times more than those emitted from SI engined vehicles (Select Committee 1987). The majority of the particulate matter exists as elemental carbonaceous particles (C_e), to which may be adsorbed various organic hydrocarbon species derived from the fuel oil or pyrosynthesis. These hydrocarbon species are known as organic carbon (C_o) and can be extracted from the elemental carbon by solvents. Compounds present in solvent extracts are usually known as the "soluble organic fraction" (SOF) of the emissions. Other components of the particulate phase include sulphate, and other material, comprising of metal from engine wear. In examining the composition of the particulate matter from filter samples Needham (1990), found 41% to be elemental carbon (C_e) forming the carbonaceous particles, 25% organic matter (C_o) derived from engine oil and oil additives, 7% derived from the diesel fuel, 14% sulphate and water, and the remainder as other material. The particulate phase is the portion of the emission currently of greatest concern (QUARG 1993), with regards to mutagenicity and health effects. This may be a reflection of the relative lack of investigation of the gaseous and vapour phases.

Probably the most apparent emission from diesel engined vehicles is smoke. Black smoke is observed under some operating conditions such as overloading, or may be due to mechanical faults such as faulty injectors or fuel pumps (Dales and Theissen 1982). Black smoke is comprised almost entirely of carbon particulate matter and associated organic species. In contrast the production of white smoke is the result of the emission of unburnt fuel as an aerosol and usually occurs at low temperatures or cold starting. Occasionally blue or grey smoke may be observed, and this is due to the burning of lubricating oil, caused by mechanical defects such as worn piston rings or overfilling.

1.6.3. Formation and composition of diesel particulate matter.

As a result of the perceived health hazards associated with the particulate emissions, these are coming under stricter legislation and this has provided the impetus for several authors to investigate the mechanisms of

formation and structure of particulate matter. Lakage and Prado (1978), after examining the carbon black formed from hydrocarbons combusted in oxygen depleted atmospheres, concluded that soot formation resulted from simultaneous association of small carbon nuclei and the deposition of large polyaromatic species on their surfaces. The adsorbed organic species may then become oxidised and/or dehydrated after exposure to the environment within the engine cylinder. The reaction and chemical cracking processes found within the engine cylinder are believed to be 200 times greater than those experienced in the exhaust, and therefore most of the particulates formed are destroyed by further oxidation reactions. Particulate emissions of turbocharged engines tend to be lower and drop with declining load. The actual sizes of the particulates themselves vary between 10-30 nm but larger soot particles have been found to consist of individual spherical units of approximately 3 μm in diameter (Haynes and Wagner 1981). The size of particle does not appear to be influenced by engine speed or loading (Amann *et al.* 1980).

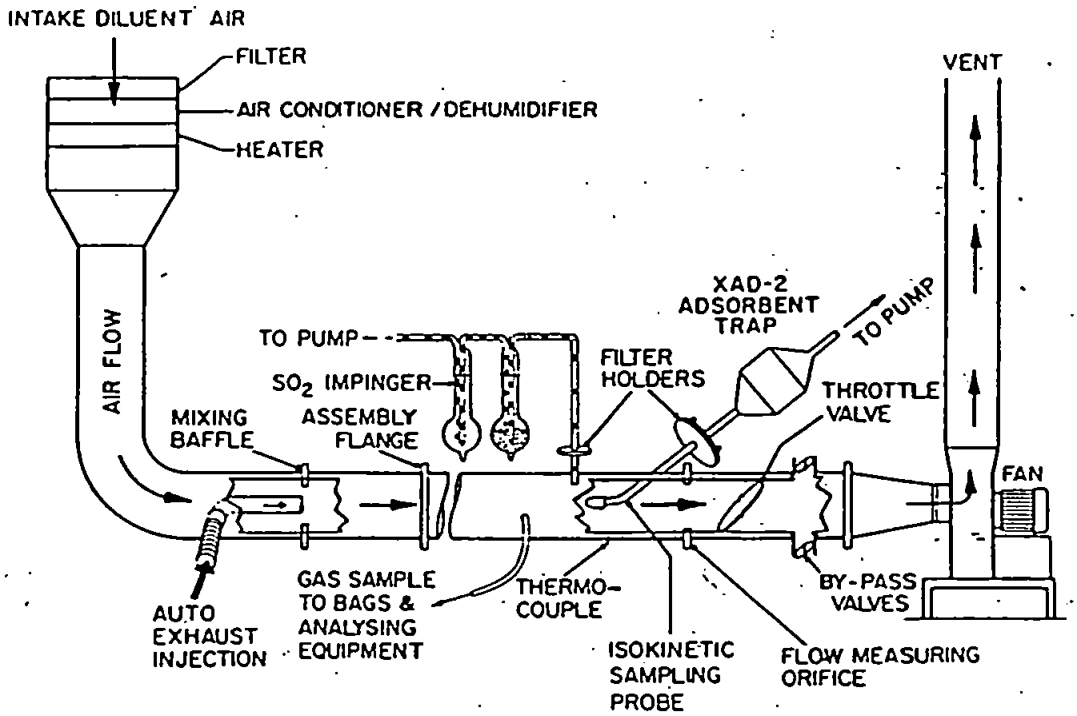
Three stages have been proposed for the formation of particulate matter. Graphite type elemental carbon particles are first formed from the incomplete combustion of fuel and sump oil, by subsequent chemical cracking, pyrolysis and subsequent dehydrogenation at temperatures of 2000-3000°C. Particles in the size region of 1-10 nm in diameter were defined as "carbonaceous nuclei" by Scheepers and Bos 1992. The subsequent collision of these nuclei appears to result in agglomeration and the formation of spherical soot particles of 10-50 nm in diameter. These soot particles then combine to form chain like structures and are further enlarged by the additional adsorption of organic species. Soot particles are estimated to have a large surface area of between 50-200 m^2 per gramme of soot, comparable with the structure of charcoal (Lies 1989).

1.7. Sampling techniques for the collection of diesel exhaust emissions.

Schuetzle (1983) described two general approaches to the collection of diesel emission samples these were (i) isokinetic sampling, using dilution tunnel methods and (ii) raw exhaust sampling by passing emissions through a cooling system. Of these two techniques, the most commonly used and most widely cited is the dilution tunnel. This method has been adopted by the US Environmental Protection Agency (EPA) as the standard method for emission sample collection, and is described in the US federal register (1975). Dilution

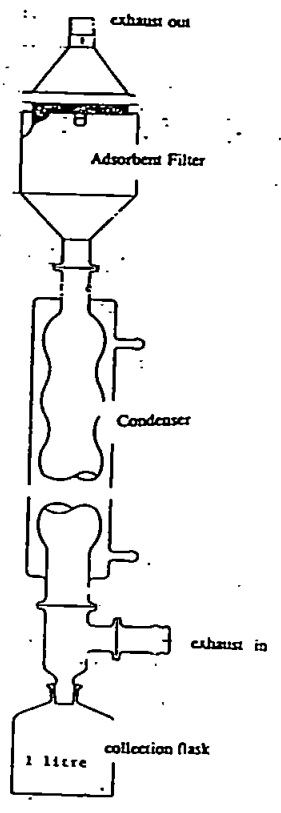
tunnels were designed to simulate conditions of the exhaust in the first few minutes after entering the atmosphere. Although several authors have used dilution tunnels for sample collection the systems used appear to vary slightly in their construction. The main components of the dilution tunnel system are shown below (figure 4). Exhaust from the engine is directed into the tunnel where it is rapidly diluted and cooled with filtered ambient air. The diluent air is controlled with respect to humidity and temperature, and a constant volume is maintained to achieve dilution ratios of between ten and fifteen to one. Once diluted the exhaust is drawn down the tunnel by the venting fan, entering sampling devices on the way. Figure 4 also illustrates some of the sampling devices used for the analysis of both the gaseous phase and particulate matter. The major features of the dilution tunnel with respect to the sample are the size and composition of the filters used for the collection of the particulate matter. In initial investigations fibre glass filters were used, but these have since been superseded by Teflon coated filters. Teflon is favoured because of its enhanced collection of vapour phase components. Collection of vapour phase organic compounds that do not adsorb to the filters is achieved by use of organic resin traps such as Pallflex (Peterson 1982), Chromosorb (Lee *et al.* 1979), or Xad-2 (Schuetzle 1983). Xad-2 was recommended by Schuetzle due to its chemical inertness to nitrogen oxides.

Figure 4: Standard EPA recommended dilution tunnel and associated equipment for sample analysis.



[a]From Schuetzle (1983)

Figure 5: Raw exhaust condensation sampling equipment.



from Grimmer 1979

More recently Ashley (1992) has described a smaller portable dilution tube developed for use in the field to meet new US emission standards. This sampling device consists of a stainless steel jacket into which dilution air is fed, surrounding a porous stainless steel tube through which the exhaust gas is passed. The dilution air diffuses through the porous tube to both dilute and cool the exhaust gas.

Despite being the major sampling method reported in the literature. There are those who consider the dilution tunnel as inefficient. The most serious drawback of the dilution tunnel is the possibility for artifact formation, resulting from prolonged exposure of adsorbed organics to the exhaust. The presence of nitrogen oxides and nitric acids has been shown to result in increased nitration of adsorbed PAH, and consequently led to higher mutagenicity of exhaust samples (Pitts *et al.* 1978, Schuetzle *et al.* 1983).

In an attempt to overcome the drawbacks of tunnel sampling, several authors, notably Grimmer *et al.* (1978) have used a direct condensation method for the collection of exhaust samples. The apparatus used by Grimmer (1978) and Kraft & Lies (1980) is shown above (figure 5). This apparatus consists of a large water cooled condensation chamber through which the exhaust gases are passed. The resulting condensed material, mainly water and the components of the gaseous phase, are collected at the base of the chamber. Particulate matter passes through the chamber, and becomes entrapped in standard particulate filters. The advantage of this scheme is that the potentially reactive species such as HNO_3, and H_2SO_4, present in the vapour/gas phase are condensed out of the exhaust stream and cannot then interact with the collected particulate matter.

Kruzel *et al.* (1991) presented a condensation method that eliminated the need for particulate filters entirely. The exhaust gases in this method were bubbled through a series of large reaction vessels containing dichloromethane (DCM). The vessels were cooled by liquid nitrogen to maintain temperatures of -30°C and -70°C. By passing the exhaust through a fritted glass disc in the first vessel the exhaust stream was broken into small bubbles, increasing the efficiency of the extraction of organic material from the exhaust. One drawback of this system was, the recovery of the SOF of the exhaust required the concentration of approximately 2 l of DCM from each reaction vessel to a volume of approximately 15ml. This was

achieved using Kuderna-Danish apparatus which requires several hours for total solvent removal..

In attempts to eliminate potential artifact formation caused by filter collection or by sample concentration and work-up, some authors have chosen to expose test organisms (usually bacteria) directly to the exhaust stream. Jones *et al.* (1985) exposed 14 cm diameter plates preseeded with Ames *Salmonella* strains to diluted gasoline exhaust through a slit sampler (see section 1.11).

1.7.1. The "total exhaust solvent scrubbing apparatus" (TESSA).

The TESSA system was designed by Petch *et al.* (1987) for the collection of engine emissions with respect to polycyclic compounds. The system is basically a condensation trap, and contains features common to those of both Grimmer *et al.* (1979) and Kruzel *et al.* (1991). Exhaust gases are scrubbed of organic species via intimate contact with an organic solvent stream of dichloromethane (DCM) and methanol. Figure 6 shows the external features of TESSA *in situ*. The sampling apparatus consists of a stainless steel tube in two sections to facilitate cleaning (figure 6 A & B). The upper section is partially packed with graded glass tubing of various diameters supported by a steel mesh, to give an estimated internal surface area of approximately $10m^2$. The base and upper regions of the tower above the glass packing are cooled by via internal and external cooling coils (figure 6 C), containing water at 4°C pumped from a cooling reservoir (figure 6 D).

In this investigation the solvent reservoir was originally gravity fed and mounted above and behind the tower that was itself located over the rear of the engine. Since the collection of the emission samples used in this investigation, the tower has been, mounted to the side of the engine. The solvent reservoir has been relocated to its current position (Figure 6 E) and pressurised with compressed air to maintain a constant flow rate during sampling. The exhaust to be sampled enters the base of the tower through the jacketed transfer line (Figure 6 F), and passes up through the tubing. This is where sample condensation and extraction occurs. The scrubbed exhaust stream then continues up through the baffles to finally exit from the top of the tower to be exhausted into the atmosphere. The sample/solvent drains to the base of the tower, where it is collected (Figure 6 G) into suitable vessels containing distilled water. Figure 7 (over) shows the internal

arrangement of cooling coils and baffles of TESSA. During sampling the solvent enters the tower at a point above the glass tubing. However, when sampling is completed the tower is rinsed by solvent entering from the tower exit, in this way the cooling baffles are also scrubbed of any remaining exhaust sample.

Due to the design of the TESSA the organic species either in free gaseous state, or adsorbed to particulate matter, are rapidly cooled and dissolved in the sample solvent. By collecting the sample into distilled water, acidic species are removed from the organic phase thus limiting any post collection modification of the sample (Trier 1988). Constant removal of the sample/solvent from the tower serves to limit potential artifact formation, caused by continuous exposure of the sample to the exhaust stream. The engine test facility as it now exists is shown below (figure 8).

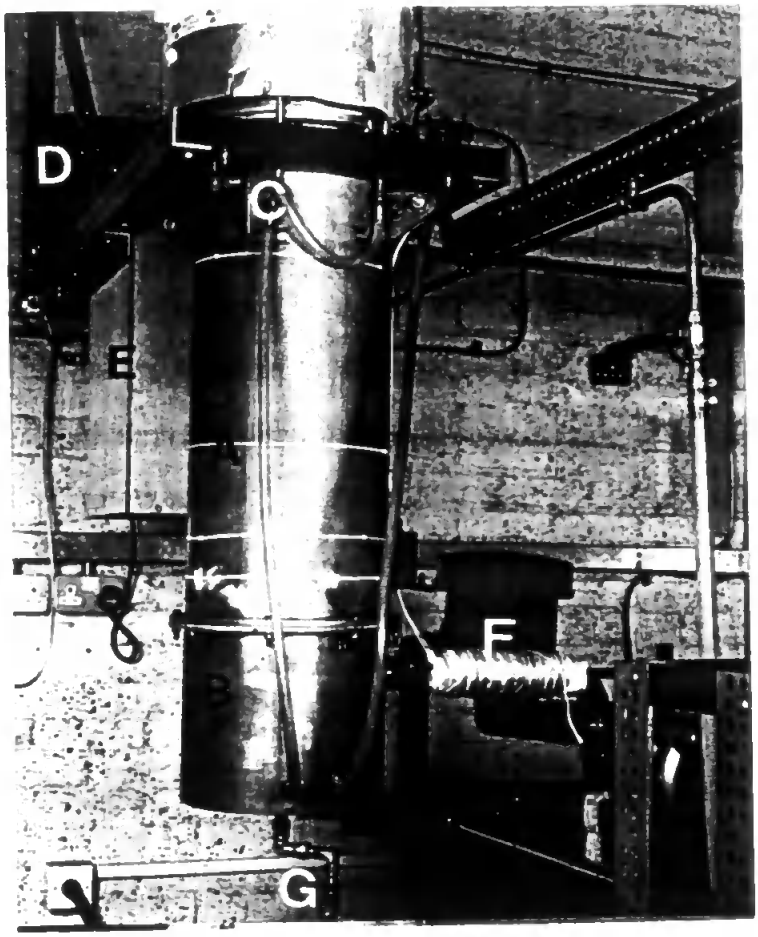


Figure: 6 External view of the Total Exhasut Solvent Scrubbing Apparatus *in situ*.

Key to TESSA.
A upper tower section containing baffles and glass tubing, **B** lower tower section, **C** external cooling coils, **D** coolant reservoir, **E** sample solvent reservoir, **F** exhaust transfer tube, **G** sample collection tap.

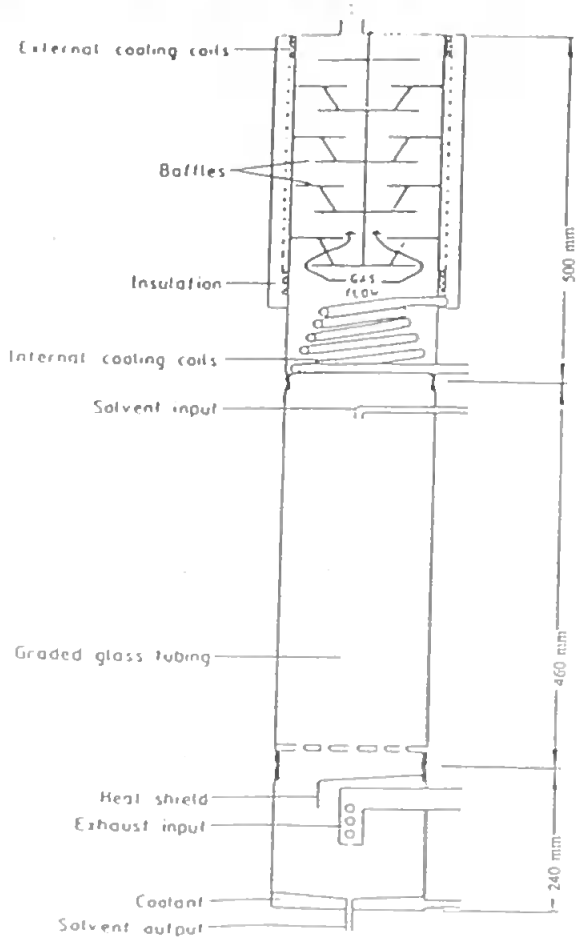
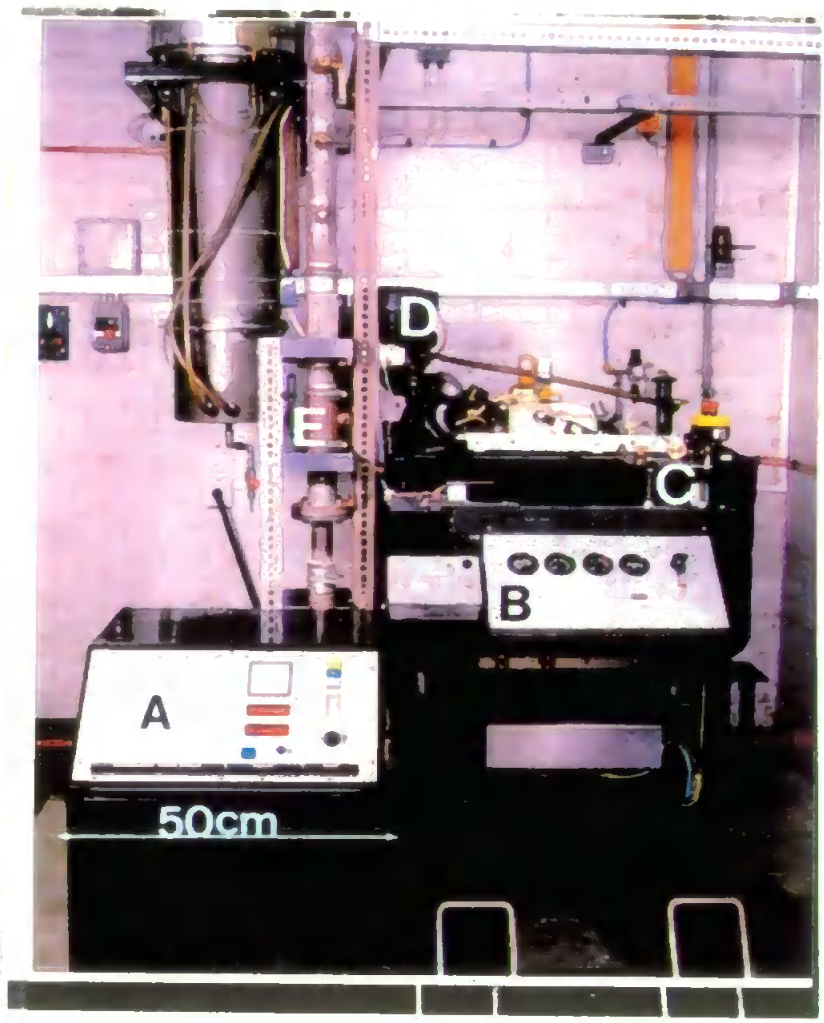


Figure 7: Schematic diagram showing the internal constrcution and dimensions of the TESSA. Reproduced from Trier 1988 with permission.

Figure 8: The diesel engine test facility at the Plymouth.



Both the engine speed and the load applied from the dynamometer are regulated from the dynamometer control (figure 8 **A**), which gives a digital display of both parameters. Engine speed is controlled manually by a control rod (figure 8 **C**). Operational parameters of the engine such temperature, oil pressure *etc.* are displayed on the panel (figure 8 **B**) which also contains the sampling operation control. The air filter/intake for the engine is located to the rear of the engine behind the sample transfer pipe (figure 8 **D**). Finally exhaust emissions from the three unsampled cylinders are removed via the exhaust pipe (figure 8 **F**).

1.8. The chemical composition of diesel exhaust emissions.

The finding that diesel exhaust emissions could cause mutations (Huisingh *et al.* 1978) led to massive effort to identify and characterise the individual chemical components of exhaust gases, particularly with respect to their concentration, and distribution between the gaseous and particulate phases. The primary products of complete diesel combustion are CO_2 (13%), and H_2O (13%) with nitrogen from the air representing 73%. The remaining 1 percent represents compounds formed by incomplete combustion of the diesel fuel or engine oil, with the emission of CO and oxygenated and nitrated derivatives of the fuel and oil. These incomplete combustion products represent thousands of chemical species found in both the vapour and particulate phases of exhaust emissions (Zaebst *et al.* 1988). Table 4 below shows some compounds and chemical groups readily identified in diesel exhaust emissions.

Table 4: Some chemical species and groups found in diesel engine exhaust emissions.

Particulate phase	Gaseous phase components.
Heterocyclics and derivatives	Acrolein
Hydrocarbons (C14-C35) and derivatives	Ammonia
Inorganic sulphates and nitrates	Benzene
Metals	1,3 - dibutadiene
Polycyclic aromatic Hydrocarbons and derivatives	Formaldehyde
	Formic acid
	Heterocyclics and derivatives
	Hydrocarbons (C1-C18) and derivatives
	Hydrogen Cyanide
	Hydrogen Sulphide
	Methane
	Methanol
	Nitric acid
	Nitrous acid
	Nitrogen oxides
	Polycyclic aromatic hydrocarbons and derivatives
	Sulphur dioxide
	Toluene

Adapted from IARC (1989)

1.8.1. Distribution of components between vapour and particulate phases.

The distribution of chemicals between these two phases is determined by several factors. These include, the vapour pressure of the compound, the temperature of the exhaust stream, and the relative concentration of particular compounds. Several investigators have calculated distribution coefficients of individual chemical species and empirical relationships between molecular masses and partition coefficients have been

described. (Schuetzle and Frazier 1986, Schuetzle 1983).

1.8.2. Chemical composition of the Gas \ Vapour phase.

The gaseous phase of diesel emissions comprises mainly C1-C18 hydrocarbons, two to four ring PAHs and nitrated and oxygenated derivatives of C10 to C12 hydrocarbons and two and three ring PAHs. C1-C10 hydrocarbons are mainly the result of pyrolytic cracking of higher molecular mass materials during combustion (IARC 1989).

1.8.3. Chemical composition of Particulate Phase emissions.

Although as shown in table 4, there appear to be few chemical groups associated with the particulate phase, the PAH group represents several hundred individual chemical species that exist either as parent PAH molecules or as chemically substitute derivatives. PAH are abundant throughout the environment, present in the atmosphere as well as terrestrial and aquatic ecosystems. They are of particular importance as some members of the group have been shown to have capability to cause cancer in both animals and man (ie benzo(a)pyrene *etc.* Grimmer *et al.* (1979).

Several authors have produced listings of the various PAH found in diesel emissions, (Schuetzle 1981 and 1983, Claxton 1983,), some of which are illustrated in table 5,with their relative carcinogenic potential. With the increasing sensitivity and resolution of both GC/MS and HPLC many other classes of compounds are being identified and characterised. The molecular structure and physical properties of some of the more abundant PAH present in diesel exhaust emissions, are illustrated in table 6. The effect of additional functional groups on the properties of some compounds is illustrated by the examples of pyrene and the nitrated derivative 1-nitropyrene (1-NP), where addition of the nitro (NO_2) group increases the boiling point from 150°C (pyrene) to 247°C (1-NP), there is also a change in the clastogenic potential with 1-NP inducing chromosome aberrations in the presence of metabolic activation. The mutagenic and carcinogenic potential of some of these compounds are discussed further (section 1.11.4)

Table 5: Some PAH species identified in diesel exhaust emissions with the corresponding carcinogenicity and mutagenicity of selected compounds.

Compound	Molecular mass	Carcinogenicity
Acenapthylene	152	0[a]
Fluorene	166	0[a]
Trimethylnaphthalene	170	
Anthracene	178	0[a]
Phenanthrene	178	0[a]
Dimethylbiphenyl	182	
Tetramethylnaphthalene	184	
Dibenzothiophene	184	
4H-	190	
2-Methylanthracene	192	0[a]
2-Methylphenanthrene	192	
3-Methylphenanthrene	192	
Trimethylbiphenyl	196	
Methyldibenzothiophene	198	
Benzaceanaphthalene	202	
Fluoranthene	202	+[a]
Pyrene	202	0[a]
2-Phenylnaphthalene	204	
Dimethylphenanthrene	206	0[a]
2-Ethylphenanthrene	206	
Dimethylphenanthrene	206	
Benzo(*def*)dibenzothiophene	208	0[a]
Ethyldibenzothiophene	212	
Benzo(*a*)fluorene	216	0[a]
Benzo(*b*)fluorene	216	0[a]
Methylfluoranthene	216	
1-Methylpyrene	216	0[a]
Ethylmethylphenanthrene	220	
Benzo(*ghi*)fluoranthene	226	
Cyclopenta(*cd*)pyrene	226	+[a]
Benz(*a*)anthracene	228	+[a]
Chrysene	228	+[a]
Benzonaphthothiophene	234	
Benzo(*b*)naphtho*(2,1-*	234	
Methylbenz(*a*)anthracene	242	+ +[a]
3-Methylchrysene	242	+[a]
Benzo(*b*)fluoranthene	252	+ +[a]
Benzo(*j*)fluoranthene	252	+ +[a]
Benzo(*k*)fluoranthene	252	+ +[a]
Benzo(*e*)pyrene	252	+ +[a]
Benzo(*a*)pyrene	254	0/+[a]
1,2-Binaphthyl	254	+ +[a]
2,2-Binaphthyl	254	
1-Phenylphenanthrene	254	
Dibenz(*a,h*)anthracene	278	+[a]
Coronene	300	0/+[a]
Dibenzopyrene	302	0/+[b]

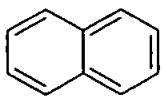
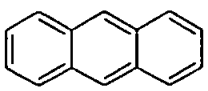
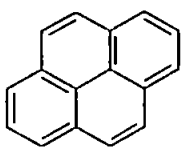
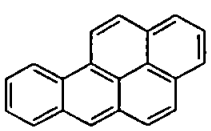
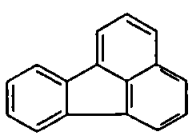
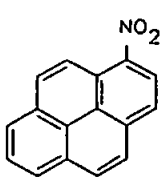
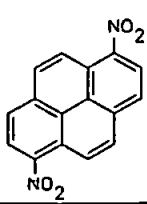
Modified from Tong and Karasek (cited by IARC 1989)

O =noncarcinogenic, + weakly carcinogenic, + + strongly carcinogenic.

N.B. These are approximations of relative activities.

[a] Adapted from Lee *et al.* (1981), [b] from Pucknat (1981)

Table 6: Structures, physical properties and mutagenicity of some common PAH.

Molecular structure	Chemical name	Structural formula	Boiling point	Melting point	Mutagenicity[#] +MA	-MA
	naphthalene	$C_{10}H_8$	87.5[+]	80.5[+]	-	+
	anthracene	$C_{14}H_{10}$	226[+]	216[+]	-	+
	pyrene	$C_{16}H_{10}$	260[+]	150.4[+]	-	-
	benzo(a)pyrene	$C_{20}H_{12}$	310[+]	178.8[+]	-	+
	fluoanthrene	$C_{16}H_{10}$	393*	110*		
	1-nitropyrene	$C_{16}CH_9NO_2$		247.2*	-	+
	1,6-dinitropyrene	$C_{16}H_8N_2O_4$		292.2*		

From * Buckingham (1991), [+] Weast & Astle (1985)

[#] Mutagenicity data from chromosome aberration assays with CHO cells both with and without metabolic activation (MA) from Ishidate, M. (1988).

1.9. Mutagenicity and carcinogenicity.

There has been some confusion in the field of genetic toxicology, due to the inconsistent application of the terminology. Mutagenic, genotoxic, and clastogenic have all been used interchangeably at some point in the literature. The DOH (1989) defines "genotoxic" as a "loose term applied to substances that are generally harmful to genetic material". Brusick (1980) defined genotoxic as substances usually having a chemical or physical properties that facilitate interaction with nucleic acids. In contrast "mutagenic" described the ability of a substance to induce mutations. Mutations have been described by Venitt and Parry (1985) as "heritable changes in nucleotide sequence or number", and by the DOH (1989) as "permanent changes in the amount or structure of genetic material". It is apparent that a mutagen can affect the DNA within a cell, causing changes varying from the base pair sequence, to the structure or number of the chromosomes.

There has been a general trend to describe compounds that cause mutations at the molecular level as "mutagens", whereas those compounds which cause chromosome aberrations are termed "clastogens". This view was supported by Brusick (1980), who identified several compounds such as, base analogues, hydroxylamine, and some intercalating agents (*eg.* acridine), that cause point mutations but which have little effect upon mammalian chromosomes. These could not therefore be defined as clastogenic, however, Arlett and Cole (1988) have suggested that there may be very few compounds that cause gene mutations that are not clastogenic. This scenario has been further complicated by another class of compounds termed "Turbagens" by Brogger (1979). Theses do not interfere with DNA directly, and so therefore fail to fit the definition of genotoxic of Brusick (1980). Turbagens interfere with normal spindle formation during cell division, this interference may result in non-disjunction of one or more chromosomes resulting in anueploidy in progeny cells, other effects include endoreduplication, which may to lead ultimately to polyploidy. Thus, as Turbagens can affect the amount of DNA at the chromosome level they can technically be classed as mutagens following the DOH definition.

1.9.1. Types of mutation detected or observed.

Mutations occurring within cells have been divided into three categories. Each category represents the types of effects observed at higher-orders of DNA structure, from molecular DNA to the entire chromosome set.

Although it is apparent that no one test can detect all three categories, the presence of chromosomal or genome aberrations may also be taken as evidence for the occurrence of mutations at the base level (Brusick 1980, UKEMS 1988).

(*i*) DNA mutations.

These are point mutations occurring in the molecular sequence of the DNA, which include base substitutions and deletion/insertion or frameshift mutations. Base substitution may have several consequences for the cell in which they occur. Substitutions can be distinguished into two types, transitional where one purine is substituted for another, and tranversional where purines are replaced by pyrimidines and *vice versa*. The effect of changing single bases may result in incorporation of the wrong amino acid in the final peptide product. Although, this may be of no direct consequence to the function of the peptide, in cases where the change affects the peptide folding, or the substrate binding site of an enzyme, the function of the protein may be lost. Occasionally, base substitution has a dramatic effect upon the peptide function *eg*. the formation of a stop codon may result in premature peptide termination and subsequent loss of the gene product in mutant cells.

Frameshift mutations occur by insertion or deletion of a single base pair. This may occur by the intercalation of certain mutagens into the DNA, subsequently distorting the structure. This results in the change of the reading frame and usually a nonsense peptide is formed. Substitution and deletion mutations are generally induced by different classes of mutagens (Brusick 1980).

(*ii*) Chromosomal mutations.

Chromosomal mutations are detectable by light microscopy of metaphase cells, as gross morphological alterations of the chromosome structure. These chromosome aberrations have been comprehensively categorised by Savage (1975). Types of aberrations vary from simple breaks, where detached fragments of either chromosomes or chromatids are observed, to complex interchanges, in which chromosomes rejoin incorrectly to form a number of configurations including triradial and dicentric chromosomes. Other chromosome mutations may only be detectable with chromosome banding methods, or more recent

fluorescent hybridisation techniques. These type of mutations include, translocations where portions of one chromosome become joined to another, and small interstitial deletions. Most gross chromosome aberrations are usually fatal to the affected cell, and therefore may be of little genetic consequence. Although some small deletions and reciprocal translocations may be compatible with life they may have serious consequences for the individual.

(*iii*) Genomic mutations.

Genomic mutations refer to changes in the numbers of chromosomes with in the cell. These can vary from conditions known as polyploidy, where the normal diploid number of chromosomes is doubled or tripled usually without any chromosome loss. The loss or gain of a single chromosome is known as aneuploid, which may arise by non-disjunction of chromosomes during mitoses or meiosis. Aneuploid cells in zygotes may be compatible with cell survival as evidenced by a number of disorders, *eg.* trisomies and rare monosomies. Aneuploidy is usually difficult to discern in cells prepared using conventional chromosome spreading techniques due to the potential loss of chromosomes from metaphase spreads preparation. The situation is further complicated in cultured cell lines, by the natural variation in chromosome numbers exhibited by individual cells. It is recommended that only increases in numbers are recorded as evidence of aneuploid (Dean and Danford 1984).

1.9.2. Consequences of mutation.

There are two factors that influence the outcome of a particular mutation, these are the level of DNA organisation at which the mutation occurs (ie. DNA or chromosomal), and the type of cell in which the mutation occurs, either somatic or germinal. Obviously mutations occurring within germinal cells, pose a high risk of expression in the offspring, and even if not expressed, they still represent an increase in the genetic burden within the population.

Brusick (1980) emphasised that a major reason for mutagenicity testing is to identify compounds that may detrimentally affect the human gene pool, and to limit the accumulation of potentially damaging genes in subsequent generations. Large increases in the genetic burden of a population, result in a situation referred

to as genetic death. Here there is general reduction of viability in the form of pre-reproductive death and sterility of individuals.

1.9.3. Genetic disorders arising from mutations in germinal cells.

Disorders found in humans, can be attributed to all three categories of mutation which have occurred in germinal cells. The most commonly observed chromosome abnormality, in both neonates and examined spontaneous terminations is aneuploidy. This is the loss or gain of chromosomal material. Estimations of the frequency of aneuploidy at conception range from 8-10% (Alberman & Creasy 1977) to 50% (Boué *et al.* 1975). Trisomies of all chromosomes have been observed although in significantly different numbers for different chromosomes, the most common in live births are trisomies of chromosomes 13, 18, and 21, whereas trisomy 16 is most common in spontaneous terminations. In contrast to the relatively frequent of trisomies, only one example of monosomy is commonly found, this is XO, (Turners syndrome), autosomal monosomy is believed to result in pre-implantation death of the zygote and consequently is not observed. Structural rearrangements of chromosomes are classified into two types, balanced or un-balanced depending on the net outcome of the genome. Balanced aberrations predominate in live-births, and unbalanced in spontaneous terminations. The most commonly cited defect associated with DNA mutations is sickle cell disease, but other haemoglobinopathies have also been shown to result from single base changes, either transition/transversion mutations or frameshift mutations arising from insertion or deletion of single bases (DOH 1989).

1.9.4 Mutations in somatic cells.

Mutations occurring in somatic cells will affect only the individual and as such poses no risk to the gene pool. The major outcome of mutations within somatic cells is believed to be the initiation and onset of cancer. Since the somatic cell mutation theory was first postulated in 1914 (Boveri) there has been an accumulation of evidence to show the role of both gene and chromosomal mutation in tumorigenesis. Despite some evidence of hereditary pre-disposition to some forms of cancer, the majority of tumours are believed to result from environmental factors. Initial studies showed evidence for an increase in the incidence of cancer in workers, associated with certain industrial processes. Yamagiwa & Ichikawa (1916,

cited by Griciute 1979) first demonstrated the tumorigenic potential of coal tar, and this led to the subsequent identification of the chemical species responsible. The first demonstrable chemical carcinogen identified was benz(a,h)anthracene in the 1930's, since this time various PAH have been identified as potent carcinogens.

In the late 1940's Berenblum & Shublik stated that cancer was the result of at least a two stage process, consisting of an initiation event that was irreversible, and a subsequent promotion event. This led to identification of groups of compounds that were specific for either tumour initiation or promotion and occasionally both. The number of stages involved in tumour formation was increased, to at least several by the publication of age/incidence curve for various cancer types by Armitage (1954 cited by MacDonald & Ford 1991). These showed that the incidence of cancer increased with increasing patient age, and was presumed to be the result of an accumulation of different mutational events. The multistage process for transformation of the benign adenomas of familial adenomatous polyposis (FAP) was shown by Fearon and Vogelstein (1990, cited by Macdonald and Ford 1991), to be the result of several mutations. Initiation was believed to be caused by the mutation of the FAP gene, subsequently followed by mutation of a cellular oncogene, and the functional loss of two tumour suppressor genes. These results suggest that the individual cell has several control mechanisms for growth and differentiation, and that several different mutations to are required overcome them.

1.9.5 Mutations within somatic cells implicated in the initiation and onset of oncogenesis.

Before the discovery of oncogenes as such, the first consistent chromosome aberration observed associated with malignancy was described as a deletion of 22q (Nowell & Hungerford 1960 cited by Hodgson & Maheer 1993). This was termed the Philadelphia chromosome. With the development of chromosome banding techniques, allowing investigations of chromosomes with greater resolution this deletion was reclassified as a translocation t.9.22 (Rowley 1973). Epstein (1986) categorised chromosomal aberrations associated with tumours into two classes; (i) balanced translocations, and (ii) aneuploidy, including trisomy and monosomy, and deletions and duplications of chromosome segments.

The numbers of aberrations observed in tumour cells are varied and most are acquired after the initiation

of the tumour. In an attempt to identify any common aberration types, Mitelman (1983, cited by Epstein 1986), compared the aberration types observed from several tumours. Approximately 50 types of aberration that occurred singly in at least two or more tumours were identified. The implication of this finding was that there were only limited number of chromosomal break points associated with tumorigenicity. In a follow-up study of over four thousand tumour karyotypes (Mitelman 1984), 96% of tumours were found to contain at least one previously identified break-points. Subsequent mapping of the chromosomal sites of known oncogenes, showed that 19 of 26 studied, were located at common break-points (Heim & Mitelman 1987 cited by MacDonald and Ford 1991). This provides stronger evidence for the roles of oncogenes and chromosomal events in the initial stages of carcinogenesis.

The classic example of a translocation intimately involved in a human malignancy is that of Burkitt's lymphoma, in which translocation of the c-myc oncogene to the heavily transcribed regions of the immunoglobulin genes, results in the increased transcription of the c-myc gene. The c-myc gene product is now thought to be implicated in initiating DNA synthesis, and overexpression may stimulate the cell into constant cell division. Introduction of c-myc alone into other cell types does not cause cell transformation, although subsequent introduction of other oncogenes or transforming agents may lead to a transformed phenotype (MacDonald and Ford 1991).

1.10 A review of short-term tests for the detection of mutagens.

To identify the induction by certain chemicals, of mutations, numerous short-term mutagenicity/genotoxicity tests have been described (Kilby and Legator). These include tests in a variety of bacteria, animals and plants, both in vitro and in vivo. The increasing popularity of short-term test is the result of the increasing cost of lifelong rodent carcinogenicty assays. These lifelong assays may take up to five years before results are obtained, and cost between £100-500 000 (Walker 1985) to perform. Despite the numbers and variety of short-term tests available, very few have been sufficiently validated either with test chemicals, or in different laboratories to enable their routine use, consequently only a small number are routinely employed. The most common reported assay is the salmonella reverse mutation assay, or Ames test.

1.10.1 An overview of the Ames Salmonella Assay.

This is the most widely used and most highly validated mutagenicity test and has been widely reviewed (Ames 1971 & 1983, and Ames and McCann 1975). Briefly summarised, several strains of *Salmonella typhimurium* were constructed, with mutations in the histidine biosynthesis gene *(eg.* TA98, TA100, and TA1535 *etc).* This mutation prevents the normal bacterial production of histidine, which is supplemented into the growth media for the bacteria to grow for a few growth cycles in the presence of the test compound. The presence of mutation hotspots within these genes, makes them particularly sensitive (depending on the strain used) to the action of both base pair substitution and frameshift mutagens. A mutation at these hotspots, reverses the original mutation allowing continued bacterial growth on the histidine deficient media. The resulting cell growth results in colony formation after incubation for 48 to 72 hours. The number of colonies is usually directly related to the concentration of the test mutagen.

This method is popular for a number of reasons, but mainly because the test is cheap, rapid, and no special training is required for scoring colonies. The scoring procedure in most testing facilities has also now been fully automated, with automatic counters available commercially. The test is also sensitive, only small sample masses are required for exposure of many thousands of cells to the test compound. This feature has lead to the development of bioassay directed fractionation of complex environmental samples (Lewtas 1988, Schuetzle & Lewtas 1986, Schuetzle & Daisey 1990). Application of metabolic activation has also allowed the testing of mutagenic metabolites of mammalian metabolism rather than bacterial. Development of *Salmonella* strains TA98 and TA100 with their particular sensitivity to PAH, has led to their almost exclusive use in PAH mutagenicity studies.

Perhaps the most significant feature of the Ames test is its reproducibility and consistency between laboratories. All strains are freely available from their original source, and as a result, if positive control data are consistent with the well-documented norms, and standard GLP procedures are followed, then data generated worldwide can be directly compared.

Despite the popularity of the Ames test the reliability and sensitivity of the test have been questioned. The initial estimates suggested that 90% of carcinogens were mutagens made by Ames (1975), has now been

reduced with the discovery of non-mutagenic carcinogens, and mutagenic non-carcinogens. Tennant *et al.* 1987, has shown that the correlation between the sensitivity of the Ames test and the carcinogenicity of some compounds that may be species, sex, or organ specific may be as low as 45% (UKEMS 1990). There has also been some debate over the relevance of extrapolation of the bacterial cell data to events occurring in vivo in humans. Consequently several authors and governing bodies, recommend the use of the "Ames assay" as an initial screen for genotoxicity to be supplemented by other assays including the mammalian cell cytogenetic assays described below (1.10.2.)

1.10.2. *In vitro* mammalian cytogenetic assays.

The most commonly employed *in vitro* cytogenetic assays are the chromosome aberration (CA) and sister chromatid exchange (SCE) assays, both have their limitations, but have advantages over bacterial systems. These include, the detection of clastogenic agents that are not mutagenic in bacteria, and the broad assumption that genotoxic agents interact with mammalian DNA of different species in the same way. Cytogenetic assays have developed from cytological examination of chromosomes, and have been standardised with the publication of treatment protocols and statistical methods for the analysis of the resulting data.

Chromosome aberration assays can be performed using a number of different cell types, ranging from long term rodent cultures, to freshly isolated human lymphocytes. The major consideration in selecting a cell line is the number and morphology of the chromosomes. Human lymphocytes are often used due to the perceived relevance of human tissue in mutagenicity studies. However, the most widely used cells are established rodent lines, usually derived from hamsters. These are preferred due to the ease of culture and the constitution of the karyotype. Most lines originating from hamster cells, have approximately 20 chromosomes that vary greatly in size and morphology. One disadvantage in the use of most rodent cultures is the requirement for a supplementary metabolic activation system (section 1.10.4).

The major disadvantage of aberration assays is associated with scoring of aberrations, this is often a long and tedious process, which also requires familiarity with the karyotype of the cells used, and the recognition

of different aberration categories. The scoring of aberrations is also highly subjective, and there is usually some variation in the results obtained from different scorers, even when using the same preparations. Other difficulties associated with aberration assays include, the adoption of slightly different procedures for conducting the assays in different laboratories. Consequently, several attempts have been made to define a standard protocol for these assays eg. Galloway *et al.* (1985) and Swierenga *et al.* (1991), although minor modifications are still made. *In vitro* cytogenetic assays are also relatively more expensive than bacterial assays, Arlett & Cole (1988) suggested that the cost of a single aberration assay is with in the region £5000. The continuing popularity of aberration assays has led to, the standardisation of the nomenclature of aberration classes (Savage 1975), and the development of statistical techniques for the analysis of data (Margolin *et al.* (1983), Margolin *et al.* (1986) Cooke *et al.* 1990).

1.10.3. The sister chromatid exchange assay.

Sister chromatid exchanges were first identified by Taylor (1958 cited by Taylor 1982) using autoradiographic techniques. However, it was not until the development of techniques using thymidine analogues and fluorescent stains by Latt (1974 cited by Latt 1982) that SCE's could be visualised using fluorescent microscopy. The most important modification to this technique, was the development of the fluorescent plus giemsa (FPG) method by Perry and Wolff (1974), which resulted in permanent preparations, this enabled the visualisation of SCE's by light microscopy. The techniques for visualising SCE's, and discussions of their origins and genetic significance, have been reviewed by Wolff (1982).

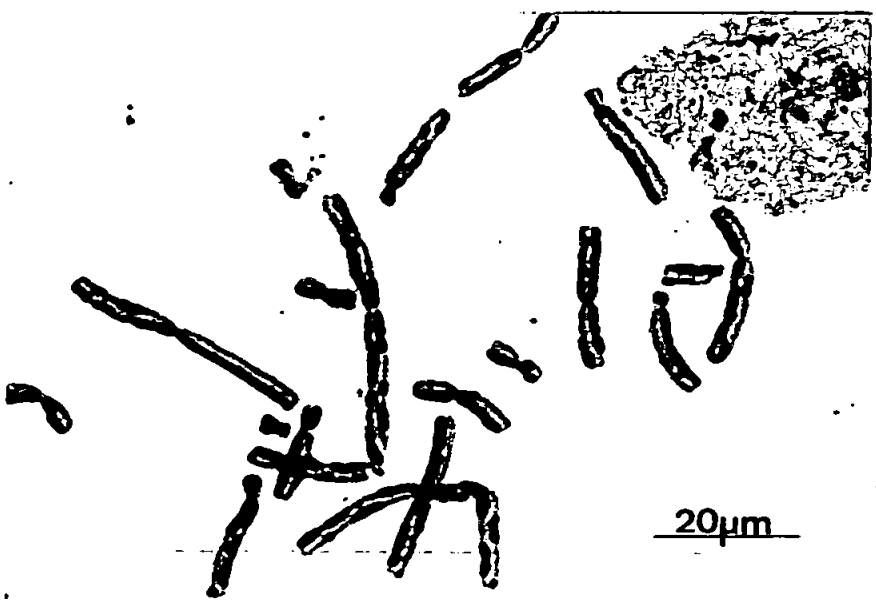
The procedure for distinguishing SCE's by the FPG technique, relies on the incorporation of the thymidine analogue 5-deoxy Bromo-uridine (BrUrd) in to the DNA of replicating cells. By culturing cells for two rounds of replication, the resulting chromosomes consist of two differentially labelled chromatids, one doubly and one singly substituted with BrUrd. The harvesting of cells and preparation of slides is performed in a similar way to that chromosome aberration assays, with the exception that all procedures are conducted under subdued lighting to prevent photodecomposition of BrUrd substituted DNA. Chromosome preparations once dyed are then incubated in a solution of Hoechst 33258, before exposure to an intense lightsource (usually U.V.) to degrade the chromatids. After exposure, the singly substituted chromatid, will be

degraded to a lesser extent than the doubly substituted one. Counter staining with giemsa results in one darkly and one palely stained chromatid. Exchanges are observed as alternating light and dark segments along the length of the chromatids (figure 9).

The underlying mechanism for the formation of SCE's is unclear, although there is evidence that it is unrelated to the formation of chromosome aberrations. This was demonstrated by Wolff (1974), who found that exposure of cells to X-rays caused large increases in the numbers of cells with aberrations, with little or no increases in frequencies of SCE's. Another requirement for SCE induction is that the cells pass through the S phase of the cell cycle. The S phase dependency led several authors (Painter, Ishii & Bender, and Ikushima, all 1980 cited by Wolff 1982) to suggest that SCE formation occurs during DNA replication and may involve strand breakage and rejoining at the replication fork.

The observed increases in SCE frequencies following cells exposure to known mutagens has led to their use as a convenient cytogenetic endpoint. In most studies, increases in SCE frequencies occur at mutagen concentrations that are below those that induce chromosome aberrations. This is taken as evidence that the SCE assay is more sensitive than aberration assays in detecting potential mutagens. The sensitivity of the assay coupled with the ease of scoring exchanges, and the reproducibility of results obtained by different scorers makes the SCE assay an attractive complement to aberration assays.

Figure 9: SCE induction in CHO cells following exposure to the known mutagen MNNG.



1.10.4. Supplementary metabolic activation.

Supplementary metabolic activation was first applied to mutagenicity testing in the Ames salmonella assay (Ames 1971), in an attempt to model the potential metabolic pathways of mutagens in mammalian cells. The activation system is usually a crude microsomal preparation of rat liver, although other rodent and mammalian species have been used (Leber et al. 1976). The microsomal fraction of liver homogenates is usually referred to as the S9 fraction (obtained after centrifugation at 9000 xg), and is composed mainly of microsomes formed from the endoplasmic reticulum of cells. These microsomes contain a group of mixed function oxidases known as the cytochrome P450 enzymes. These are named after the characteristic absorbance spectrum of the reduced enzymes when complexed with carbon monoxide that has a λ maximum at 450 nm.

Investigation of these cytochromes has revealed a number of closely related iso-enzymes with overlapping substrate specificities, which show marked species differences, and inducible expression with different iso-forms induced by different categories of agents. Inducing agents are routinely used in mutagenicity assays to enhance the potential of the metabolism of test agents and to increase the P450 concentration within the liver. The most commonly used inducing agent was a mixture of Polychlorinated biphenyls (PCBs, Arochlor 1254). However, there has been considerable effort to find more environmentally friendly alternative to PCB's. The most successful has been a combination of ß-naphthoflavone and 5-methyl cholanthrene, which have been shown to induce a similar range of cytochromes at equivalent concentrations.

The use of metabolic activation is essential in bacterial tests to simulate the exposure of test compounds to mammalian metabolism, it is also required in most established mammalian cell lines. Most cell lines routinely used in mutagenicity assays (CHO, V79 etc) have been shown to have little intrinsic P450 metabolic capacity (Hsie *et al.* 1981). Freshly isolated human lymphocytes do have some inherent P450 activity but are usually incubated with an S9 preparation.

Despite the benefits of S9 fractions, there has been much criticism of the relevance of rodent liver preparations, particularly because of the imbalance of activating and deactivating metabolic pathways. The

result of this imbalance is to enhance the production of potentially mutagenic metabolites. In an attempt to overcome these drawbacks, several authors have used metabolically competent cells. Freshly isolated hepatocytes, or metabolically active, feeder cells that are co-cultured with the cells have been used for detecting mutagenic events. More recently techniques have been applied to clone P450 genes and maintain their expression in rodent cell lines (Doehmer *et al.* 1988, Crespi *et al.* 1991).

Probably, the most significant development in metabolic activation has been the use of the transgenic rodent assay, which combines both in vivo tissue specific metabolism, with the ease of detecting and scoring mutations of *in vitro* tests. The major drawback of this assay is the cost. It is likely that the use of rat liver S9 fraction, will continue to be the most cost effective if less than adequate solution for use as a metabolic activation system.

1.11. Review of the literature on the mutagenic and health effects of diesel emissions.

The report by Huisingh *et al.* in 1978 is generally regarded as the first to record the mutagenic potential of diesel engine emissions (Lewtas 1986), although diesel emissions had been shown to contain potentially mutagenic compounds *ie.* polycyclic aromatic hydrocarbons (PAHs) as early as 1950's (Kotin *et al.* 1955 cited by IARC 1989). Husingh's *et al.* discovery in 1978 resulted in a massive research effort particularly in the U.S. to develop techniques for identifying and quantifying potential mutagens in all aspects of diesel emissions. Despite the apparently large volume of literature published on the mutagenic effects of diesel emissions, there is surprisingly little concerned with the effects of whole emissions samples in mammalian short-term assays either cytogenetic or mutagenic. Most of the work has been performed using the Ames *Salmonella typhimurium* assays, and most of this using individual component compounds identified in diesel emissions.

The available literature concerning the genotoxicology of whole diesel emissions can be divided into several categories; (*i*) The mutagenic potential of whole diesel exhaust emission samples in a variety of short term bioassays; (*ii*) the influence of operational and other factors on the composition and subsequent mutagenicity of diesel emissions; (iii) the isolation and identification of individual chemical groups in diesel emissions,

and testing of these for mutagenic potential; (iv) the effects of long term exposure to diesel emission, usually by inhalation studied in rats, and (v) epidemiological studies of potential risk groups of the population *ie;* garage workers, truck drivers etc.

The first of these categories, is the main concern of this work and so will be dealt with in more detail than the others. Both the IARC (1989) and Grasso *et al.* (1988) have published extensive reviews of the available literature for all categories described above, these will be briefly reviewed including the addition of recent information.

1.11.1 Genotoxicity of diesel emissions in short-term cytogenetic assays.

Although Husingh (1978) reported mutagenic potential in bacterial assays, when diesel emissions were tested in mammalian CHO cells for toxicity the samples were described as "non-toxic", and on the basis of this result, no apparent further investigation using mammalian cells was performed. This may be the reason there is so little data for the mutagenic affects of diesel emission in mammalian cells. In 1982 Lewtas cited data for the mutagenic effects of diesel emissions in number of short-term bioassays. These included *in vitro* mammalian cytogenetic assays for both Sister chromatid exchanges (SCE) and chromosome aberration assays in both Chinese hamster ovary Cells (CHO), and phytohaemagglutinin (PHA) stimulated human lymphocytes. Samples tested were collected using a standard dilution tunnel, from four engines from different manufacturers. Results from SCE and CHO aberration studies were presented as the gradients of the dose response curves obtained. Result from SCE assays showed increases with three of the four samples without metabolic activation, whereas with the presence of MA the previously inactive sample caused an increase, and one active sample showed no significant effect. Similar results were obtained in chromosome aberration assays in cultured CHO cells exposed to one sample only for six hours and then harvested at 12, 15 and 21 hours without MA. Dose related increases in numbers of aberrations were observed at all three harvesting times. A sample obtained from an Oldsmobile engine, when tested in human lymphocytes at concentrations of 0.1 to 100 μg/ml, caused chromatid and chromosome breaks. Chromosome exchanges were observed at concentrations of 5μg/ml, although chromosome gaps were only observed at concentrations of 100μg/ml. In the presence of MA there was no significant increase in the numbers of aberrant cells,

although an increase in some specific chromosome aberration types was observed.

Morimoto (1986) presented data from SCE tests using PHA stimulated lymphocytes and hypersensitive cultured human lymphoblastoid lines including those from patients with Blooms syndrome and Xeroderma pigmentosum. Two emission samples were tested described as, "a Light Duty (LD) and Heavy Duty (HD)" from an unspecified source. Both normal lymphocytes and cultured lymphoblastoid cells showed dose related increases in the numbers of SCE's per cell. Significant increases were observed at concentrations of greater than 30μg/ml with lymphocytes, and at greater than 50μg/ml with lymphoblastoid lines. Both assays were performed without the presence of a metabolic activation system. *In vivo* SCE assays were also performed, using rats exposed to concentrations of up to 4mg/kg bodyweight day for up to 30 months. Results of this study showed no significant increase in numbers of SCE's after 30 months. From these result Morimoto concluded that although significant increases could be obtained from as variety of *in vitro* test systems *in vivo* studies produced only negative results.

In 1987 Shore *et al.* presented a thorough review of mutagenicity testing on diesel emissions, including details of engine types used, dilution techniques and chemical analysis. Samples were collected from four engines two of which were diesel operated using both U.S. and European test cycles. Samples collected were tested in several short term mutagenicity tests including, the Ames salmonella assay, and chromosome aberration assays in rat RL4 cells, which were chosen due to their metabolic capability. Results of testing with a light duty diesel engine sample, caused significant increases in the percentage of cells with aberrations at concentrations as low as 25 μg/ml. A reduction in the mitotic rates was also observed at this concentration. Results from exposure to two other diesel engine samples showed similar trends although the cytotoxicity varied from as little as 100 μg/ml to 200 μg/ml. From that work, it was suggested that preliminary cytotoxicity assays, tended to underestimate the toxic effects of the emission samples (Shore *et al.* 1987).

1.11.2. Mutagenicity of whole diesel emission extracts in other short-term mutagenicity assays.

As stated, the majority of data for mutagenic effects of diesel engine emission is derived from short-term

point mutation assays such as, the Ames salmonella assays, although there are increasing numbers of reports using mammalian point mutation assays *ie;* Thymidine kinase and HGPRT assays. The following section lists some of the results obtained to date using these assays.

Whole diesel extracts have been reported to be mutagenic in TA98 (Rappaport *et al.* 1980), TA98 and TA100 (Wei *et al.* 1980). Particulates extracted with several organic and physiological solvents have been shown to have mutagenic activity in strains TA98 and TA 100 (Siak *et al.* 1981). Clark *et al.*(1981, 1982a, 1982b and 1983) reviewed the effects of car type, temperature and test cycle on the mutagenicity of diesel emission samples tested in both TA98 and TA100 strains. Claxton 1983 presented a review of the diesel emission mutagenicity data of that time and concluded with several observations. These included, the predominant use of the Ames test as the mutagenicity test used, and the almost exclusive use of *Salmonella* strains TA98 and TA100. This latter feature of the literature was presumably due to the small sample masses available for testing. In 1982 the first influential symposium on the toxicological properties of diesel emission was held (Lewtas 1982). Here, most of the papers were concerned with risk assessment and the results of preliminary inhalation experiments. Lewtas (1982) presented a summary of the effects of diesel emissions in several short-term bioassays, including the Ames salmonella assay, the *E.coli* mutagenesis assay, the mammalian cell mutation assay, and DNA damage repair assays in both yeast and mammalian cells. These tests were performed using samples generated from several diesel and gasoline engines, with samples collected using standard dilution tunnel technology. Bacterial mutagenesis assays gave positive results for each sample tested, with a reduction of the mutagenic effects of samples when tested in the presence of a metabolic action system. Results from mammalian mutagenesis assays were similar in that, almost all, of the samples tested produced mutagenic responses in almost all the tests. In DNA damage tests three emission samples were found to induce positive dose responses when tested for mitotic recombination events in yeasts, although, it had been shown that PAH such as benzo(a)pyrene (BaP) do not induce recombination events (Mitchell *et al.* 1980, cited by Lewtas 1982). None of the samples tested, appeared to induce DNA strand breaks in Syrian hamster cells, as demonstrated by alkaline sucrose sedimentation (Casto *et al.* 1978 cited by Lewtas 1982). However, this technique was considered by Casto to be the least sensitive indicator of DNA damage.

Rannug (1983) presented results of tests in which bacterial strains TA98 and TA100 were treated with emission samples collected using a U.S. federal test cycle. Samples were collected from a dilution system, with three engine types including, light duty diesel, gasoline, and engines modified to run on liquid petroleum gas and methanol. All three engine types gave positive results with either one, or both of the strains used, and Rannug classified the mutagenic potential into three groups based on the number of revertants per litre (revs/l) of exhaust. The highest reversion rate was observed with the diesel fuelled engines, giving between 50-250 revs/l, gasoline engines produced between 10-50 revs/l and LPG/methanol engines less than 10 revs/l. In contrast the same engines tested using the European test cycle produced consistently less revs/l although the relative order of the engine types remained unchanged. The mutagenic potential of the emissions was also increased when the engines were operated from a cold start.

The majority of the work on vehicle emission after 1983 was concerned primarily with fractionation and identification of mutagenic components of the emissions. In 1986 there was a second major symposium on the mutagenic effects of vehicle emissions. This reflected the move towards fractionation and identification of mutagenic compounds and developments in the techniques for their isolation.

In 1986 several authors presented results of both *in vitro* short-term bioassays and *in vivo* studies. Morimoto *et al.* showed that the mutagenicity of diesel samples in the Ames test could be enhanced by differing methods of inducing the metabolic activating system. S9 induced by PCB (ie Arochlor 1254) administration, induced the highest responses, with both light duty and heavy duty samples. Mammalian gene mutation assays were also conducted using azoguanine and ouabain resistance as markers. These gene mutation assays gave similar results to those cytogenetic assays (described previously) where mutagenic effects were observed at sample concentrations in excess of 50 μg/ml. Morimoto also noted the differing effects of engine types used for sample collection. Other short-term tests performed included, the micronucleus test using ICR mice. These were exposed to particulate laden air at concentrations of 400μg/ml from four weeks to 18 months. Results of this assay showed no significant increase in numbers of micronucleated cells at any test dose.

Matsushita 1986 presented data from direct exposure methods suggesting the presence of highly mutagenic components in the gas phase of diesel exhaust emissions. Although these groups of mutagens were not identified at the time, tentative characterisation showed the presence of nitrated derivatives of benzene and naphthalene.

In 1987 Shore *et al.* compared the mutagenicity of gasoline and diesel engines, in bacterial strains TA98, TA100 and a nitroreductase deficient strain and expressed the numbers of revertants cells as numbers per mile of engine travel (revs/mile). Dilution tunnel samples from each engine type produced numbers of revs/mile that were similar. The mutagenic effects were again reduced in the presence of metabolic activation, which Shore described as indicative of "the inactivation of direct acting mutagens", although he did not rule out the possibility of mutagen/protein binding.

In 1992 the International Program on Chemical safety (IPCS) (Claxton *et al.* 1992) published results of a collaborative study on complex mixtures. One of the mixtures was the US National Institute of Science and Technology (NIST) standard reference material for diesel particulate matter (designated SRM 1650). This sample was collected from the inside of a dilution tunnel apparatus after a series of sequential engine test cycle runs. It was intended to contain representative compounds found in diesel exhaust emissions, rather than to be a definitive emission sample. Results of this study using identical samples distributed to twenty laboratories throughout the world, showed that the diesel reference material caused increases in the number of revertants in both TA 98 and TA 100 with and without metabolic activation, with TA100 generally being the more sensitive strain (as judged by gradients of dose responses). Reproducibility of results between laboratories varied from 33% to 152% depending on the method of sample extraction (*ie;* sonication or soxhlet extraction) and depending on bacterial strain used (TA 98 or TA100).

1.11.3. Factors influencing the chemical composition and mutagenicity of diesel exhaust emissions. Several factors have been recognised which can directly affect the composition of engine emissions and consequently the mutagenicity of such emissions. These include, obvious differences between engine and vehicle types, fuels used, and more subtle differences such as engine timing and operating temperature. A

common difficulty encountered when attempting to compare data from different sources, is the use of different engine test cycles, in different laboratories. As a result, several studies have been conducted to determine, the extent of variation between different driving cycles. One drawback of the data cited in the following studies, is that the majority was produced in the early 1980's. Since this time legislative and engineering advances have produced more modern engines, whose emissions may not be characteristic of these earlier samples. One indication of the types of changes that have occurred is illustrated in the work of Seizenger *et al.* (1982), who presented emission data from a number of vehicles from 1979, 1980, and 1981 model years. One noticeable feature of the data is the reduction in particulate emissions collected, the mean masses collected dropped from 0.68 grammes per mile (g/mile) for 1979 model year vehicles to 0.27 g/mile for 1981 model year vehicles. Although this illustration is not intended to suggest that this early data should be disregarded, it could be argued, that development in diesel engine design also requires new evaluation of the nature of the emissions now produced.

Clark *et al.* (1981) were some the first investigators to examine the exhaust emissions from different diesel vehicle types. They tested six vehicles from both European and US manufacturers representative of the technology of the early 1980's. All vehicles were tested using the same driving cycle, and dilution tunnel for sample collection, with one fuel stock. The samples collected were then compared regarding their mutagenicity in *Salmonella typhimurium* strains TA98. Replicate samples collected on different days from each vehicle showed good reproducibility for emissions when expressed as g/mile and as numbers of revertants per μg of sample (revs/μg) collected. A comparison of the six vehicle types in four bacterial strains, showed a much greater variation between emission samples, e.g. the mutagenicity observed in strain TA98 varied from 4 to 12 revs/μg extract. As expected, there was clearly a difference between the mutagenicity of emissions from different vehicle types.

Although there have been some attempts to standardise the method for the collection of engine emissions in both the U.S. and Europe (*ie.* use of the dilution tunnel method), the actual operating conditions of the engine during this sampling are very different. In the US, the standard procedure is that of transient engine testing, under which the test engine is taken through a cycle of different engine loads and speeds, in an

attempt to replicate on road conditions. Several of these test cycles have been developed to represent different modes of driving, commonly cited examples include the Federal Test Procedure (FTP) and Highway Fuel Economy Test (HFET), testing cycles. In these tests, emissions are collected continuously throughout the duration of the test, and are controlled on chassis dynamometers

In contrast to the U.S. transient testing, the favoured test cycle in Europe is the steady state test cycle. Here the engine is operated at thirteen different modes of load and speed each derived from a U.S. test cycle. The engine is conditioned at each mode for a maximum of six minutes with emissions collected during the final three minutes. This method of testing, does not allow for periods of engine acceleration and deceleration, as are found in transient tests. At these conditions the emissions from engines are at their most uncontrollable due to effects of overfuelling, which is enhanced in turbocharged vehicles by a lag period between fuel injection and increase in turbo-speed during rapid acceleration. There is also an argument against the use of steady state testing, because of the possibility of engine manufacturers attempting to beat emission legislation. This can be achieved by employing electronic devices to limit engine emissions at conditions of the test cycle, and to exceed emission limits at untested engine conditions. The inclusion of periods of acceleration and deceleration in transient tests would make it more difficult to control emissions using electronic controls..

The major deficiency of the steady state test is the lack of cold starting conditions that are the largest source of smoke emission of diesel engines. To investigate the effects of driving cycle on the emission from engines Clark *et al.* (1982a) tested three engines from the 1980-81 model years. Two of these engines were operated on three different test cycles, the US Highway fuel economy test (HFET), the US EPA Federal test procedure (FTP), and the New York city cycle (NYCC). These tests represent several different driving states, including, steady state driving averaging 50 mph (HFET), slower urban travelling at an average of 20 mph, with several periods of acceleration and deceleration (FTP), and congested urban travelling, at an average of 7 mph (NYCC). Gaseous exhaust emissions were significantly affected by the different driving cycles, with the NYCC producing the highest concentrations of CO, HC, and NO_x emissions. The fuel economy of the NYCC cycle was reduced to that observed in the HFET, however, in mutagenicity tests

the numbers of revs/μg of exhaust sample extract were almost identical for each cycle, with both strains TA98 and TA100. Also apparent from this study, was the decrease in the percentage mass of extractable organic material from the total exhaust sample, as the total mass of the sample increased, the total extractable organic mass decreased.

To investigate the effects of acceleration and deceleration compared to constant speed, on the mutagenicity of emissions Bechtold *et al.* (1984), collected samples from individual sections of the FTP cycle. The mutagenicity of the samples obtained was compared to that of samples collected following the whole cycle. Initial results showed that, samples collected during a period of initial acceleration from standing to 50 mph, and at the subsequent cruise at 50 mph, both produced higher numbers of revertants than were obtained from running the whole cycle. Although they could not explain this occurrence directly, Bechtold *et al.* showed that the levels of mutagenicity were highest when the maximum emission of NOx was observed.

The effects of engine timing on the extent and mutagenicity of diesel emissions, appear to vary between engines and vehicle types. Zweidinger (1982) has shown that poor fuel injection timing may lead to an increase in the mutagenicity of the emissions. Du *et al.* (1986) has shown that advanced timing in an IDI engine resulted in the increased formation of particulate emissions. In contrast Scheutzle and Frazier (1986) stated that the changes in fuel injection timing had no significant effect on mutagenicity.

1.11.4. Mutagenicity of some chemical components identified within diesel emissions.

Although parent PAH have been identified, they are only mutagenic in assays in which a metabolic activation system is present. Some mutagens have also been identified that are "direct acting". In that, they do not require the presence of metabolic activation for activity.

Schuetzle *et al.* (1980) presented a comprehensive analysis of the various PAH derivative groups identified in diesel extracts. The presence of direct acting mutagens in several subfractions of diesel exhaust samples led Rappaport *et al.* (1980) to conclude that the direct acting mutagenicity was probably the result of several different chemical groups of mutagens. They also identified one of these chemical groups as those of

dicarboxylic acid anhydride derivatives of PAH. The subsequent testing of pyrene dicarboxylic acid anhydride in bacterial assays showed that this derivative was a relatively weak mutagen causing only 220 revs/mile.

Isolation and identification of nitrated derivatives of PAH was reported by several authors in the early 1980's. These included Pederson 1981, Salmeen *et al.* 1982, Salmeen *et al.* 1983, Tokiwa *et al.* 1983, Nakagawa *et al.* 1983. This group of PAH derivatives was found to contain several potent direct acting mutagens, including 1-nitropyrene, 1,6 di-nitropyrene, and 9-nitro-anthracene, which caused increased numbers of revertants at concentrations of 0.4μg/ml (Salmeen *et al.* 1984). The presence of six of these nitrated PAH was estimated to account for between 30-40% of the total mutagenic activity of a diesel sample (Salmeen *et al.* 1983) and the presence of 1-nitropyrene alone 9-24% (Salmeen 1982). Based on the evidence Nakagawa *et al.* (1983) concluded that nitrated PAH "must be the major mutagens" in diesel exhaust samples.

Other nitrated groups were identified by Manabe *et al.* (1985) including nitrohydroxypyrenes and nitroacetoxypyrenes, which accounted for nine and 12% of the mutagenicity of a crude diesel extract in TA98 respectively. Bechtold 1986 identified 2-nitro-9-fluorenone which was found to be between five and 10 times less mutagenic than 1-nitropyrene. One parent PAH, fluoranthrene although mutagenic was also found by Bos *et al.* (1988) to be volatile at 37°C in a taped plate assay. This finding has important consequences regarding the loss of this mutagen from samples during extraction and subsequent work-up procedures. The mutagenic activities of methyl derivatives of PAH, including 1,4-, 1,3-, 2,4-, and 3,4-dimethyl phenanthrenes (DMP) were investigated by Sinsheimer *et al.*(1991). All gave positive responses in both TA98 and TA100 and in *in vivo* SCE assays in mice. Of the four tested 1,4-DMP was the most potent.

1.12. Aim of this Investigation.

The aim of this study was to test engine emission samples collected using the TESSA, by in vitro cytogenetic assays. Using samples collected at various engine speeds and loads a "mutagenicity map" of the

engine was to be constructed, to determine under what conditions potentially mutagenic emission are produced. Finally the most clastogenic samples were to be fractionated into aliphatic, aromatic and polar subfractions to determine which groups of compounds were responsible for the clastogenic activity observed.

2.0 Methods.

2.1. Diesel emission sample collection.

2.1.1. Diesel engine and test equipment.

Exhaust emissions were produced from a Perkins 'Prima 65', 2 litre (1993 ml), four cylinder direct injection, naturally aspirated engine. This engine type was used in commercial light duty Austin Maestro diesel vans. The engine was mounted on a test bed, and connected by a short drive shaft to an FA1000 Eddy current dynamometer. This was in turn controlled by a 2000 CE compact controller, both by Test Automation LTD. The dynamometer controller was used to control the load applied to the engine (Torque Nm) and also displayed the engine speed (rpm). The engine throttle was controlled manually by a threaded control rod with adjusting screw.

2.1.2. Fuel and Oil.

During this investigation the fuel stock was changed after collection of the first four samples (ES1-ES4). Both fuel stocks were analysed by Perkins Technology Limited (results shown below table 7) the second fuel stock was a standard No 2 Derv fuel (British Petroleum). Engine oil used throughout this investigation was Shell advanced Rimula X heavy duty lubricating oil (15W/40).

2.1.3. Emission sampling system.

Details of the Total Exhaust Solvent Scrubbing Apparatus (TESSA) sampling system are described elsewhere (Petch *et al.* 1987, Trier 1988). Before sampling, the engine was conditioned from starting, by running at a high load and speed (approximately 4000 rpm at 80 Nm) for one hour. When engine conditioning was completed, emission samples were collected by sequential runs of TESSA, allowing fifteen minutes for equilibration at the new load/speed conditions before collection of each sample.

Sampling of exhaust emissions consisted of three stages. First, just before redirecting the exhaust flow to the TESSA, 500 ml of sample solvent (methanol: dichloromethane, 1:1) was used to wet the internal TESSA packing. Second, both the exhaust flow, and the solvent reservoir were opened into the TESSA simultaneously for two minutes, the solvent flow rate was estimated to be approximately 1 litre/min during sampling. Finally, when sampling was completed, the exhaust flow to TESSA was stopped, and the inside

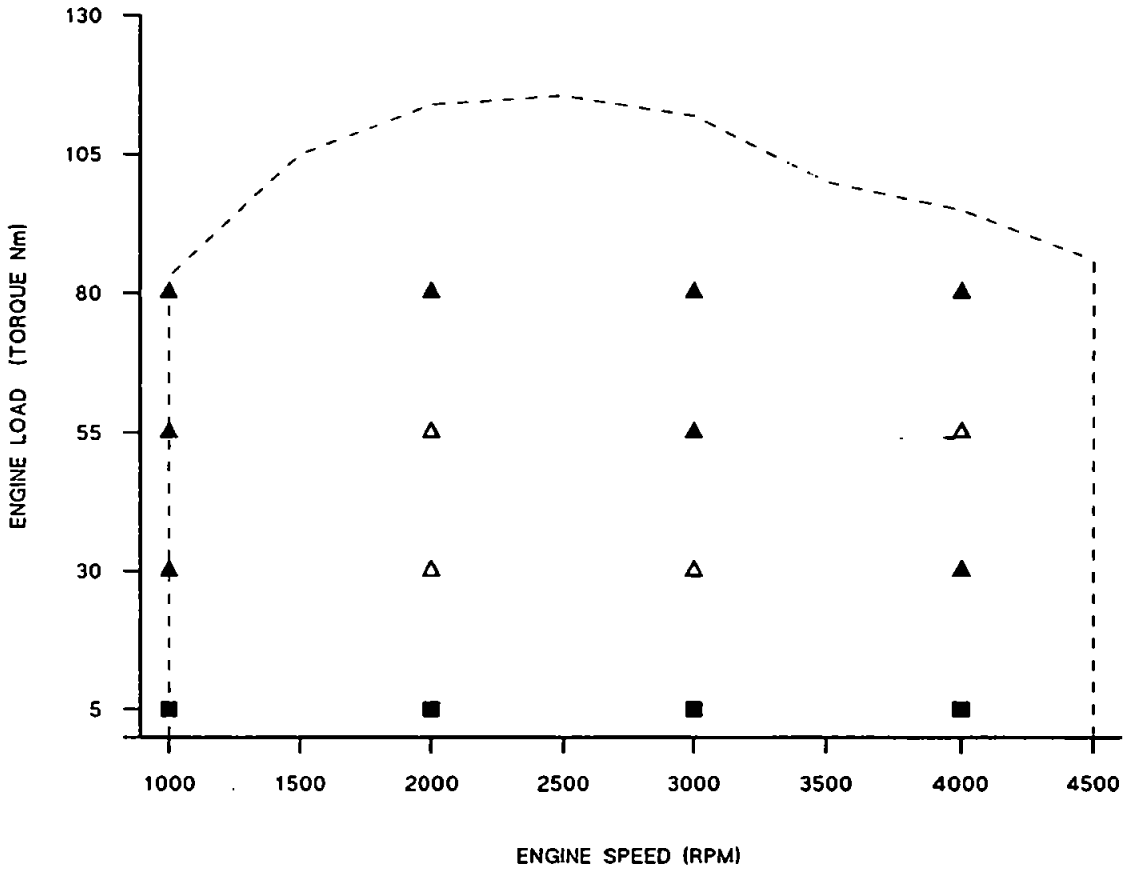
of the tower rinsed with a further 500ml of solvent. This was introduced into the tower at the exhaust gas outlet assuring the whole internal surface of the TESSA was rinsed.

The sample/solvent and tower washings (approximately 3.0l) were collected into a 5l conical flasks containing 1l of distilled water. Flasks' were protected from light, and stored at 4°C until ready for sample work-up. All solvents used during sample collection and subsequent work-up procedures were of glass distilled HPLC grade (Rathburns). All disposables used *ie.* filters, disposable tips, anti-bumping granules, sodium sulphate *etc.*, were soxhlet extracted overnight in HPLC grade dichloromethane (DCM), before use. Samples were collected over a variety of speeds and loads as shown below (figure 10).

Table 7: Comparison of boiling points and specific gravities of test fuels used.

Property	Fuel 1 (5/3/91)	Fuel 2 (14/1/93)
Specific gravity	0.849	0.863
Viscosity	3.36 cS	3.58cS
Distillation		
Initial boiling point	170°C	185°C
5%	211°C	217°C
10%	226°C	231°C
20%	245°C	250°C
30%	260°C	264°C
40%	270°C	275°C
50%	281°C	286°C
60%	293°C	298°C
70%	304°C	310°C
80%	318°C	323°C
90%	336°C	343°C
Final boiling point	359°C	364°C
Recovery	99%	99%
Cetane index	53	49
Aromatic content	Not recorded	29%

Figure 10: Load and speed conditions of the Prima diesel engine, at which samples were collected for testing in mutagenicity assays.



Legend:

Dashed lines represent the limits of the operating parameters of the engine. All points represent conditions at which samples were collected. Squares represents samples collected using original fuel stocks. Triangles represent samples collected with new fuel stock. Filled symbols represent samples tested for mutagenicity in one or more assays.

2.1.4. Sample work-up and storage.

Following collection, samples were protected from light and stored at 4°C until the work-up procedure was performed. The total sample was allowed to separate into two phases in a 5 l separating funnel. The lower DCM phase was collected into an 2 l conical flask, and the remaining aqueous phase was reextracted a further three times with 50ml aliquots of DCM. The collected DCM phases were then combined and filtered through glass micro-fibre filters (Whatman GF/F), under vacuum, to remove carbon particulate matter. Samples were transferred to 1 litre round bottomed flasks and rotary evaporated down to a volume of approximately 100 ml. Sample solutions were allowed to dry overnight by the addition of approximately 5g of DCM extracted anhydrous sodium sulphate (BDH).

After drying, the sodium sulphate was removed by filtering the sample through DCM prewetted discs (Whatman) into 100ml flasks. Filters were then washed with a further 10 to 15ml of DCM to ensure maximum recovery of sample. Samples were then further concentrated by rotary evaporation to a volume of approximately 2ml. The concentrate, and subsequent 1ml washings of the flask were then transferred in small volumes, to a pre-weighed sample vial (approximate volume 5 ml). Residual DCM was removed by placing the vial into a 40°C waterbath under a gentle stream of GC grade Nitrogen. Vials were reweighed at intervals, and blowdown stopped when a constant mass was achieved (over an interval of five minutes). Samples were then capped under nitrogen and stored at -70°c until required.

Initial samples (ES1-ES2) were dissolved in Analar DMSO to a concentration of 10mg/ml, subsequently samples of masses less than 60 mg were prepared in DMSO to give a solution of 100 mg/ml. Samples with masses of greater than 60 mg were redissolved in DCM to a final concentration of 100 mg/ml.

2.1.5. Sample fractionation.

Several emission samples were fractionated by column chromatography into three sub-fractions, these were known as aliphatic, aromatic, and polar fractions. Fractionation was undertaken for two reasons; First several reports have shown that most of the mutagenicity of emission samples resides in particular fractions namely the aromatic and polar fractions. Due to the large sample masses available it was thought that bio-

assay directed fractionation could be performed with some of the emission samples. The second reason for fractionation of samples was an attempt to identify which sub-fractions were responsible for the cellular toxicity observed in all samples.

Most of the emission samples collected were fractionated into the three subfractions, using open column elution chromatography, to enable the composition of the samples to be characterised by gas chromatography and in bioassays. Samples collected with masses greater than 100 mg, were fractionated twice in aliquots of 30 mg for analysis by both gas chromatography (GC) and mutagenicity testing. All other samples were fractionated only once with an initial mass of 10 mg for GC analysis only.

Stock solutions of whole emission samples were prepared in DCM to concentrations of 100 mg/ml. Approximately 30 mg aliquots (300μl) were placed into preweighed vials and placed under nitrogen until free from DCM. The mass of the sample for fractionation was then recorded to within 0.1 mg. Fractionation was performed using 30cm x 1cm diameter (approx.) glass columns fitted with glass frits and teflon taps. The columns had a volume of approximately 25-30 ml with a dead volume of approximately 1ml. Columns were slurry packed with 3g of 5% deactivated silica (60-120 mesh, BDH) in 20 ml of hexane, and covered with approximately 0.5g of fine DCM extracted sand (20-60 mesh). Columns were then conditioned by running through approximately 25ml of hexane.

Prior to each fractionation, vials to be used for GC analysis were spiked with 100 μl of the deuterated internal standards (D10 phenanthrene and D8 naphthalene, 2 mg/ml in hexane), which were added to assess sample losses during fractionation. The fractionation procedure was as follows, samples were resuspended in a small volume (200 μl) of hexane, and were then loaded onto the column using a glass long tip Pasteur pipette. The sample vial was rinsed three times more with hexane, each rinse being added to the column. Finally the sample was drawn into the column and eluted with 10ml of hexane. Fractions were collected into 100 ml round bottomed flasks. Aromatic and polar fractions were obtained similarly after first rinsing the sample vial with DCM and methanol respectively. The aromatic fraction was eluted with 15ml of DCM and the polar fraction eluted with 25ml of methanol.

All fractions were rotary evaporated to a volume of approximately 2ml and then transferred to pre-weighed vials (approximate volume 1.75ml) for nitrogen blow-down. Fractions that were not rotary evaporated on the same day as collection were stored at 4°C, whereas samples undergoing nitrogen blow-down were stored temporarily at -20°C.

The polar methanol fractions were found to contain suspended fine silica particulates, and an attempt to remove these was made by filtration of the fraction through a 0.2μm (pore size) cellulose acetate disposable filters. Filters were connected to 10ml glass Luer locked syringes and conditioned by filtering 2ml of methanol. The sample was then added to the syringe barrel and allowed to drain, under pressure from the plunger, into round bottomed flasks. The syringe and filter were then rinsed with a further 20ml of methanol, and the particle free filtrate rotary evaporated down to a volume of 2ml. the fractions were then transferred to vials for blow-down.

Following the failure of the filtration procedure to remove all the suspended silica, silica was prepared by soxhlet extraction overnight in methanol, in an attempt to remove fine particulates before deactivation.

2.2. Cell stocks and maintenance.

2.2.1 Sources of cell lines used.

During this investigation three different Chinese hamster ovary (CHO) cell lines were used. All lines originated from a CHO-K1 cell line from the European Collection of Animal Cell Cultures (ECACC) (culture number 85051005). Initial work was performed using a CHO-K1 cell line maintained at the University. In the first detailed experiments, this line was found to be unstable, with approximately 40% of cells examined in untreated cultures showing chromosome aberrations. This was originally thought to be a result of overextended passage of the line, as cells had then been subcultured approximately twenty times after resuscitation from frozen stocks. Fresh cultures were resuscitated, and assessed for spontaneous occurrences of aberrations. Again, spontaneous damage was observed in approximately 40% of cells. From this it was believed that these cultures were possibly contaminated with a mycoplasmal infection, which would explain the high incidence of damage. The original stocks were discarded and a new CHO-K1 line purchased from the ECACC. This line was found to be no better and a second replacement was obtained.

This second line designated CHO-K1-PD2 was found to have a reduced level of spontaneous damage of approximately 20%.

Using the PD2 stock, an investigation of the tissue culture media and other materials was initiated to determine if this was the source of the increase in spontaneous chromosome damage. Several batches of sera, media, culture flasks, and new storage glassware were obtained. Cells were cultured for two passages in various combinations of media etc., and then examined for occurrence of chromosome aberrations. The best combination of materials, did not significantly reduce levels of damage although the frequency of spontaneous aberrations was reduced to approximately 12 to 15%. This line was used for initial testing with both stock mutagens and two emission samples. Dilution cloning experiments (2.2.1.), were also established in an attempt to reduce the spontaneous level of damage to more acceptable levels (ca. 5%). Subsequently a stable cell clone was isolated with a spontaneous damage rate of 0 to 4%. All further work was conducted using this clonal line, which was designated CHO-K1-G6.

The CHO-K1-G6 line was characterised with respect to chromosome stability and number by scoring metaphase cells. This lines was then expanded, and stock vials stored at -80°C (2.2.4.) at the sixth passage after cloning. Working cell lines were subcultured for several weeks up to the fifteenth passage, and then discarded in an attempt to maintain karyotypic stability. Fresh cultures were then resuscitated from frozen stocks.

2.2.2. Routine maintenance of cell cultures.

All manipulations of cells for regular subculturing and for initiating cultures for genotoxicity assays were performed in a horizontal laminar flow hood. All CHO cells were maintained in Ham's F12 media supplied without L-glutamine and with bicarbonate buffer (Flow Laboratories). This was supplemented with 10% foetal bovine serum (FBS), and 1% of a 200mM solution of L-glutamine. Cells were cultured in 25 cm^2 tissue culture flasks (Falcon), at 37°C in a 5% CO_2 atmosphere. Gassing of cells was achieved by adding 3ml of CO_2 directly to culture flasks through a sterile plugged Pasteur pipette connected to a gas cylinder. Volumes were measured in a 20ml syringe.

2.2.3. Routine subculture of cells.

Cells were subcultured approximately twice weekly, by harvesting using trypsin to detach the cells from flasks. The culture media was removed from culture flasks, and the cell monolayers were washed with 5ml of pre-warmed phosphate buffered saline (PBS) (Oxoid). This was subsequently poured off, and 1ml of cold trypsin (2.5%) in Hanks balanced salt solution (Sigma) was added, and washed over the cells. Excess trypsin was poured off, and the flasks incubated at 37°C for 2-5 minutes to allow cell rounding and detachment. When all the cells were detached from the flask, 5ml of pre-warmed Ham's F12 media was added to "neutralise" the action of the trypsin. New flasks were seeded with 0.5ml of the cell suspension (containing approximately $1\text{-}2\times10^5$ cells) in 8ml of F12. These were then gassed and incubated for a maximum of four days.

2.2.4. Storage of cell lines.

Cell stocks were stored frozen at -80°C. For freezing, cells were expanded by subculturing into flasks, and grown to late log phase (3-4 days). Cells were washed with prewarmed PBS, detached from flasks by trypsin, and washed again by resuspending in 5ml of prewarmed PBS. Cells were then transferred to sterile centrifuge tubes, and pelleted by centrifugation for ten minutes at 1000 rpm. The PBS was then aseptically removed and the cell pellet resuspended in 2ml of pre-warmed F12 media containing 10% sterile glycerol (Sigma). The resulting cell suspension (approximately $1\text{-}2\times10^6$ cells/ml) was then transferred to sterile cryovials (Nunc), and placed into a cooling box in an -80°C freezer. This served to reduce the cell temperature by approximately 1°C per minute. Cells were resuscitated from frozen stocks by rapidly defrosting in a waterbath at 37°C, and then transferring to flasks containing 8ml of pre-warmed and pre-gassed F12 media. Cultures were incubated for 48 hours, before subculturing into fresh flasks.

2.3. Supplementary methods used in cell culture.

2.3.1. Dilution cloning.

For dilution cloning, cells were suspended in F12 media supplemented with FBS, glutamine, and antibiotics, and diluted to a concentration of 10 cells/ml. 100μl aliquots of the suspension were placed into each well

of a 96-well microtitre plate (Linbro). Plates were incubated overnight at 37°C in a 15% CO_2 atmosphere in airtight containers. Plates were examined, and wells containing single colonies of one to two cells, (allowing for overnight division), were marked, and a further 100μl of media added. Cells were incubated until confluent over the base of the well (1-2 weeks) with a change of media every four days. Cell clones were then selected according to their appearance, *ie.* epithelial morphology, with few or no giant cells. The cells were detached from the wells with trypsin (100μl), and seeded into 25cm^2 area flasks. Clonal cells were incubated for approximately 7-10 days until confluent, and then subcultured into three flasks. One flask was used to determine the chromosome complement and spontaneous aberration frequency. The remaining flasks of the selected clone were expanded and frozen as described previously (2.1.3).

2.3.2. Growth assays of CHO-K1 cells.

The cell doubling time of the CHO cells was determined from growth curve experiments based on the protocol described by Freshney (1987). Cells were inoculated into 24 well multiwell plates (Linbro), at a density of 2x10^4 cells per well, in 1ml of Hams F12 with supplements, and incubated for 24 hours at 37°C. At approximately 24 hour intervals after seeding, cells in four of the wells were harvested for cell counting. Monolayers were washed with prewarmed PBS, and then detached by addition of 0.1ml of trypsin. When the cells were rounded, the trypsin was gently pipetted up and down to dislodge the cells. Cells were counted in a haemocytometer, and the mean number of cells per well calculated. The results were expressed as log10 numbers of cells against time (days) after seeding. This method gave an estimate of the generation time of the cells allowing subsequent calibration of exposure times for subsequent mutagenicity assays.

2.3.3. Staining for the presence of Mycoplasma infection.

To determine if the cell stocks were infected with Mycoplasma species, cultures were inoculated onto large (22x50 mm) glass coverslips, previously sterilised by dry heat. Three coverslips were aseptically placed into 90mm diameter, sterile, bacterial grade petri-dishes, and inoculated directly with 1ml of a 2x10^4 cells/ml cell suspension. The plates were then incubated for two hours at 37°C to allow cell attachment to the coverslips, before the addition of a further 7ml of culture media. The plates were placed into 1 litre airtight boxes, gassed with 50 ml CO_2, and then incubated for a further three days at 37°C, after incubation

coverslips were fixed and stained for the presence of mycoplasma infection.

Cells were stained for the presence of mycoplasma infection following the protocol described by Chen (1977). Culture media was partially drained from coverslip cultures, and replaced with 8 ml of Carnoy's fixative (3.1 methanol. glacial acetic acid), and fixed for five minutes. The fixative was then replaced with 100% Carnoy's fixative, and cultures fixed for a further ten minutes. The coverslips were allowed to air dry, before covering with 1ml of a 0.05μg/ml solution of Hoechst 33258 (Sigma) in PBS for 15 minutes at room temperature. After incubation, cells were rinsed three times in distilled water, and then the coverslip blotted dry. Coverslips were then mounted onto microscope slides with a drop of glycerol mountant (citric acid 4.6g/l, Na_2PO_4 8.2 g/l and 500 ml glycerol).

Positively and negatively infected Human Vero cell preparations were purchased (ECACC) prefixed, and were stained and mounted as described above. Cells were examined under a x100 objective using the reflected fluorescent light source as described below (2.2.4)

2.3.4. Cell harvest and slide preparation for examination of chromosomes.

Prior to cell harvest, colcemid (0.1ml of a 200μg/ml solution) was added to cultures, which were then incubated for a further 2 hours. Following incubation with colcemid, cells were harvested as follows. Culture flasks were coded alphabetically and then, mitotic cells were dislodged by gently shaking the flask. The suspended cells and culture media were then transferred into correspondingly coded 10ml centrifuge tubes. The cells were pelleted by centrifugation at 1000 rpm for 10 mins. The media was discarded and the cell pellet resuspended. The cells remaining in the flasks were rinsed with warmed PBS, and detached from the flask by the addition of 0.4ml of trypsin. When finally detached, further trypsin action was prevented by the addition of 5 ml of fresh media.. The resulting cell suspension was then transferred back into the respectively labelled centrifuge tube and the cells again pelleted by centrifugation. This method was used to reduce the loss of mitotic cells, which were already suspended in culture fluid and decreased further loss during the washing of cells with PBS. The cell suspensions were centrifuged at 1000 rpm for ten minutes, and the pelleted cells then resuspended in hypotonic KCl (0.56%) for 15 to 20 minutes at 37°C. The cells

were again centrifuged and the pellet resuspended in 5ml of freshly prepared, chilled acetic methanol fixative (3.1 absolute methanol. glacial acetic acid). Cells were immediately centrifuged and the fixative replaced. Cells were fixed for at least one hour, in at least three changes of fixative. Cultures were usually harvested and treated up to the second fixation, and then stored overnight at 4°C. With the final change of fixative, and slide preparation, performed the next day.

Metaphase spreads were prepared by dropping the translucent cell suspension onto pre-chilled, grease free slides, which were then allowed to air dry. Initial slides were examined under phase contrast to assess the degree of chromosome spreading. When acceptable slides from all cultures had been prepared, slides were stained in 3-5% giemsa in buffer (pH 6.8, Gurr) for 15-20 minutes.

2.3.5. Karyotypic analysis of cloned CHO-K1 cells.

Due to variations in both slide preparation and natural variation between cells in the population, the number of chromosomes observed in different metaphases usually differ. To determine how many chromosomes constitute the "normal" cell, untreated cells were harvested and examined to determine the distribution of chromosomes. The most frequently observed number (modal number) was assumed to represent the normal cell complement. In genotoxicity assays the cells with the modal number +/- 2 chromosomes were selected for scoring.

The modal chromosome number of the G6 clonal line, was determined from the numbers of chromosomes in approximately 500 randomly selected cells. Several cells with the modal number of chromosomes were selected and photographed for preparation of tentative karyotypes, based on the size and centromere position of the chromosomes. Banding techniques were not used to compile karyotypes, due to the extensive modification of the chromosomes compared with those in original Chinese hamster cells.

2.3.6. Microscopy and Photomicrography.

Cell preparations were examined on a Reichert-Jung "Polyvar", microscope equipped with x40 and x100 planapochromat objectives. For light microscopy, metaphase chromosomes of interest were photographed

on the Polyvar microscope, equipped with a Konica 35mm camera with automatic exposure control. Chromosome spreads were photographed using KB 14 black and white film, against a green filter to enhance contrast. The maximum exposure time was kept to one second to avoid vibration. All photographs were developed in IDII developer (1.1 in water) for approximately six minutes with 30 seconds agitation at approximately two minute intervals.

For fluorescence microscopy, cells stained with Hoescht 32258, were examined under a x100 objective, using a reflected fluorescent light source (mercury vapour) equipped with a 320-330nm excitation filter and 418nm long pass barrier filter. Cells were photographed using Kodak T-MAX 400 black and white film, pre-exposed at ASA 6400, and then exposed for two to four seconds at ASA 400. This film was developed as for a film speed ASA 100 for six minutes in IDII.

2.4. Metabolic activation.

Metabolic activation of test samples and positive control mutagens was achieved by using a crude preparation of rat liver homogenate. The metabolic enzymes of interest (cytochrome P450's) are isolated from hepatic tissue debris by centrifugation at approximately 9000g for 15-20 minutes. The resulting supernatant is known as the post mitochondrial fraction or S9 fraction. This fraction contains microsomes derived mainly from disruption of the endoplasmic reticulum of the cells and is rich in both P450 enzymes and glucose-6-phosphate dehydrogenase. This latter enzyme is used to regenerate NADPH in the test system.

2.4.1 Preparation of the S9 fraction.

S9 fractions were obtained using a protocol described by Maron & Ames (1983). Initially male Wistar rats were used to provide the S9 fraction. However, later batches were prepared from Sprague-Dawley rats. Male rats of approximately 200g were intraperitoneally injected with 500mg/kg body weight Arochlor 1254 in sterile corn oil. After five days the rats were sacrificed and the livers removed aseptically. The livers were washed in ice cold saline in a sieve to remove all traces of blood. Whole livers were weighed and three times this mass of KCl solution (0.15M) was kept on ice for use in homogenation. The livers were finely minced with sterile scissors and homogenised (handheld Potter Homogeniser) with the addition of the

reserved KCl solution. When fully homogenised the remaining KCl solution was added and the suspension transferred to a sterile 250ml centrifuge bottle. The suspension was centrifuged (MSE 18) in a pre-chilled rotor at 9000g for twenty minutes at 4°C. After centrifugation the resulting supernatant was decanted off and dispensed into sterile cryovials, quick frozen in a dry ice/methanol bath or liquid nitrogen, and then transferred to a liquid nitrogen freezer. A sample of the S9 was retained and streaked onto tryptone soy agar (TSA) for sterility testing.

2.4.2. Cytochrome P-450 determination in S9 fraction.

To determine the functional quality and quantity of the P450 enzymes in the S9 fractions, the enzymes were assayed spectrophotometrically, using a method described by Lake (1987). This assay utilises the characteristic absorbance spectrum of the reduced P450 enzymes when complexed with carbon monoxide (CO). Enzyme activity was measured from frozen samples rather than before freezing to allow for loss of activity associated with the freezing process. The S9 fractions were allowed to completely defrost at room temperature and were then stored on ice until required. Dilutions of S9 fraction were made in pre-chilled 0.2M phosphate buffer (pH 4), and reduced by addition of a small amount of solid sodium dithionite. The reduced enzyme suspension was then divided into two matched polystyrene cuvettes, and an absorbance baseline measured between 400 - 500 nm in a twin beam spectrophotometer (PYE UNICAM). Carbon monoxide from a gas generator, containing concentrated sulphuric and formic acids, was bubbled through the test cuvette for one minute. The sample was then re-scanned and the absorbance spectra recorded. The concentration of active cytochrome P-450 per ml of S9 fraction was calculated from the difference in absorbance maxima as observed between 450 nm and 490 nm (ΔA 400-490nm). The quantity of P-450 in nano-moles (nmol) per ml of the S9 fraction was calculated from equation **1.** The molar extinction coefficient of cytochrome P450 is given as 91 cm^2/nmol after Omura and Sato (1964) cited by Lake (1987).

$$\frac{\Delta A(450\text{-}490)nm}{1} \times \frac{1000}{91} \times \frac{vol\ of\ S9\ sample(ml) + volume\ of\ buffer(ml)}{vol\ of\ sample(ml)} = n\,mol\,P450/ml\ of\ S9\ fraction$$

2.4.3. Composition of S9 mix.

Besides the presence of the S9 described above, (2.4.1) several cofactors are required to enhance enzyme activity. Usually, these form an NADPH regenerating system consisting of NADP and glucose-6-phosphate

(G-6-P). The S9 fraction plus co-factors is the referred to as the "S9 mix".

Several authors have described widely different formulae for the composition of the S9 mix. One of the simplest was that described by Benford and Hubbard (1987), and consisted of culture media, S9 fraction, and G-6-P and NADP (monosodium salt). This S9 mix was adopted for use in this investigation. The composition of the S9 mix is shown below (table 8) both for the original mix, referred to as 1xS9, and a subsequent modification containing twice the volume of S9 fraction referred to as 2xS9.

Table 8: Composition of S9 mixes used in *in vitro* genotoxicity assays

Component	1xS9	2xS9
Serum free F12 Media	28.7 ml	27.7 ml
S9 (fraction)	1.0 ml	2.0 ml
NADP solution (80 mM)	0.15 ml	0.15 ml
G-6-P solution (100 mM)	0.15 ml	0.15 ml
Total	30.0 ml	30.0 ml

2.4.4. Biological characterisation of S9 activity.

Three batches of S9 mix were prepared although only two were used in assays after enzyme activity assays. To establish the suitability of the S9 fractions to be used in genotoxicity assays, they were tested for their ability to metabolise the activation dependent mutagen cyclophosphamide (CP). CP was chosen as a stock mutagen because it was readily available and more importantly was soluble in water, rather than organic solvents, reducing the risks involved of handling of the solutions. Assays of the initial S9 fraction, were conducted in sister chromatid exchange assays, as these were considered easier and more rapid to score. Four concentrations (1.5, 3.0, & 6.0 μg/ml) of CP were tested with the 1xS9 mix, but only one CP concentration (3.0 μg/ml) was tested with the 2xS9 mix. Negative and positive controls contained CP without S9 mix and distilled water with both S9 mixes. Later S9 fraction preparations were tested in chromosome aberration assays, where four concentrations of CP were tested with both the 1xS9 and 2xS9 mixes. The dose responses obtained were compared to those published by Galloway *et al.* (1985) to assess the effectiveness of the S9 fraction.

2.5. Genotoxicity assays.

2.5.1. Emission sample cytotoxicity tests.

Cytotoxicity tests were conducted on all samples to discover the maximum tolerated concentrations. The method chosen was based on that described by Truehaft (1983). This is dependent on the uptake of neutral red stain by viable cells. Assays were conducted in 24 well multiwell plates (Linbro), each well having a diameter of 16mm and a surface area of approximately $2cm^2$. Plates were seeded at a density of $2x10^4$ cells per well and incubated for 24 hours at 37°C. Various concentrations of each sample were dissolved in dimethyl sulphoxide (DMSO, Sigma), and then introduced into wells in volumes of 10μl. DMSO was used as a solvent control. Duplicate wells were inoculated for each concentration tested. Cells were further incubated for 14-18 hours. After exposure the culture media was removed and the cell monolayer washed with 1ml of PBS. This was then replaced by 1 ml of a 33mg/l solution of neutral red in PBS. Cells were incubated for a further two hours to allow dye uptake, after which the residual neutral red was discarded and the monolayers then washed twice with 1ml of PBS. Intracellular dye was released by the addition of 1ml of extraction buffer (50% ethanol in 0.1M NaH_2PO_4). The amount of dye uptake was detected spectrophotometrically against an extraction buffer blank at 540nm in a twin beam spectrophotometer (PYE Unicam). The cytotoxic effects of exposure were derived from the comparison of the absorbance readings of treated wells to that of the controls.

2.5.2. Chromosome aberration assays.

The protocol used for performing chromosome aberration assays was based on that described by Galloway *et al.* (1985). Cells for aberration assays were seeded at a density of approximately $2.5x10^5$ cells/ml in 8ml of Ham's F12 supplemented with FBS (10%), l-glutamine (1%), and with solutions of 200 IU/ml Penicillin and 200μg/ml Streptomycin (both 1%), and incubated for 24 hours. In almost all experiments, four sample concentrations, solvent (DMSO) and positive controls (N-methyl-N-N-nitrosoguanidine and cyclophosphamide) were tested. Duplicate cultures were seeded for all sample concentrations and solvent controls, with single cultures used for positive controls. Cultures were treated, both with and without the presence of a supplementary metabolic activation system (S9 mix) as described below (2.4.3).

In cultures not requiring metabolic activation, after 24 hours, 80μl of samples dissolved in dimethyl sulphoxide (DMSO) at various concentrations, and solvent controls, were directly introduced into flasks. This was achieved by tilting culture flasks and ejecting the sample directly into the media, avoiding direct contact of the solvent/sample with the walls of the flask or the cell monolayer. This served to prevent direct toxicity to the cells and to prevent the DMSO solubilising components of the flask material. Inoculated flasks were then gently rocked to ensure even dispersal of the test chemicals, and incubated for some further 14-18 hours. Colcemid (100μl of a 200μg/ml solution per flask) was added for the final two hours.

Cultures requiring metabolic activation were treated as follows, 24 hours after culture initiation, the growth media was discarded, and the cell monolayer washed with 5ml of pre-warmed PBS. This was then replaced by 8ml of prewarmed "S9 mix", into which samples and solvents were directly inoculated. Cells were exposed to the S9 mix plus test agents for two hours, after which the S9 was discarded into a storage container, and the cells washed twice with 5ml of PBS. After washing, 8ml of pre-warmed F12 media was added to the cultures, which were then incubated for some further 12-14 hours, with colcemid present (as above) for the final two hours.

2.5.3 Scoring of cells for chromosome aberrations.

Stained slides were coded to prevent bias during scoring. Slide labels were masked with tape and randomly labelled. Metaphase cells with the modal number of chromosomes, were selected by the appearance, and degree of spreading of the chromosomes. Cells with the original modal number of 20 $\pm$ 2 chromosomes were scored as suggested by Galloway (1985). In later assays, where the modal number fell to 19 chromosomes the selected range of 18-22 chromosomes was still used. Cells in which the chromatids were not separated, were not scored. Cells in which chromosomes were poorly spread, were examined to determine if this was due to the presence of complex aberrations (as suggested by Savage 1975).

Metaphase cells for scoring were selected from successive fields of view under x40 magnification and then examined in detail under a x100 oil immersion objective. One hundred cells (where possible), were scored from each flask. In cases where 100 cells could not be scored from one slide, successive slides were

prepared from each culture. Aberrant cells were recorded on data sheets with their location and types of aberration. Observed aberrations were classed into several categories based on the description given by Galloway *et al.* (1985). These are described as follows; chromatid and chromosome gaps (which were defined as any non-staining region at which the chromatid arm at either side of the region remained aligned), chromatid and chromosome breaks (including terminal deletions), chromatid and chromosome interchanges (resulting in triradials, dicentrics, *etc.*). Chromosome damage such as pulverised chromosomes and cells with multiple aberrations were recorded as 'other' aberrations. Results were expressed as percentages of cells with chromosome aberrations excluding gaps.

The mitotic indices of treated cultures were also calculated, by counting the numbers of mitotic cells from a random sample of 2000 cells, as suggested by Dean and Danford (1984). Results were expressed as number of metaphase cells per 1000 cells.

2.5.4. Sister chromatid exchange assays.

Cultures for sister chromatid exchange assays were prepared and inoculated with test samples, essentially as in chromosome aberration assays as previously described (2.3.2). In SCE assays without supplementary metabolic activation, two hours after sample inoculation 0.1 ml of a 0.3 mg/ml aqueous solution of 5-bromo-deoxy uridine (BrdUrd) was introduced. In cultures with supplementary metabolic activation the BrdUrd was added directly to the replacement media as described in previously (2.3.3). After inoculation of the BrdUrd, all cultures were placed into light proof boxes and incubated for some further 24-26 hours. Colcemid was added under subdued light conditions for the final two hours of incubation. All procedures for cell harvesting and slide preparation were performed in subdued light as described above (2.3.3).

2.5.5. Differential staining for sister chromatid exchanges.

Cells were harvested and fixed as previously described with the exception that all procedures were performed under subdued lighting conditions, which were maintained until the slides were dry. The Hoescht plus giemsa technique (FPG), used for differential chromatid staining was a modified version of that described by Dean and Danford (1985). Air dried slides were immersed in room temperature solutions of

PBS pH 7.0 for five minutes, then transferred to a 0.5μg/ml solution of Hoescht 33258 in PBS for 10 minutes. Slides were then rinsed in PBS and placed face up in a shallow tray and covered with a thin layer of PBS to prevent drying during UV exposure. Slides were exposed to a 356nm UV light source (Camlab) at a distance of approximately 15-20 cm for two hours. After exposure, slides were rinsed in a solution of 2xSSC (0.03M sodium citrate /0.30 M NaCl) at room temperature, and incubated for 15 minutes in this solution at 65°C. Slides were finally rinsed in distilled water and stained in 2-3% giemsa for 15-20 minutes.

2.5.6. Scoring of cells for sister chromatid exchanges.

Cells were selected based on chromosome spreading and contrast between the chromatids. Thirty cells were randomly selected from each culture and the numbers of exchanges counted in each, using a x100 oil immersion objective. Results were expressed as both the mean number of exchanges per chromosome and per cell.

2.6. Statistical analysis of cytogenetic data.

Data obtained from chromosome aberration and sister chromatid exchange assays were analysed by protocols suggested in the UKEMS guidelines (Kirkland 1989). Variation between replicate cultures was assessed by using appropriate dispersion statistics, and dose responses were tested for significance by linear trend tests described by Margolin (1986) and Cooke *et al.*(1989).

2.6.1. Analysis of the variation in numbers of cells with chromosome aberrations between replicate cultures.

Before testing dose responses for significance, the variation between numbers of aberrant cells in duplicate cultures at each sample concentration, was tested using a binomial dispersion test as described Richardson *et al.*(1989). This test was used to determine the inter-duplicate variation between the numbers of aberrant cells observed. If there was no significant variation between duplicate cultures then it was statistically valid to combine the values of numbers of cells scored and numbers of aberrant cells, from duplicate cultures. The test statistic X^2 (calculated from equation 1), when compared to the upper percentage points of the chi squared distribution with $\sum_{i=0}^{t} (m_i - 1)$ degrees of freedom, indicates whether there is excess variation between

replicate cultures.

$$X^2 = \sum_{i=0}^{t} \sum_{j=1}^{m_i} \frac{(r_{ij} - n_{ij} p_i)^2}{n_{ij} p_i (1 - p_i)} \qquad (1)$$

In experiments where the number of sample treatments are labelled from *0* to *t*, and where *t*0 is the solvent control. In the i^{th} treatment where $i=0$ to t, there are m_i numbers of replicates (in most cases $m = 2$ in this work). From the j^{th} replicate ($j = 1$ to m) there are n_{ij} numbers of cells scored, of these r_{ij} are found to contain aberrations. The proportion (p_i) of aberrant cells at the i^{th} treatment, was calculated from the summing the numbers of cells scored and the number of aberrant cells observed from the duplicate cultures ($p_i = r_i/n_i$).0 to give those samples in which no excess variation between cultures were observed, the data for numbers of aberrant cells and total number of cells scored were pooled.

2.6.2. Analysis of chromosome aberration data for dose related responses.

After determining the variation between duplicate cultures, the presence of significant dose responses of numbers of aberrations with increasing sample concentration, was assessed using the linear trend test described by Margolin *et al.*(1986). Preliminary analysis of the aberration data required the grouping of aberrations into three categories defined by Galloway (1985). These categories were.

(*i*) Simple; including chromosome / chromatid breaks and terminal deletions.

(*ii*) Complex; including exchanges and rearrangements.

(*iii*) Total; including both types *i* and *ii*, and cells containing multiple aberrations and pulverised cells.

The numbers of aberrant cells from each duplicate culture at each sample concentration were pooled and the proportion of aberrant cells per sample treatment ($\hat{p}_{0ij}$) were calculated using $\hat{p}_{ij} = Y_{ij}/n_i$. In experiments where there are *r* doses labelled d_0 to d_r, d_o is the solvent control. n_i numbers of cells are scored at the i^{th} dose. Y_{ij} represents the number of cells at the i^{th} dose with category *j* of aberrations where *j* is equal to the categories described above (section 2.6.2.). The overall trend statistic for each category of damage *j* is given by equation 2.

$$Z_j = \frac{\sum_{i=0}^{r} n_i(\hat{p}_{ij} - \bar{p}_j)\, x_i}{[\bar{p}_j(1-\bar{p}_j)s_x^{2}]^{1/2}} \qquad (2)$$

The value of Z_j obtained is one tailed, and is referred to a table of the standard normal distribution, to obtain a P value. Values were then corrected by tripling the obtained P value to compensate for the multiplicity of comparisons against the control data. Margolin *et al.* concluded that it is preferential to score 200 cells per treatment culture rather than 100, Richardson *et al.* suggested that there was no statistical advantage in scoring 200 cells from one flask over 100 cells from two flasks as long as the in the latter case the cell populations were consistent with the binomial distribution of aberrations. Trend statistics were calculated using a spreadsheet, the format of which was constructed using the data presented by Galloway (1986).

2.7. Analysis of sister chromatid exchange data.

Analysis of SCE data is dependent of the on the assumption that the exchanges occur randomly over all the chromosomes within a cells, and between all cells thus following a Poisson distribution within the culture. Prior to analysis of the linear trend of any dose response, preliminary confirmation of the distribution of SCE following those predicted by a Poisson model was required. This was achieved by employing a dispersion test as described by Snedecor and Cochran (1980, cited by Cooke 1990). This compares the mean values of the of SCE's obtained against the variance of their distribution, as in the Poisson model these values are equal. The test is applied to the SCE distribution between cells within single cultures and the SCE distribution between different cultures, thus enabling a estimate of both the intra- and inter- flask SCE distributions.

2.7.1. Analysis of SCE distribution between individual cells.

This test was performed using the values of SCE's per cell and is based on the test statistic $\chi^2 = \sum_{k=1}^{n} (y_k - \bar{y})^2 / \bar{y}$, where there are n cells each with y numbers of SCE's. The mean numbers of SCE's per culture, $\bar{y}$ is given by $\bar{y} = \sum_{k=1}^{n} y_k / n$ (k = individual cells). On the basis of that the data are consistent with a Poisson model, the χ^2 statistic has a distribution with (n-1) degrees of freedom. The values

obtained from each culture were then summed to give an estimate for the whole experiment and increase the degrees of freedom. In this test the number of cells scored per culture need not be equal. The total χ^2 statistic obtained was then compared to the critical value of the χ^2 distribution with the appropriate degrees of freedom at (P=0.05) and if less than the critical value, was assumed to indicate that there was no excess variation between intracellular SCE distribution.

2.7.2. Analysis of SCE data for replicate culture effects.

After testing of the individual cultures, a similar dispersion test was performed on the data from replicate cultures with n numbers of cells scored and *r* numbers of replicates at each sample concentration tested. The test statistic was derived from $\chi^2=\sum_{j=1}^{r_i}(T_j-n_j\bar{y})^2/n_j\bar{y}$ where, the total SCE count of a replicate culture *j* (*j*= 1 to *r*) is defined as *Tj*. The mean number of SCE's at each sample concentration is given by $\bar{T}=\sum_{j=1}^{r}T_j/r$. $\bar{y}$ represents the mean number of SCE's for all replicate cultures at a particular sample concentration, and is found from $\bar{y}=\sum_{j=1}^{r_i}T_j/\sum_{j=1}^{r_i}n_j$. The test statistic obtained from each set of replicate cultures was referred to the X^2 distribution with (*r*-1) degrees of freedom. The values were again summed over all sample concentrations to achieve a value for the whole experiment.

2.7.3. Analysis of sister chromatid exchange data for dose related responses.

Two methods were used to calculate values for the linear trend statistics of the SCE data, depending on whether the data was compatible with the Poisson model. For those datasets which were compatible with a Poisson distribution of SCE's in both individual cell counts and between replicate cultures, the linear trend test described by Galloway *et al.* (1985) was used. Due to the slight variation in the numbers of chromosomes within cells scored, the trend statistic was based in the numbers of SCE's per chromosome rather than per cell. The linear trend statistic (Z) is derived from $Z=\sum_{i=0}^{r}x_i(Y_{i\cdot}-kn_i\bar{Y})/[\bar{Y}s_x^2]^{1/2}$, the components of which are described below (table 9.)

The other linear trend test used was that described by Cooke *et al.*(1989), and is based on the failure of the replicate data to meet the requirements of the Poisson model. In this case the linear trend was calculated using the between cultures analysis of variance (ANOVA) the error was estimated from the between cultures mean square value obtained (Appendix 3). This analysis requires transformation of the SCE numbers to

Table 9: Notation used for the analysis of SCE data for dose related linear trends.

The SCE experiments consist of a control and r doses of a chemical , where n_i cells are scored after exposure to the i^{th} dose d_i the response Y_{ij} denotes the SCE count for the j^{th} cell scored after exposure to the i^{th} dose d_i , $j=1,....n_i$, $i=,0,1,....r$. here $d=0$ is the control and it is assumed that $0<d_i<d_2<...<d_r$. The n_i cells scored after exposure to the i^{th} dose may represent results obtained from replicate flasks.

Reproduced from Margolin et al 1985.

attempt to achieve a constant variance for the data.

A square root transformation was selected and the trend statistic calculated from b/SE(b) where

$$b=\frac{\sum_{i=1}^{t}(x_i-\bar{x})\bar{z}_i}{\sum_{i=1}^{t}(x_i-\bar{x})^2} \qquad SE\ of\ b\ =\sqrt{[s^2/r\sum_{i=1}^{t}(x_i-\bar{x})^2}$$

In the above x = the coded sample concentration, $\bar{x}$ = the sum of coded dose/number of doses, $\bar{z}$ = the transformed mean numbers of SCE's per culture. S^2= the between cultures mean square. Test statistics obtained were compared to the t distribution (one-tailed) with between cultures degrees of freedom (Appendix 3).

2.8. Analysis of engine emission data.

2.8.1. Calculation of emission rates from given data for the conditions sampled.

Emission data obtained from the Prima engine while on site at Plymouth, during the initial period of this study, were obtained from Perkins Engineering LTD. The speeds and load at which emissions were collected were not the same as those selected for mutagenicity sample collection. To calculate values of each emission at the conditions tested, regression analysis of the Perkins data was performed. Data was plotted using "Fig

P" graphical software (Biosoft), which was then used to calculate best fit and 95% confidence limits for regression lines. The values for load and speed used for mutagenicity sample collection, were then entered, and the results of emission at each of theses points calculated by the software. The results of this analysis are included in appendix 3.

3.0. Results.

3.1. Cytogenetic evaluation of CHO cell lines used.

3.1.1. Initial investigation of the CHO-K1 cell line.

After preliminary studies with known mutagens, the original CHO cell line was used to assay samples ES1 and ES2. The results of these studies (figures 11 & 12) showed that an extremely high (ca. 40 to 50%) percentage of the cells scored in control cultures contained aberrations, the majority of which were chromatid and chromosome breaks. No dose related increase in percentage of cells with aberrations was observed in cultures exposed to either emission sample. However, the actual numbers of individual aberrations scored in cultures did increases in a dose related manner for both samples. Numbers of aberrations in cultures exposed to ES1 increased from 30 in control flasks to 40 in cultures exposed to 100 μg/ml, and those exposed to ES2 from similar control values to a maximum of number 80 at a sample concentration of 50 μg/ml.

These high levels of damage were originally believed to be the result of overextended passage of the cell line. As described previously (methods 2.1.1) attempts were made to identify the factors responsible for the high incidence of damage. Changes in media, sera, and culture vessels had no significant effect on spontaneous damage levels (data not shown). Slide cultures were tested for the presence of a mycoplasmal infection using the fluorescent intercalating stain Hoechst 33258 the results of which are shown below (figures 13, 14, &15). Figure 13 illustrates a non--infected cell line with intense nuclear staining with minimal cytoplasmic fluorescence. In contrast figure 14, illustrates pinpoints of fluorescence located in the cytoplasm, this extranuclear DNA staining is a feature of cells infected with mycoplasmas. The final figure in this sequence (figure 15) shows the effects of staining the CHO-K1 cell line, no evidence of fluorescent staining within the cytoplasm was observed and from this observation it was concluded that this line was not apparently infected with a species of mycoplasma.

A third cell line obtained from the ECACC (designated CHO-K1-PD2), had a reduced rate of spontaneous damage (approximately 10-15 %), and produced a dose related increase in percentages of aberrant cells when treated with MNNG. This line was used to perform repeat assays of emission sample ES1, ES2, and of ES3, whilst cell cloning experiments to isolate a stable cell line were undertaken.

Figure 11: Percentages of cells with chromosome aberrations and total numbers of observed aberrations, in the original CHO-K1 cell line, following exposure to ES1 (1000 rpm @ Nm load).

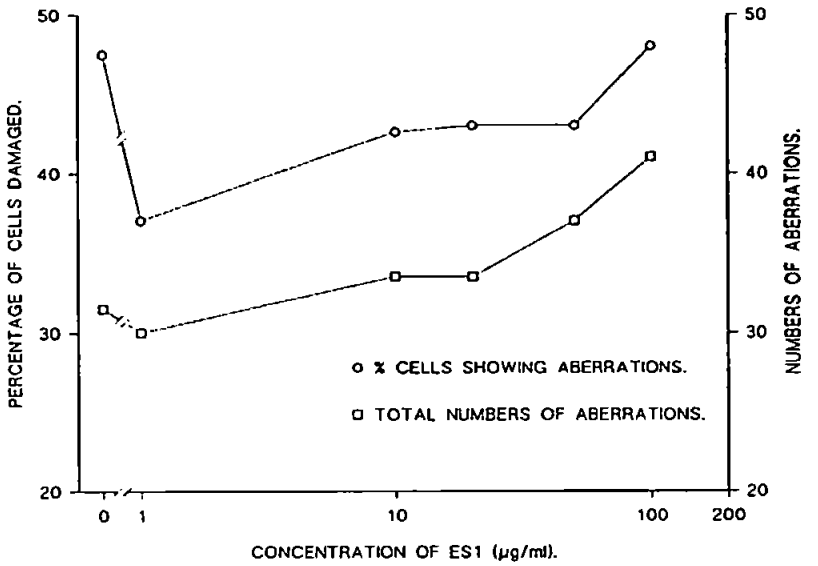


Figure 12: Percentages of cells with chromosome aberrations and total numbers of aberrations observed after exposure of the CHO-K1 cell line to diesel sample ES2 (2000 rpm @ 5Nm load).

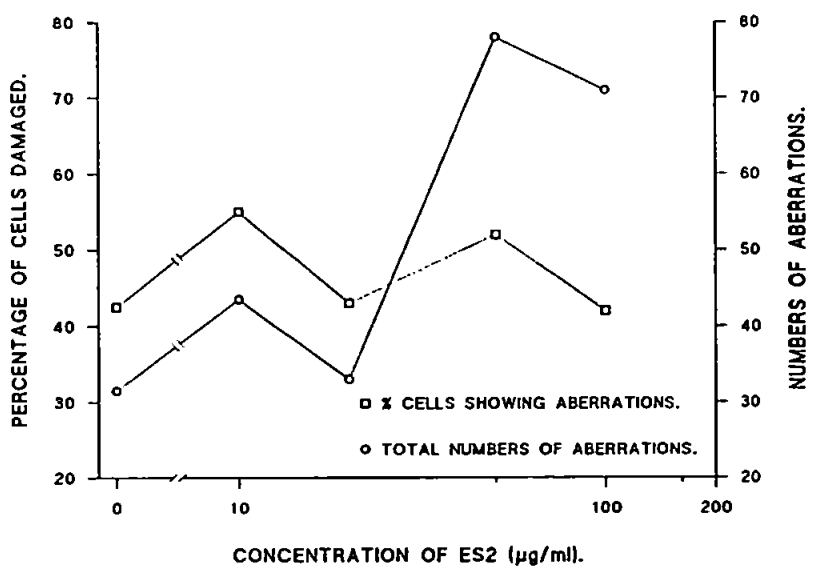


Figure 13: Uninfected Vero cells stained for the detection of mycoplasmal contamination with Hoechst 332158.

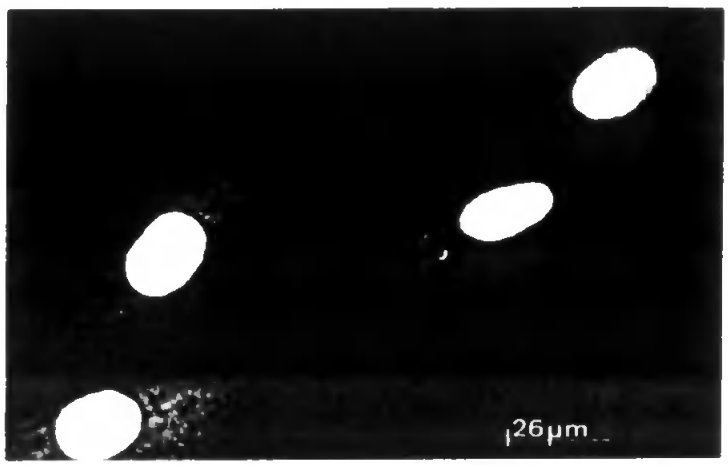


Figure 14: The detection of mycoplasmal infection by Hoechst 33258 fluorescent staining, in Vero cells infected with *M. orale*.

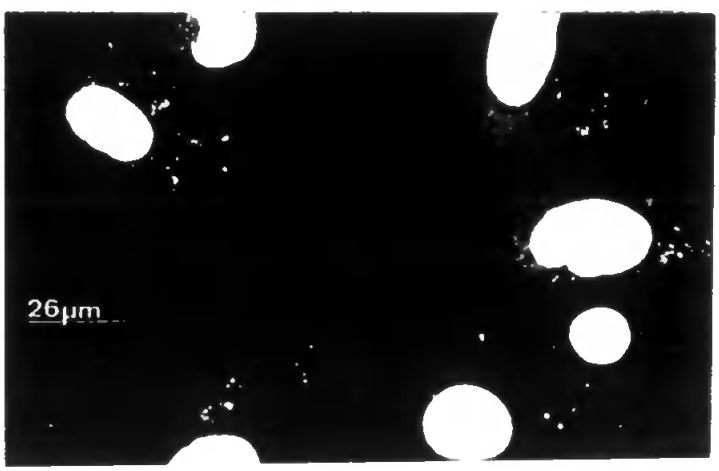
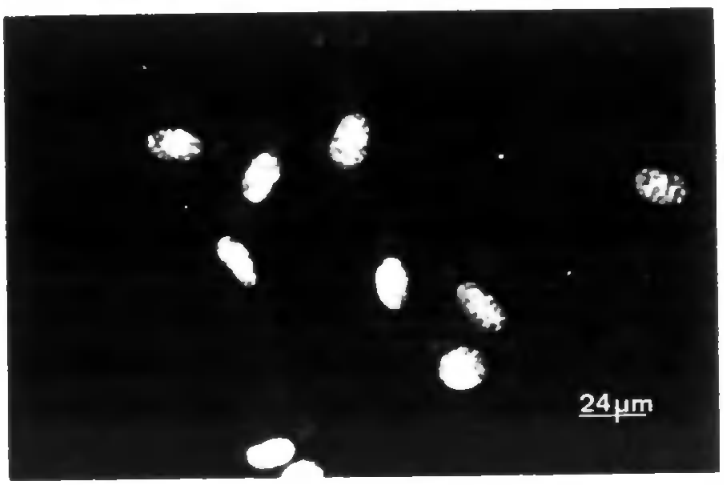


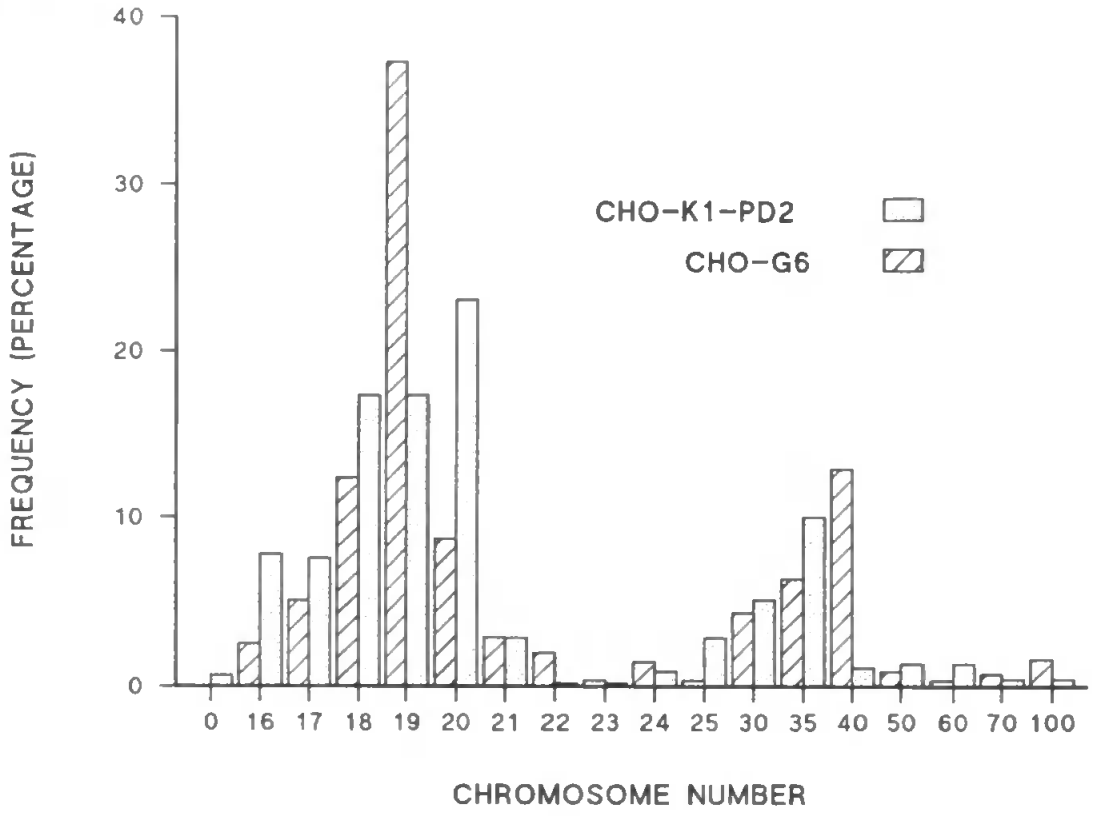
Figure 15: CHO-K1 cells stained for the presence of mycoplasmal infection with Hoechst 33258.



3.1.2 Characteristics of the clonal line designated CHO-K1-PD2-G6.

A clonal cell line was isolated and designated CHO-K1-PD2-G6 (subsequently referred to as G6). This was selected for use in genotoxicity assays due to reduced levels of spontaneous aberrations. This line was then characterised by comparing the distribution of chromosome numbers within cells, with that of the parental (CHO-K1-PD2) line, the results of this comparison are shown in figure 16. The modal number of chromosomes was found to have decreased from 20 in the PD2 line to 19 in the G6 clone. The distribution had also altered from approximately equal number of cells with 18,19 and 20 chromosomes in PD2, to a majority of approximately 40% with 19 chromosomes in G6. There was also a relatively high percentage of cells (approximately 15%), with between 30 and 40 chromosomes in both cell lines.

Figure 16: Chromosome number distribution within cells from both the PD2 and G6 lines from 500 observations.



Tentative karyotypes (based on chromosome size) of cells with the modal number of chromosomes, from each cell line are shown below (figure 17). There are several significant features of the two lines. First, the difference in modal numbers is not the result of the loss of a single chromosome from the G6 line, there are extensive differences in the morphologies of some chromosomes in the two lines. There are, also

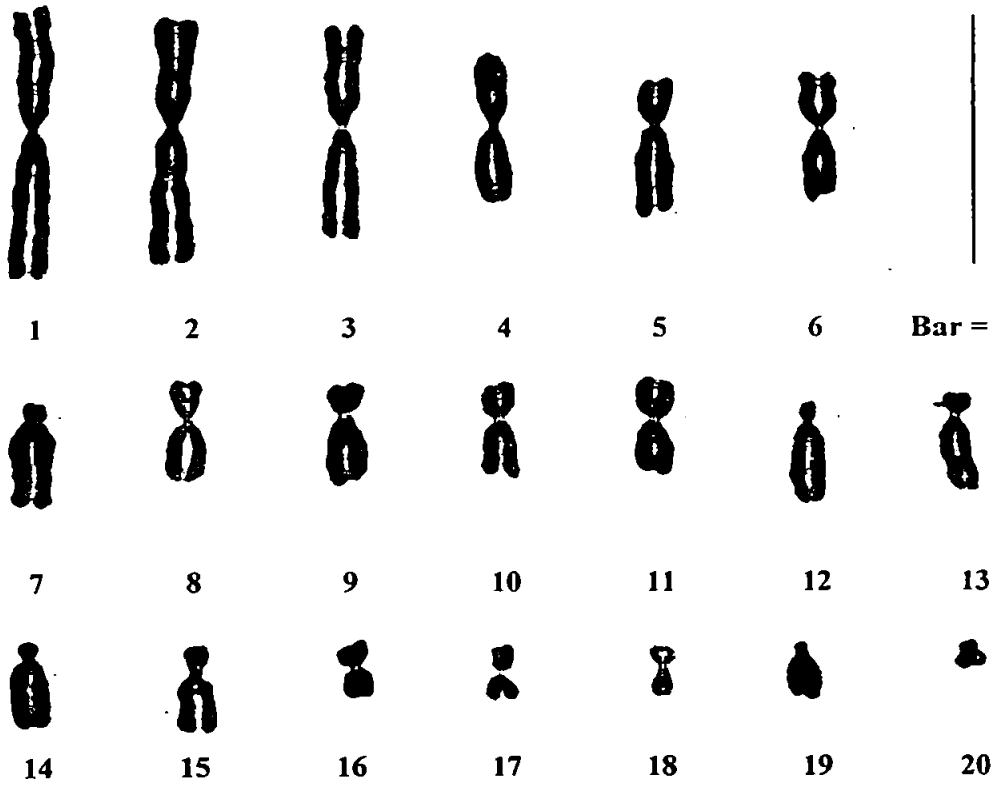
chromosomes which are recognisable as similar between both cell lines. In the CHO-K1-PD2 line there are few chromosomes which could be tentatively considered as homologues, those that are similar in size and morphology are, the three acrocentric (7,12,13), and the sub-metacentrics (8,9,10,11). In contrast the G6 line has several chromosomes which could be paired, based on their size and morphology, these are, 6a with 7a, 8a with 9a, 10a with 11a, and 12a with 13a.

There are several distinctive chromosomes common to both cell lines, these include the larger metacentrics 1, 1a, 2, 2a, 3, 3a, the sub-metacentrics 5 and 5a, the small metacentrics 16, 16a, 17, 17a ,18 , 18a, and the smallest chromosome (20, 19a). The largest chromosomes (1, 1a, 2, and 2a) may represent the large metacentric chromosomes found in the original CHO karyotype, but in both cell lines chromosome 1 and 1a, appear to be slightly longer and more submetacentric than the number 2 chromosomes in respective lines.

The loss of two acrocentric chromosomes from the PD2 line, could be explained by a simple centric fusion, which would reduce the chromosome number in the G6 line. However, there is no candidate chromosome in the G6 line which could arise from such a fusion event. One distinctive chromosome, in the G6 line (4a in figure 17) appears to have a contracted q arm with a pale staining region at approximately two-thirds of the total length of the chromosome. This marker is apparent in all clonal G6 cells with the modal number of chromosomes, and may be identified in other cells of the G6 line with 20 chromosomes. Five of these chromosomes, with pale staining regions are shown below (figure 18). The pale diffuse region was found to be relatively consistent in the chromosomes studied occurring at between 75 and 80 % of the total length of the chromosome (table 10). In metaphase spreads where the pale region was less discernible, this chromosome could be readily identified by the difference in separation of the chromatids of the p and q arms. The q chromatids were narrower than those of the p arm and closer together, whereas the chromatids of the p arm were similar in diameter and separation as the other chromosomes within the cell. This chromosome has been tentatively identified as the X chromosome, which is known to have a heterochromatic staining region approximately halfway along the length of the q arm.

Figure 17: Tentative karyotypes of two cells with modal chromosome numbers, one of the CHO-K1-PD2 cell line, the other from a clonal line designated G6, derived from the parental PD2 line.

CHO-K1 cell line



G6 clonal line

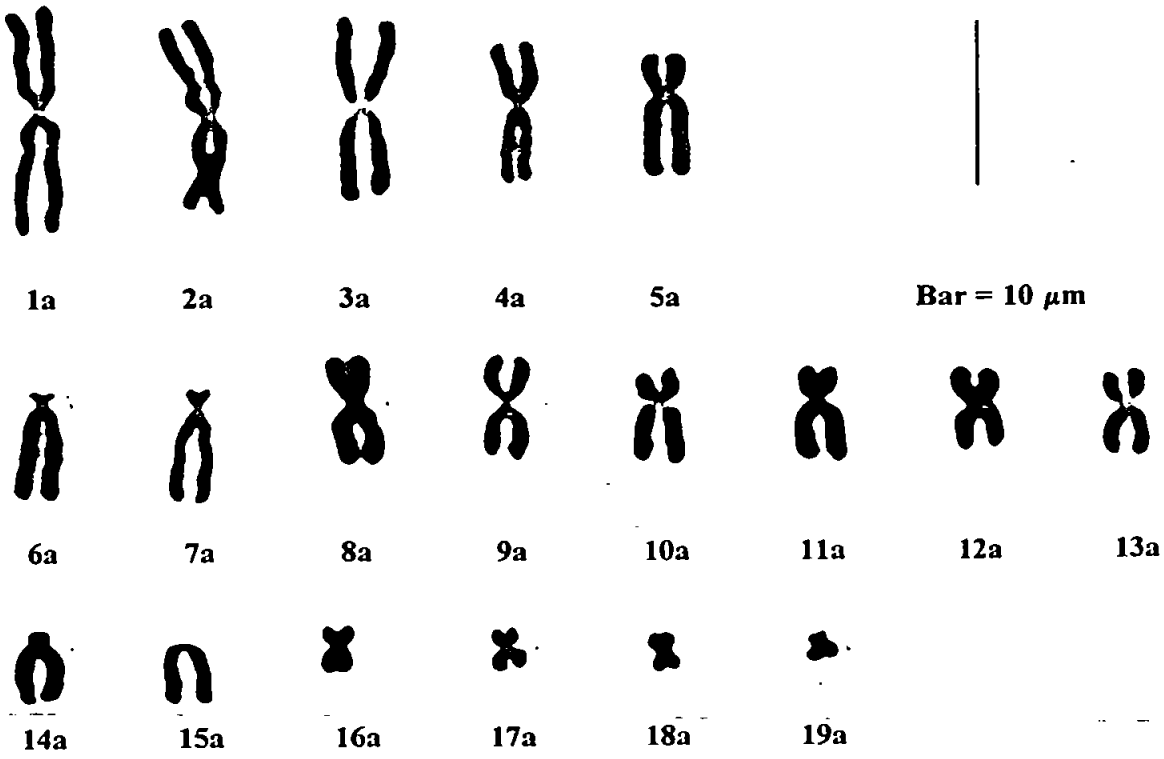


Figure 18: Representative chromosomes from five CHO-K1-G6 cells showing heterochromatic staining regions.

Table 10: Distribution of pale staining region along the lengths of chromosome 4, from 5 representative CHO-K1 cells.

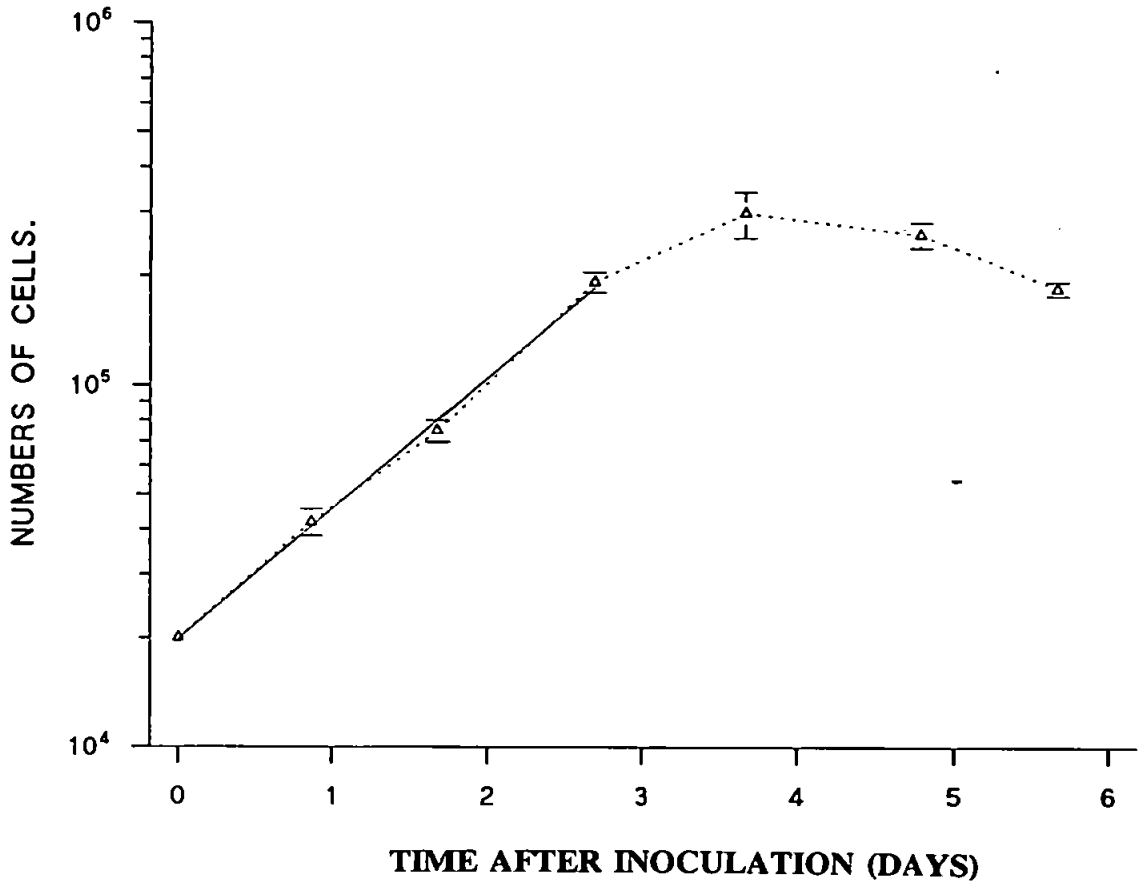
Chromosome (from figure 18)	Total chromosome length (μm)	Length from p to gap (μm)	Distance from p to gap as percentage of total length
1	6.64	4.56	75
2	8.3	5.81	75
3	9.5	5.81	77
4	8.3	6.64	80
5	7.47	5.81	70

N.B: Total chromosome lengths were measured using eyepiece and stage graticules. The length from the telomere of the p arm to the midpoint of the pale staining region, referred to as the "gap" in table ? was also measured. This length was then expressed as a percentage of the total length of the chromosome to allow for variations due to different levels of contraction of the chromosomes.

3.1.3. Growth assay.

The growth kinetics of the G6 cells, when grown in multiwell plates was determined (figure 19), and the length of the cell doubling time then calculated from the linear regression equation of the exponential phase of the growth curve. The cell doubling time was found to be approximately 19 hours. This time was longer

Figure 19: Growth of CHO-G6 cells in 24 well microtitre plates, cell doubling time was calculated from the linear regression equation derived for the exponential phase as indicated.



than reported in other published studies which indicated that the cell doubling time of CHO cells is between 14 and 18 hours. Based on the large volume of published data, cultures used in genotoxicity assays were harvested approximately 14 to 16 hours after exposure to test compounds, to ensure accumulation of cells at the first mitosis after exposure.

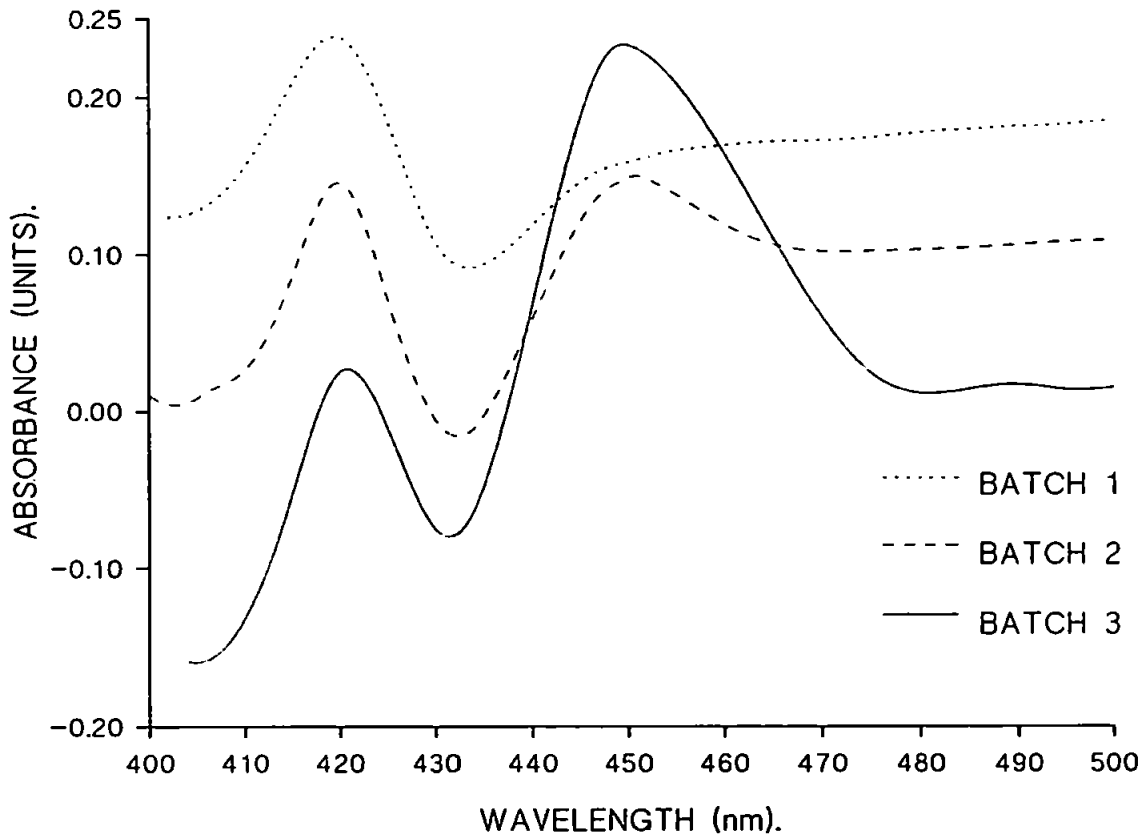
3.2. Metabolic activation and testing of known mutagens

3.2.1 Preparation and assay of the metabolic activating system (S9 fraction).

Several batches of S9 fraction, had to be prepared before a suitable level of cytochrome activity was obtained. Levels of active cytochrome content was determined from the absorbance spectra of reduced cytochromes complexed with CO. The absorbance spectra from three different batches of S9 fraction are shown below (figure 20). It can be seen that all three batches differ in maximum absorbance (λ max) at 450 nm. The absorbance peaks observed at 420 nm represent both contaminating haemoglobin and

denatured cytochrome P-450. The concentration of active P450 enzymes estimated from the difference in absorbance at 450 nm and 500 nm (see methods 2.4.2.). The first batch was found to contain very little P450 in active form and was discarded. A second batch contained a measurable quantity of enzyme (approximately 11 nmol/ml of S9), and induced increases in numbers of SCE's when tested with cyclophosphamide, and was therefore used in genotoxicity assays. The final batch prepared (Batch 3) was found to have the highest concentration of active P450 ca. 24 nmol/ml. This final batch was only used for the assay of four emission samples, ES4, ES12, ES15, and ES19.

Figure 20: Absorbance spectra of reduced cytochrome P450 mixed function oxygenase in three batches of S9 fraction.

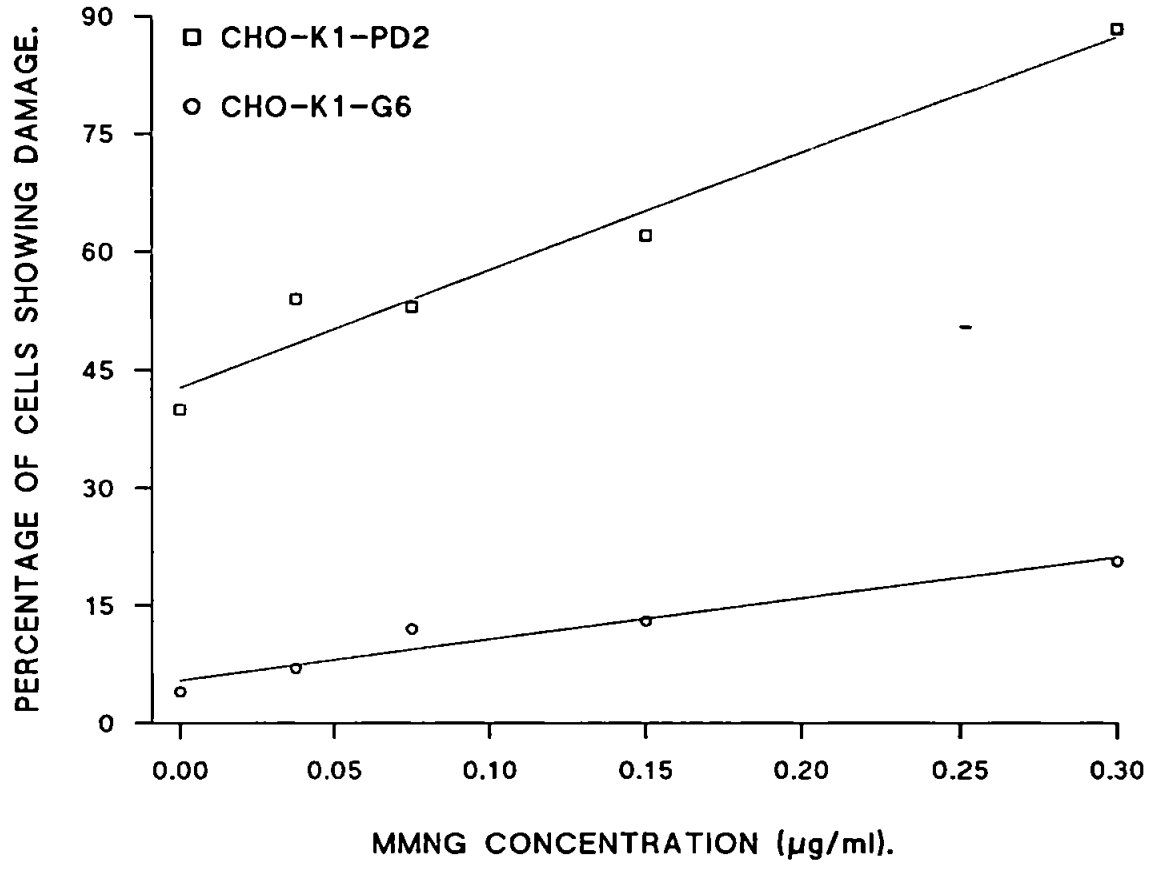


3.2.2. Results of chromosome aberration assays with known mutagens.

Aberration assays using the test mutagen MNNG were performed to assess the sensitivity of the assays and the scoring of aberrations. Results of assays of using both the PD2 and G6 cell strains are shown below (figure 21). The high incidence (approximately 45 %) of aberrations observed in solvent controls in the PD2, were largely due to the occurrence of "simple" aberrations. The PD2 line did produce a linear response

when treated with MNNG and was used for the assay of three emission samples. Subsequent isolation of the G6 clone with reduced levels of spontaneous damage (ca. 5%), also produced a MNNG dependent dose

Figure 21: Comparison of dose responses obtained from exposure of two CHO K1 cell lines to increasing concentrations of MNNG.



response comparable to published data (i.e. 15 to 20 % aberrant cells at 0.3 µg/ml MNNG, Galloway et al 1985). The extent of this response differed markedly from that obtained with PD2. Comparison of the regression lines obtained from the data in figure 21, was conducted to determine if the dose response obtained were similar despite the initial increases due to high background damage in PD2. If dose responses were similar, then it would be valid to compare dose response obtained from assays conducted using both cell lines. Results of this analysis indicated that the gradients (slope) of the dose responses of the two samples were significantly different ($P<0.05$). This may be indicative of a synergistic effect between the MNNG and the factor responsible for the spontaneous damage. On the basis of the responses obtained from the exposure of the G6 cell to MNNG and, the favourable comparison with published data it was assumed that the G6 line was suitable for use in chromosome aberration assays of diesel emission samples.

3.2.3. Activity assay of the S9 fractions.

The activity of the S9 fractions collected, with respect to their potential for metabolising cyclophosphamide, was determined in both SCE and aberration assays. the initial batch of S9 fraction with a measurable P450 content (batch 2, figure 20) was assayed in SCE assays as this yielded results more quickly than aberration assays. The results of this assay using two S9 mixes with high and low concentrations of S9 fraction (see methods 2.4.3) are shown below (figure 22). The use of the 1xS9 mix appeared to have little effect on the numbers of SCE's per cell, at all concentrations of CP, other than to increase the numbers of SCE's in control cultures in the absence of CP to 15 per cell. The use of the 2xS9 mix caused a dramatic increase in the numbers of SCE's per cell (approximately 60) at a CP concentration of 3 μg/ml, while the control values remained at approximately 7 SCE per cell. These values both compare favourably with results presented by Galloway *et al.* (1985), where 3 μg/ml of CP was found cause between 40 to 80 SCE's per cell, and where control values were approximately 10 SCE's per cell.

Figure 22: Induction of sister chromatid exchanges in CHO-PD2 cells following exposure to increasing concentrations of cyclophosphamide in the presence of two S9 mixes containing different concentrations of S9 fraction.

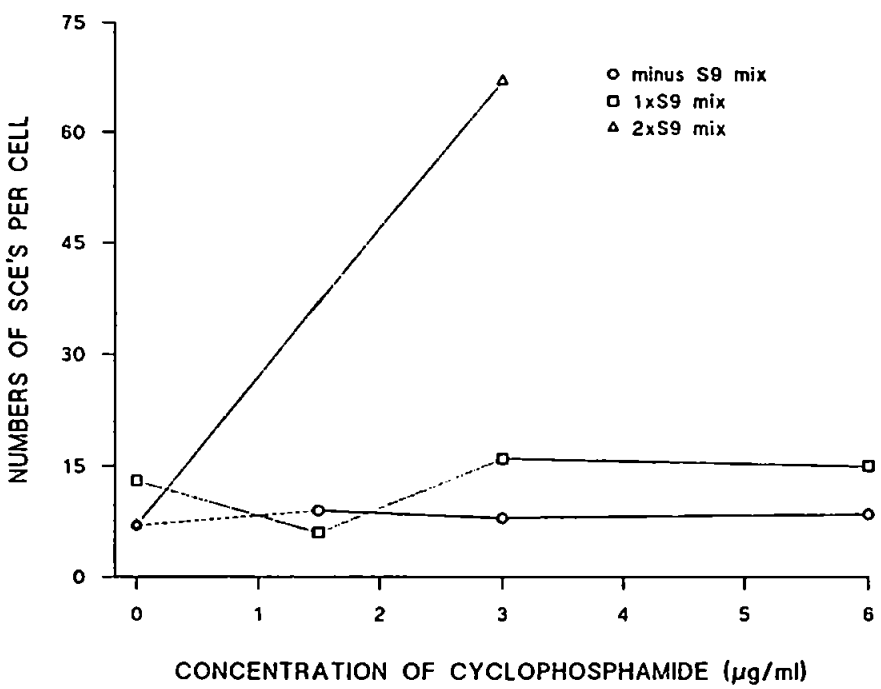
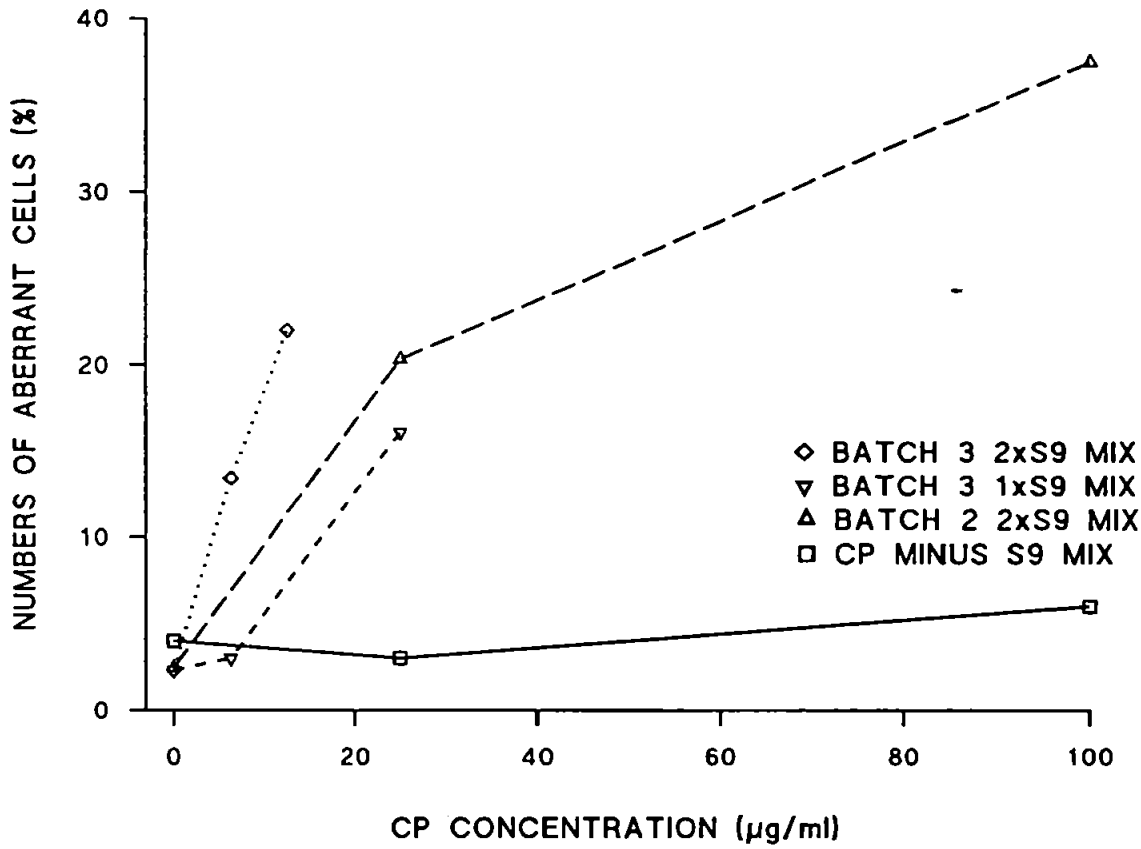


Figure 23: Chromosome aberration induction in CHO-G6 cells following exposure to cyclophosphamide with two batches of S9 and different S9 mixes.



The activity of the second active batch of S9 fraction (Batch 3 figure 20), was compared with the activity of batch 2 (figure 20) in chromosome aberration assays, the results of which are shown in figure 23. The percentage of cells with aberrations remained fairly constant in all control cultures both with and without metabolic activation at approximately 4%. The 2xS9 mix of batch 2 was found to induce an increase in percentage of cells with aberrations to approximately 20% at a concentration of 25 µg/ml. In contrast, the 1xS9 mix of batch 3 increased the percentage of cells with aberrations to 17% at a CP concentration of 25µg/ml. Whereas the 2xS9 mix of batch 3 further increased the percentage of cells with aberrations to 22%, at a CP concentration of only 12.5 µg/ml. This suggested that the activity of the third batch of S9 fraction was superior to that of the second batch. On the basis of these results the third batch of S9 fraction was also used as a 2xS9 mix in subsequent aberration assays of emission samples.

The results obtained in the aberration assays were also comparable to the data of Galloway et al. (1985), where a CP concentration of 25 μg/ml was found to cause aberrations, in between 25 to 30% of cells. These preliminary studies of the response of G6 cells to known mutagens, have shown that both MNNG and CP, induced increases in numbers of cells with aberrations that were comparable to published data. Also, both S9 fractions used in the testing of emission samples, elicited responses to CP, which were also consistent with available literature.

3.3. Emission sample collection.

Diesel emission samples were collected at the engine and load conditions described previously (figure 10). The final masses obtained after sample-workup and corresponding load/speed conditions are shown below in table 11. Emission samples ES1 to ES4 were collected during one week in 1991, the remaining samples were collected over a period of three weeks during 1992. Replicate samples were collected on the same day

Table 11: Engine conditions, final masses, and dates of collection of diesel emission samples collected for genotoxicity testing.

Emission sample number	Engine load (Nm)	Engine Speed (rpm)	Sample mass collected (mg)	Date of sample collection
ES 1	5	1000	31.9	16/04/91
ES 2	5	2000	28.2	16/04/91
ES 3	5	3000	39.2	16/04/91
ES 4	5	4000	111.2	16/04/91
ES 5	30	1000	17.7	01/10/92
ES 6	30	1000	LOST	01/10/92
ES 7	30	2000	23.0	01/10/92
ES 8	30	3000	138.5	01/10/92
ES 9	30	4000	103.2	09/10/92
ES 10	55	1000	67.4	09/10/92
ES 11	55	2000	138.0	09/10/92
ES 12	55	3000	136.2	09/10/92
ES 13	55	4000	LOST	12/10/92
ES 14	80	1000	38.7	12/10/92
ES 15	80	2000	151.7	12/10/92
ES 16	80	3000	260.0	12/10/92
ES 17	5	1000	20.5	21/10/92
ES 18	55	4000	79.2	21/10/92
ES 19	80	4000	235.5	21/10/92

(ES5 and ES6). However ES6 became contaminated during workup and was discarded. Another sample that was lost was ES13, which formed an unrecoverable emulsion during the final stages of rotary evaporation. Consequently a replicate sample was collected (ES18). To assess interday variation in emissions, sample ES17 was collected under the same conditions as those of ES1, over a year later. The final masses of the samples differ by approximately 10 mg. However, ES17 has not yet been tested in aberration assays. The largest mass collected was for ES19 at the most extreme engine conditions tested. This mass was collected after only one minute of sampling. The sample time was reduced due to the engine overheating at these conditions of speed and load. When all solvent was removed and samples achieved a final mass, stock solutions were prepared in either DMSO or DCM. Samples ES1, and ES2, were dissolved in DMSO to a final concentration of 10 mg/ml. All other samples of mass less than 30 mg were dissolved in DMSO to a concentration of 100 mg/ml. The remaining samples were dissolved in DCM at a concentration of 100 mg/ml.

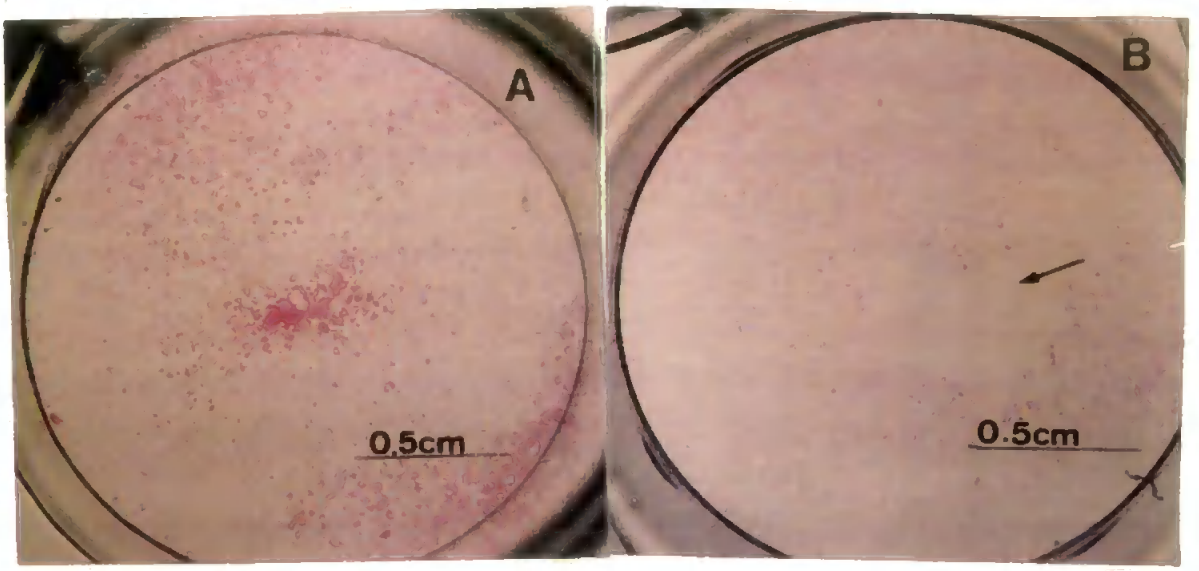
3.4. Results of cytotoxicity assays.

Toxicity assays were performed on all samples collected. An initial method used, of determining the plating efficiency of cells was found to be susceptible to fungal contamination, and so was discontinued in favour of the viability assay (described in section 2.5.1). The viability assay was also easier to score, gave results after a shorter timespan (*ie.* three days rather than seven), and was less prone to contamination. The inoculation of 10 μl aliquots of emission samples dissolved in DMSO, did result in a localised cytotoxic effect at the point of sample introduction. This localised toxicity is illustrated in figure 24 (B), and resulted in a lack of cells directly at the point of inoculation (arrow), which was presumably due to osmotic effects or direct toxicity of the DMSO. It also proved difficult to calibrate the viability assay regarding estimates of the number of viable cells per absorbance unit at 540 nm. This was because the extraction buffer (50% ethanol in 0.1M NaH_2PO_4) prevented the removal of the cells with trypsin, and mechanical removal of cells by scraping was difficult in the small culture wells.

The toxicity of the solvent was illustrated in the viability assay of ES1 (figure 25) in which increasing

volumes of the sample solution were added, and where the control (0μg/ml) culture was inoculated with the same volume of DMSO as was introduced in the 200μg/ml culture.

Figure 24: The effect of direct inoculation of DMSO on CHO-G6 cells, in (A) an untreated culture, and (B) a culture following the introduction of 10μl of DMSO, showing localised cytotoxicity (arrow).



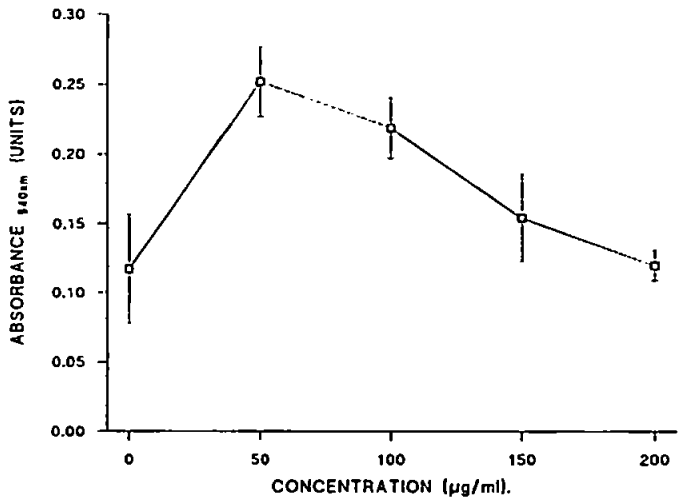
The increase in absorbance from cultures exposed to intermediate sample concentrations was a result of the reduced volume of solvent used in these cultures. From this result it was obvious that solvent volume at each sample concentration should be kept constant, and that the overall volume of solvent added to cultures should be as small as possible. On this basis the concentrations of stock solutions of emission samples was increased from 10 to 100 mg/ml to allow a smaller sample inoculum (80μl) to be used..

From figure 25, the effect of ES1 on the viability of cells was found to be indistinguishable from the effects of the solvent. Cultures exposed to both the solvent control and the highest sample concentration both had mean absorbance values of approximately 0.125 units. This lack of effect suggested that ES1 could have been tested at higher concentration, although this was difficult because of the limitations of the volumes of solvent required. The stock solution could not be concentrated due to the high boiling point of DMSO (ca. 80 °C), which would have also led to the loss of volatile components of the sample. The results of viability assays of other samples are included in appendix 1.

All samples excluding ES1 and ES2 showed evidence of toxicity (reduced cell viability) at concentrations

of less than 100 μg/ml. The majority of the samples collected in 1992 (ES5-ES19) were toxic at concentrations of between 25 and 50 μg/ml. This feature of the samples limited the effective dose range available for testing in subsequent aberration assays.

Figure 25: Reduction in neutral red uptake in CHO cells following exposure to increasing concentrations of ES1 (1000 rpm @ 5 Nm load).

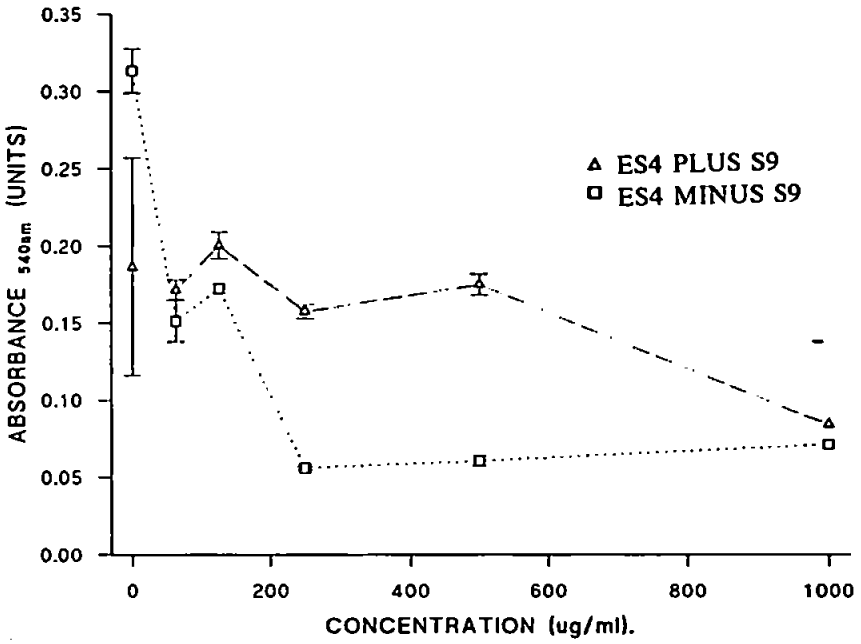


A viability assay of ES4 in which metabolic activation was also used (figure 26), showed that ES4 was toxic at concentrations in excess of 200 μg/ml (at which absorbance values fell to 0.05 units). However, the shorter exposure time (2 hours) encountered in the metabolically activated cultures allowed much higher sample concentrations (500 μg/ml) to be tolerated with little reduction in the neutral red uptake over the comparative controls. The presence of S9 mix did however increase the variation between control cultures (0.11 to 0.26 units), and also reduced the mean value of the control as compared to the non S9 control from 0.32 units to 0.17 units. The neutral red absorbance values in all cultures with metabolic activation remained between 0.15 and 0.20 units, decreasing to background levels (0.06 units) at a concentration of 1 mg/ml.

Due to the marked differences in toxicity observed among the first series of emission samples (ES1 to ES4) which were toxic of concentrations of between 100 and 200 μg/ml, and those of the second series that were mostly toxic at concentrations of approximately 50 μg/ml, it was hypothesised that the extreme toxicity

observed may be due to residues of solvents in the samples not removed after blowdown.

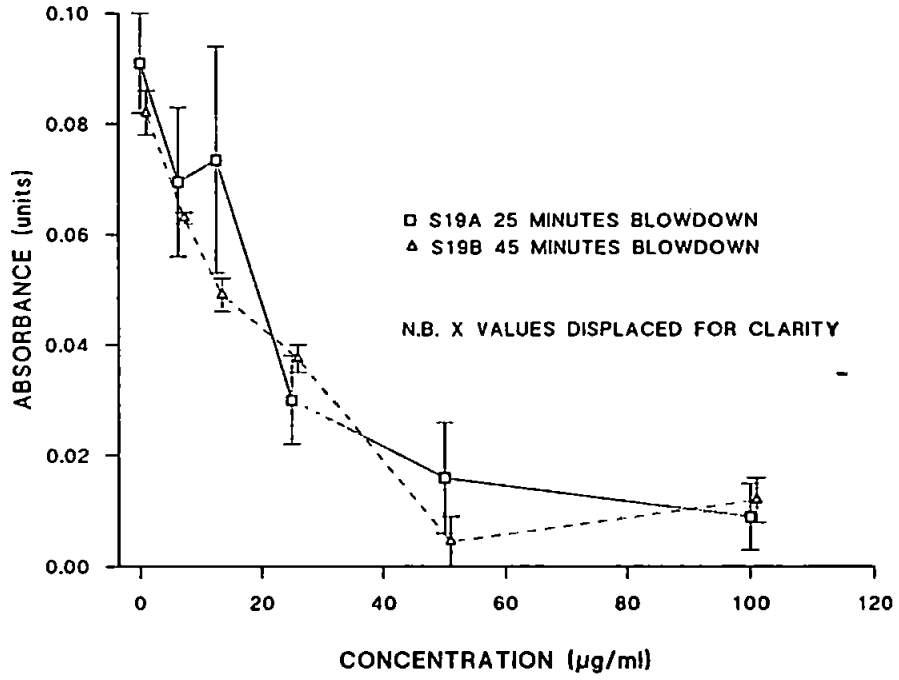
Figure 26: Reduction in neutral red uptake of viable cells following exposure to increasing concentration of ES4 (4000 rpm @ 5 Nm load) both in the presence and absence of supplementary metabolic activation.



These residues may have been absent from samples ES 1 and 2, due to the differing method of blowdown employed for these samples. To determine if this was possible, two aliquots of ES 19 were prepared from the stock solution and subjected to nitrogen blowdown at 60 °C for 20 minutes and 45 minutes respectively. The samples were then dissolved in DMSO and used in parallel toxicity assays. Results were then compared to determine if there were any significant differences between the two samples (figure 27).

From figure 27 it would appear that increased nitrogen blowdown has little effect upon the toxicity of ES19, both samples cause a rapid decline in the neutral red absorbance at 540 nm, indicating a reduction in cell viability. Both samples reduced the neutral red absorbance to negligible values at a concentration of 50 μg/ml, although there appears to be some variation between replicate wells at each concentration. From this evidence it would appear that no benefit would be achieved by increasing the blowdown time, and in fact any increase would probably enhance the loss of volatile components of the sample.

Figure 27: Cell viability as measured by neutral red uptake of CHO cells exposed to increasing concentrations of ES19 subjected to differing periods of Nitrogen blowdown.

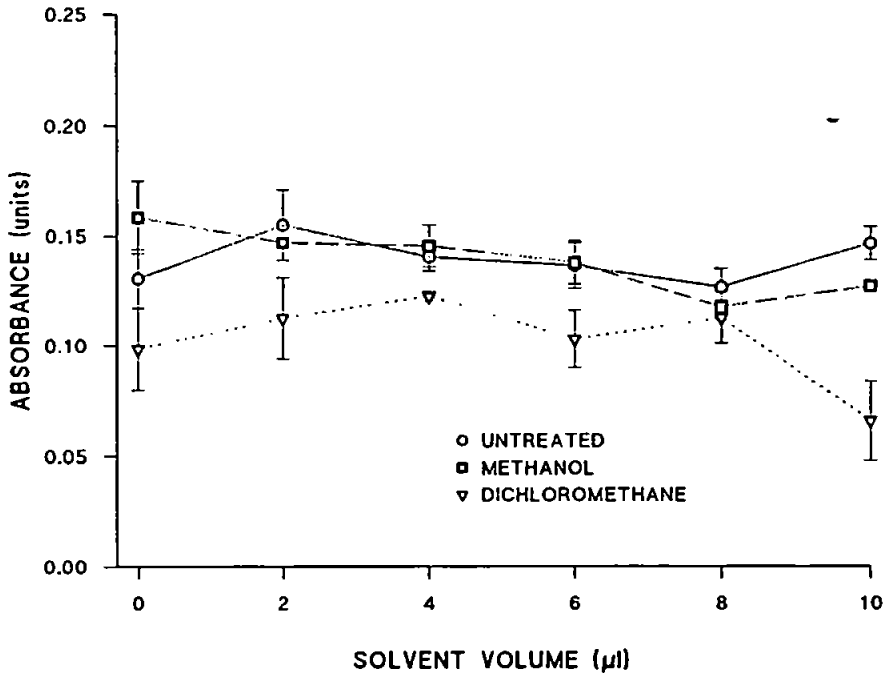


To further test the hypothesis that the increased toxicity was a consequence of inefficient removal of organic solvents rather than the sample itself, aliquots of the solvents used in sample collection and preparation (*ie.* DCM and methanol), were also tested in toxicity assays to determine their individual effects (figure 28)

Duplicate wells were inoculated with increasing volumes of the test solvent, and the absorbance of neutral red compared to duplicate wells that were untreated. From figure 28 it can be seen that the variation observed between all the untreated cultures was quite small indicating that similar numbers of cells were located in each well. The effect of methanol on the absorbance of neutral red as compared to untreated controls is minimal, although there appears to be a very slight reduction at volumes of 8 and 10 μl. In contrast DCM causes an obvious reduction in absorbance at volumes of 10 μl although absorbance values obtained at all solvent volumes were reduced compared to those of the untreated controls.. In either case the toxic effects occur at volumes far in excess of those that could possibly be retained in the test sample,

consequently it is assumed that the extreme toxic effects observed were the result of the inherent toxicity of the sample rather than contamination by solvent. The reduced toxicity of the initial samples (ES1-ES4) may therefore be the result of loss of volatile components due to prolonged sample blowdown.

Figure 28: Cell viability as determined by neutral red uptake by CHO-G6 cells exposed to increasing concentrations of methanol or dichloromethane.



3.5. Results of chromosome aberration assays.

3.5.1. Chromosome aberration assays of emission samples collected at the 5Nm engine load series.

Emission samples ES1, ES2 and ES3 (collected at 1000, 2000, and 3000 rpm respectively), were all tested before the availability of a suitable S9 fraction. Consequently, there was then insufficient quantities of sample remaining to retest these samples with metabolic activation. Results of preliminary cytotoxicity tests for ES1 and ES2 (appendix 1) showed that there was no loss in cell viability at the maximum concentrations (200 μg/ml) tested. Both ES1 and ES2, when tested in aberration assays caused reductions in the mitotic indices of cultures at the highest concentrations. But at all sample concentrations sufficient numbers of cells were available for scoring.

ES1 had little effect on the mitotic cultures, with a mitotic rate of approximately 45 mitoses per 1000 cells (mptc) at a concentration of 200 μg compared to 60 cells per 1000 in solvent controls (figure 29). A dose related increase in the percentages of cells with aberrations was observed with a maximum increase from the control level of 15% to 21% at a concentration of 100 μg/ml (figure 29). The linear trend statistics obtained for cultures exposed to ES1 (table 12) showed significant values in both the total and simple aberration categories.

The effect of exposure to ES2 was even greater than that of ES1, the mitotic rates were reduced from $75^0/_{00}$ in controls to only $20^0/_{00}$ in cultures exposed to 200 μg/ml. There was also an observed dose related decrease in the mitotic rates at concentrations of greater than 10 μg/ml (figure 30). The percentage of cells with aberrations also reflected the decrease in mitotic rates, with increases from control values of 15% to 25% observed at 100 μg/ml and further to 40% at 200 μg/ml (figure 30). The linear trend statistics obtained for this dataset were highly significant ($P < 0.01$) for all three damage categories (table 13).

The high levels of damaged cells observed in the solvent control cultures of these two samples may detract from the significance of the dose responses obtained, however the levels are reproducible between the two experiments, both having mean values of approximately 15% cells with aberrations. The lack of any significant effects observed by concentrations as low as 1 μg/ml in the ES1 assays, led to the exclusion of

sample concentrations of less than 10μg/ml in subsequent assays.

Figure 29: Mean numbers of CHO-PD2 cells with "total" aberrations, and mitotic indices of replicate cultures after exposure to diesel emission sample ES1 (collected at 1000 rpm @ 5Nm load).

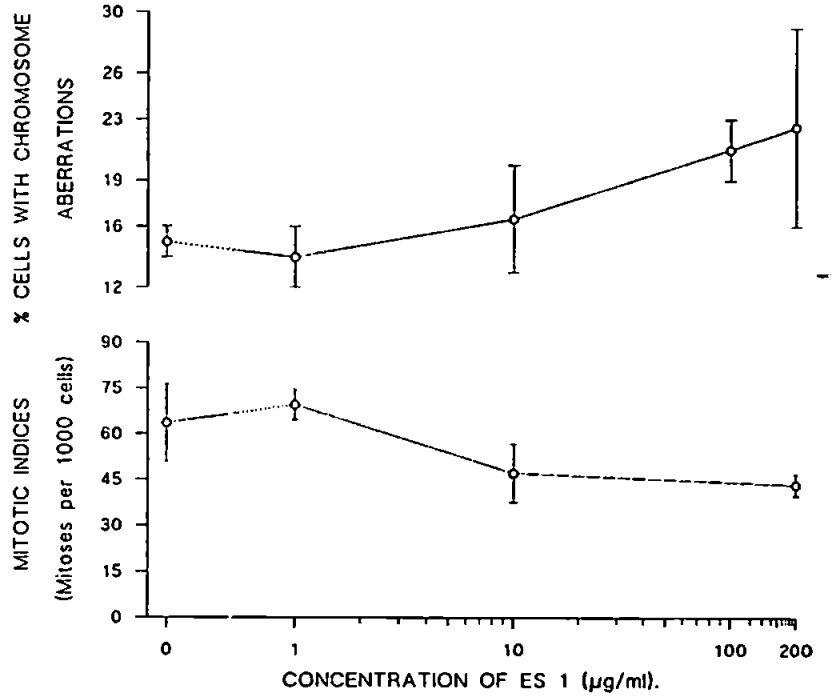


Table 12: Chromosome aberrations observed in CHO-PD2 cells exposed to diesel emission sample ES1 (collected at 1000 rpm @ 5Nm load), combined values from duplicate cultures.

Concentration (μg/ml)	Number of cells scored	Aberration category		
		Total	Simple	Complex
0.0	200	30	21	9
1.0	200	28	21	7
10.0	200	33	19	14
100	200	42	33	9
200	200	45	33	12
Linear trend statistic		2.473	2.421	0.798
Probability		6.8×10^{-3}	8.0×10^{-3}	2.2×10^{-1}

N.B.
Values of replicate cultures at each sample concentration were combined.
"Aberration category" refers to the numbers of cells scored containing damage from each of the three categories described by Galloway *et al.* (1985, see section 2.6.2).

Figure 30: Mean percentages of CHO-PD2 cells with "total" aberrations, and mitotic indices of replicate cultures exposed to diesel emission sample ES2 (collected at 2000 rpm at 5 Nm engine load).

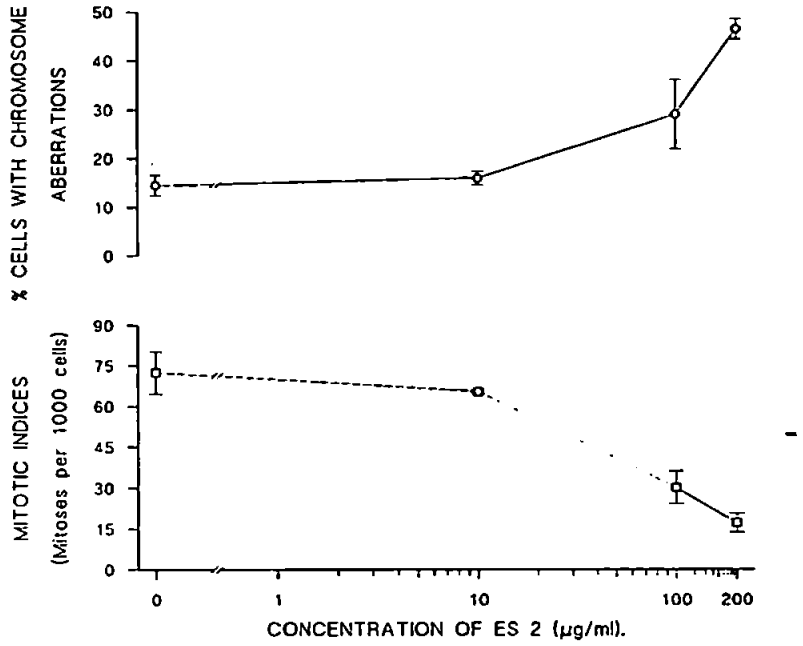


Table 13: Chromosome aberrations and observed in CHO-PD2 cells exposed to diesel emission sample ES2 (collected at 2000 rpm @ 5 Nm load) combined values from duplicate cultures.

Concentration	Numbers of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	200	29	24	5
10	200	32	30	2
100	200	49	49	9
200	200	93	71	22
Linear trend statistic		7.460	5.928	3.982
Probability		6.8×10^{-14}	1.8×10^{-9}	4.8×10^{-5}

In contrast to ES1 and ES2, ES3 (figure 31) was the first sample to be tested with the clonal G6 cell line. consequently the control levels of damage were reduced to approximately 5-10% as compared to the 10-20% observed with the previous samples. The preliminary toxicity assays (appendix 1) showed that this ES3 did reduce cell viability at concentrations of 200 μg/ml. Subsequent testing of this sample in aberration assays resulted in insufficient cells for analysis at 200 μg/ml. From figure 31, a reduction in the mitotic rate from $50^0/_{00}$ in controls to $40^0/_{00}$ at a concentrations of 10 μg/ml occurs. The mitotic rate was further reduced to half that of control values at 100 μg/ml. Due to the failure to score any metaphase cells at 200 μg/ml, statistical analysis of only three sample points should be considered with caution. However linear trend statistics were calculated for the available data (table 14). Both increases in "total" and "simple" aberrations were significant ($P < 0.01$) although the increase in ""complex"" aberrations was not ($P > 0.05$).

The final sample in this engine series (ES4), was also assayed at the time of the other samples (ES1, ES2, & ES3). However, poor slide preparation and a lack of suitable mitotic figures for analysis, meant that this sample was not scored. Consequently a repeat experiment was performed on the remaining sample both with and without metabolic activation. This assay was limited by the small sample mass available and the highest achievable concentration was only 54 μg/ml (figure 32). Using these small sample concentrations, no effects were observed on either the numbers of cells with aberrations or on the mitotic rates of treated cultures. Linear trend statistics (table 15) reflect the lack of activity. Exposure to the positive controls (MNNG and CP, Appendix 2) both induced approximately twice the numbers of damaged cells as observed in solvent control cultures, confirming that the lack of activity of ES4 was a consequence of the sample rather than the test system.

In summary, the emission samples collected at the 5Nm engine load showed increasing cytotoxicity with increasing engine load as determined from viability assays and mitotic indices. All samples with exception of ES4 induced chromosome aberrations in a dose related manner. The result of ES4 (4000 rpm) was inconclusive due to the small sample mass available for the repeat testing.

Figure 31: Mean percentages of CHO-G6 cells with "total" aberrations, and mitotic indices of replicate cultures exposed to diesel emission samples ES3 (collected at 3000 rpm at 5 Nm load).

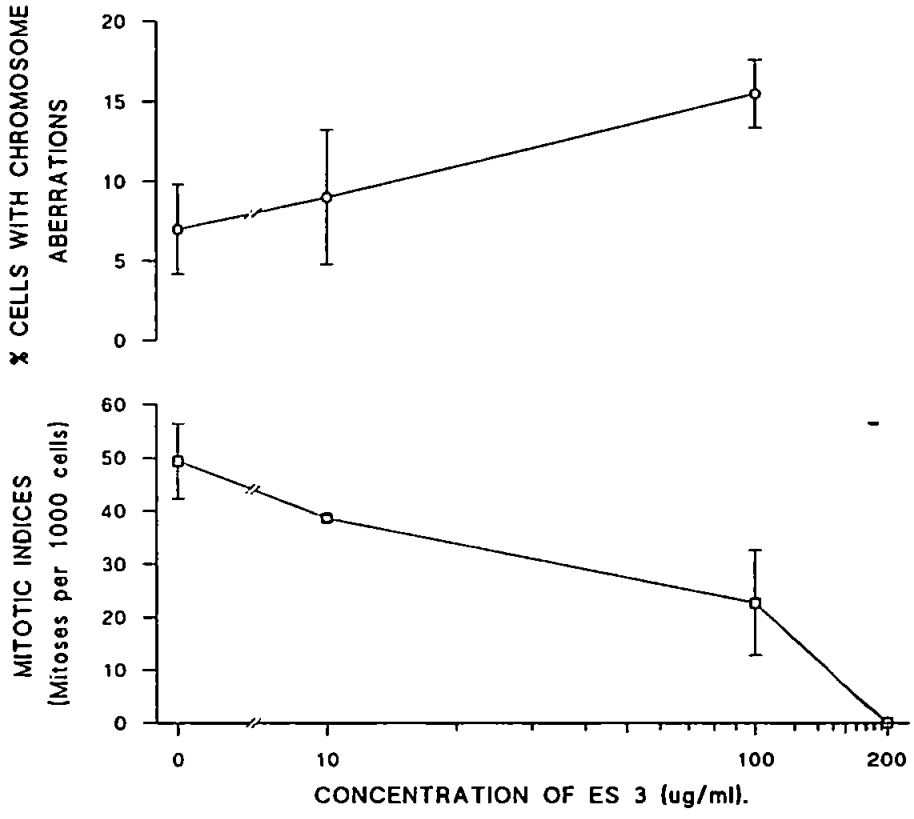


Table 14: Chromosome aberration observed in CHO-G6 cells following exposure to diesel emission sample ES3 (collected at 3000 rpm @ 5 Nm load), combined values from duplicates cultures.

Sample concentration	Numbers of cells	Abberation category		
(μg/ml)	scored	Total	Simple	Complex
0	200	14	10	4
10	200	18	14	4
100	200	32	24	8
200	Cytotoxic	-	-	-
Linear trend statistic		2.916	2.580	1.241
Probability		1.8×10^{-3}	4.9×10^{-3}	0.111

Figure 32: Mean percentages of CHO-G6 cells with "total" aberrations in replicate cultures exposed to diesel emission sample ES4 (collected at 4000 rpm @ 5Nm load), both with and without metabolic activation.

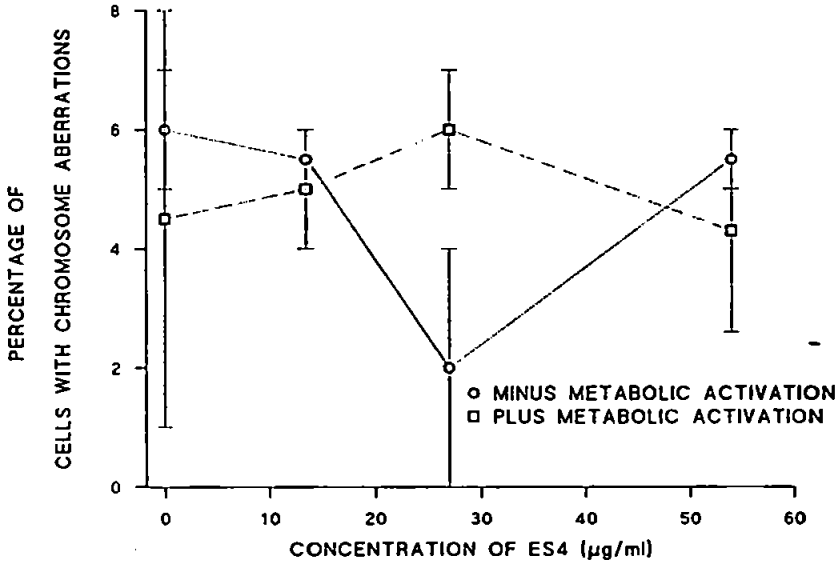


Table 15: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission samples ES4 (collected at 4000 rpm @ 5 Nm load) both with and without supplementary metabolic activation. Showing combined values from duplicate cultures.

Sample concentration	Number of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
ES 4 minus MA				
0	200	12	10	2
13.5	130	11	9	2
27	200	4	4	0
54	200	11	10	1
Linear trend		-0.457	-0.141	-0.875
Probability		6.7×10^{-1}	5.6×10^{-1}	8.1×10^{-1}
ES 4 plus MA				
0	200	9	8	1
13.5	200	10	9	1
27	200	12	11	1
54	176	8	8	0
Linear trend		0.195	0.413	-0.714
Probability		4.3×10^{-1}	3.4×101^{-1}	7.9×10^{-1}

3.5.2. Results of assays of samples collected at 30 Nm engine load.

ES5 was the first sample of the second series to be assayed. One feature of the second series of samples was their increased cytotoxicity as demonstrated by cell viability assays. Due to smaller dose range available for testing, and the lack of effect observed previously with sample concentrations of less than 10 μg/ml, the sample concentrations used in this assay were equally spaced rather than logarithmically spaced as in other assays.

ES5 (1000 rpm) was tested up to maximum concentration of 125 μg/ml in aberration assays, which was estimated to be the maximum tolerated limit from viability assays (apppendix 1). Exposure of cells to ES5 without metabolic activation resulted in a bell shaped dose response (figure 33), with the highest percentage of cells with aberrations (12.5%) observed at a concentration of 75 μg/ml. In all replicate cultures of each sample concentration there was little variation in mitotic rates (figure 33). There was a dose related decrease in the mitotic rates from control values for approximately 95mptc to approximately 50mptc at 50 μg/ml. There was a further decrease in rates to 40% at 100 μg/ml before reaching only 25 at 125 μg/ml.

Linear trend statistics (table 16) were found to be significant ($P<0.05$) for total number of cells with aberrations and highly significant ($P<0.01$) for cells containing complex aberrations. The reduction in mitotic rates observed at concentration of greater than 75 /μg/ml were found to limit the numbers of cells available for analysis at these concentrations.

In contrast, exposure of cells to ES5 with metabolic activation had no consistent effect, either on percentages of cells with total aberrations or on mitotic indices (figure 34). Both remained at control levels of approximately 8% cells with aberrations, and 55 mptc. There was some considerable variation in the mitotic indices of the replicate cultures tested at a concentration of 50 μg/ml which varied from 45mptc to 105mptc. This did not appear to affect the number of cells available for scoring or the number of aberrations observed (table 17).

Figure 33: Mean percentages of CHO-G6 cells with "total" aberrations, and mitotic indices from replicate cultures exposed to diesel emission sample ES5 (collected at 1000 rpm @ 30 Nm load).

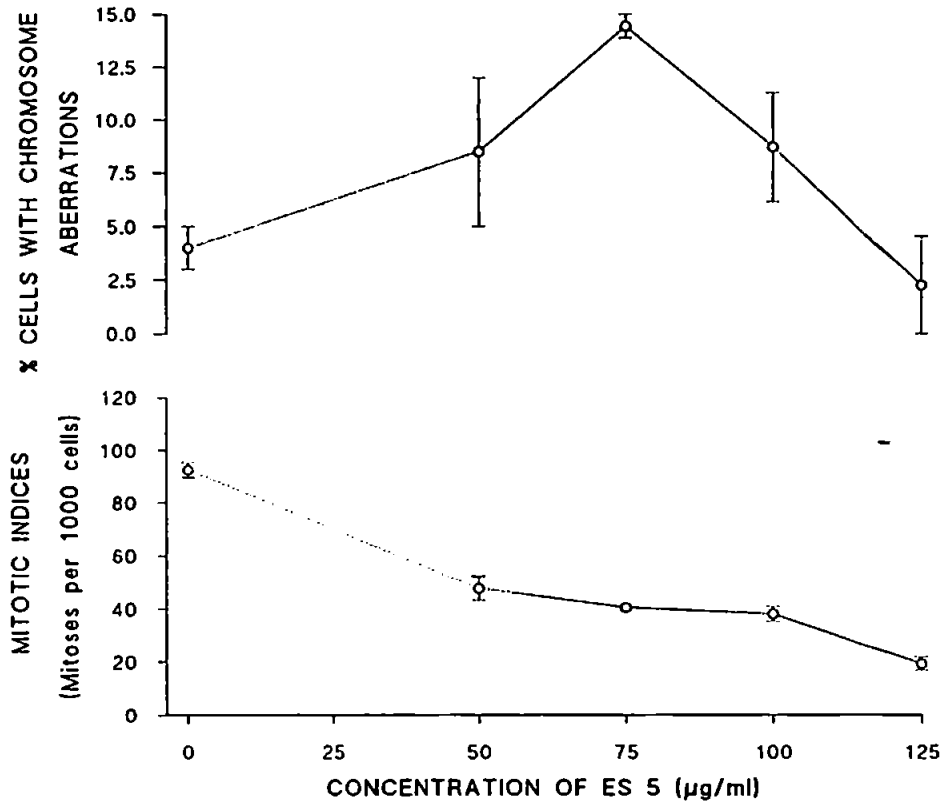


Table 16: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES5 (collected at 1000 rpm @ 30 Nm load). Combined values from duplicate cultures.

Sample concentration	Number of cells	Aberration Category		
(µg/ml)	scored	Total	Simple	Complex
0	200	8	8	0
50	200	17	16	1
75	172	25	20	5
100	127	11	9	6
125	33	1	1	0
Linear trend		1.847	1.241	3.033
Probability		3.34×10^{-2}	0.108	1.23×10^{-3}

Figure 34: Mean percentages of CHO-G6 cells with total aberrations, and mitotic indices of replicate cultures exposed to diesel emission sample ES5 (collected at 1000 rpm @ 30 Nm load) with supplementary metabolic activation.

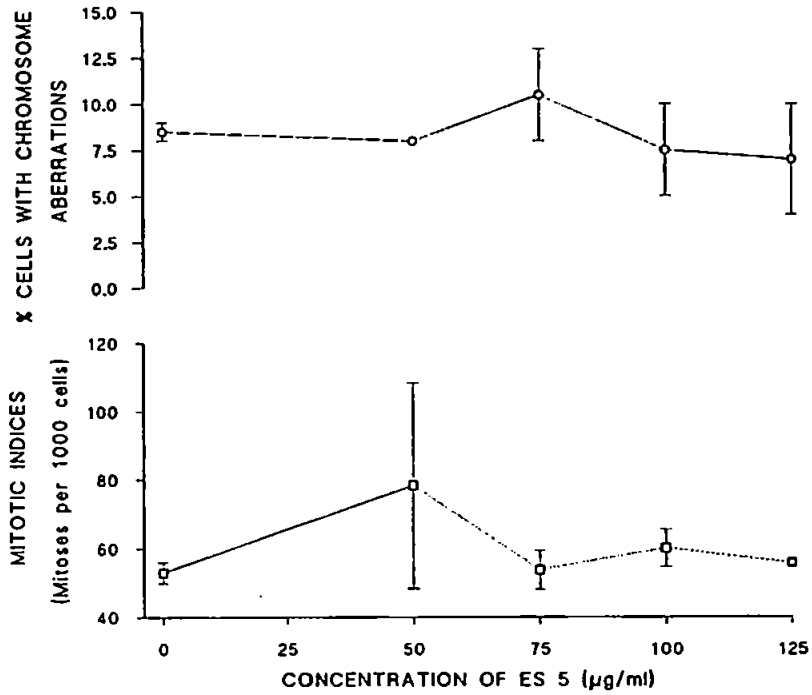


Table 17: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES5 (collected at 1000 rpm @ 5 Nm load), with supplementary metabolic activation. Combined values from duplicate cultures.

Sample concentration	Number of cells	Aberration Category		
(µg/ml)	Scored	Total	Simple	Complex
0	200	17	15	2
50	200	16	12	4
75	200	21	18	3
100	200	15	11	4
125	200	14	10	4
Linear trend		-0.471	-0.875	0.676
Probability		0.684	0.809	0.253

ES9 represents the high speed (4000 rpm) sample collected in this engine series. This sample was found to be extremely cytotoxic at concentrations in excess of 50 μg/ml (appendix 1). A considerable variation was seen the mitotic indices of the control cultures in exposed to ES9 without metabolic activation (figure 35) which varied from approximately 20 to 160mptc. Again, little effect was observed in the numbers of aberrant cells due to this variation (figure 35). The mitotic rates decreased from a mean control value of approximately 90mptc to 40mptc at a concentration of 25 μg/ml before rising slightly to 60mptc at 50 μg/ml. These lower mitotic rates reduced the numbers of cells available for scoring at concentration 25 and 50 μg/ml. There was little effect upon the numbers of cells with aberrations and this reflected in the lower trend statistics (table 18).

Exposure of G6 cells to ES9 with metabolic activation (figure 36) resulted in a slight increase in the percentage of cells with aberrations from control values of 3.5% to 5% at concentrations of 100 μg/ml. There was little effect on the mitotic rates of cultures which remained at values of between 30 to 40 mptc at all sample concentrations. There was considerable variation between mitotic indices of replicates cultures exposed to concentrations of 50 and 100 μg/ml.

A significant linear trend ($P < 0.05$) was observed for simple aberrations, and again a lower number of cells was scored at the highest sample concentration (table 19).

Figure 35: Mean percentages of CHO-G6 cells with "total" aberrations and mitotic indices of replicate cultures exposed to diesel emission sample ES9 (collected at 4000 rpm @ 30 Nm load).

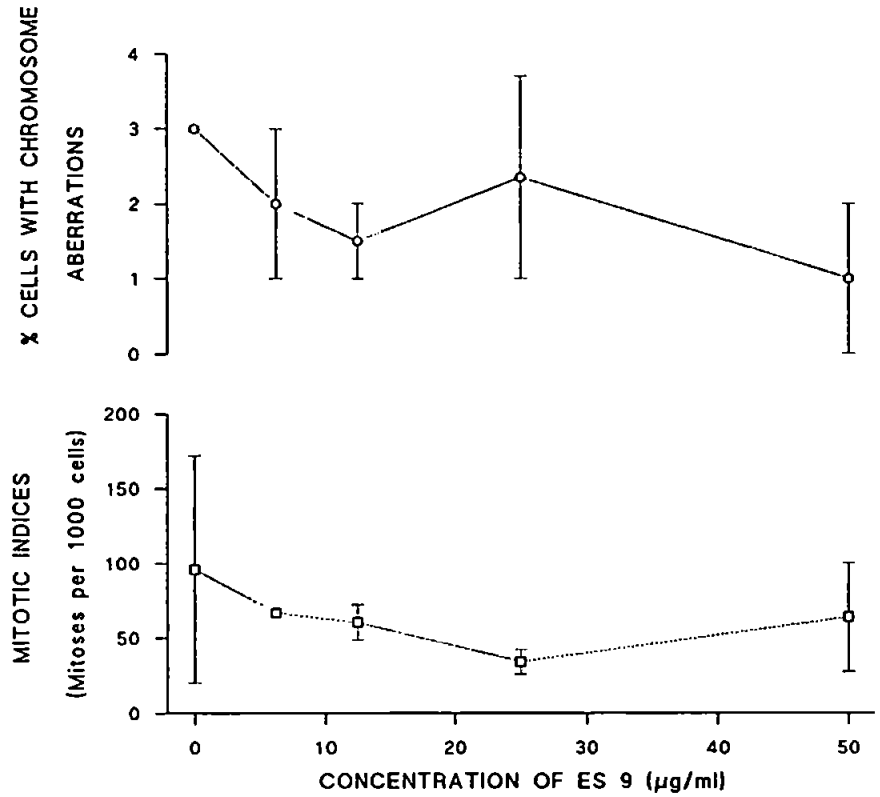


Table 18: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES (collected at 4000 rpm @ 5Nm load). Combined values from duplicate cultures.

Sample concentration	Numbers of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	200	6	2	2
6.25	200	4	1	2
12.5	200	3	2	1
25	181	4	3	1
50	119	2	1	0
Linear trend		-0.722	0.454	-0.853
Probability		0.765	0.328	0.803

Figure 36: Mean percentages of CHO-G6 cells with "total" aberrations and mitotic indices of replicate cultures exposed to diesel emission sample ES9 (collected at 4000 rpm @ 30 Nm load) with supplementary metabolic activation.

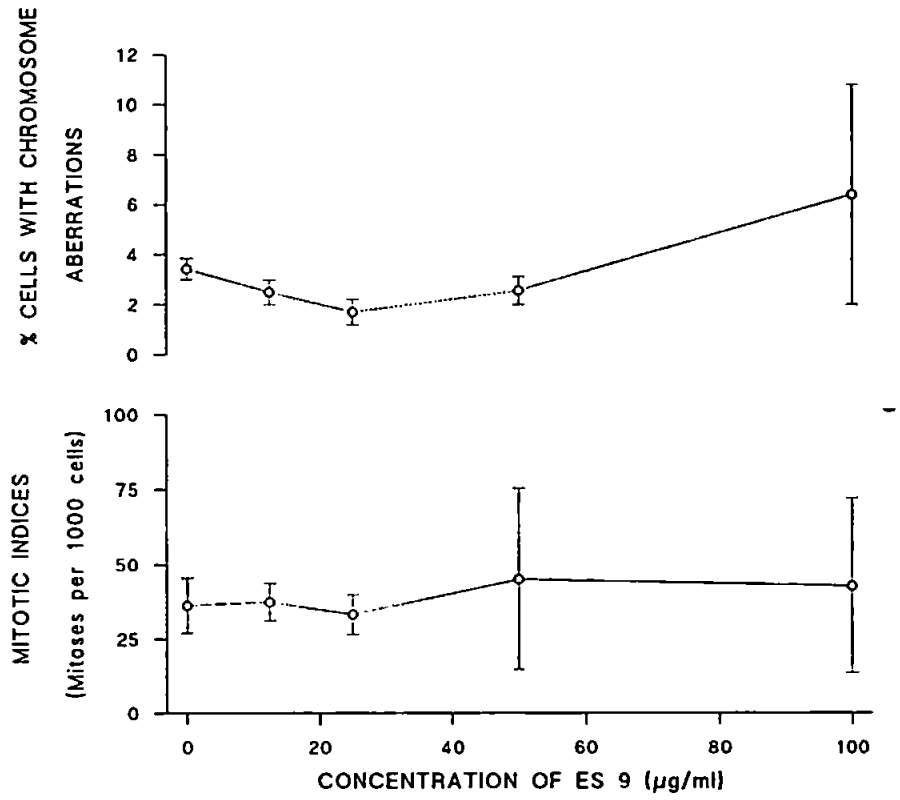


Table 19: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES9 (collected at 4000 rpm @ 30 Nm load) with supplementary metabolic activation. Combined values from duplicate cultures.

Sample concentration	Numbers of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	178	6	2	3
12.5	200	5	2	0
25	173	3	2	2
50	196	5	4	0
100	137	6	5	0
Linear trend		0.361	1.804	-2.460
Probability		0.359	3.62×10^{-2}	0.993

3.5.3. Assays of samples collected 55 Nm load.

Two emission samples collected at this engine load were tested in assays, these were ES10 (1000 rpm) and ES12 (3000 rpm). ES10 although cytotoxic at 50 μg/ml (Appendix 1), was found to induce a highly significant dose response ($p<0.001$) in both the "total" and "simple" damage categories (table 20). An increase in the percentages of cells with aberrations from mean solvent control values of 2% to a maximum of 10.5% at a concentration of 50 μg/ml (figure 37) was observed. The mitotic indices of exposed cultures declined almost linearly with increasing sample concentration, from mean control levels of approximately 80-85 mptc to 37 mptc at a concentration of 50 μg/ml (figure 37). From table 20, it can be seen that sufficient numbers of cells were scored from all cultures except those at the highest sample concentration. Highly significant ($P<0.01$) linear trends were obtained for both the total and simple aberration categories. The numbers of cells with complex aberrations reached a maximum of 7% at a concentration of 25 μg/ml before falling to control level (2%) at 50 μg/ml.

Exposure of cells to ES10 with metabolic activation had a similar effect (figure 38). A dose related increase in the percentage of cells with aberration was observed from mean control values of 5% to a maximum of 10% at a concentration of 50 μg/ml. There was also a slight increase in the mitotic rates of cultures from control values of approximately 30 mptc to 40 mptc at 50 μg/ml. Linear trend statistics (table 21) were again found to be significant ($P<0.05$) for both total and simple aberration categories, although at the higher concentrations (25 & 50 μg/ml) a reduced number of cells was scored.

In contrast to the results obtained with ES10. Exposure of cells to ES12 had little effect either with or without metabolic activation (figures 39 & 40). There were dose related decreases in the mitotic rates of exposed cultures from control values of approximately 70 mptc to 25 mptc at concentrations of 50 μg/ml (without metabolic activation) and 100 μg/ml (with metabolic activation). It can be seen from tables 22 and 23 that sufficient cells were scored for all sample concentrations except at the highest selected.

Figure 37: Mean percentages of CHO-G6 cells with "total aberrations" and mitotic indices of replicate cultures following exposure to diesel emission sample ES10 (collected at 1000 rpm @ 55 Nm load).

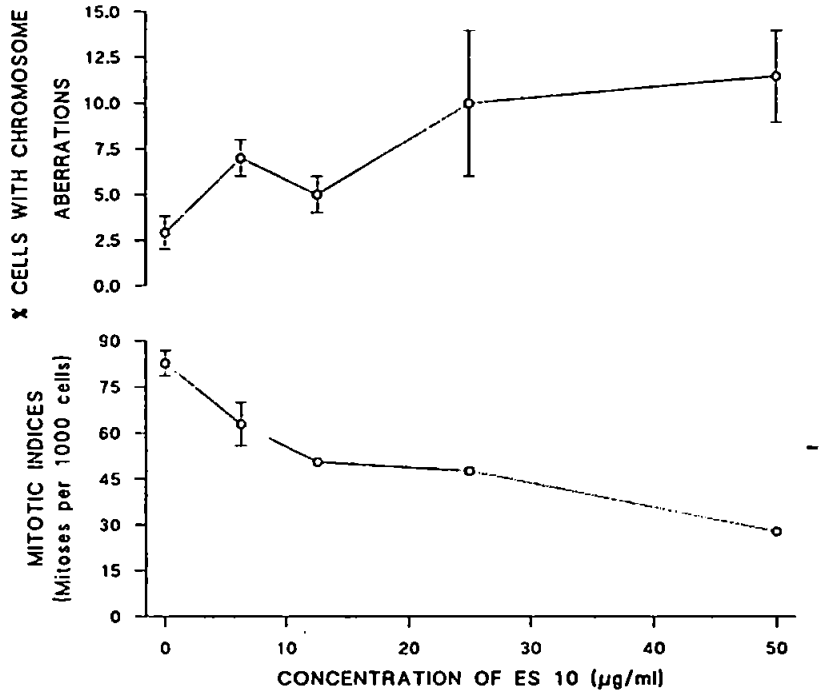


Table 20: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES10 (collected at 1000 rpm @ 55 Nm load). Combined values from duplicate cultures.

Sample concentration	Numbers of cells	Aberration Category		
(µg/ml)	scored	Total	Simple	Complex
0	205	6	4	2
6.25	200	14	13	1
12.5	200	10	6	4
25	200	20	13	7
50	178	21	18	2
Linear trend		3.509	3.073	1.082
Probability		2.33×10^{-4}	1.11×10^{-3}	0.141

Figure 38: Mean percentages of CHO-G6 cells with "total" aberrations, and mitotic indices of replicate cultures exposed to diesel emission sample ES 10 (collected at 1000 rpm @ 55 Nm load) with supplementary metabolic activation.

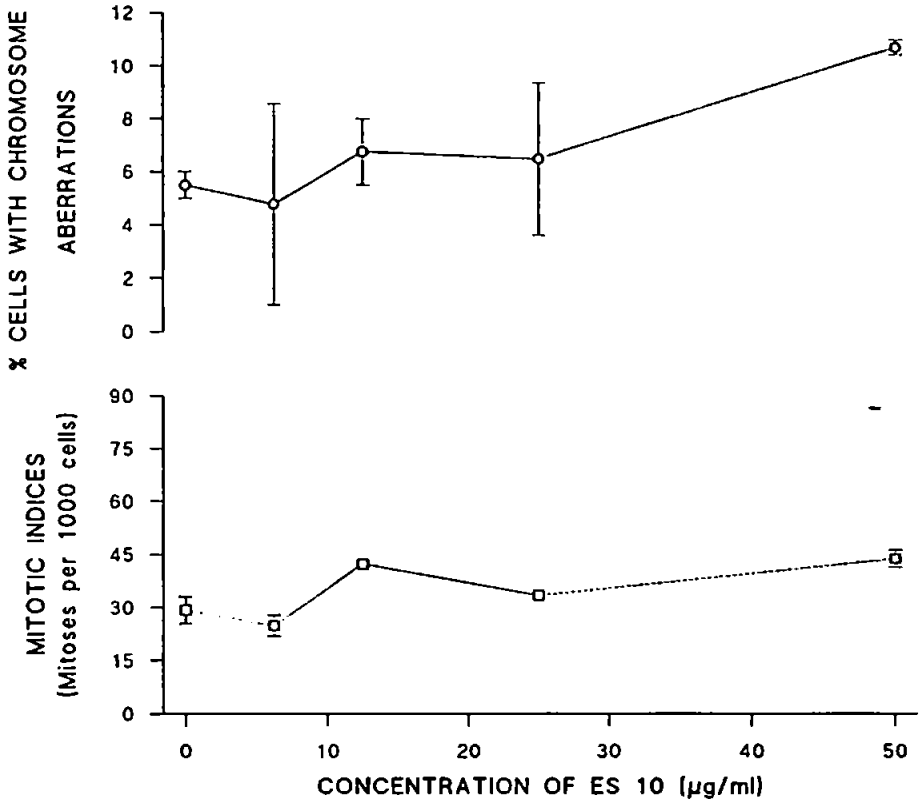


Table 21: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES10 (collected at 1000 rpm @ 55 Nm load) with metabolic activation. Combined values from duplicate cultures.

Sample concentration	Numbers of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	200	11	10	1
6.25	205	10	5	2
12.5	208	14	13	1
25	97	8	6	2
50	167	18	16	3
Linear trend		2.111	2.023	1.320
Probability		1.79×10^{-2}	2.18×10^{-2}	9.34×10^{-2}

Figure 39: Mean percentages of CHO-G6 cells with "total chromosome aberrations" aberrations, and mitotic indices of replicate cultures exposed to diesel emission sample ES12 (collected at 3000 rpm @ 55 Nm load)

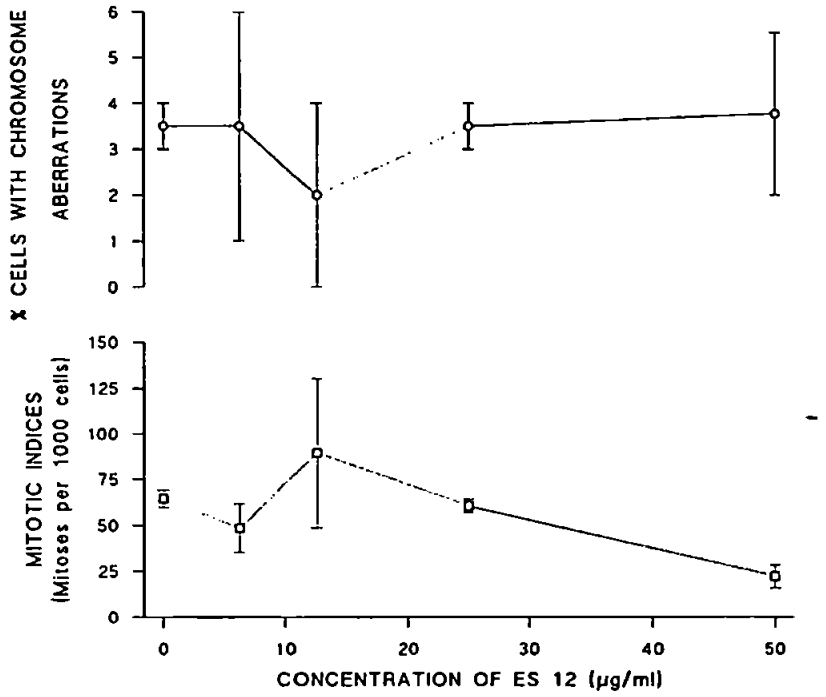


Table 22: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES12 (collected at 3000 rpm @ 55 Nm load). Combined values from duplicate cultures.

Sample concentration	Numbers of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	200	7	7	0
12.5	200	7	5	2
25	200	4	3	0
50	200	7	5	2
100	175	6	4	2
LINEAR TREND		-0.054	-0.707	1.265
Probability		0.539	0.760	0.104

Figure 40: Mean percentages of CHO-G6 cells with "total" chromosome aberrations, and mitotic indices of replicate cultures exposed to diesel emission sample ES12 (collected at 3000 rpm @ 55 Nm load) with supplementary metabolic activation.

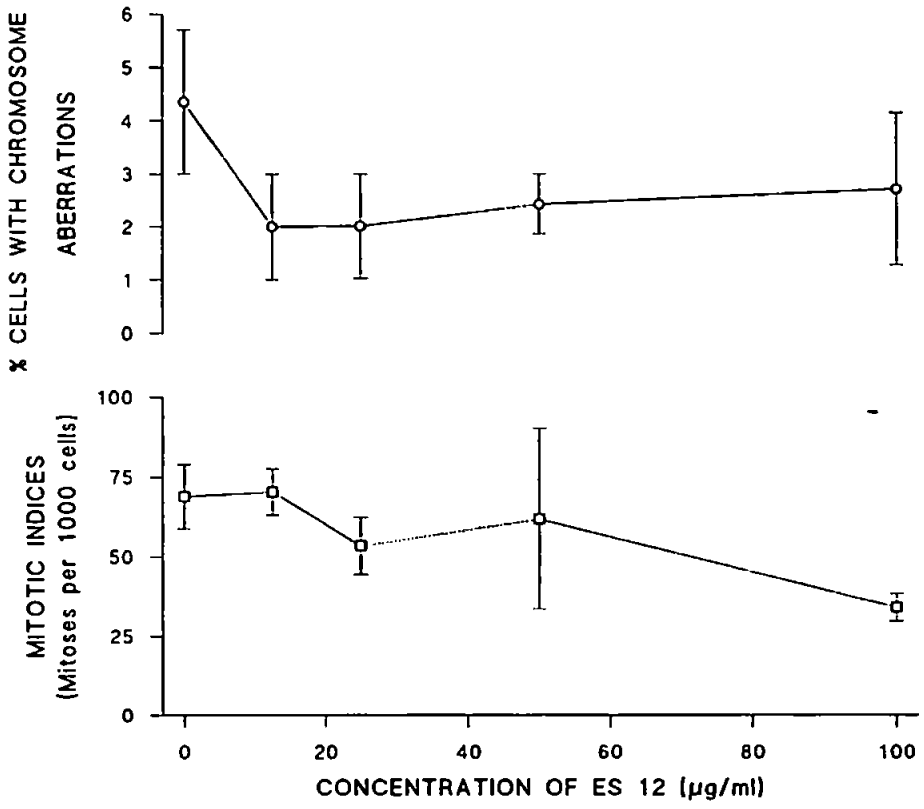


Table 23: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES12 (collected at 3000 rpm @ 55 Nm load) with metabolic activation. Combined values from duplicate cultures.

Sample concentration	Numbers of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	205	9	9	0
12.5	200	4	3	1
25	197	4	3	0
50	207	5	5	0
100	173	5	4	0
Linear trend		-0.759	-0.978	-0.675
Probability		0.776	0.836	0.750

3.5.4. Assays of the 80 Nm engine load series.

All four samples collected at this engine load were tested in both viability and aberration assays. All were found to be cytotoxic at concentrations of below 25-50 μg/ml in viability assays (appendix 1). Metaphase spreads from the aberrations assay of ES 16 (3000 rpm) were relatively few and of poor quality, hence this sample was considered unsuitable for scoring. An initial assay of ES19 (4000 rpm) was also performed but when scored was found to have a higher number of cells with aberration in control cultures than was expected (appendix 2, table 48). This sample was then retested using freshly resuscitated G6 cells.

Exposure of cells to ES14 without metabolic activation caused no increase in the percentage of cells with aberrations. Although there was a dose related decrease in the mitotic rates from mean values of 45 mptc in control cultures to approximately 10 mptc in cultures exposed to sample concentrations of 50 μg/ml (figure 41). The reduction in mitotic indices also affected the numbers of cells available for scoring which were reduced at concentrations of 25 and 50 μg/ml (table 24).

Exposure to ES14 with metabolic activation did cause an increase in the numbers of cells with aberrations. The mean percentage rose from control values of 4.5% to 7.5% at 25 μg/ml before falling to control levels at 50 μg/ml. There was a further increase in the numbers of cells with aberrations to approximately 9% at concentrations 100 μg/ml (figure 42). Mitotic indices were lower in control cultures at approximately 20 mptc which increased to a mean of 60mptc at a concentration of 50 μg/ml which corresponded to the decrease in the percentage of cells with aberrations at this concentration, before falling to control levels at 100 μg/ml. Linear trend statistics (Table 25) were found to be significant ($P<0.05$) for both total and complex aberration categories. Due to the reduced mitotic rates of most cultures there was again difficulty in scoring the required numbers of cells.

Exposure to ES 15 (2000 rpm) showed a small elevation in the percentage of cells with aberrations form a control level of 1.5% to maximum of 3 % (figure 43). Mitotic indices of control cultures were relatively high at approximately 60, and although increasing to a mean value of 125 at 6.25 μg/ml fell to a value of only 24 at the highest concentration (50 μg/ml). Linear trend statistics reflect the general lack of response

Figure 41: Mean percentages of CHO-G6 cells with "total" chromosome aberrations, and mitotic indices of replicate cultures exposed to diesel emission sample ES14 (collected at 1000 rpm @ 80 Nm load).

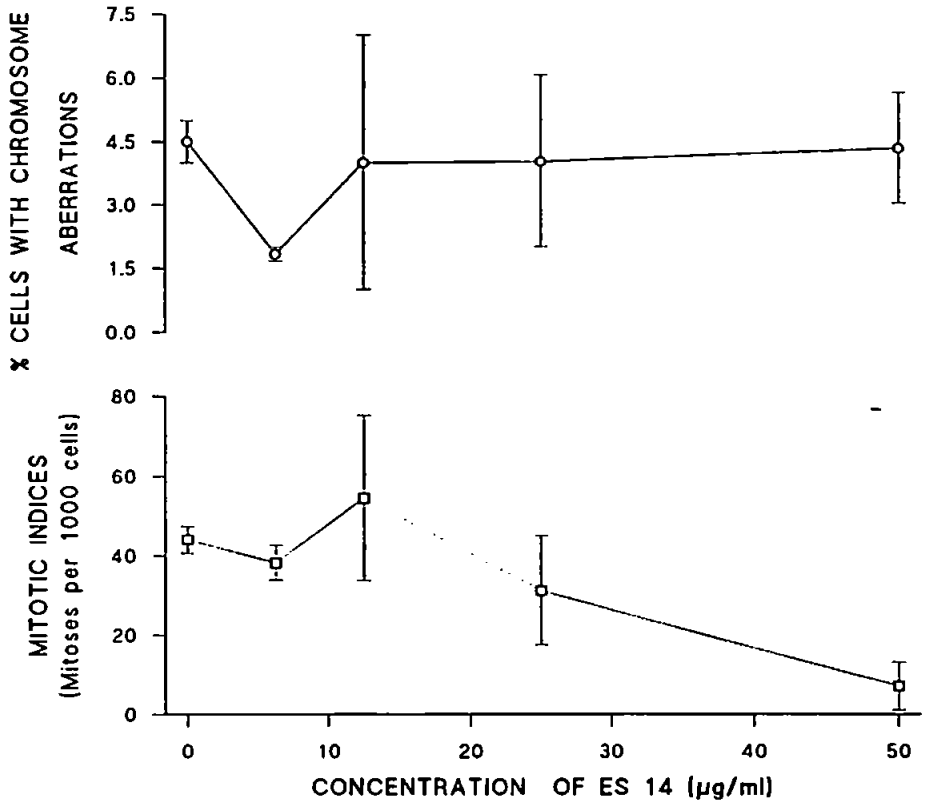


Table 24: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES14 (collected at 1000 rpm @ 80 Nm load). Combined values from duplicate cultures.

sample concentration	Number of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	200	9	6	0
6.25	109	2	2	0
12.5	200	8	7	0
25	166	6	5	1
50	139	7	5	1
Linear trend		-0.041	-0.208	1.026
Probability		0.512	0.523	0.845

Figure 42: Mean percentages of CHO-G6 cells with "total chromosome aberrations, and mitotic indices of cultures exposed to diesel emission sample ES14 (collected at 1000 rpm @ 80 Nm load) with supplementary metabolic activation.

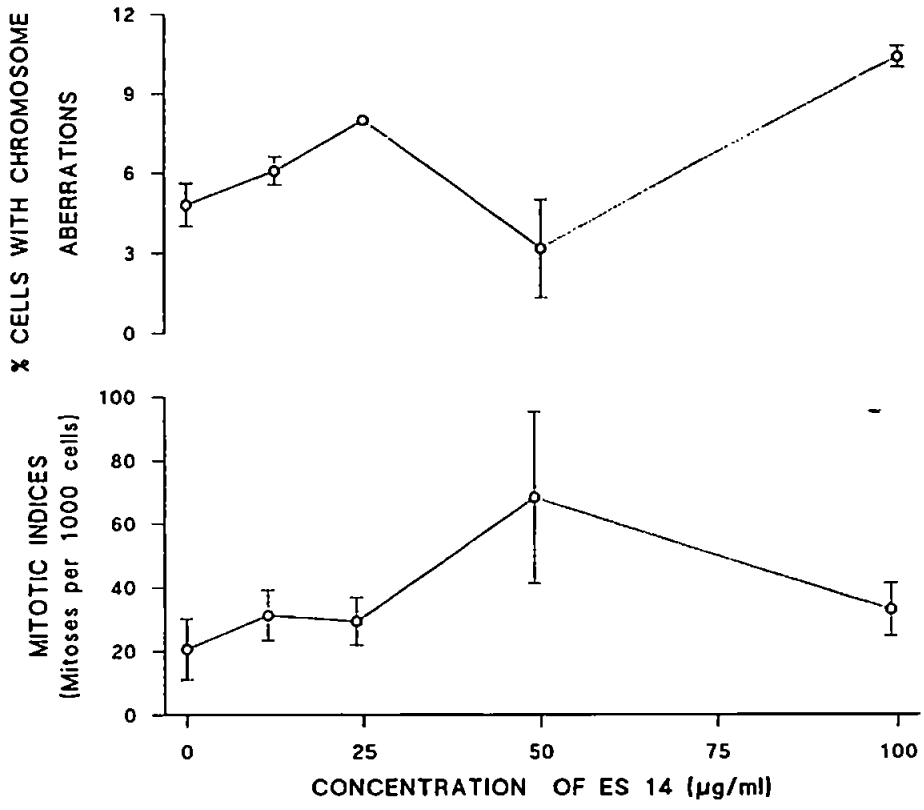


Table 25: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES14 (collected at 1000 rpm @ 80 Nm load) with metabolic activation. Combined values from duplicate cultures.

Sample concentration	Numbers of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	189	9	8	0
12.5	190	11	11	0
25	178	14	14	0
50	175	6	5	1
100	146	15	13	2
Linear trend		1.808	1.467	2.00
Probability		3.7×10^{-2}	7.7×10^{-2}	2.3×10^{-2}

Figure 43: Mean percentages of CHO-G6 cells with "total" chromosome aberrations, and mitotic indices from replicate cultures exposed to diesel emission sample ES15 (collected at 2000 rpm at 80 Nm load).

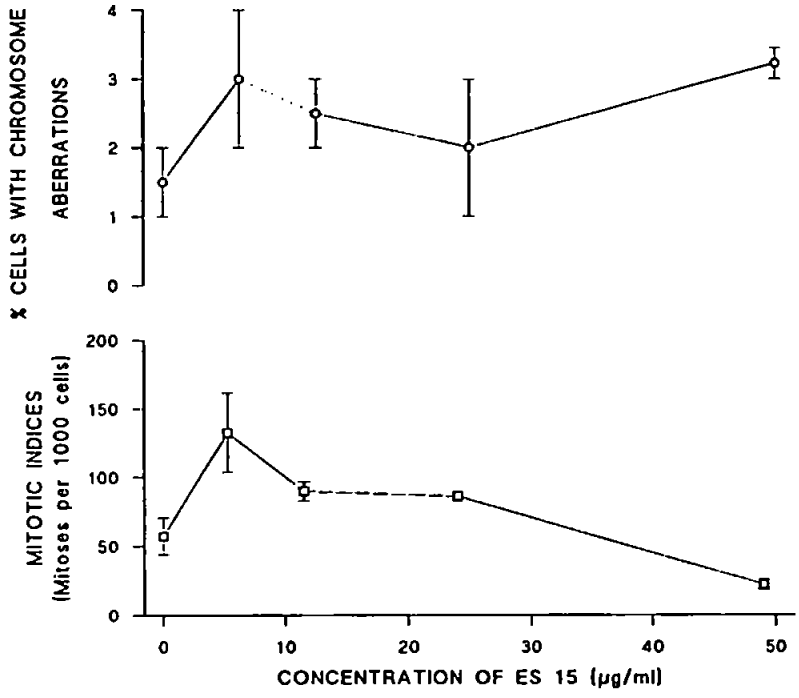


Table 26: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES15 (collected at 2000 rpm @ 55 Nm load). Combined values from duplicate cultures.

Sample concentration	Numbers of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0.0	200	3	3	0
6.25	200	6	4	0
12.5	200	5	5	0
25	200	4	3	0
50	158	5	4	0
Linear trend		0.614	0.448	0.000
Probability		0.272	0.174	0.500

and this is reflected in the low significance vales (table 26).

Exposure of cells to ES15 with metabolic activation again had little effect with an increase from control levels of 1% to a maximum of 3% both at concentrations of 12.5 and 100 μg/ml. The mitotic indices of cultures remained fairly constant at approximately 65mptc at all sample concentrations (figure 44). A significant linear trend ($P<0.01$) was obtained for cells with complex aberrations although this was based only on the observation of four cells with complex damage at 100 μg/ml (table 27).

The final sample collected (ES19) was found to be cytotoxic at a concentration of 50 μg/ml in the aberration assays and insufficient numbers of cells were recovered for analysis. the contents of one replicate were also lost (Appendix 2). The resulting linear trends calculated using the remaining data are of dubious value, but show a significant ($P<0.05$) increase in the number of cells with "simple" aberrations (table 28). Figure 45 illustrates the increase in aberrant cells with concentration up to 25 μg/ml, although there was little effect on the mitotic rates over this dose range.

Exposure of cells to ES19 with metabolic activation (figure 46) showed no increase in the numbers of aberrant cells were observed or in the mitotic indices of cultures, although there was considerable variation between replicate cultures at concentrations of 50 and 100 μg/ml. The reduced mitotic rates at 50 μg/ml reduced the numbers of cells available for scoring (table 29).

Figure 44: Mean percentages of CHO-G6 cells with "total" chromosome aberrations, and mitotic indices from replicate cultures exposed to diesel emission sample ES15 (collected at 2000 rpm @80 Nm load) with supplementary metabolic activation.

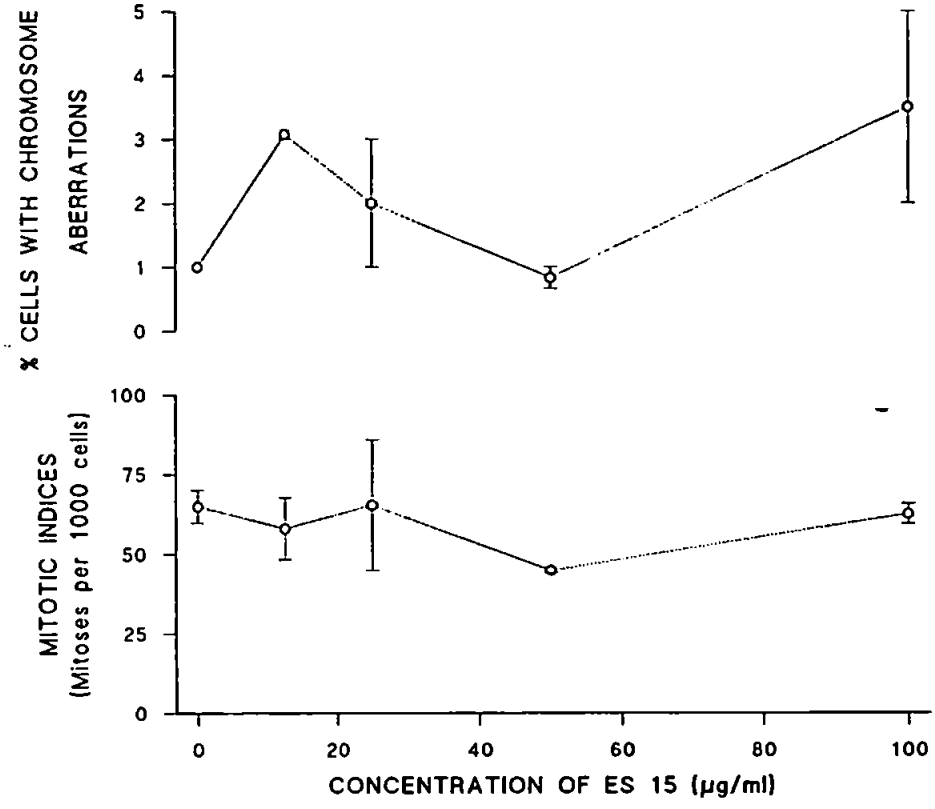


Table 27: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES15 (collected at 2000 rpm @ 80 Nm load). Combined values from duplicate cultures.

Sample concentration	Numbers of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	200	2	1	0
12.5	195	6	6	0
25	200	4	4	0
50	250	2	2	0
100	200	7	3	4
Linear trend		1.077	0.134	2.862
Probability		0.144	0.450	2.2×10^{-3}

Figure 45: Mean percentages of CHO-G6 cells with "total" chromosome aberrations, and mitotic indices of replicate cultures after exposure to diesel emission sample ES19 (collected at 4000 rpm @ 80 Nm load).

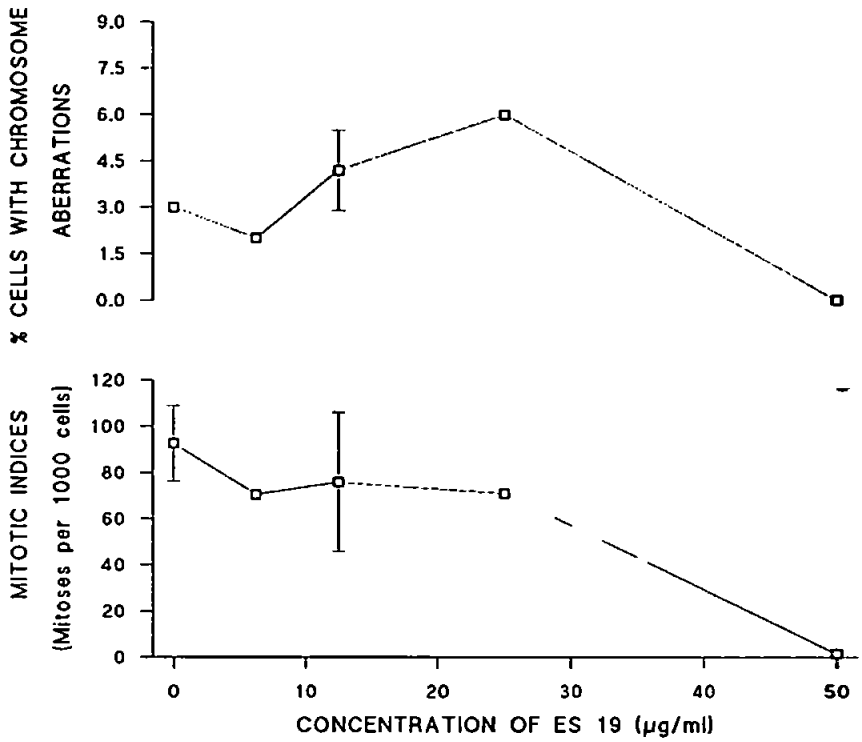


Table 28: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES19 (collected at 4000 rpm @ 80 Nm load). Combined values from duplicate cultures.

Sample concentration	Number of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	200	6	4	2
6.25	200	4	3	0
12.5	367	16	13	2
25	100	6	6	1
50	0	-	-	-
Linear trend		1.574	2.057	0.054
Probability		5.9×10^{-2}	2.0×10^{-2}	0.502

Figure 46: Mean percentages of CHO-G6 cell with "total" chromosome aberrations, and mitotic indices of replicate cultures exposed to diesel emission sample ES19 (collected at 4000 rpm 80 Nm load) with supplementary metabolic activation.

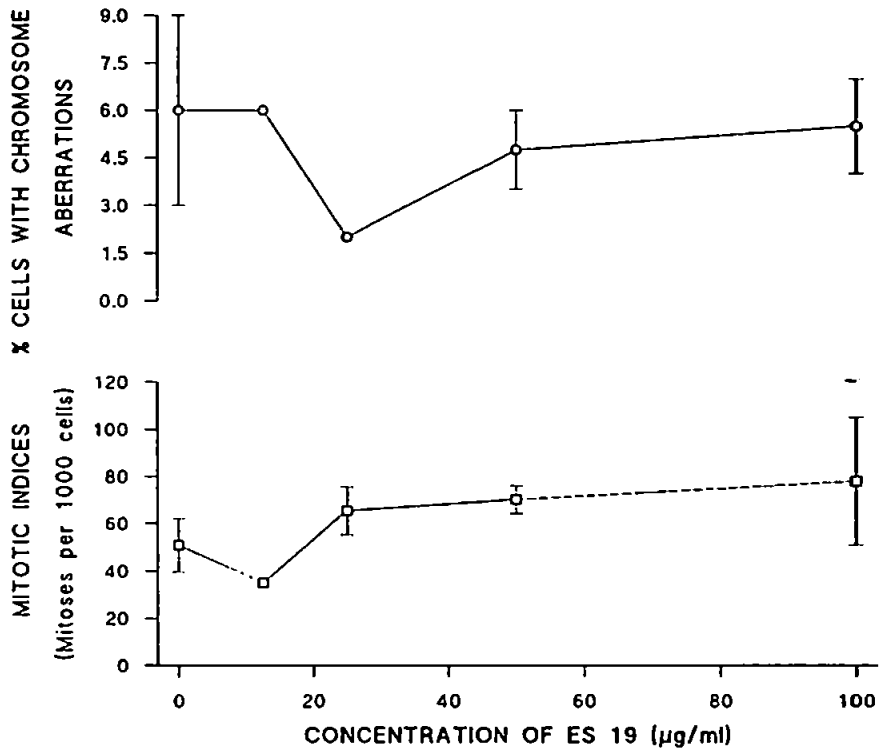


Table 29: Chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES19 (collected at 4000 rpm @ 80 Nm load) with metabolic activation. Combined values from duplicate cultures.

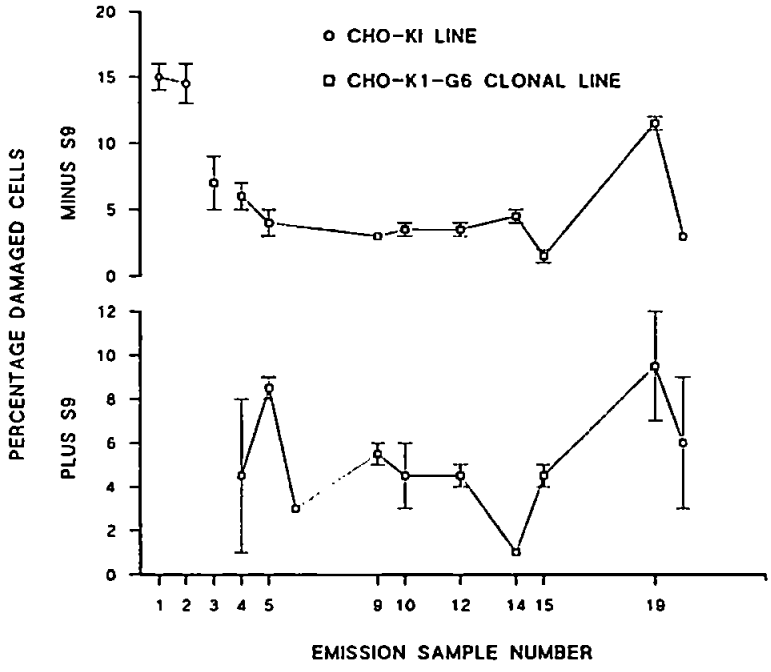
Sample concentration	Numbers of cells	Aberration category		
(µg/ml)	scored	Total	Simple	Complex
0	200	12	10	2
12.5	200	12	10	2
25	200	4	4	0
50	157	8	7	1
100	200	11	10	1
Linear trend		-0.0407	-0.129	-0.782
Probability		0.658	0.547	0.783

3.5.5. Comparison of the control data from chromosome aberration assays.

Despite the variation observed in the magnitude of the mutagenic responses to the various emission samples, a comparison of the solvent control data obtained from the whole series of experiments has shown that there was little variation between solvent control cultures in each experiment. This would suggest that the variable dose responses obtained were the result of the clastogenic potential of the samples, and that there was no difference in the responses to samples due to the day of testing. Figure 47 (below) shows the percentage of damaged cells in solvent control cultures from the series of experiments. The mean percentage of aberrant cells observed in solvent control cultures (excluding ES 1 and ES 2) was 4% with a standard deviation of 1.5. In cultures with metabolic activation the mean percentage of aberrant cells observed was slightly higher at 4.66% with greater variation between both intra- and inter experimental cultures emphasised by the higher standard deviation of 2.046.

The control values obtained with the PD2 cell line were approximately three tines those observed for the G6 clonal line. A large increase in the percentage of cells with aberrations was observed in cultures exposed to ES19, (approximately 10 and 9% with and without metabolic activation respectively). This doubling of the expected background suggested a problem with the cell line which was subsequently discarded. A repeat assay of ES19 was performed using a freshly resuscitated G6 cell culture, the result of which was a reduction to the expected levels of spontaneous damage (ie approximately 4% and 5% without and with metabolic activation respectively).

Figure 47: Intra-experimental variation in numbers of aberrant cells observed in solvent control cultures.



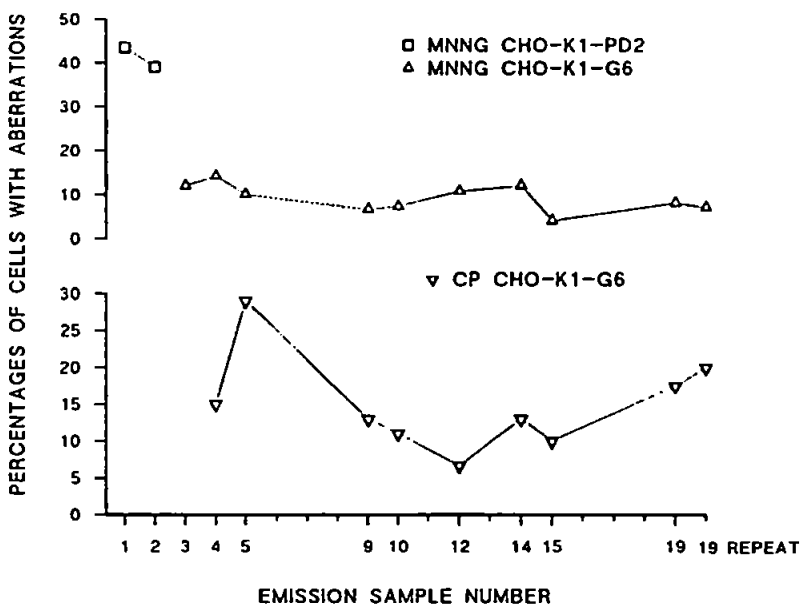
A comparison of the percentages of cells with aberrations in positive control cultures exposed to either 0.075 μg/ml MNNG of 12.5 μg/ml cyclophosphamide, are shown below (figure 48). Although exposed to the same concentration of MMNG, the mean number of cells with aberrations in PD2 cultures was approximately three times higher than those in CHO-G6 cells. The mean percentage of cells with aberrations over all experiments conducted with the PD2 line was 41.25% with a standard deviation of 2.2. Whereas in the G6 line the mean value was only 9.18 with an deviation of 2.9. In all experiments the presence of MNNG caused an approximate doubling of the percentage of cells with aberrations as compared to the respective solvent control cultures.

The mean percentage of cells with aberrations in cyclophosphamide treated controls was approximately 15%, there also appears to be a greater variation between values from different experiments (figure 48), and this is reflected by a higher standard deviation of 6.2. In most experiments the percentage of cells with aberrations was between 10-15%, the most extreme variation was observed in the assays of ES5 where 30%

percent of cells were found to have aberration in the CP control culture. Overall the presence of CP increased the percentage of cells with aberration to between 3 and 4 times that of the respective control cultures.

Analysis of data from control cultures suggests that a steady background level of spontaneous aberrations was maintained, although the presence of metabolic activation tended to increase the background level slightly. In all experiments, positive control cultures showed 2-3 times the number of cells with aberrations compared with respective control cultures, indicating the sensitivity of the cells to clastogenic agents. Responses to positive control were generally similar between experiments indicating that the G6 line was sensitive to clastogenic agents and that the differing responses obtained with emission samples were the result of the samples rather than a failure of the test system.

Figure 48: Variation in the numbers of cells with aberrations in successive positive control cultures of CHO cells.



3.6. Results of Sister chromatid exchange assays.

Although SCE assays are easier and quicker to score than aberration assays, the ambiguous nature of the lesions that cause SCE's, meant that it was preferable to concentrate more on chromosome aberration assays. Consequently, only three emission samples were tested in SCE assays which were conducted using both the original CHO-K1 cell line and the cloned line G6. The PD2 line, although found to be unsuitable for chromosome aberration assays due to high frequencies of background damage, was found to have consistently lower spontaneous SCE rates than the clonal G6 line. Emission samples ES1, ES2 were tested with the CHO-K1-PD2 line, while an assay of ES3 and a repeated assay of ES1 were conducted using the G6 clonal line, with the presence of supplementary metabolic activation.

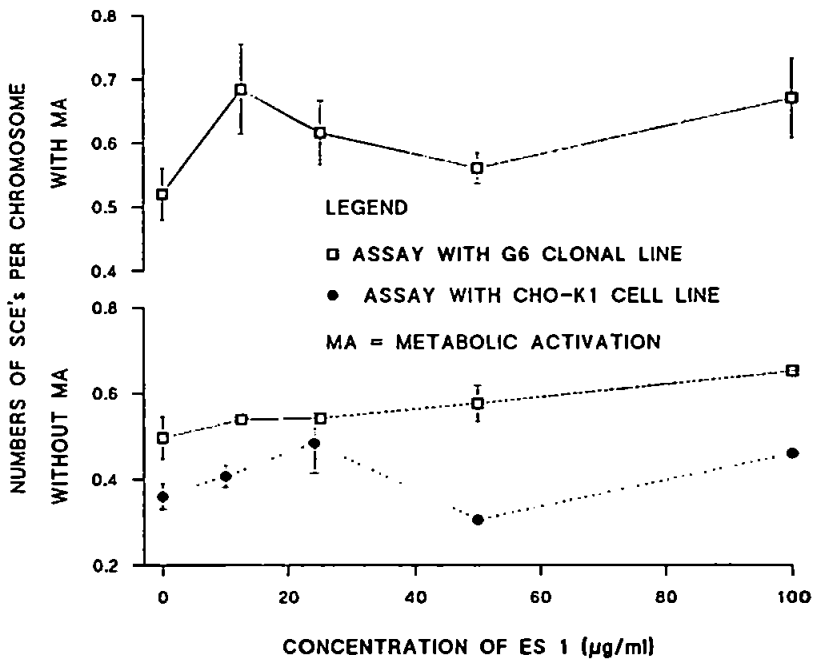
The results obtained following exposure of PD2 cells to ES 1 are shown below (figure 49). Despite an initial increase in the numbers of SCE's per chromosome (SCE/chrom) from mean control levels of 0.35 to 0.45 at a concentration of 25 μg/ml, there was a subsequent reduction in the frequency of exchanges per chromosome at 50 μg/ml to approximately 0.3, however the frequency of exchanges again increased to 0.4 at a ES1 concentration of 100 μg/ml. Statistical analysis of the data was performed (see section 2.7), and after preliminary analysis of the SCE distribution data (methods 2.7.1) it was found that excess intra-replicate variation occurred at a concentration of 25 μg/ml. Consequently the linear trend calculated for the CHO-K1 data was derived from an analysis of variance (ANOVA methods 2.7.3). The critical values obtained (appendix 3) were not significant. This lack of a dose response was presumably due to the decrease in SCE frequency observed at a concentration of 50μg/ml.

The repeat test of ES1 using the G6 cell line both with and without supplemental metabolic activation are also shown in figure 49. Exposure of G6 cells to ES1 induced a dose related increase in the frequencies of exchanges per chromosome. The SCE frequency rose from a mean control values of 0.5 SCE/chrom in control cultures to 0.646 SCE/chrom at an ES1 concentration of 100 μg/ml. Preliminary analysis of the data (appendix 3) indicated that there was no difference in the distribution of the SCE's between cells within cultures and between cells in different cultures. The linear trend statistic was calculated (table 30) and found to be highly significant ($P<0.01$).

In contrast, exposure of G6 cells to ES1 with metabolic activation (figure 49) caused an increase in the SCE/chrom frequency over the mean control value of 0.477, at all sample concentrations. However, these increases were not dose related. The maximum frequency of SCE/chrom (0.66) was obtained at a sample concentration of 12.5 μg/ml with the lowest (0.589) occurring at 25 μg/ml.

Again, analysis of the data indicated that a poisson model for SCE distribution was applicable and data from replicate cultures was combined to calculate a linear trend statistic (table 30). The significance of the linear trend (6.0×10^{-4}, table 30) was found to be greater than that of the G6 cells exposed without metabolic activation, despite the lack of a strict dose related increase in the SCE frequency. This presumably due to the greater frequencies of SCE's induced by exposure to ES1 with metabolic activation.

Figure 49: Numbers of SCE's per chromosome induced in both CHO-K1 and G6 cell lines after exposure to ES 1 (1000 rpm @ 5 Nm).



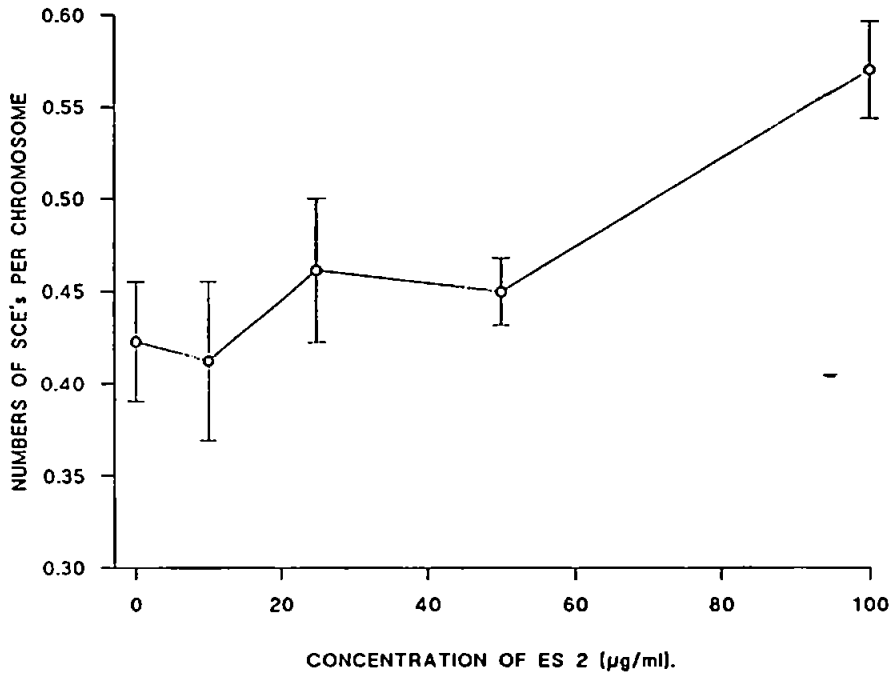
The results of the SCE assay of ES2 are shown in figure 50. A dose related increase in the numbers of SCE's/cell was observed at all samples concentrations, with the maximum increase being observed at a concetration of 100 μg/ml (elevated from control values of 0.42 to 0.55 SCE's/cell at a concentration of 100 μg/ml).

Table 30: Summary statistics, linear trend values, and linear trend probabilities from the exposure of CHO-K1-G6 cells to emission sample ES1 (collected at 1000 rpm @ 5 Nm engine load) in SCE assays both with and without metabolic activation.

Sample concentration	Number of chromosomes scored	Numbers of SCE's scored	Numbers of SCE per chromosome	Numbers of SCE's per cell scored
Minus MA				
0	1162	577	0.497	9.617
10	843	452	0.536	10.512
25	1180	640	0.542	10.667
50	1171	711	0.607	11.850
100	1166	751	0.644	12.517
Linear trend	4.03			
Probability	2.7×10^{-5}			
Plus MA				
0	1036	494	0.477	9.321
10	689	455	0.660	12.639
25	925	567	0.613	12.064
50	920	542	0.589	11.292
100	679	425	0.626	12.143
Linear trend	4.37			
Probability	6.0×10^{-6}			

Despite this increase there was little effect observed over a concentration range of 25-50 μg/ml where the SCE frequency remained at 0.44 SCE's/cell. Preliminary analysis of the SCE distribution indicated that a Poisson model was applicable although there was evidence for excess replicate variation in cultures exposed to 25 μg/ml. Consequently the linear trend statistic was determined from an ANOVA analysis using the logarithmically transformed SCE/cell data. From this the variation between replicate cultures was not found to be significant and a significant ($P < 0.05$) value for the linear trend statistic was obtained. Exposure of cells to ES3 without metabolic activation (figure 51) resulted in an increase in the frequencies of SCE/cell from 0.46 to 0.53 at a concentration of 100 μg/ml. Thereafter, there was a slight decrease in SCE frequency to 0.52 at an ES3 concentration of 150 μg/ml. Analysis of this data showed that despite the apparently large variation between replicate cultures, particularly at concentrations of 25 and 150 μg/ml, there was no significant ($P < 0.05$) variation between replicate cultures. The significant linear trend statistic ($P = 1.62 \times 10^{-2}$) obtained (table 31) reflects the reduction in the numbers of SCE's observed.

Figure 50: Numbers of SCE's induced by exposure of CHO-K1 cells to increasing concentrations of ES2 (2000 rpm @ 5 Nm engine load).



In contrast exposure of the cells to ES3 with metabolic activation was complicated by the loss of two replicate cultures at concentrations of 100 and 150 µg/ml. Despite an initial increase in the SCE/chromosome frequency from 0.49 in control cultures to 0.57 at a concentration of 50 µg/ml, there was a decline to below control levels at a concentration of 150 µg/ml. Again, there was no evidence of culture of replicate effects on the distribution of SCE's, although the decline in SCE frequencies at the higher sample concentrations reduced the magnitude of the linear trend statistic to a non-significant level.

Figure 51: Numbers of SCE's per chromosome induced by increasing concentrations of ES 3 (3000 rpm @ 5 Nm load) both with and without supplementary metabolic activation.

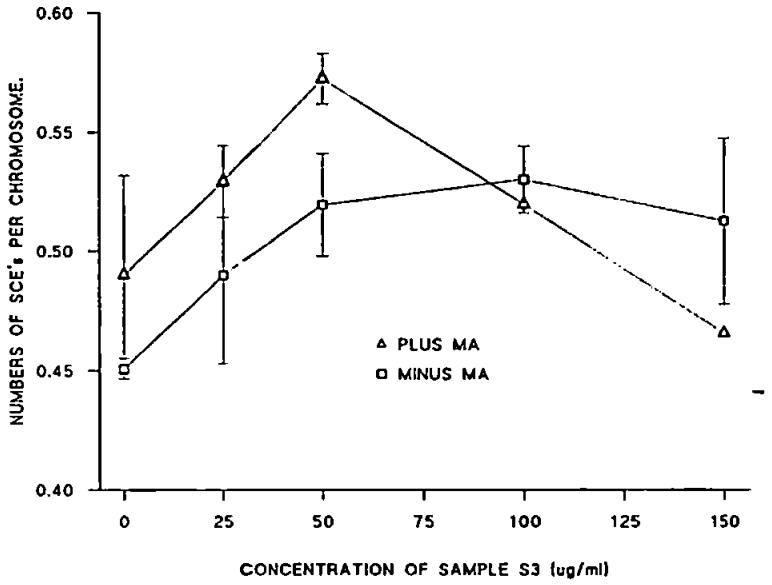


Table 31: SCE frequencies and linear trends of CHO-G6 cells exposed to increasing concentrations of ES3 (3000 rpm @ 5Nm load).

Sample concentration	Numbers of chromosomes scored	Numbers of SCE's scored	Number of SCE's per Chromosome	Numbers of SCE's per cell
Minus MA				
0	1134	523	0.461	8.717
25	1132	555	0.490	9.250
50	1143	617	0.540	10.283
100	1126	611	0.543	10.183
150	1131	580	0.513	9.667
Linear trend	2.14			
Probability	1.62×10^{-2}			
Plus MA				
0	1132	555	0.490	9.250
25	1135	600	0.529	10.000
50	1127	645	0.572	10.750
100	562	292	0.520	9.733
150	569	255	0.448	8.500
Linear trend	-0.89			
Probability	0.81			

3.7. Analysis of trend data.

Following the accumulation of aberration data for a number of the samples collected, attempts were made correlate the mutagenicity of the emissions with the known physical composition, at each sample condition. Values for the various emissions at each sample point were extrapolated by linear regression from the provided data (appendix 4). Table 32 (below) shows the linear trend statistics obtained from cultures exposed to the samples both with and without MA.

Table 32: Values of linear trend statistics of all categories and sum and mean values of all categories from cultures exposed to emission samples both with and without metabolic activation.

Emission	Linear trend statistics from cultures minus MA		
sample	Total	Simple	- Complex
ES1	2.473	2.421	0.798
ES2	7.46	5.928	3.912
ES3	2.916	2.58	1.241
ES4	-0.457	-0.141	-0.875
ES5	1.847	1.241	3.033
ES9	-0.772	0.452	-0.853
ES10	3.509	3.073	1.082
ES12	-0.054	-0.707	1.265
ES14	-0.041	-0.208	1.026
ES15	0.614	0.951	0
ES19	2.135	2.511	0.218
	Linear trend statistics from cultures with MA		
ES1	-	-	-
ES2	-	-	-
ES3	-	-	-
ES4	0.195	0.413	-0.714
ES5	-0.471	-0.875	0.676
ES9	0.361	1.804	-2.46
ES10	2.111	2.023	1.32
ES12	-0.759	-0.978	-0.675
ES14	1.808	1.467	2
ES15	1.077	0.134	2.862
ES19	-0.407	-0.129	-0.782

- Not tested.

The linear trend values obtained were compared by regression analysis against each of the emissions in turn. For this correlation it was assumed that the linear trend statistic obtained for each emission sample is a direct reflection of its inherent mutagenic capability. The larger the trend statistic the greater the mutagenic potential. Due to the nature of the responses observed, samples resulting in negative trend statistic are assumed to have no mutagenic activity in this particular test system. To account for the separate effects of varying engine speed and loading the emission values were calculated as units per gramme of fuel consumed.

Fuel consumption and fuel density data were used to calculate the rate of fuel consumption in grammes per minute (g/min). This correction is analogous to expressing the mutagenicity as mutants per mile travelled, as used by other authors (see section 1.11.3). Gaseous emission were then expressed as emissions (ppm) per gram of fuel consumed per minute (ppm/gf), and particulate emissions were expressed as Bosch smoke units (which are an optical measure of particulate emissions) per minute (units/gf). Table 33 (below) shows the adjusted emissions for each sample condition.

Table 33: Emission values for each sampling condition expressed in units per gram of fuel consumed.

Emission sample	Fuel consumption (g/min)	CO (ppm/gf)	HC (ppm/gf)	NOx (ppm/gf)	Bosch (units/gf)
ES1	14.700	26.871	56.600	18.912	0.018
ES2	29.446	7.777	26.794	26.794	0.006
ES3	45.936	6.944	18.830	5.050	0.005
ES4	68.186	5.514	14.387	5.089	0.011
ES5	25.655	17.463	37.888	21.127	0.033
ES9	119.061	3.158	7.022	5.493	0.007
ES10	38.528	14.976	24.943	21.620	0.078
ES12	109.965	2.910	5.920	6.602	0.007
ES14	52.364	16.328	17.913	20.224	0.095
ES15	93.384	3.223	5.804	9.584	0.02
ES19	220.162	2.798	1.653	4.778	0.017

The results of regression analysis using the linear trend statistics for the "total" damage category for non MA cultures are shown below (figure 52). Regression analysis of CO, NOx and HC against the trend statistic, show positive trends of increasing sample mutagenicity with increasing emission concentration, although only the NOx trend is significant ($P<0.05$). However, this result is dependent on the presence of the highest linear trend value, omission of this point reduces the significance to ($P>0.05$). A further investigation of the regression of the linear trend data against the NOx concentrations was made using the values from all the linear trend categories (figure 53). The result of this shows that the P values obtained for "total", "complex" both significant ($P<0.05$).

Figure 52: Linear regression of linear trend statistics from the total chromosome damage category against the emissions (ppm) of NOx, HC, CO, and (units) of Bosch smoke per gramme of fuel consumed.

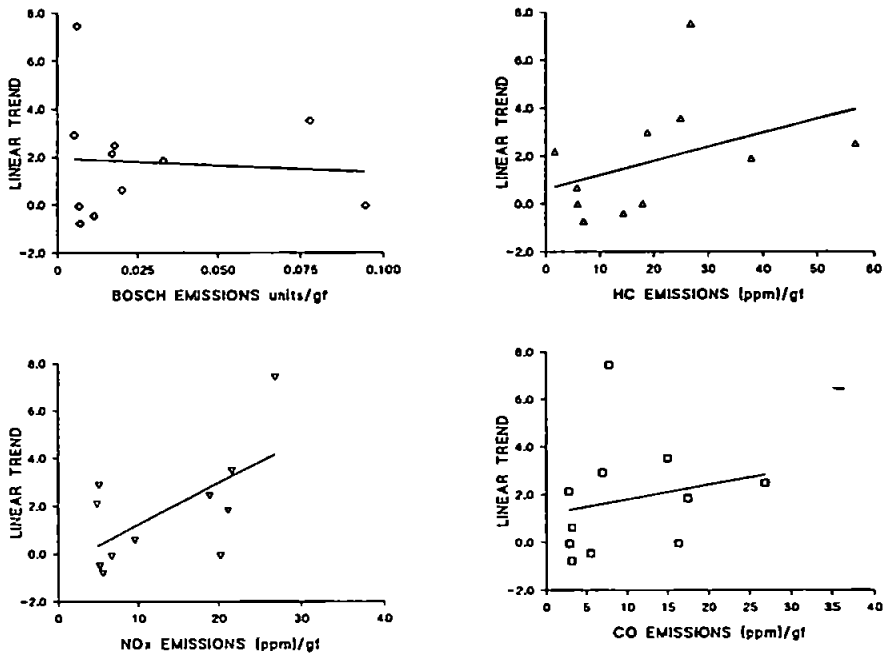
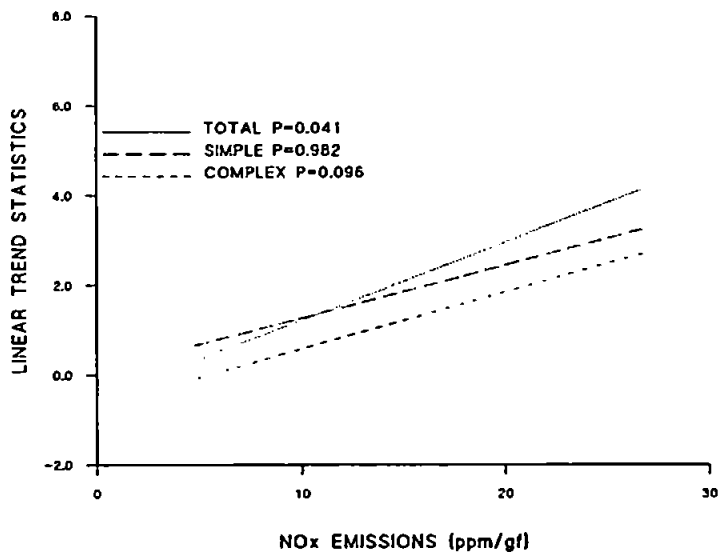


Figure 53: Linear regression of linear trend statistics of all chromosome damage categories against NOx emissions per gramme of fuel consumed.



A similar analysis of the "total" damage category linear trend statistics obtained after cellular exposure to emission samples with MA, showed a general increase in mutagenicity with increasing emission concentrations (figure 54). However the only significant trend ($P<0.05$) observed was for Bosch smoke emissions. These emissions were evaluated further and figure 55 shows the regression analysis of the trend statistics from all the damage categories against Bosch smoke emissions per gram fuel consumed. Significant ($P<0.05$) regression trends were obtained for the "total" damage category.

Figure 54: Linear regression of linear trend statistics from the total chromosome damage category of cultures exposed to emission samples with metabolic activation, against the emissions (ppm) of NOx, HC, CO, and (units) of Bosch smoke per gramme of fuel consumed.

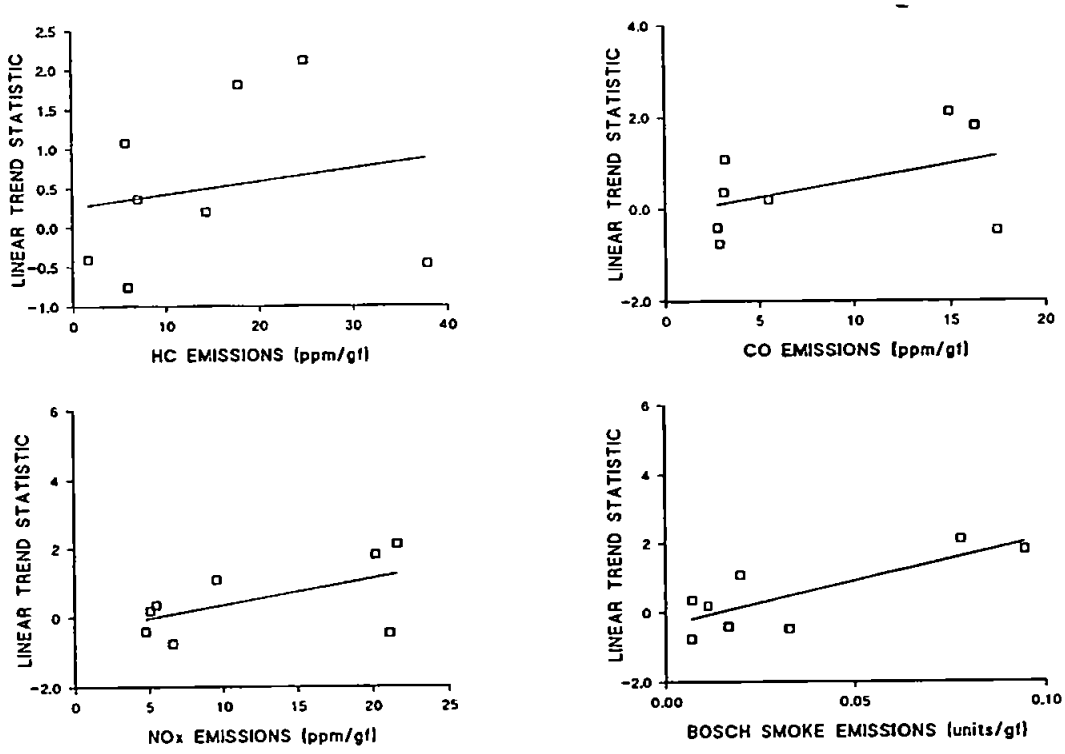
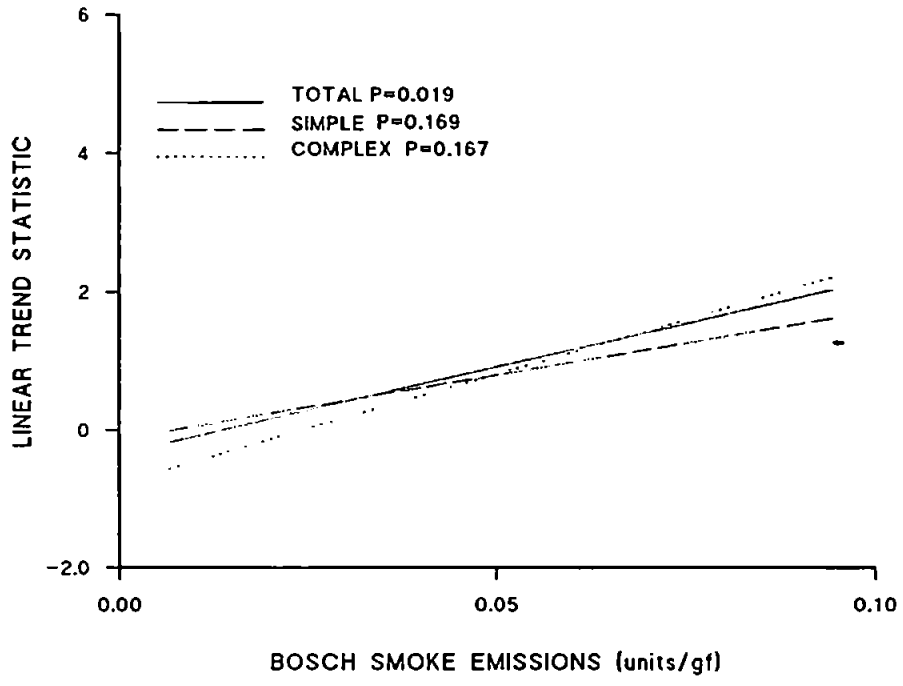


Figure 55: Regression analysis of Bosch smoke emission per gram of fuel consumed against the linear trend statistics obtained for three categories of aberrations and the summed totals, from cultures exposed to emission samples with supplementary metabolic activation.



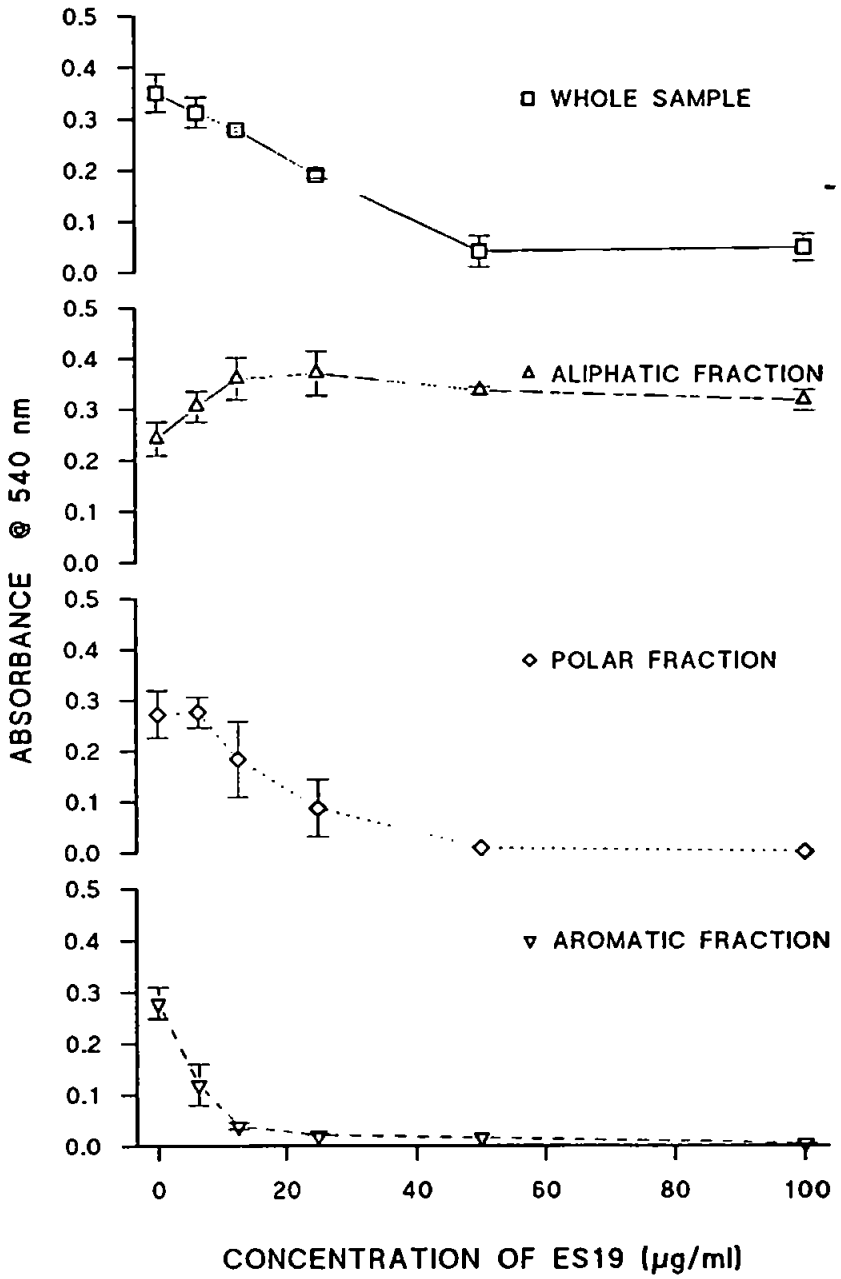
3.8. Fractionation studies.

As outlined in the methods it was hoped to perform assays on emission sample fractions obtained from open column chromatography. Almost all samples of masses of over 30 mg were fractionated in to three subfractions on silica gel columns using three elution solvents, hexane , DCM and methanol. However some samples of less than 100 mg in mass were prepared in stock solution of DMSO, consequently a re-extraction procedure using acetone was performed but was liable to large sample losses. Table 64 (appendix 4), shows the relative masses of the subfractions obtained following chromatography on silica columns. Problems were encountered in removing silica fines from methanol fractions, in these cases the mass of the methanol fraction was assumed to be equal to the total sample mass less the combined masses of the hexane and DCM fractions. This assumes 100% recovery from the fractionation which was unlikely to have been achieved as some colouration was still observed in the silica column after elution of the methanol fraction. This compromise was accepted because attempting to remove the silica from the fraction by filtering through a glass wool column was as likely to cause as much error by sample loss.

Fractions obtained from ES19 were subjected to preliminary toxicity assays. It was suggested that the toxicity of the emission samples may be related to the components of the aliphatic (hexane) fraction., and that subsequent removal of this fraction may reduce the cellular toxicity, enabling higher sample concentrations of the other fractions known to contain potentially mutagenic chemical species (Polar fractions contain PAH) to be tested. The results of the toxicity assays of the subfractions of ES19 are shown in figure 56 (below). Each data point represents the mean of two replicate wells. It is apparent that exposure to the hexane (aliphatic) fraction alone has little cytotoxic effect as determined by this viability assay, absorbance readings were relatively constant and remained at control levels at the highest concentration tested. The increase over control values depicted by the aliphatic fraction was the result of considerable variation in the control cultures for that series. In contrast both the aromatic and polar fractions cause a considerable reduction in the neutral red uptake with the aromatic fraction causing reductions at the lowest sample concentration tested (12.5 μg/ml). The toxicity of the unfractionated sample closely resembles that of the polar fraction (both reduce cell viability to below control values at a sample concentration of 25 μg/ml). However, in most samples the polar fraction represents approximately 10-20% of the sample mass

(table 64). In contrast, both the aromatic and aliphatic fractions represent approximately equal proportions of the whole sample (*ca.* 40% each, table 64) thus reducing the highly toxic effect of the aromatic fraction. This toxicity assay was repeated and similar results were obtained, with negligible toxic effect associated with the aliphatic fraction.

Figure 56: Viability assay of neutral red uptake of CHO-K1-G6 cells after exposure to increasing concentrations of the whole sample and three subfractions of ES19 (4000 rpm @ 80 Nm load).



After the initial toxicity assay it was hoped to assay the sample fractions in aberration assays, however the

increase in spontaneous aberrations observed in the first assay of the whole ES19 and the requirement for a repeat assay, made this impracticable.

3.9. Summary of results.

Of the sixteen emission samples collected, twelve were tested in chromosome aberration assays, and eight of these with a supplementary metabolic activation system. Five samples tested without MA and two with gave significant dose related increases in the "total" numbers of aberrations, which was reflected in also the numbers of cells with "simple" aberrations. Only one sample caused a significant increase in the "complex" damages category without increase in the other categories (ES5) without exposure to MA, whereas ES15 caused a significant increase in the "complex" aberrations with MA.

Results of the three sister chromatid exchange assays performed were consistent with aberration assays in that all three samples gave increases in the numbers of exchanges with increasing sample concentration without MA. Whereas of those performed with MA one gave a significant linear trend (ES1) and the other had little effect (ES3).

Comparison of the linear trend statistics with the available data on the concentrations of NOx, HC, CO, and Bosch smoke emissions of the exhaust at similar engine conditions showed a general increase in the mutagenicity with HC and CO concentration, whereas NOx emission were significantly correlated with linear trend statistics. In contrast linear trend statistics obtained from cultures treated with MA showed a significant trend to increase with increasing Bosch smoke emissions.

Fractionation of one sample, revealed that the toxicity of the emission samples is located in both the aromatic and polar fractions as derived by silica column chromatography. With the aromatic fraction being the most toxic. There is no evidence of cytotoxicity in the aliphatic fractions. All samples tested showed evidence of cytotoxic effects at concentration of less 200 μg/ml, with most being toxic at a concentration of approximately 50 μg/ml. This toxicity was shown to be an inherent property of the samples rather than the result of solvent contamination.

4.0 Discussion.

4.1. Appraisal of the total exhaust solvent scrubbing apparatus (TESSA).

The TESSA was designed specifically to determine the origins of the components of engine exhaust emissions. A consequence of this original specification was that the sampling apparatus should be as close to the engine as possible (Trier 1988). The close proximity of the TESSA to the combustion chamber enabled the collection of emission samples, and limited the effects of pyrosynthesis/pyrolysis associated with the exhaust system. This was an important consideration as (Nielson 1984, cited by Trier 1988) has shown that PAH associated with mutagenicity undergo thermal decomposition at temperatures in excess of 200 °C. As samples collected by the TESSA are sampled almost directly from the combustion chamber, it has been suggested that unlike samples obtained from dilution tunnels, emissions collected using the TESSA may not be representative of exhaust emissions from the tailpipes of vehicles.

In the initial evaluation of the TESSA, Trier (1988) showed that by increasing the length of the sample transfer tube (in order to simulate an exhaust system) considerable changes in the emissions samples collected occurred. He found that there was marked decrease in the proportion of the aliphatic fraction of the emission samples. This phenomena was also reported by Hayano *et al.* (1985), who observed a 40% reduction in the content of aliphatic compounds in samples collected directly from the combustion chamber as compared to those collected from the exhaust. Trier (1988) concluded that the observed reduction of aliphatic compounds was due to, extended combustion of these compounds along the length of the exhaust system. A comparison of the aromatic and polar fractions of these samples with samples obtained from nearer the combustion chamber indicated that they were of similar composition, although no detailed analysis of individual compounds was performed.

One interesting question raised by the differences in the proportions of aliphatic and aromatic compounds is the effect that this has on the mutagenicity of the sample. From the results of this investigation the reduction of the aliphatic fraction of the emission would have little effect on the toxicity of the whole samples, and if as suggested by Lewtas (1988) there is little mutagenic activity associated with the aliphatic fraction, this reduction would not affect the mutagenicity of the samples. However, there are known to be several reactions which occur in the higher temperature environment of the exhaust stream which may lead

to the formation of highly mutagenic species of PAH. This is obviously an area open for further investigation.

Another consideration important in the design of the TESSA, was the removal of collected sample material from the exhaust stream in an attempt to limit further chemical modifications. Several authors have shown that continual exposure of the samples to the exhaust stream may influence the chemical composition of the samples. Lindskog (1983) found that the presence of NO_2 and HNO_3 in the exhaust caused significant degradation of PAH adsorbed to particulate matter. This loss was accompanied by an increase in the mutagenicity of solvent extracts of these particulates. Scheutzle (1983) illustrated some of the pathways by which sampling with dilution tunnel could influence the composition of the samples. These included, oxidation of PAH in the gas phase by the NO_2 and HNO_3 in the exhaust stream, and further degradation of PAH once trapped on filters or adsorbed to resin traps. On the basis of this evidence, the removal of collected exhaust from the exhaust stream in the TESSA, offers advantages over the dilution tunnel, particularly for reducing the potential of artifact formation during sample collection.

There is however some question over the use of aqueous extraction of the TESSA sample preparations. This has been seen as a possible source of loss of organic material with failure to recover all components collected from the exhaust. Trier (1988) quantified losses of various PAH during sample workup, by the addition of known standard PAH to the extraction solvent. Recoveries of in excess of 90% were achieved for most PAH species, the lowest recovery observed (40%) was of naphthalene. Trier suggested that these losses were the result of a combination of the low boiling point of naphthalene and its relatively high solubility in water (31.2 mg /l) as compared to other PAH such as phenanthrene (1.29 mg/ml) and benzo(a)pyrene (3.8 μg/l) (Mackay and Shiu 1977). Lee *et al.* (1981) recommended the use of solvent extraction for the recovery of PAH from aqueous solution. Solvents of choice include C5-C8 alkanes and dichloromethane. Other methods, include the filtering of the sample through adsorbent polymeric resins and subsequent stripping of PAH by elution with organic solvents. However, these the use of these adsorbents tends to increase the likelihood of contamination, due to their slight solubility in some organic solvents (Lee *et al.* 1981).

The most obvious problem associated with the use of the TESSA for the collection of emission samples is the lack of comparative data for the samples collected by the TESSA and dilution tunnel methods. Despite the claim made by Trier at al. (1988) that the recovery of low molecular mass, and presumably volatile organic compounds is enhanced in the TESSA, there is little published evidence to support this. Trier 1988) did compare the aliphatic fraction of diesel emission samples collected using the TESSA with those of a dilution tunnel. This has shown that the TESSA sample did contain low mass components not found in the filter sample. However, the dilution tunnel samples were extracted from filters, and not combined with the solvent extracts from adsorbent traps (see section 1.7), which are commonly used in dilution tunnels to collect volatile compounds not trapped on Teflon filters. It is therefore possible that the TESSA samples are of similar composition to those obtained by dilution tunnels, using both filters and adsorbent traps. It is recommended that a further comparison of the samples collected by the TESSA and those from dilution tunnels should be conducted.

Other than the chemical nature of the emission samples collected by the TESSA, there are some other minor disadvantages with the system, mainly the lack of environmental control in the engine test bay. In most cases ambient temperature and humidity are controlled or at least measured (*eg.* Clark *et al.* 1982, had vehicles mounted on an environmentally controlled dynamometer). This is particularly important for the testing of naturally aspirated engines as changes in air density may influence the quality of combustion (see section 1.5). One drawback of the TESSA is that the combustion air intake is situated over the engine itself. This is of consequence, as during engine runs cooling fans cut in intermittently, blowing warm air over the engine into the intakes. In one such run there was found to be a 10 °C change in the temperature of air entering the intakes when the cooling fans cut in. The effects of this change on the combustion process or the composition of the emission is not known.

As this intermittent heating effect occurred during collection of all samples it is unlikely to have significant effects on individual samples. Indeed, the effect of increasing ambient temperature has not been shown to increase the mutagenicity of diesel samples (Clark *et al.* 1982). Nevertheless, if it is impractical to control the temperature of the air for combustion, it should be at least monitored. It is therefore suggested that some

methods for measuring the temperature of the air be instigated, this may involve the use of computer linked thermocouples. Other areas of the TESSA which may be of interest to monitor with respect to temperature are the transfer tube between the manifold and the TESSA, the inside of the TESSA itself, and the outlet of TESSA.

Overall evaluation of the TESSA suggest that it is an effective and relative cheap method for sampling vehicle emissions. The main benefits include the mass of sample which can be collected, the ability to collect several samples during one day, and the speed at which samples can be prepared and used. There are several minor drawbacks associated with the technique, which could be overcome with further experimentation. Further evaluation of the TESSA samples with respect to potential sample loss is also required, and the comparison of the mutagenicity of TESSA and dilution tunnel samples is needed to establish the use of TESSA for routine sample collection. Other areas for future study include the influence of the length of transfer tube on the mutagenicity of the emission samples, characterisation of the aqueous phase which is usually discarded during sample work-up to determine whether this has any mutagenic activity, and an investigation of the effects of acceleration and deceleration on the mutagenicity of the emissions.

4.2. Factors affecting the outcome of *in vitro* cytogenetic assays.

Several factors can be identified which may influence the potential result of *in vitro* cytogenetic assays. These factors can range from subtle modifications of the procedure, such as the length of sample exposure time or the duration of incubation with supplementary metabolic activation, to more substantial inter-laboratory differences, the most important of which is probably the source and strain of cells used.

4.2.1 Variation between cell lines.

One of the major difficulties in the use of long term cell cultures, is that there is the opportunity for chromosomal evolution within the cultures. Consequently, lines derived from the same source may, after several passages have distinctly different karyotypes. These inherent chromosome changes also result in chromosome aberrations in a number of cells. In order for cytogenetic assays to have the sensitivity–

required for the detection of chemical mutagens, the spontaneous aberration rates should be as low as possible.

The most important problem encountered in this work was the high level of background chromosome aberrations observed in the stock cell lines. Much time was spent trying to determine the causes of spontaneous damage, and it would appear from this work that the damage was a consequence of a subpopulation of cells within the CHO-K1 line with unstable karyotypes, rather than the result of an mycoplasma infection or media incompatibilities.

The clonal line (G6) isolated from the parental CHO line was found to have a reduced level of spontaneous damage with a mean frequency over all the experiments performed of approximately 4%. This figure correlates well with published data from other sources, Margolin et al 1986 presented historical chromosome aberration control data from 2 laboratories where the mean values obtained for the total numbers of cell with abberation from 66 and 102 experiments were 3.06 and 2.18 respectively. In general, results for most (Galloway *et al.* 1985, Loveday *et al.* 1990) studies indicate that aberrations rates of between 2 and 5% are the norm.

Another problem associated with establishing a clonal cell line with a stable karyotype is that the cells tend to vary from laboratory to laboratory. These inter-strain differences are most easily illustrated by the modal numbers of chromosomes in different clones. In this investigation alone, a marked shift from modal chromosome numbers from 20 in the PD2 line to 19 in the G6 line were observed. Other strains with modal numbers ranging from between 19 to 21 chromosomes have been described (Kirkland 1992). Examination of the karyotypes of cells from the G6 and PD2 lines with the modal numbers of chromosomes, showed a number of unique chromosomes in each, thus indicating that the clonal line was derived from a sub population of cells in the original culture rather than a recent mutant line, originating from the cloning procedure.

Many CHO lines have been studied in detail in order to determine the origin of their karyotypes from that

of the original diploid hamster karyotype, and there have been several published karyotypes based on various chromosome banding techniques (*ie.* Kakati & Sinha, 1972, Deaven & Peterson 1973, Ray & Mohandas 1976). Characterisation of the G6 line was not performed in this work because of the extensive rearrangement of chromosomes in the G6 line. One feature of the G6 cell line used was the heterochromatic staining region common to the chromosome labelled number 4. This site may represent the heterochromatic region of the X chromosome which has been identified in the CHO karyotypes of Worton *et al.* (1976), Ray *et al.* (1986), Simi *et al.* (1988 & 1990), Drets, *et al.* (1992), Kato & Yosida (1972) and Martinez *et al.* (1989). Chromosome banding analysis is required to determine if the chromosome labelled 4 in this investigation is the X chromosome, and whether there has been any extensive modification of this chromosome, as with other chromosomes or whether it has remained conserved. The heterochromatic region did not appear to be preferentially associated with chromosome aberrations, such as breaks and interchanges although, a thorough examination of chromosome breakpoints was not undertaken.

Kirkland (1992) has also suggested that there may be quantitative differences in the responses of different CHO strains to some chemicals. Consequently, a comparison of the dose response of the G6 line used in this study to that obtained by other authors was made, using the known mutagens MNNG and CP as controls. The results of exposing the G6 cells to these mutagens were similar to those described by Galloway *et al.* (1985). Concentrations of 0.075 μg/ml of MNNG and 12.5ug/ml of CP induced mean values for total numbers of cells with aberrations in the G6 line of approximately 9% and 15% respectively as compared to 5-15% and 17-30% in Galloway's work.

4.2.2 Metabolic activation.

The presence of supplementary metabolic activation system (S9 mix), or more accurately choice of S9 inducing agent has also been demonstrated to have a significant influence on the spontaneous aberration rate of CHO cells (Kirkland 1993). Table 34 (below) illustrates the effects of S9 inducing agent upon the spontaneous damage rates observed in various strains of CHO cells. Two clonal lines CHO"UK" and CHO"WBL" show marked increases in the numbers of cells with spontaneous aberrations to 30% and 40.5% respectively when exposed to S9 mix induced with Arochlor 1254. In contrast the CHO "ATCC"

line has a lower background level of 1.5%. This ATCC line is also apparently the same line as was used for the isolation of the G6 line used in this work. The use of B naphthaflavone/phenobarbitone mix as an induction agent results in similar levels of spontaneously damaged cells (<5%) in all three cell lines.

Table 34: Spontaneous aberration frequencies for different clones of Chines hamster ovary cells in the presence of "S9 mix" induced by different agents.

	Percentage of cells with aberrations.		
S9 Inducing agent	CHO"UK"	CHO "ATCC"	CHO"WBL"
Phenobarbitone / β-naphthaflavone	3.3	1.4	4.0
Arochlor 1254	29.5	1.5	41.0

Adapted from Kirkland (1992).

Data from S9 treated CHO cells published by Margolin (1986) showed a slight increase in the mean percentages of cells with aberrations as compared to non-S9 controls. These increases were recorded as 3.74% and 2.26% as compared to the minus S9 control values of 3.06% and 2.18%. There was also an increase in the associated variance indicating that the presence of S9 increases the variability of control results in different experiments. In this work the presence of S9 was also found to increase the mean numbers of cells with spontaneous aberrations from 4% to 4. 66% , also with an associated increase in the variance of control values between experiments (section 3.6).

4.2.3 The effect of sampling time and mitotic delay on aberration yields.

Bean *et al.* (1992) investigated the effects of increasing the length of sample exposure time using the WBL stain of CHO cells. The outcome of this work was the finding that an exposure time of 9 to 12 hours was sufficient for detecting positive responses. However, Galloway *et al.* (1987) tested three compounds which showed no effects after exposure for 10-12 hours but which demonstrated positive clastogenic activity after extended cell exposure for 18-20 hours. Loveday *et al.* (1989) also observed increased numbers of cells with chromosomes aberrations following exposure of cells to for 18-20 hours than were observed after 10 hours of exposure.

Scott *et al.* (1989) have illustrated the hypothetical effects of mitotic delay on the resulting dose response

curve obtained for a model mutagen. Chemical agents that cause mitotic delays to cells, which are then sampled after only one exposure time may not produce dose related increases in aberrations at all, or at least, a reduction in the numbers of cells with aberrations seen at higher sample concentrations. The effect of this downturn is of particular importance if the analysis of data involves the use of the linear trend test proposed by Margolin *et al.* (1986) which is less sensitive for responses in which the maximum numbers of aberrations occurs at a test concentration other than the maximum tested. It is beneficial to be able to apply a simple linear model to aberration data, as this is much less complicated than attempting to determine the significance of a non linear responses using other statistical methods.

If the outcome of aberrations assays does not yield a dose related increase in aberrations, then the recommendation of the UKEMS (1990) is that a repeat experiment be performed. Prior to retesting, and investigation of the mitotic delay encountered is also recommended. Procedures for estimating the mitotic delay include, preliminary sister chromatid exchange experiments, in which the numbers of first and second division cells are recorded (Galloway 1987).

4.3. Results of cytogenetic assays.

4.3.1 Results of cytotoxicity assays .

The most significant feature of the viability assays performed, was the difference between the toxicity of the first four samples collected when compared to the toxicity of the later samples. The majority of the samples collected during the second period of sampling were toxic at concentrations of between 25 to 50 μg/ml. compared to the initial samples (ES1-ES4) which exhibited toxicity at concentrations of greater than 100 μg/ml. There are several possible explanations for this variation. The most likely cause is the loss of volatile toxic components from the samples during nitrogen blow-down, and this illustrates one of the difficulties of working with complex organic mixtures such as, determining the point at which removal of residual solvent is also removing the volatile compounds within the sample. It was the recognition of this step as a source of potential sample loss that led to the adoption of heated waterbaths to facilitate solvent removal as described by Peterson and Chuang (1982, methods 2..1.4). There appear to be few available techniques by which solvent removal can be standardised, Lee *et al.* 1981 suggested the use of Kuderna-

Danish evaporation as a satisfactory way to remove solvents with minimal losses of PAH. One drawback of this method is that the sample concentration time is increased (Peterson and Chuang 1982). The use of amber glassware was also suggested to reduce the possibility of photochemical degradation of the sample.

Another potential source of variation which may affect the toxicity of the samples is the changes in fuel stocks used in the two sampling periods. The second fuel was assumed to have a higher aromatic content than the first based on its higher boiling point. Fuel aromaticity has been implicated as a factor which may affect the mutagenicity of diesel samples (Scheutzle 1986), although there is little information concerning the toxicity of samples obtained from different aromatic fuels, and this is another area for investigation. The presence of contaminating residual solvent has been shown not to be a significant factor in the toxicity of the samples. For all of the solvents used their was little evidence of cytotoxicity at volumes of 8μl, which would be far in excess of any residual solvent within the sample.

There has been little description of the toxicity of whole diesel emission samples in mammalian cells. Lewtas (1978) stated that their emission samples were toxic to CHO cells and consequently further tests were conducted in bacterial assays. Later in 1982 Lewtas indicated that exposure of cells to 80 μg/ml of diesel emission from a Nissan diesel engine was cytotoxic to CHO cells. Shore *et al.*(1987) described the toxicity of three samples, one was toxic at a concentration of 100μg/ml, whereas the others were tolerated in cultures up to concentrations of 200 μg/ml.

4.3.2. Results of chromosome aberration assays.

The results of chromosome aberration assays performed in this work reflect the general findings of other authors in that both positive and negative dose responses were obtained depending on the sample tested. The advantage of the data generated in this study, is that the only variation between samples should be the engine conditions at which they were collected, rather than as in most other studies where different engines or vehicle types were used.

In most samples, the actual increase in the numbers of cells with aberrations was small. The maximum increases of between 3 and 4 times the control values were observed in only three samples (ES2, ES5, and ES10). The maximum increase in the numbers of cells with aberrations was observed usually at the highest concentration tested. This feature of the dose response obtained has important significance in that, there is some debate over the in vivo effects of clastogenic agents which act only at toxic concentrations Kirkland (1992).

On two occasions bell shaped dose responses curves were obtained (ES5 plus MA and ES19), where the maximum numbers of cells with aberrations occurred at intermediate sample concentrations, with a reduction to control levels at the highest concentrations. This effect may be a consequence of mitotic delay induced by these samples, the significance of which has been discussed. Lewtas (1983) has shown that there was an increase in the numbers of aberrations observed in cells treated with increasing concentrations of a diesel emission sample, and that the numbers of aberrations increased with increasing sampling time. In this case however a linear dose response was obtained at all sampling intervals.

The linear trend probabilities obtained were used to estimate the clastogenic potential of the emission samples. For this classification the recommendations of Galloway *et al.* (1985) were adopted, whereby the results of the trend statistics of the individual aberration categories are considered independently. Thus, any sample having a significant trend statistic in any damage category is considered to be clastogenic. To allow for the multiplicity of comparisons associated with this assumption, the trend probabilities obtained were adjusted following the recommendations described by Margolin (1986), in this case the linear trend statistic probabilities obtained were tripled. Consequently, samples with significant ($P<0.05$) adjusted trend probabilities were considered to have weak clastogenic activity, whereas those with trend probabilities ($P<0.01$) were considered as clastogenic in the CHO cells used.

Table 35 below illustrates the significance levels of the corrected trend probabilities. Of the 11 samples tested in aberration assays without metabolic activation only ES2 (2000 rpm @ 5Nm load) produced significant ($P<0.01$) positive responses in all aberration categories. Both ES3 and ES10 are also considered

positive clastogens due to their significant ($P < 0.01$) trend probabilities in the total aberration category, ES5 was also shown to be clastogenic on the basis of an increases in the numbers of cells with complex aberrations. ES1 was considered to be a weakly clastogenic samples due to the significant ($P < 0.05$) trend probabilities obtained for both total and simple damage. Due to the lack of available sample, the repeat assay of ES4 was not tested to the maximum tolerated concentration (as recommended by the UKEMS 1990), the consequence of which may be the lack of clastogenic effects. However, all other samples were tested at concentrations which did induce both toxicity and reduced the mitotic indices of cultures.

In contrast, of the eight samples tested with metabolic activation only ES14 (1000 rpm @ 80Nm load) caused a significant ($P < 0.01$) increases in the complex damages category. However, this result was based on the observation of several cells with complex aberrations only in cultures exposed to the highest sample concentration tested. Consequently, this lack of dose response suggests that further evaluation of this sample is required, and hence this sample is considered at this time to be only weakly clastogenic.

Table 35: Summary of emission sample clastogenicity classification based on linear trend probabilities adjusted for multiplicity of comparisons.

	Significance of linear trend statistic of each damage category without exposure to S9 mix			Significance of linear trend statistics of each damage category with exposure to S9 mix.		
Sample	Total	Simple	Complex	Total	Simple	Complex
ES1	+	+	-			
ES2	+ +	+ +	+ +			
ES3	+ +	+	-			
ES4	-	-	-	-	-	-
ES5	+	-	+ +	-	-	-
ES9	-	-	-	-	-	-
ES10	+ +	+ +	-	-	-	-
ES12	-	-	-	-	-	-
ES14	-	-	-	-	-	-
ES15	-	-	-	-	-	**?
ES19	-	-	-	-	-	-

Key
- indicates no clastogenic activity.
+ indicates weak clastogenic activity trend probability ($P < 0.05$)
+ + indicates strong clastogenic activity trend probability ($P < 0.01$)
**? indicates weak clastogenic activity trend probability ($P < 0.01$) based on data from only one sample concentration

Comparison of this data with other CHO aberration data is difficult as other authors have usually compared samples obtained from different engine or vehicle types. The data presented by Lewtas (1982) was for the exposure of CHO cells to engine samples collected from a Nissan diesel engine. In that study none of the sample tested with metabolic activation showed any evidence of clastogenic activity. One feature of that data was that increasing sample exposure time also increased the numbers of cells with abberations. The maximum percentage of cells with aberrations exposed to a concentration of 60 μg of exhaust extract increased from 18% after 12 hours exposure to 66% after 15 hours, before falling to 15% after 21 hours exposure.

It is also difficult to make comparison between this work and the aberration data presented by Shore et al.

1987, as this was performed using metabolically competent RL4 cells which did not require the addition of supplementary metabolic activation. The samples tested were also obtained from filter samples collected after running an entire driving cycle. Of the three diesel samples tested two were found to be toxic at concentrations of 100 μg/ml and both induced approximately 12-17% cells with aberrations at this concentration, the other sample was tested to a maximum concentration of 200 μg/ml and induced aberrations in approximately 50% of cells at this concentration. Assuming these assays represent the effects of metabolic activation of the diesel samples, they are in direct contrast to the results of TESSA samples in which little clastogenic effect was observed in metabolically active cultures.

The UKEMS recommends the use of a second sampling interval, if a non-linear dose response is obtained, and generally repitition of assays no matter what the outcome of the first (UKEMS 1990). In some cases increased numbers of cells with aberrations were observed at the lowest concentrations tested. This may suggest that longer sampling intervals are required for the detection of aberrations with these samples. On this basis it is suggested that the effects of mitotic delay and increased sampling intervals be examined to fully validate the mutagenic potential of these emission samples.

4.3.3. Results of SCE assays.

All samples (ES1, ES2 and ES3) tested in SCE assays gave positive results with significant linear trends, with the exception of G6 cells exposed to ES3 with metabolic activation. The outcome of the SCE assays was compatible with that of aberration assays, in that samples tested in both assays gave the same results.. The excess variation between the numbers of SCE's observed in replicate cultures exposed to ES2, was subsequently found to be insignificant when the data was transformed and a significant linear trend was obtained from the ANOVA test.

Table 36: Criteria for defining the mutagenic potential of samples tested in single trials in *in vitro* SCE assays.

Numbers of sample concentrations where SCE counts were elevated by 20% above controls	Significance level for trend test		
	P>0.025	0.025>P>0.005	0.005>P
0	-	-	?w
1	-	?w	?+
≥2	+b	+	+

Key: - is negative, + is positive, ?w and ?+ are questionable weak and questionable positive., due to only one concentration being elevated above control values, +b is positive lacking in dose response.

From Galloway *et al.* (1985)

To determine whether the exhaust samples could be classed as mutagenic in the SCE assay, the significance of the linear trend statistics obtained, coupled with the numbers of concentrations at which the SCE frequency was elevated to 20 % above control values were compared to the criteria described by Galloway *et al.* (1985), as shown in table 36. The repeat assay of ES1 with metabolic activation indicated that this sample was mutagenic both with and without metabolic activation. In contrast, a weak positive result was obtained for the induction of SCE 's by ES2. Finally the results of the assay of ES3 both with and without metabolic activation, failed to meet the criteria, and so ES3 was declared to be negative on this basis. However, the decline in SCE frequencies at the higher sample concentrations obtained with ES3 suggest that a repeat assay of this sample may be required.

The SCE data obtained from this study is comparable with the few published examples. Both Brooks *et al.* (1984 cited by Lewtas & Williams 1986), and Mitchell *et al.* (1981, cited by Lewtas 1982) have shown that diesel particulate extracts induce SCE's in CHO cells at concentrations of between 100 to 400 μg/ml. Morimoto *et al.* (1986) found significant increases in the SCE frequencies from control levels of 15 SCE/cell to 20 SCE/cell in human lymphocytes at a concentrations in excess of 100 μg/ml. However, when tested in hypersensitive lymphoblastoid lines significant increases were observed at concentrations of only 20 μg/ml. Geurnero & Rounds (1991) has also shown that exposure of Syrian hamsters to diesel particulate matter, leads to increased SCE frequencies in lung cells.

4.4. Trends between mutagenicity and composition of diesel emissions.

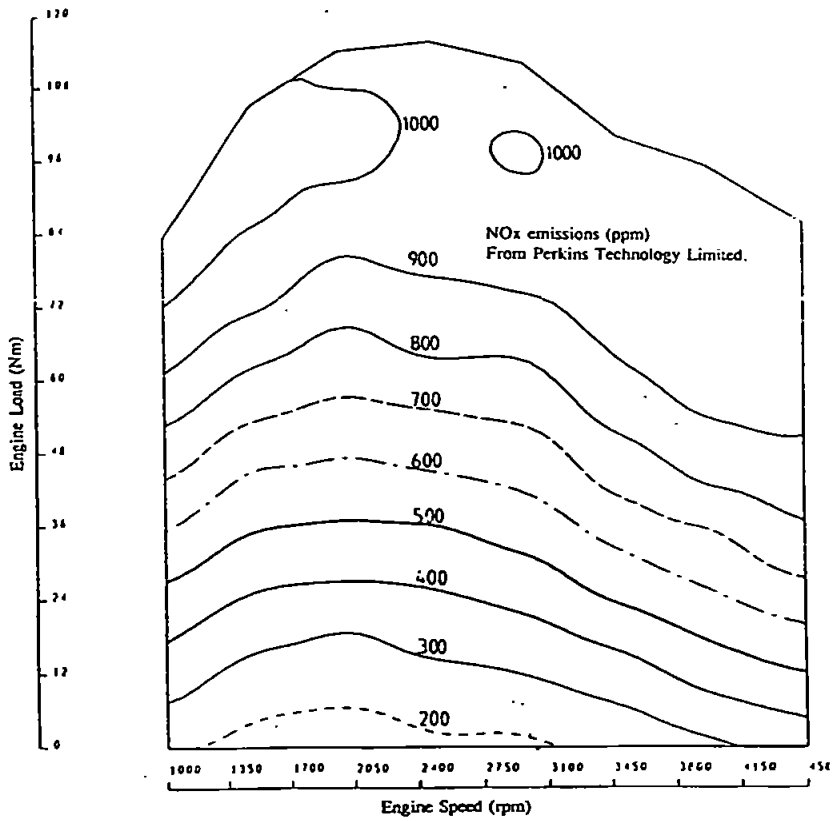
The linear trend statistics obtained for each emission sample were assumed to re-present the mutagenic potential of each sample. The lack of datapoints (maximum number of four) tested at each engine load and speed made the comparison of trend statistics with these factors impractical. In order to counter the effects of engine loading and speed, values of gaseous and particulate emissions were calculated as concentrations per unit of fuel consumed. Fuel consumption was found to have an approximately linear correlation with both engine speed and load at the conditions tested (Appendix 4).

In the majority of studies where bacteria were used as test organisms, the numbers of mutant cells induced by exposure to emission samples are usually expressed as revertants per mile travelled (revs/mile) or as revertants per unit of sample mass (revs/μg or revs/g). The use of percentages of mammalian cells with aberrations, precludes such extrapolations Consequently in most studies using mammalian cells with cytogenetic endpoints the relative mutagenic potential of samples has been presented as the magnitude of the gradient (slope) obtained from linear regression of the dose response. This statistic is essentially similar to the trend statistic, however the trend statistic is preferred due to the reduced reliance on the values obtained at the higher sample concentrations (Margolin *et al.* 1986).

Regression analysis of the linear trend statistic from cultures incubated with emission samples without supplementary metabolic activation, with the exhaust emission per gramme of fuel consumed, has shown weak positive correlation between linear trend and HC and CO emissions, and a negative correlation with Bosch smoke emissions. There was a significant ($P<0.05$) correlation between linear trends and emission of NOx. This is perhaps to be expected, as bacterial studies by both Bechtold (1986) and Scheutzle (1983) have suggested that the mutagenicity of an extract increases with increasing NOx emissions. Scheutzle found that increased NOx emissions were associated with enhanced formation of the mutagen 1-nitropyrene in samples. This led to a higher mutagenic response of these samples. A more recent study by (Courtois *et al.* 1993) using a similar approach as in this study (collection of samples from a number of different speeds and loads) has also suggested that there is a direct correlation between mutagenicity and NOx emissions, again samples were tested using the "Ames" test.

The correlation between increasing mutagenicity and NOx emissions is likely to be the result of the formation of highly mutagenic nitrated derivatives of PAH in the exhaust, although this suggestion requires confirmation, by the analysis of the nitrated PAH content of the samples. Assuming that NOx concentration is an important factor which affects the mutagenicity of the diesel emission as is suggested by this work and that of other including Bechtold (1993), it is of interest to identify under what conditions of engine use will high levels of NOx be emitted. High NOx are formed from the nitrogen and oxygen of the combustion air at high combustion temperatures. Reducing the temperature of combustion reduces NOx emissions but also increases particulate emissions. This is because highly efficient combustion is required to burn the majority of the fuel and subsequently reduced particulate formation takes place at temperatures high enough to promote NOx formation. For the engine used in this study a emission profile of NOx emissions is shown below (figure 57).

Figure 57: Emissions of Oxides of Nitrogen (NOx) from the Prima diesel engine.

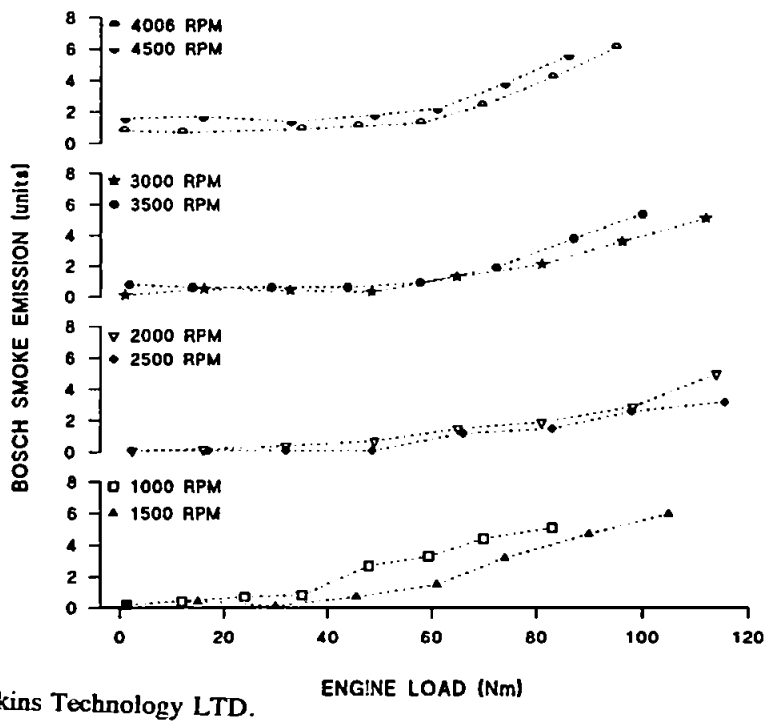


From figure (57) NOx emissions can be seen to increase generally with increasing engine load. But at each specific load, NOx emissions then tend to be higher at the lowest and highest speeds. For example the NOx emissions at zero load (0 Nm) are approximately 225 ppm at 1000 rpm, this values falls to approximately

150 ppm at 2000 rpm and then increase to 190 ppm and 300 ppm at speeds of 3000 and 4000 rpm respectively. This trend is repeated with increasing engine load, until the highest loads, where there is then little difference between NOx emissions at different speeds.

In contrast the regression of the linear trend statistics of cultures treated with metabolic activation with exhaust emissions, show a highly significant correlation between the linear trend statistics and Bosch smoke emissions. Again, weak positive responses were also observed with HC and CO emissions. Bosch smoke is a measure of the particulate matter emitted from the engine. The correlation of particulate emissions and indirect mutagenicity may represent metabolic activation of parental PAH compounds which are known to be associated with the diesel particulate matter. The Bosch smoke emissions from the Prima engine at several speeds are illustrated below (figure 58).

Figure 58: Emissions of diesel particulate matter as measured by the Bosch smoke test, from the Prima engine.



Source Perkins Technology LTD.

From figure 58 the values of Bosch smoke emissions can be seen to increase with engine load at all speeds. Generally, at speeds greater than 1500 rpm, emissions patterns are similar, although it appears that the

smoke emissions above two units appears to occur at higher loads, as engine speed is increased. This delay is most dramatic at speeds of 1000 and 1500 rpm. At 1000 rpm Bosch smoke emissions increase above 2 units at a load of approximately 45 Nm, whereas at 1500 rpm, this rise is delayed until an engine load of 65 Nm is reached.

One other factor which may influence the results of the regression analysis is the use of the trend test itself as measure of sample mutagenicity. The linear trend test is powerful only for detecting linear responses, however non-linear responses including considerable down-turns in the dose responses (as were obtained for some samples in this investigation) may reduce the power of the test for detecting significant increases in cells with damage. On this basis consideration must be given to achieving a linear dose response for all samples tested, in an attempt to maximise the sensitivity of the trend test.

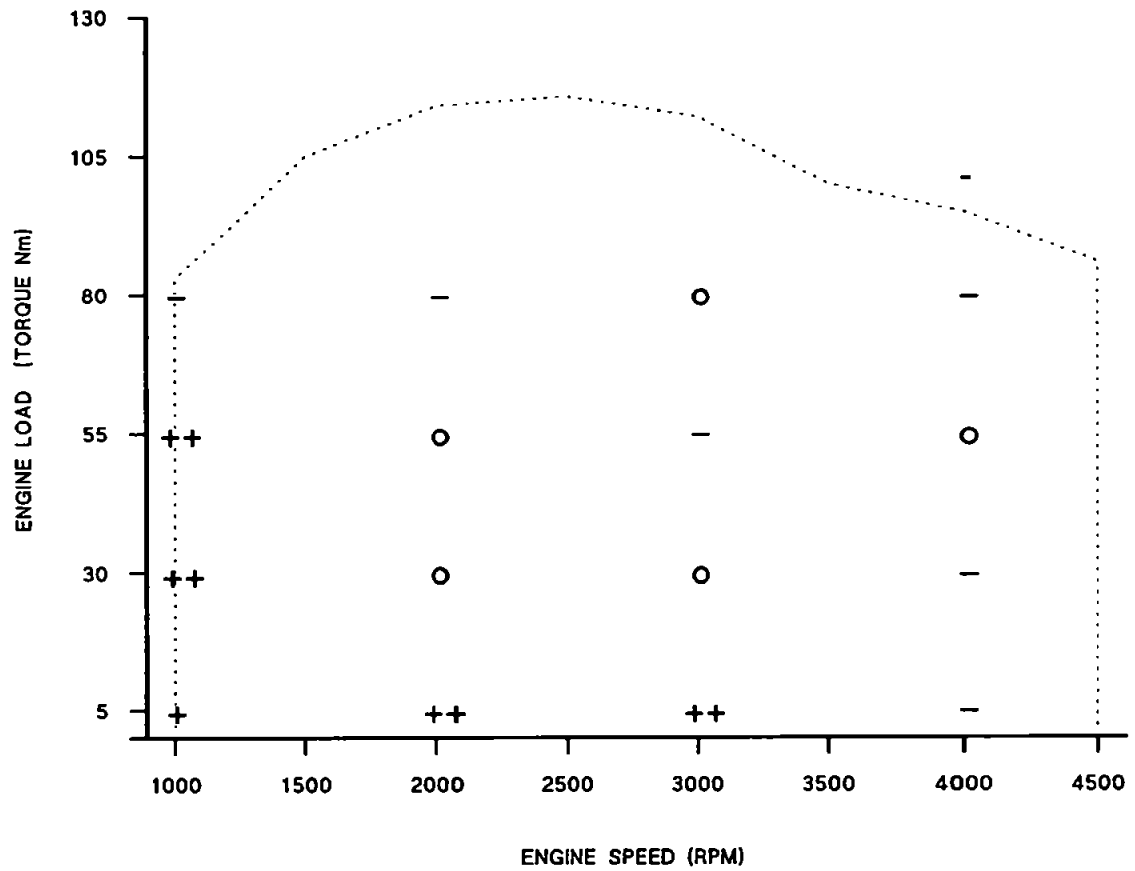
4.5. Mapping of potentially mutagenic engine emissions.

The primary objective of this investigation was to construct a mutagenicity map of the Prima engine in an attempt to identify the particular engine conditions at which the highest mutagenic potential of the emissions was greatest. The construction of this map was complicated by identifying a unit of measure for the emission mutagenicity. To this end, the clastogenic significance of the emission samples collected, as determined from table 35. were used to indicate the potential clastogenicity of the emission at the various engine conditions tested. The map constructed from this data is shown in figure 59. The symbols indicate the relative clastogenicty of the samples, as determined from the results of the chromosome aberrations assays without metabolic activation.

From this map using the current data, the clastogenicity of diesel exhaust emissions appears to be associated with the lower half of the operating range of the engine *ie.* low speed (1000 rpm) with low to intermediate load (5-55 Nm) and low load (5 Nm with low to intermediate speeds 1000 -3000 rpm). The clastogenicity of the emissions can separated by a diagonal from conditions of 1000 rpm @ 80 Nm load to 4000 rpm at 5 Nm load. Below this line all samples tested were found to have clastogenic activity, and those tested above showed no evidence of clastogenicity. The samples found to be positive, were collected at various times, were tested in both PD2 and G6 cell lines, and varied greatly in their toxicity, so there is little evidence

of bias introduced due to these factors. Further assays of the untested samples are required to give support to these observed trends. Chemical analysis of these samples is also required to identify any changes in emission composition that may account for these observations.

Figure 59: Mutagenicity map of the Prima direct injection diesel engine, showing the clastogenic potential of emission samples collected at a number of engine loads and speeds.



Legend

..... Represents the maximum operating conditions of the Prima engine.

+ + strong clastogenic activity

+ weak clastogenic activity

- no evidence of clastogenic activity

O samples not tested for clastogenic potential

4.6. Relevance of results of mutagenicity tests to in vivo exposures.

There has been considerable discussion of the role of short-term mutagenicity tests and their value for predicting the carcinogenicity of test compounds. The shortcomings of each assay have been largely over come by the adoption of test batteries, whereby a number of tests to detect different genetic endpoints are used simultaneously (DOH 1990). This has reduced the requirement for long-term rodent carcinogenicity tests. However the repeated testing of the same compounds in a number of different test systems may become as expensive as performing long-term carcinogenicity tests. There is also some delay in obtaining the results from a battery of tests, as few institutions have the resources to conduct the tests simultaneously. In general only a few tests are performed and of these the Ames *Salmonella* assay is usually used as an initial screen. Both the UKEMS and the DOH recommend the use of a second assay, particularly if negative results are obtained in bacterial assays. Mammalian cytogentic assays are usually then used as a second screen and it is widely believed that the use of both these tests will detect most types of chemical mutagens (DOH 1990).

Kirkland (1992) has questioned the relevance of *in vitro* responses in which aberrations were only induced at concentrations at which there was evidence of cytotoxic effects. He suggested that high toxicity clastogens (HTC's) have less *in vivo* importance than compounds that exert clastogenic effects at concentrations which show little evidence of cytotoxicity (low toxicity clastogens: LTC's). Data presented by Scott *et al.* (1990) has shown, that whereas LTC's such as mitomycin C do show potent clastogenic and carcinogenic activity *in vivo*, most HTC's (*eg:* 2-aminobiphenyl) are negative, or at best are weak carcinogens.

The evidence from this investigation, would suggest that the diesel emission samples collected from the TESSA, could be classed as high toxicity clastogens. This classification is based on the observation that increases in the frequency of aberrations above control levels were generally small and occurred at concentrations which approached the maximum tolerated in cell viability assays, which reduced the miotic indices. Whether the reduction in the mitotic indices observed is the direct result of the toxicity of the sample reducing the numbers of viable cells within the cultures or alternatively is an effect of mitotic inhibition requires further investigation. This would require the testing of samples with delayed harvesting

times to determine when peak yields of aberrations occur.

4.7. The potential health risks associated with diesel exhaust emissions.

Prior to the initiation of this investigation (1990) the most recent literature available seemed to be contradictory. The IARC (1989) classified the diesel exhaust emissions as a potential carcinogen. In contrast, Grasso *et al.* (1988) after reviewing the literature concluded that, the evidence for carcinogenicity of diesel exhaust emissions was sparse and " the overall risk of cancer was very small even in highly exposed populations". Since this time, a number of long term studies have been published and reanalysed. Most chronic inhalation studies have shown little evidence for the induction of lung cancer by diesel particulates. The positive increases that have been observed (Ishinishi 1986, Mauderly *et al.* 1987, and Brightwell *et al.* 1989), have occurred only when the test organisms were rats, and then only at highly elevated particulate concentrations. Stober (1992) reviewed the findings of several studies including those of Garshick *et al.* (1987 & 1988) that appeared to indicate that small, but significant increases in the numbers of lung cancers were associated with particulate exposure. However in most of these studies the relative risk of cancer induced by smoking is an overwhelming factor which tends to mask any small increase which may be due to diesel exposure.

Heinrich (1993 cited by Pepelko and Chen 1993), has shown that the inhalation of carbon black, (which is structurally similar to the carbonaceous core of diesel particulate matter but without adsorbed organic material), induces a similar magnitude of lung cancers in rats exposed to high concentrations. Other workers (Mauderly *et al.* 1991, Pott *et al.* 1991) have shown similar results. Since the presence of adsorbed carcinogenic PAH species represents only a small percentage of the total mass of diesel particulate (Tong and Karasek 1984), they are likely to be only of minor importance to the overall carcinogenic effects which have been observed. Pepelko and Chen also suggested that the adsorbed species may have a secondary role in tumour initiation which is further promoted by the presence of the particles themselves.

Despite the lack of evidence for the carcinogenic effects of diesel emissions, recent interest has been stimulated in the physical health effects of particulate matter inhalation. Wade and Newman (1993) have

published conclusive evidence that exposure to elevated concentration of diesel particulate matter from diesel locomotives has induced at least three incidences of asthma. Another recent report (Clench Aas *et al.* 1993) has led to the association of increased mortality and morbidity with increasing particulate concentrations in the urban atmosphere, these findings have stimulated further investigation in the U.K. with the publishing of a report on the influence of diesel emissions on the air quality in urban areas (QUARG 1993).

From a health perspective the association between increasing NOx emissions and direct acting mutagenicity has again been demonstrated, and this correlation identified, in at least three different studies, must be indicative of a causal relationship. However, the contribution of these direct acting mutagens to the clastogenic effects observed in cultures exposed to *in vitro* metabolic activation requires investigation. In contrast the association of indirect mutagenicity with emission of particulate matter lends support to the recent claims for the health effects associated with urban emission of particulate matter. As has been mentioned (Pepelko 1993) there may be an secondary role for particulate adsorbed, organic material in the initiation or promotion of lung tumours. Further investigation is required to determine if this so.

One area for future concern involves the current use of technology to reduce emission, The particulate traps fitted to new diesel vehicles have a limited life span and require regeneration; this is achieved by burning off the collected particulate matter, either while the vehicle is in motion or stationary. The time required for the burning off, can be envisaged as a transitory period during which potentially harmful compounds associated with the particulate matter are likely to become present at high concentrations in a localised area. The outcome of these regeneration sessions with regard to the composition of their gaseous emissions and potential health effects require investigation. A preliminary chemical analysis of the emissions of trap regeneration have recently been reported (Li *et al.* 1994).

The main indication from this study is that, the most clastogenic exhaust emissions appear to occur at engine conditions likely to be encountered in an urban environment. Driving conditions of low speed, at various loads, are typically associated with buses, which probably contribute most to urban pollution. The clastogenicity of samples collected at conditions of low load but with increasing speeds are less likely to

encountered in city centres, although these may be experienced on some urban roads and dual carriageways. The findings that clastogenic emissions are produced under urban driving conditions, the association between indirect mutagenicity and increasing particulate emissions, and direct mutagenicity and NOx emissions, all tend to support the growing evidence of the detrimental health effects associated with diesel exhaust emissions in urban areas. The engine conditions associated with these effects are not only associated with urban traffic. Similar conditions are also experienced in stationary traffic queues. The concentrations of emissions under these conditions does not appear to have been investigated, although it is suggested here that local vehicle pollutant levels may become sufficiently high so as to pose a potential health risk. Obviously this statement requires verification, and this a potential area for further study.

Another area for investigation is to determine what compound or compounds present in the aromatic fractions are directly responsible for the observed cellular cytotoxicity. The exhaust emission cytotoxicity may have a direct bearing upon the chronic respiratory effects observed in some individuals. As aromatic compounds are likely to be emitted adsorbed to particulate matter under environmental conditions, this may explain the observed association between particulate emissions and respiratory disorders reported by Pope (1992).

4.8. Conclusions.

The direct comparison of the results of this study with the available literature are complicated, by the use of transient test cycles by other authors, for the collection of samples. The mutagenic potential of such samples would be expected to be positive if particular segments of the test cycle result in the formation of highly mutagenic emissions. This view is supported by the finding of Bechtold *et al.* (1984) that a twelve fold variation in the amount of mutagens was produced during various sections of the FTP cycle. The most mutagenic section of the test cycle was found to be associated with high speed cruising and rapid acceleration. It is therefore not surprising to find a combination of both positive and negative mutagenic results, in engine samples collected under different conditions.

In-vitro cytogenetic assays were used in this study, to supplement the lack of *in vitro* chromosome aberration

data concerning the effects of diesel emissions. Very few actual studies had previously been performed, and in some cases the same data has been presented on more than one occasion (Lewtas 1982 ,1983). Despite this, there is a general belief that the results of cytogenetic tests are adequately represented in the literature. One feature of the existing data is that results tend to be contradictory. Samples obtained from different engines show great variation in their clastogenic potential. In contrast to other work, the results of this study have shown that there are also significant differences in the clastogenic potential of emissions obtained from the same engine under different operating conditions.

APPENDIX 1.

RESULTS OF TOXICTY ASSAYS.

The following figures illustrate the responses of CHO-G6 cells following expsoure to diesel emissions samples. The viability of cells was determined by the uptake of Neutral red dye in a solution of phosphate buffered saline, in a period of 2 hours after overnight exposure to the emission samples. In most cases there were few cells remaining in wells containing the highest sample concentrations. There did appear to be a residual amount of neutral red remaining in wells after washing, and this accounts for the non-zero readings in wells. In general absorbance values of less than 0.05 are regarded as containing no viable cells. Data from duplicate wells are presented with the mean absorbance values, and with error bars of the standard error of the means. Toxicity assays previously included in the text (section 3) are not repeated.

Figure 60: The effects of exposure to diesel emission samples ES2 (2000 rpm @ 5Nm) on the cells viability of CHO- K1 cells as indicated by uptake of neutral red dye.

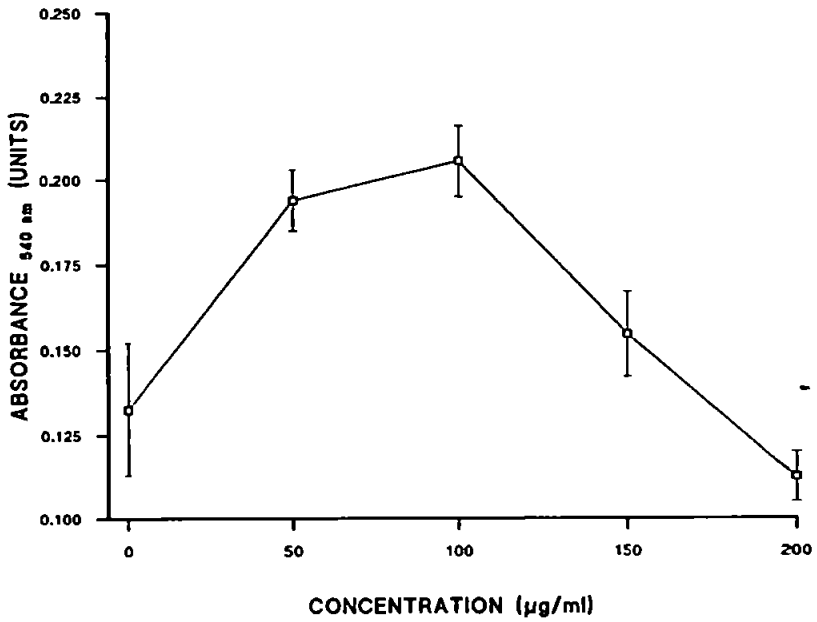


Figure 61: The effects of exposure to diesel emission samples ES3 (3000 rpm @ 5Nm) on the cells viability of CHO- K1 cells as indicated by uptake of neutral red dye.

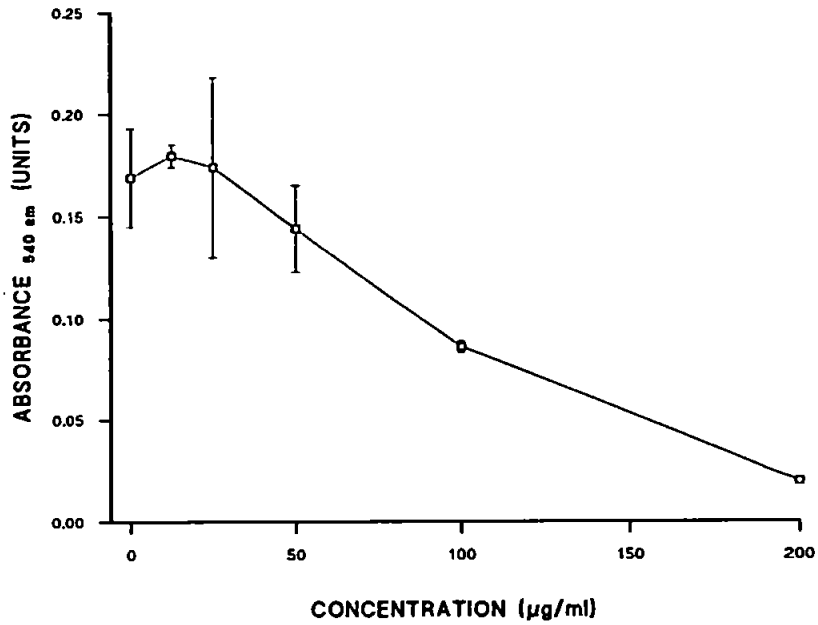


Figure 62: The effects of exposure to diesel emission sample ES5 (1000 rpm @ 30 Nm load) on the cell viability of CHO-G6 cells as indicated by uptake of neutral red dye.

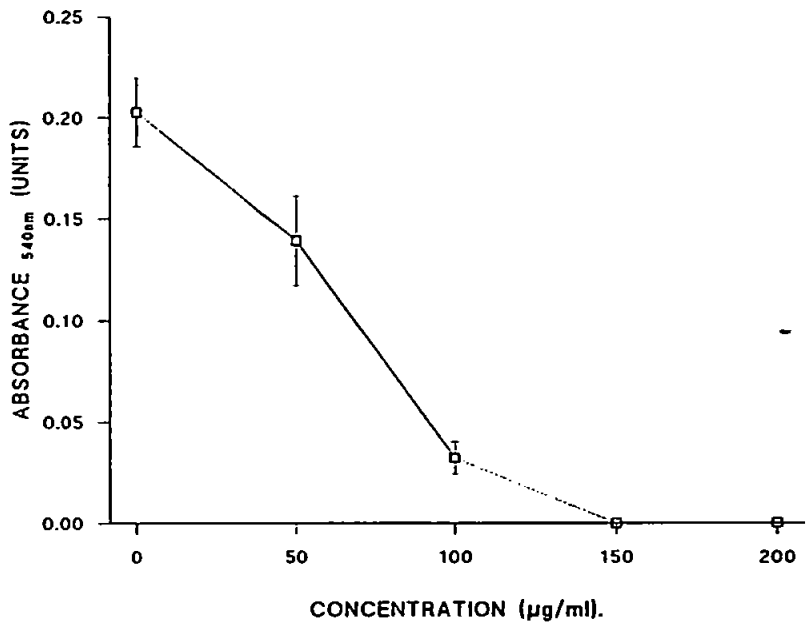


Figure 63: The effects of exposure to diesel emission sample ES7 (2000 rpm @ 30 Nm load) on the cell viability of CHO-G6 cells as indicated by uptake of neutral red dye.

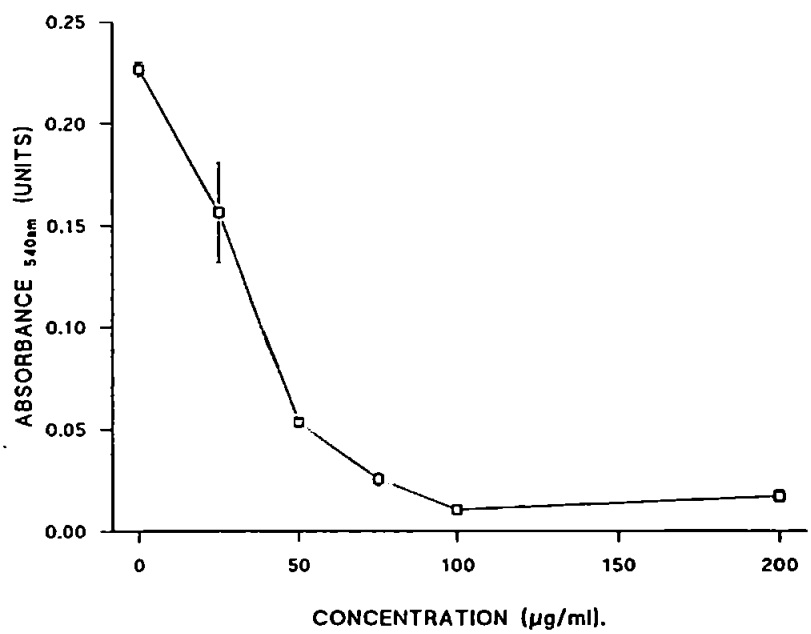


Figure 64: The effects of exposure to diesel emission sample ES8 (3000 rpm @ 30 Nm load) on the cell viability of CHO-G6 cells as indicated by uptake of neutral red dye.

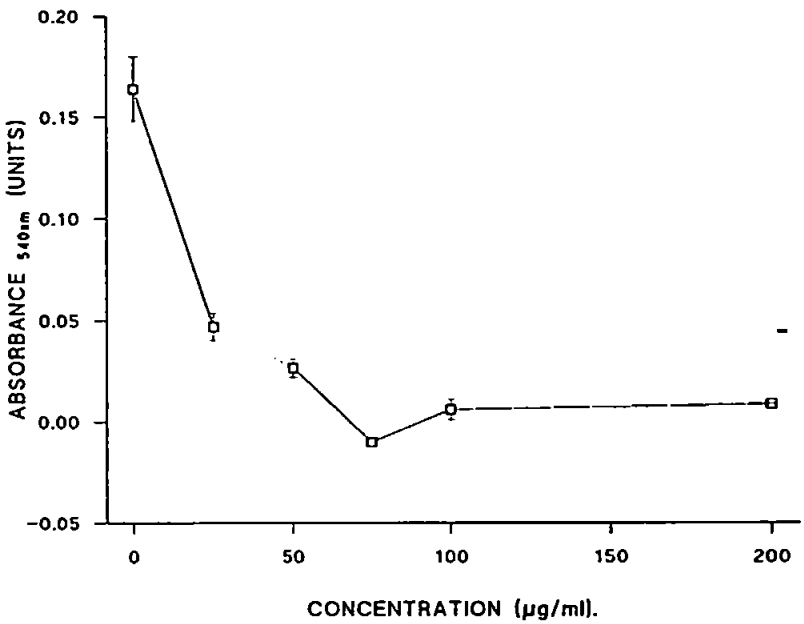


Figure 65:The effects of expsoure to diesel emisson samples ES9 (4000 rpm @ 30 Nm load) on the viability of CHO-G6 cells as indicated by the uptake of neutral red dye.

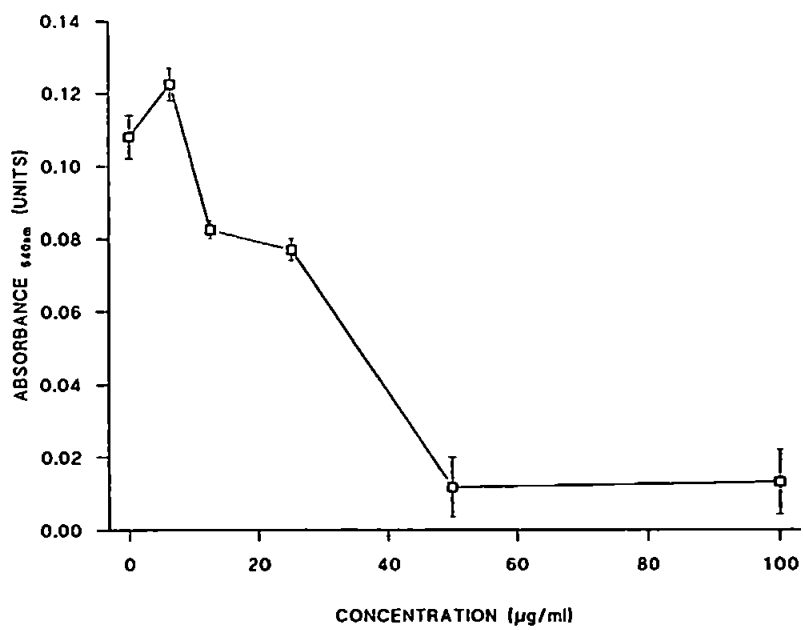


Figure 66: The effects of exposure to diesel emission sample ES10 (1000 rpm @ 55 Nm load) on the cell viability of CHO-G6 cells as indicated by uptake of neutral red dye.

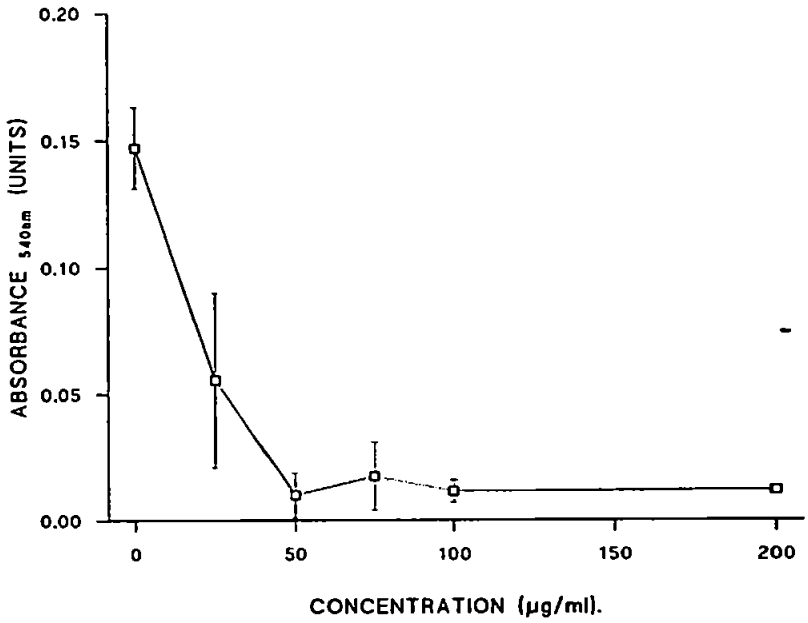


Figure 67: The effects of exposure to diesel emission sample ES11 (2000 rpm @ 30 Nm load) on the cell viability of CHO-G6 cells as indicated by uptake of neutral red dye.

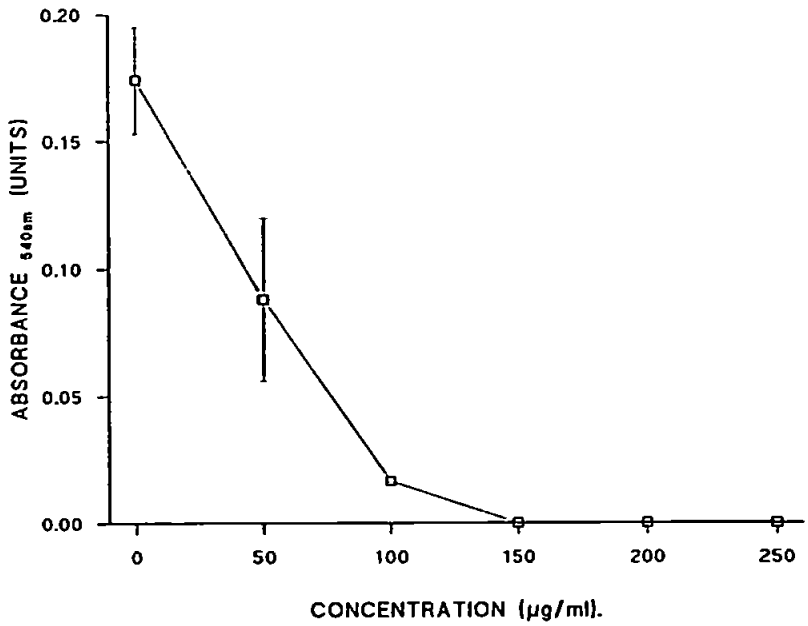


Figure 68: The effects of exposure to diesel emission sample ES12 (3000 rpm @ 55 Nm load) on the cell viability of CHO-G6 cells as indicated by uptake of neutral red dye.

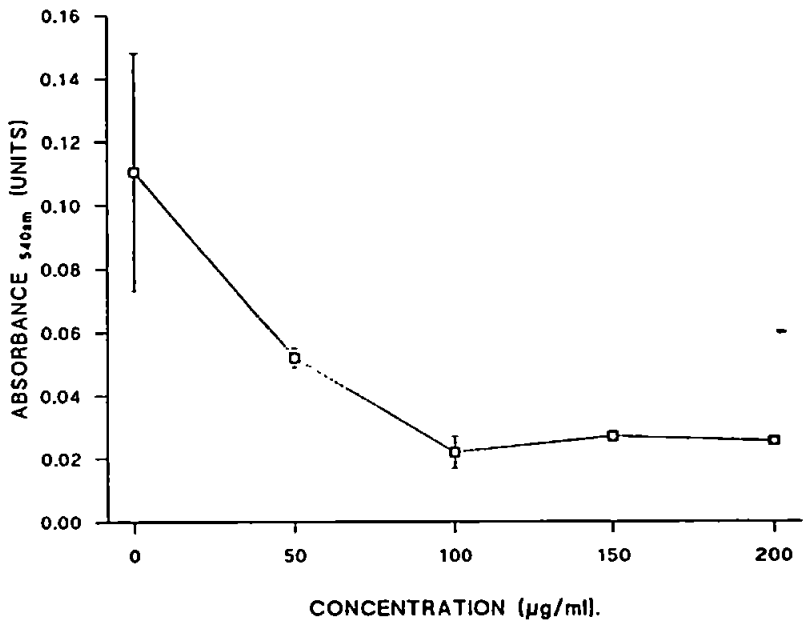


Figure 69: The effects of exposure to diesel emission sample ES14 (1000 rpm @ 80 Nm load) on the cell viability of CHO-G6 cells as indicated by uptake of neutral red dye.

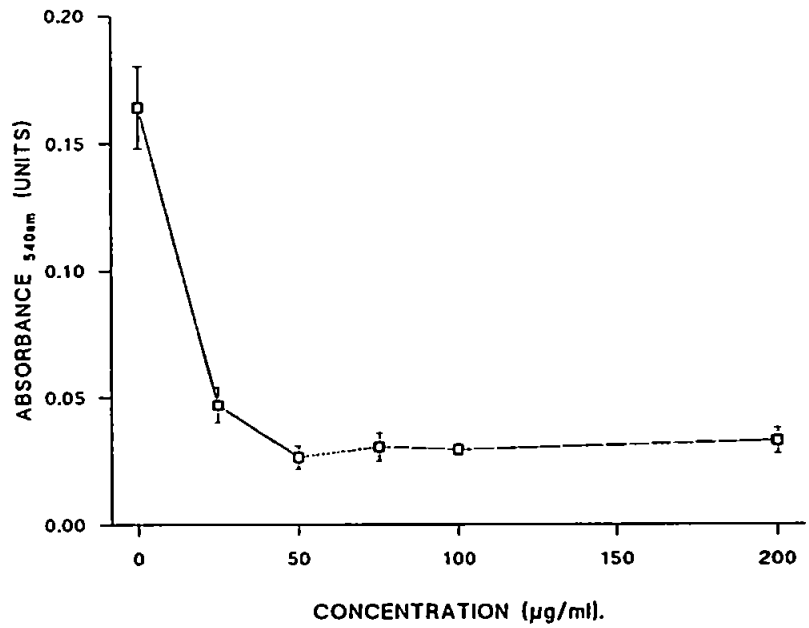


Figure 70: The effects of exposure to diesel emission sample ES15 (2000 rpm @ 80 Nm load) on the cell viability of CHO-G6 cells as indicated by uptake of neutral red dye.

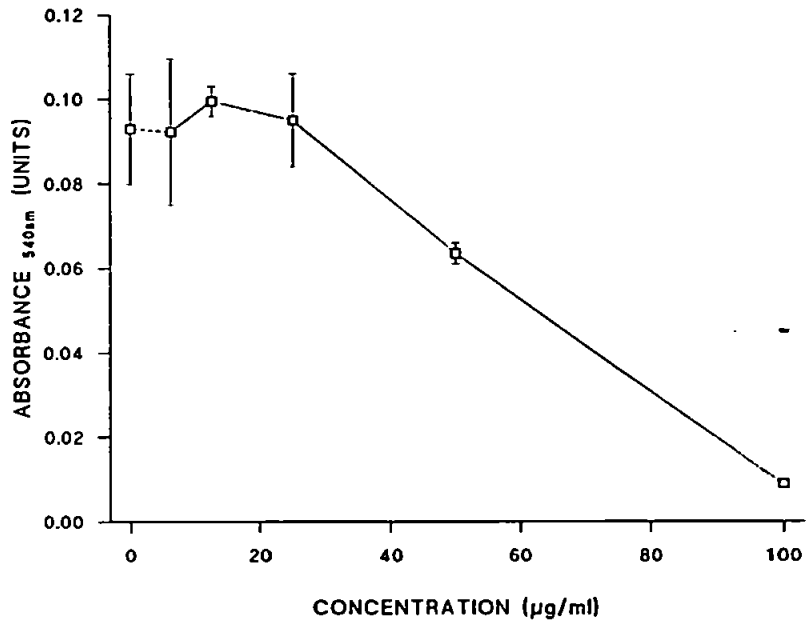


Figure 71: The effects of exposure to diesel emission sample ES16 (3000 rpm @ 80 Nm load) on the cell viability of CHO-G6 cells as indicated by uptake of neutral red dye.

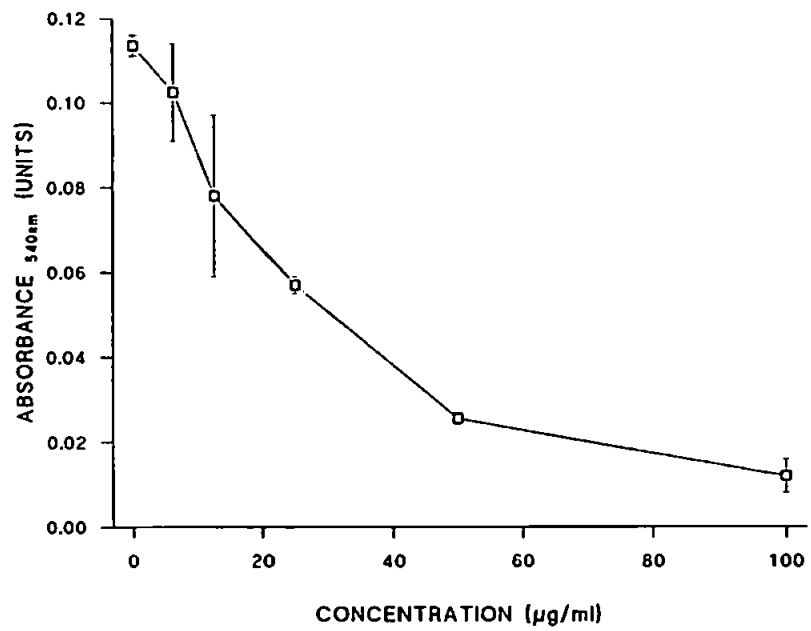
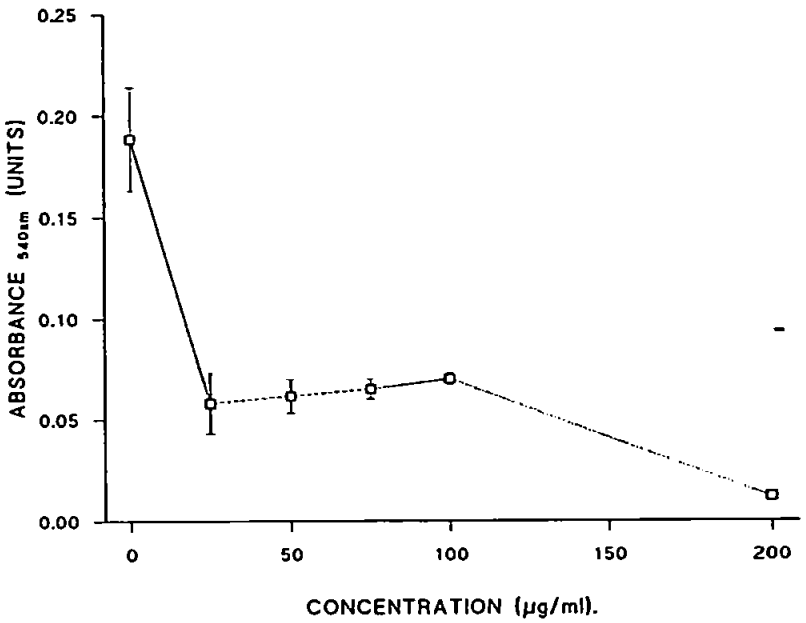


Figure 72: The effects of exposure to diesel emission sample ES17 (1000 rpm @ 5 Nm load) on the cell viability of CHO-G6 cells as indicated by uptake of neutral red dye.



APPENDIX 2

CHROMOSOME ABERRATION DATA.

The following tables illustrate the chromosome aberration data collected from exposure of CHO cells to diesel emission samples. The numbers of cells scored in each replicate culture is shown with the numbers of cells containing aberrations from the three categories. Values are also given for the numbers of aberrations observed in both positive control cultures exposed to MNNG and CP. The data was used for the calculation of both the dispersion test of between replicate differences and the linear trend statistics which were presented earlier (section 3).

Table 37: Numbers of chromosome aberrations observed in CHO-PD2 cells following exposure to diesel emission sample ES1 collected at (1000 rpm @ 5Nm engine load).

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
0	100	14	10	4
0	100	16	11	5
1	100	12	8	4
1	100	16	13	3
10	100	13	10	3
10	100	20	9	11
100	100	19	14	5
100	100	23	19	4
200	100	29	21	8
200	100	16	12	4
MNNG	72	31	20	9

Table 38: Numbers of chromosome aberrations observed in CHO-PD2 cells following exposure to diesel emission sample ES2 collected at (2000 rpm @ 5Nm engine load).

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
0	100	16	14	2
0	100	13	14	3
10	100	15	14	1
10	100	17	16	1
100	100	34	30	4
100	100	24	19	5
200	100	48	36	12
200	100	45	35	10
MNNG	100	39	32	6

Table 39: Numbers of chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES3 collected at (3000 rpm @ 5Nm engine load).

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
0	100	5	4	1
0	100	9	6	3
10	100	6	14	3
10	100	12	10	1
100	100	17	14	4
100	100	14	10	4
MNNG	100	12	9	3

Table 40: Numbers of chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES4 collected at (4000 rpm @ 5Nm engine load) both with and without supplementary metabolic activation.

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
Without metabolic activation				
0	100	7	5	2
0	100	5	5	0
13.5	100	6	4	2
13.5	100	5	5	0
27	74	4	4	0
27	56	0	0	0
54	100	5	4	1
54	100	6	6	0
MNNG	56	8	7	1
With metabolic activation				
0	100	1	1	0
0	100	8	7	1
13.5	100	4	4	0
13.5	100	6	5	1
27	100	5	5	0
27	100	7	6	1
54	76	2	2	0
54	100	6	6	0
CP	86	13	9	3

Table 41: Numbers of chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES5 collected at (1000 rpm @ 30 Nm engine load).

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
Without metabolic activation				
0	100	3	3	0
0	100	5	5	0
50	100	12	12	0
50	100	5	4	1
75	100	15	11	4
75	72	10	9	1
100	65	4	4	4
100	62	7	5	2
125	22	1	1	0
125	11	0	0	0
MNNG	100	10	8	2
Plus metabolic activation				
0	100	8	7	1
0	100	9	8	1
50	100	8	6	2
50	100	8	6	2
75	100	8	6	2
75	100	13	12	1
100	100	10	6	4
100	100	5	5	0
125	100	10	9	1
125	100	4	1	3
CP	100	29	21	8

Table 42: Numbers of chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES9 collected at (4000 rpm @ 30 Nm engine load) with and without supplementary metabolic activation.

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
Without metabolic activation				
0	100	3	1	2
0	100	3	1	0
6.25	100	1	0	1
6.25	100	3	1	1
12.5	100	1	0	1
12.5	100	2	2	0
25	81	3	2	1
25	100	1	1	0
50	19	0	0	0
50	100	2	1	0
MNNG	75	5	3	1
With metabolic activation				
0	100	3	2	1
0	78	3	0	2
12.5	100	2	1	0
12.5	100	3	1	2
25	90	2	2	0
25	83	1	0	0
50	96	3	2	0
50	100	2	2	0
100	100	2	1	0
100	37	4	4	0
CP	100	13	5	6

Table 43: Numbers of chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES10 collected at (1000 rpm @ 55Nm engine load) both with and without supplementary metabolic activation.

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
Without metabolic activation				
0	105	4	3	1
0	100	2	1	1
6.25	100	6	5	1
6.25	100	8	8	0
12.5	100	6	4	2
12.5	100	4	2	2
25	100	6	2	4
25	100	14	11	2
50	100	14	11	2
50	78	7	7	0
MNNG				
With metabolic activation				
0	100	6	6	0
0	100	5	4	1
6.25	105	9	4	2
6.25	100	1	1	0
12.5	100	8	7	0
12.5	108	6	6	1
25	64	6	5	1
25	55	2	1	1
50	100	11	9	2
50	67	7	7	1
CP	18	2	2	0

Table 44: Numbers of chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES12 collected at (3000 rpm @ 55 Nm engine load).

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
Without metabolic activation				
0	100	3	3	0
0	100	4	4	0
6.25	100	1	0	1
6.25	100	6	5	1
12.5	100	4	3	0
12.5	100	0	0	0
25	100	3	2	1
25	100	4	3	1
50	100	2	1	1
50	75	4	3	1
MNNG	112	12	9	3
With metabolic activation				
0	105	6	6	0
0	100	3	3	0
12.5	100	1	1	0
12.5	100	3	2	1
25	97	1	1	0
25	100	3	2	0
50	107	2	2	0
50	100	3	3	0
100	77	1	1	0
100	96	4	3	0
CP	59	4	3	1

Table 45: Numbers of chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES14 collected at (1000 rpm @ 80 Nm engine load) both with and without metabolic activation.

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
Without metabolic activation				
0	100	4	3	0
0	100	5	3	0
6.25	60	1	1	0
6.25	50	1	1	0
12.5	100	1	1	0
12.5	100	6	6	0
25	66	4	3	1
25	100	3	3	0
50	21	1	0	0
50	33	1	0	0
MNNG	100	12	12	0
With metabolic activation				
0	100	4	4	0
0	89	5	4	0
12.5	100	6	6	0
12.5	90	5	5	0
25	100	8	8	01
25	78	6	6	0
50	100	1	0	1
50	75	5	5	0
100	48	5	4	1
100	100	10	9	1
CP	91	12	9	2

Table 46: Numbers of chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES15 collected at (2000 rpm @ 80 Nm engine load) both with and without metabolic activation.

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
Without metabolic activation				
0	100	2	2	0
0	100	1	1	0
6.25	100	4	3	0
6.25	100	2	1	0
12.5	100	3	3	0
12.5	100	2	2	0
25	100	1	0	0
25	100	3	3	0
50	58	2	1	0
50	100	3	3	0
MNNG	100	4	3	1
With metabolic activation				
0	100	1	0	0
0	100	1	1	0
12.5	95	3	3	0
12.5	100	3	3	0
25	100	3	3	0
25	100	1	1	0
50	100	1	1	0
50	150	1	1	0
100	100	2	1	1
100	100	5	2	3
CP	100	10	5	4

Table 47: Numbers of chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES19 (collected at 4000 rpm @ 80 Nm engine load).

Concentration	No scored	total	simple	complex
Without metabolic activation				
0	100	3	1	2
0	100	3	3	0
6.25	100	2	2	0
6.25	100	2	1	0
12.5	200	11	9	2
12.5	167	5	4	1
25	100	6	6	0
25	lost	-	-	-
50	ct	-	-	-
50	ct	-	-	-
MNNG	100	7	6	1
With metabolic activation				
0	100	3	3	0
0	100	9	7	2
12.5	100	6	5	1
12.5	100	6	5	1
25	100	2	2	0
25	100	2	2	0
50	100	6	6	0
50	57	2	1	1
100	100	4	4	0
100	100	7	6	1
CP	20	4	2	2

Table 48: Numbers of chromosome aberrations observed in CHO-G6 cells following exposure to diesel emission sample ES19 collected at (4000 rpm @ 80 Nm engine load) both with and without metabolic activation.

Sample concentration (μg/ml)	Numbers of cells scored	Total numbers of cells with aberrations	Numbers of cells with simple aberrations	Numbers of cells with complex aberrations
Without metabolic activation				
0	100	11	10	0
0	100	12	9	3
6.25	100	4	3	1
6.25	100	7	7	0
12.5	100	5	5	0
12.5	100	2	0	1
25	100	11	10	1
25	100	10	10	0
50	100	9	9	0
50	33	3	2	1
MNNG	100	8	7	1
With metabolic activation				
0	100	12	12	0
0	129	7	7	0
12.5	7	0	6	0
12.5	100	3	3	0
25	100	5	0	0
25	100	4	4	1
50	100	6	5	0
50	100	5	5	0
100	100	11	11	1
100	100	10	8	1
CP	63	11	5	6

APPENDIX 3

DATA FROM SISTER CHROMATID EXCHANGE ASSAYS.

Analysis of SCE data was performed on three levels. An analysis of distribution of exchanges between both, cells within a single culture and the distribution of SCE's between replicate cultures was required, before the analysis of the dose response via a linear trend test could be performed. The initial distribution tests ware per formed as described by Cooke *et al.* (1989), and the summary results of these are shown below The results obtained from the distribution of the assays of ES1 and ES2 without metabolic activation in the CHO cell line showed excess variation between replicate cultures. Consequently these datasets were analysed using an ANOVA test using the between cultures error and transformed SCE values. Linear trends were calculated for these non-Poisson data tests using the methods illustrated by Cooke et al. Linear trends for the remaining data from cultures of G6 cells exposed to both ES1 and ES3 with and without metabolic activation were analysed using the linear trend described by Galloway et al. (1985)

Table 49: Summary statistics for poisson model data test for between cells error in cultures of CHO-K1-PD2 cells exposed to diesel emission sample ES1 (collected at 1000 rpm @ 5Nm engine load).

	1	2	3	4	5	6	7	8	9	10
$\sum_{k=1}^{a}(y_k-\bar{y})^2$	252.7	224.1	209.3	303.7	341.4	609.3	198.4	184.3	127.1	146.1
Mean $\bar{y}$	6.50	7.70	8.30	7.50	8.20	10.93	6.10	6.43	9.23	8.97
χ^2	38.85	29.10	25.22	40.50	41.62	55.75	32.52	28.65	13.77	16.29
Degrees of freedom	29	29	29	29	29	29	29	29	29	29

Total χ^2 = 325.24

Total degrees of freedom = 290

Table 50: Summary of Poisson distribution test for replicate effects of distribution of SCE's in cultures of CHO-K1-PD2 cells exposed to diesel emission sample ES1 (collected at 1000 rpm @ 5Nm engine load).

Treatment	0	10	25	50	100	Totals
$\sum_{j=1}^{r}(T-\bar{T})^2$	648	288	3362	50	32	
$\bar{T}$	213	237	287	188	273	
χ^2	3.04	1.21	11.71	0.26	0.117	16.35
D.F	1	1	1	1	1	5

Table 51: ANOVA using between cultures error for estimating linear trend of SCE data from cultures of CHO-K1-PD2 cells exposed to diesel emission sample ES1 (collected at 1000 rpm @ 5 Nm engine load).

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square	*F*
Treatments	4	42.35	10.59	4.55
Between Replicates	5	11.67	2.33	
Total	9	54.02		

Table 52: Summary statistics for Poisson model fitted to SCE data induced by exposure of CHO-K1-PD2 cells to increasing concentrations of diesel emission sample ES2 (collected at 2000 rpm @ 5 Nm engine load).

	1	2	3	4	5	6	7	8	9	10
$\sum_{k=1}^{n}(k_k-\bar{y})^2$	301.2	300.4	192.6	238.0	376.5	100.8	238.6	133.7	451.8	283.9
Mean $\bar{y}$	8.77	7.60	8.80	7.17	9.73	8.33	9.13	8.43	11.6	10.7
χ^2	34.35	39.50	21.88	33.2	38.7	12.1	26.0	15.86	38.8	26.4
Degrees of freedom	29	29	29	29	29	29	29	29	29	29

Total χ^2 = 286.9

Total degrees of freedom= 290

Table 53: Summary of Poisson distribution test for replicate effects of distribution of SCE's in cultures of CHO-K1-PD2 cells exposed to diesel emission sample ES2 (collected at 2000 rpm @ 5 Nm engine load).

Treatment	0	10	25	50	100	Totals
$\sum_{j=1}^{r}(T-\bar{T})^2$	612.5	1220.5	882.0	220.5	364.5	
$\bar{T}$	245.5	239.5	271.0	263.5	335.0	
χ^2	2.49	5.01	3.25	0.80	1.08	12.65
Degrees of freedom	1	1	1	1	1	5

Table 54: ANOVA using between cultures error for estimating linear trend of SCE data from cultures of CHO-K1-PD2 cells exposed to diesel emission sample ES2 (collected at 2000 rpm @ 5Nm engine load).

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square	*F*
Treatments	4	28.37	7.0925	3.589
Between Replicates	5	9.88	1.976	
Total	9	39.25		

Table 55: Summary statistics of test of the SCE distribution between cells in cultures of CHO-K1-G6 cells following exposure to diesel emission sample ES1 (collected at 1000 rpm at 5Nm engine load).

Culture	1	2	3	4	5	6	7	8	9	10	Total
$\sum (y_i-\bar{y})^2$	262.7	329.4	101.2	287.5	473.4	224.7	265.4	410.7	249.0	628.3	
Mean (	8.67	10.57	10.54	10.50	10.40	10.90	11.57	12.10	12.63	12.70	
χ^2	30.31	31.17	9.61	27.38	45.37	20.61	22.94	33.94	19.71	49.47	291
D.F	29	29	12	29	29	29	29	29	29	29	273

Table 56 : Inter-replicate dispersion test for excess variation from Poisson distribution of SCE's induced in CHO-K1-G6 cells following exposure to diesel emission sample ES1 (collected at 1000 rpm @ 5Nm engine load).

Treatment	0	1	2	3	4	5	Total
$\sum_{i=1}^{n} (y_i-\bar{y})^2$	1200.5						
mean $\bar{y}$	9.62	10.51	10.67	11.85	12.52		
χ^2	5.62	0.00	0.31	0.40	0.16		6.49
D.F.	1	1	1	1	1		5

Table 57: Summary statistics of test of the SCE distribution between cells in cultures of CHO-K1-G6 cells following exposure to diesel emission sample ES1 (collected at 1000 rpm at 5Nm engine load) with supplementary metabolic activation.

Culture	1	2	3	4	5	6	7	8	9	10	Total
	280.0	337.7	446.3	42.5	100.9	32.4.	426.0	233.9	335.8	308.0	
mean	8.80	9.55	12.70	15.50	12.94	11.70	11.67	11.07	11.80	14.2	
X	31.91	35.00	35.14	2.94	7.8	27.72	36.51	21.13	30.24	30.24	259
D.F	29	22	29	5	16	29	27	29	29	4	219

Table 58: Inter-replicate dispersion test for excess variation from Poisson distribution of SCE's induced in CHO-K1-G6 cells following exposure to diesel emission sample ES1 (collected at 1000 rpm @ 5Nm engine load) in the presence of supplementary metabolic activation.

Treatment	0	1	2	3	4	5	Total
$\sum_{k=1}^{n}(y_k-\bar{y})^2$	1200.5						
mean $\bar{y}$	9.63	12.64	12.06	11.29	12.14		
χ^2	0.26	0.05	0.91	0.36	2.04		3.62
D.F.	1	1	1	1	1		5

Table 59: Summary statistics of test of the SCE distribution between cells in CHO-K1-G6 cultures following exposure to diesel emission sample ES3 (collected at 3000 rpm at 5Nm engine load).

Culture	1	2	3	4	5	6	7	8	9	10	Total
$\sum_{k=1}^{t}(y_k-\bar{y}.$	150.8	363.0	196.0	253.5	253.0	140.7	197.0	282.0	146.7	158.0	
mean	8.80	8.63	10.00	8.50	10.03	10.33	10.37	10.00	10.33	9.00	
X	17.14	42.04	19.60	28.92	25.21	13.61	19.00	28.20	14.19	17.56	225
D.F	29	29	29	29	29	29	29	29	29	29	290

Table 60: Summary statistics of test of the SCE distribution between cells in cultures after exposure to diesel emission sample ES3 (collected at 3000 rpm at 5Nm engine load) with supplementary metabolic activation.

Culture	1	2	3	4	5	6	7	8	9	10	Total
	287.9	151.4	296.3	240.3	236.7	145.2	321.9	239.5			
mean	10.07	8.43	9.70	10.30	10.90	10.60	9.73	8.50			
X	28.60	17.95	30.55	23.33	21.72	13.70	33.07	28.18			197
D.F.	29	29	29	29	29	29	29	29			232

Table 61: Inter-replicate dispersion test for excess variation from Poisson distribution of SCE's induced in CHO-K-G6 cells following exposure to diesel emission samples ES3 (collected at 3000 rpm @ 5 Nm engine load).

Treatment	0	1	2	3	4	5	Total
$\sum_{k=1}^{n}(y_k-\bar{y})^2$	12.5	1012.5	4.5	60.5	800		
mean $\bar{y}$	261.5	277.5	308.5	305.5	290		
χ^2	0.048	3.649	0.015	0.198	2.759		6.669
D.F.	1	1	1	1	1		5

Table 62: Inter-replicate dispersion test for excess variation from Poisson distribution of SCE 's induced in CHO-K1-G6 cells following exposure to diesel emission sample ES 3 (collected at 3000 rpm @ 5Nm engine load) with supplementary metabolic activation.

Treatment	0	1	2	3	4	5	Total
$\sum_{k=1}^{n}(y_k-\bar{y})^2$	1200.5	162.5	220.5	0.0	0.0	0.0	
mean $\bar{y}$	277.5	300.0	322.5	292.0	255		
χ^2	4.362	0.542	0.684	0.0	0.0		5.588
D.F.	1	1	1	0	0		3

Appendix 4.

Engine emission data.

The following data were collected by Perkins Technology Ltd., using the Prima engine used in this investigation at the Plymouth site in April 1991. These values were therefore assumed to be representative of the emission produced at the engine conditions used for sample collection. Due to the slight differences in the engine speeds and load applied for the collection of the emission data, estimates of the emission values at the condition sampled for mutagenicity testing were derived from best fit models for the data and extrapolation with the experimental load conditions. it was also assumed that the emission values obtained at 4006 rpm were the same as those of 4000 rpm (the speed used for mutagenicity sampling). The calculated values for Bosch smoke, HC, CO and NOx emissions are shown below (figures 72,73,74, and 75) and were calculated from model line fits at each engine speed. In all figures the extrapolated values are shown as shaded symbols.

Table 63: Engine emissions recorded from the Prima engine on 04/04/91 by Perkins technology.

Engine speed (rpm)	Engine load (Nm)	CO (ppm)	NOx (ppm)	Bosch smoke (units)	HC (ppm)
1000	83.0	916	1077	5.1	938
1000	70.0	690	985	4.4	938
1000	59.4	625	885	3.3	937
1000	48.0	530	752	2.7	955
1000	35.0	488	598	0.8	1011
1000	24.0	417	468	0.7	915
1000	12.0	428	354	0.4	898
1000	1.4	369	231	0.2	803
2000	114.0	1516	974	5.0	513
2000	98.2	374	1038	2.9	516
2000	81.0	258	905	1.9	521
2000	65.0	284	766	1.5	608
2000	49.0	311	617	0.7	628
2000	32.0	341	452	0.4	719
2000	16.0	301	273	0.2	756
2000	2.4	300	168	0.1	798
3000	112	1905	958	5.1	403
3000	96.2	488	1007	3.6	477
3000	81.0	305	955	2.1	522
3000	64.8	316	813	1.3	614
3000	48.4	305	664	0.3	675
3000	32.8	321	515	0.4	747
3000	16.2	341	336	0.5	842
3000	1.0	343	196	0.1	871
4006	95	2671	899	6.1	115
4006	83	784	934	4.2	320
4006	69.6	347	998	2.4	499
4006	57.8	317	933	1.3	609
4006	45.8	325	835	1.1	984
4006	34.8	467	693	0.9	777
4006	12.0	477	442	0.7	975
4006	0.8	481	294	0.8	995

Figure 73: Nitrogen oxides (NOx) emissions from the Perkins Prima diesel engine actual and predicted values.

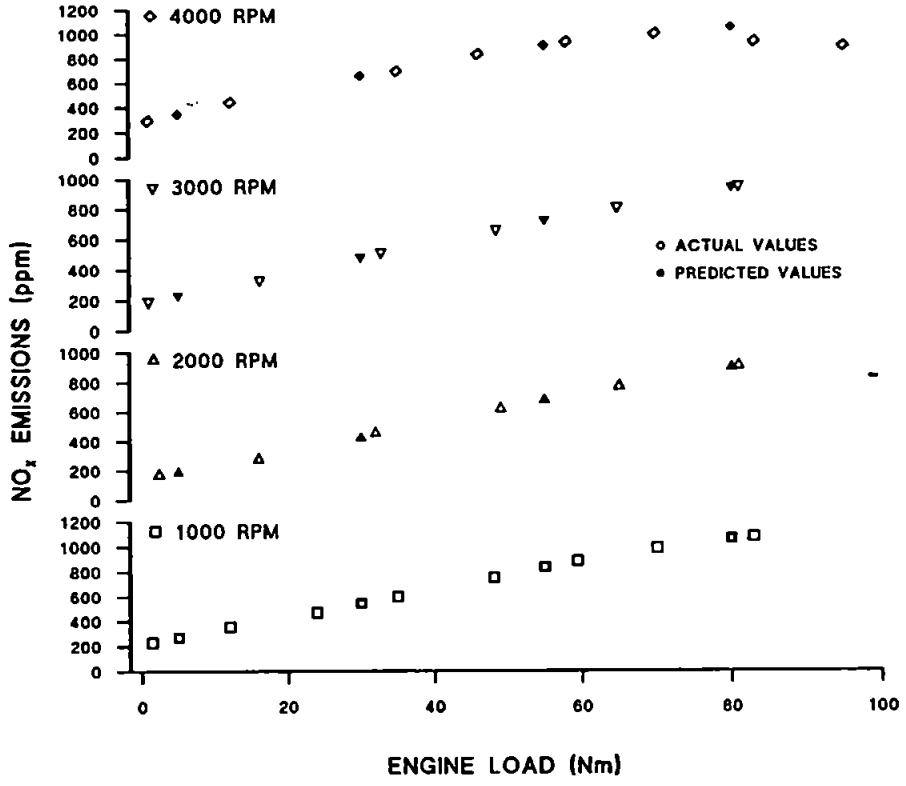


Figure 74: Bosch smoke emissions from the Perkins Prima engine showing actual values and predicted values for sampling conditions used for mutagenicity assays.

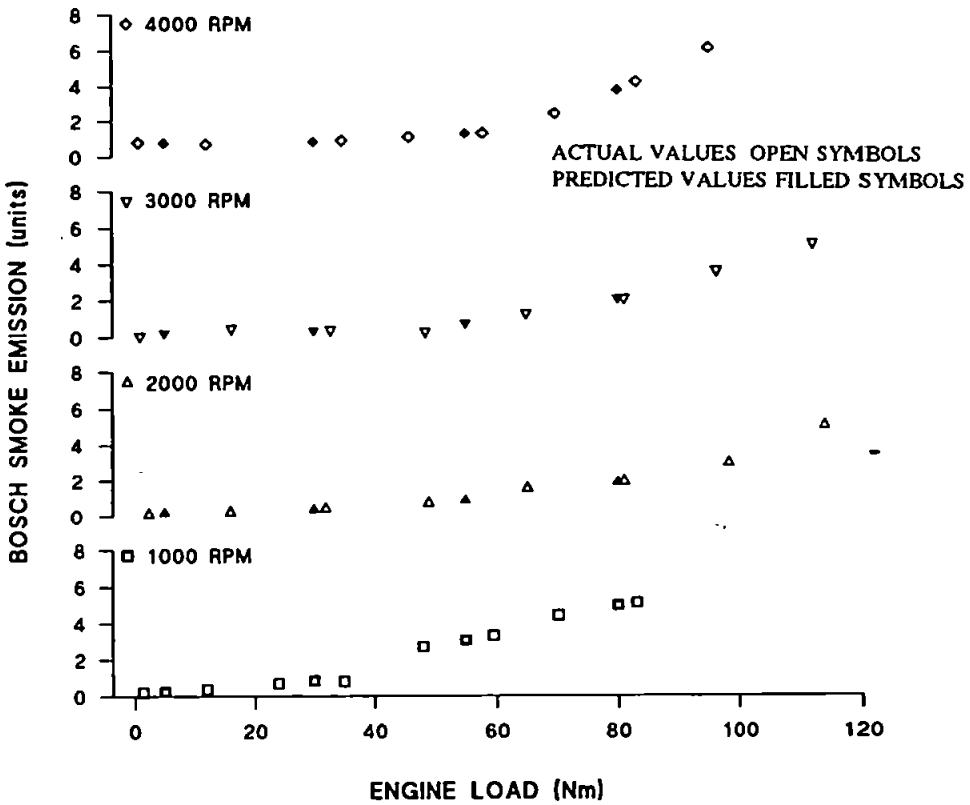


Figure 75: Hydrocarbon (HC)emissions from the Perkins Prima diesel engine, actual and predicted values.

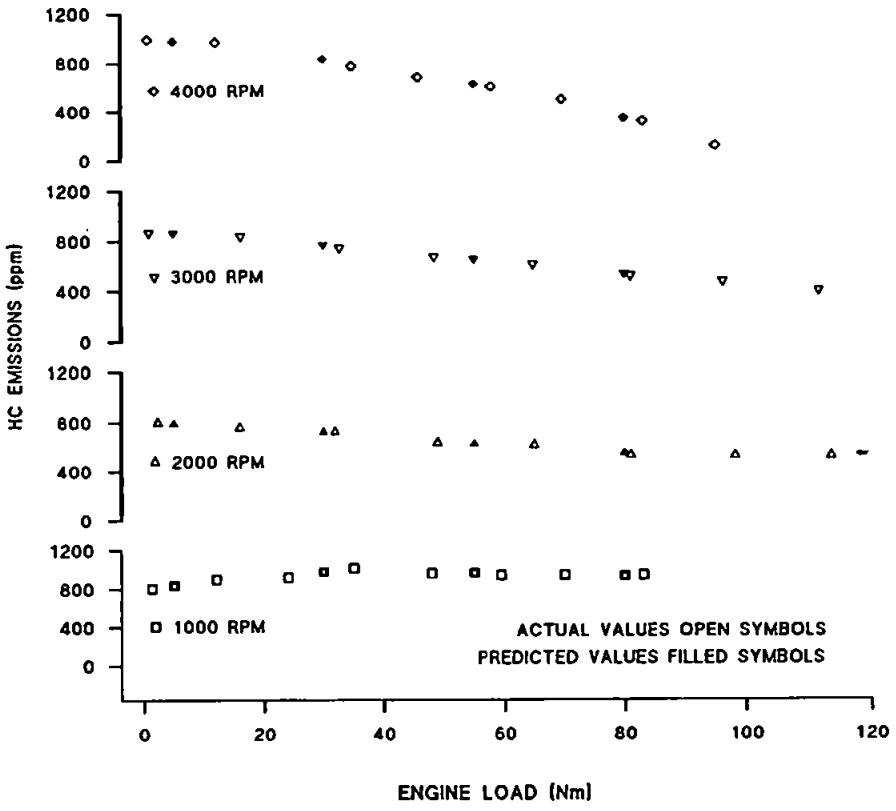


Figure 76: Carbon monoxide (CO) emissions from the Perkins Prima diesel engine, actual and predicted values.

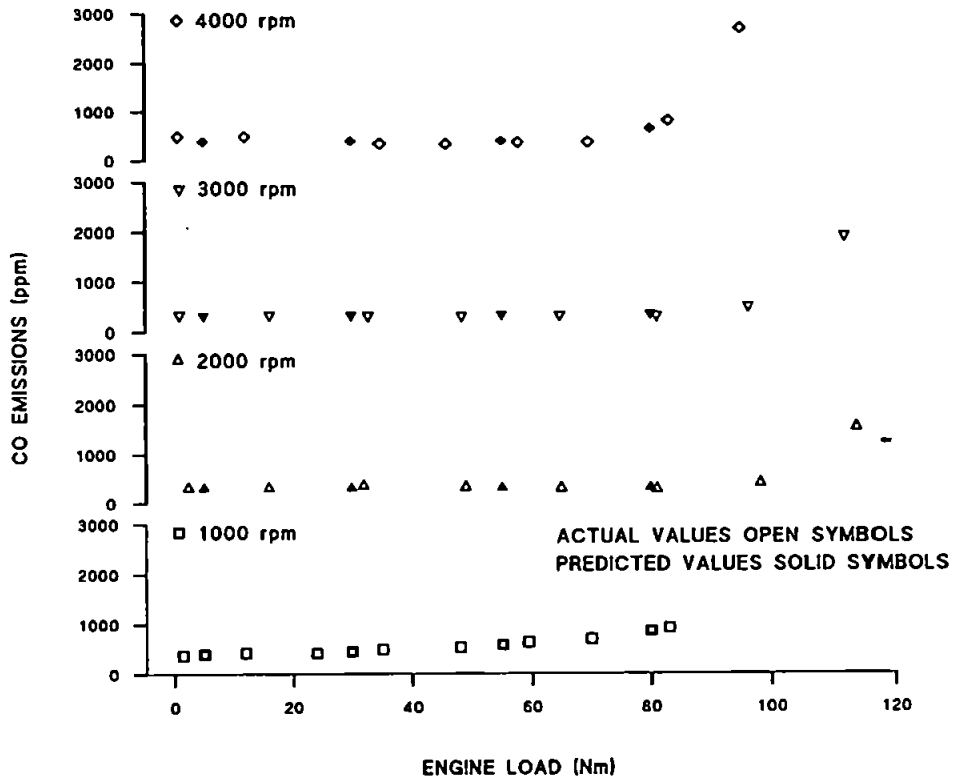


Figure 77: Fuel consumption in the Prima diesel engine at four speeds as a function of increasing engine load.

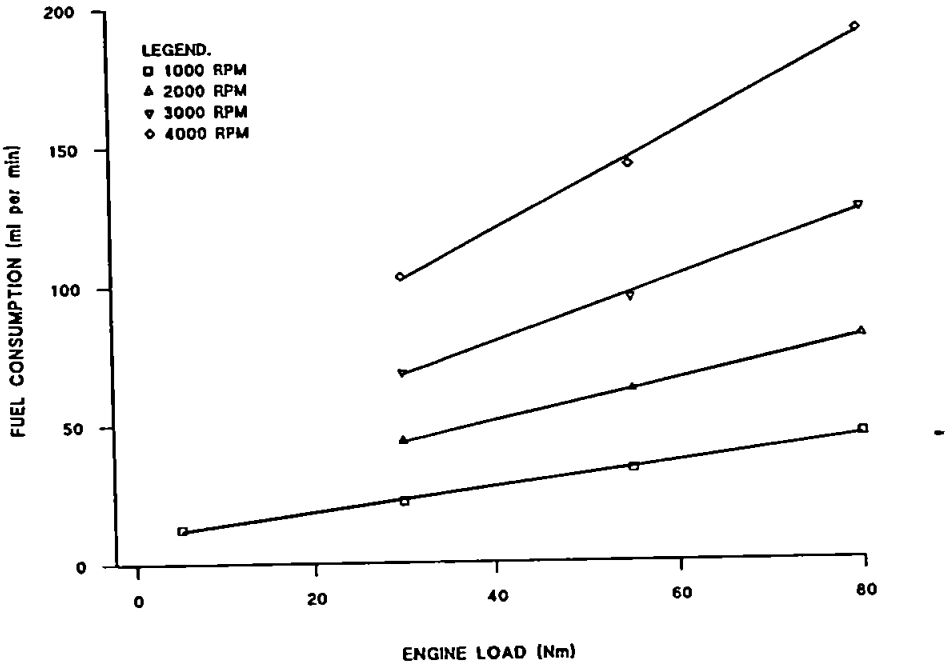


Table 64: Masses and percentage contribution of individual subfractions, of diesel emission samples obtained following open column elution chromatography on 5% deactivated silica.

Emission Sample Number	Sample fraction masses in mg and corresponding percentages of the total recovered masses in parenthesis		
	Aliphatic	Aromatic	Polar
ES8	6.8 (42.5)	7.6 (47.5)	1.6 (10)
ES9	14.49 (53.1)	8.6 (31.5)	4.22 (15.5)
ES11	11.99 (40.7)	11.7 (39.7)	5.8 (19.7)
ES12	11.8 (41.0)	12.9 (44.8)	4.09 (14.2)
ES15	11.9 (47.6)	8.6 (34.4)	4.5 (18.0)
ES19	11.1 (48.7)	9.9 (43.4)	1.8 (7.9)

NB: Masses of polar fractions were calculated by subtracting the combined masses of the aliphatic and aromatic fractions from the initial starting mass. This action was necessary due to the difficulty of removing silica fines from the polar fractions.

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