In Vitro Genotoxicity of Particulate and Semi-Volatile Organic Compound Exhaust Materials from a Set of Gasoline and a Set of Diesel Engine Vehicles Operated at 30°F

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The Analysis of Genotoxic Activities of Exhaust Emissions from Mobile Natural Gas, Diesel, and Spark-Ignition Engines

An Interagency Agreement Study by

the US Centers for Disease Control and Prevention –
National Institute for Occupational Safety and Health

and

the US Department of Energy –
Office of Freedom Car and Vehicle Technologies

Engine Exhaust Samples

Gasoline or diesel engine autos and light-duty trucks
chassis dynamometer run at 30°F ambient,
California Unified Driving Cycle (SwRI)

- Exhaust particulate was filter collected, acetone wash recovered
- SVOC was sorbent resin-collected, acetone extraction recovered (DOE-NREL, LRRI, DRI)
- Standard comparison = NIST standard diesel exhaust particulate 1650a
  (1980's technology automotive diesel particulate)

In vitro genotoxicity assays

Gene mutation -

Salmonella typhimurium reverse mutation

Chromosomal damage -

micronucleus induction in V79 mammalian cells

DNA damage -

single-cell gel electrophoresis for single and double DNA strand breaks in V79 cells

Mutagenicity Assay: “Ames” *Salmonella typhimurium* histidine reversion test

- Test for reverse-mutation to histidine independence
- Tester strains YG 1024 and YG 1029 for frameshift and for base-pair substitution mutations
- +/- S9 microsomal enzyme activation of the test materials
- Acetone extracts of materials assayed as suspensions in Tween 80

Salmonella test protocol

Experiments for each sample:

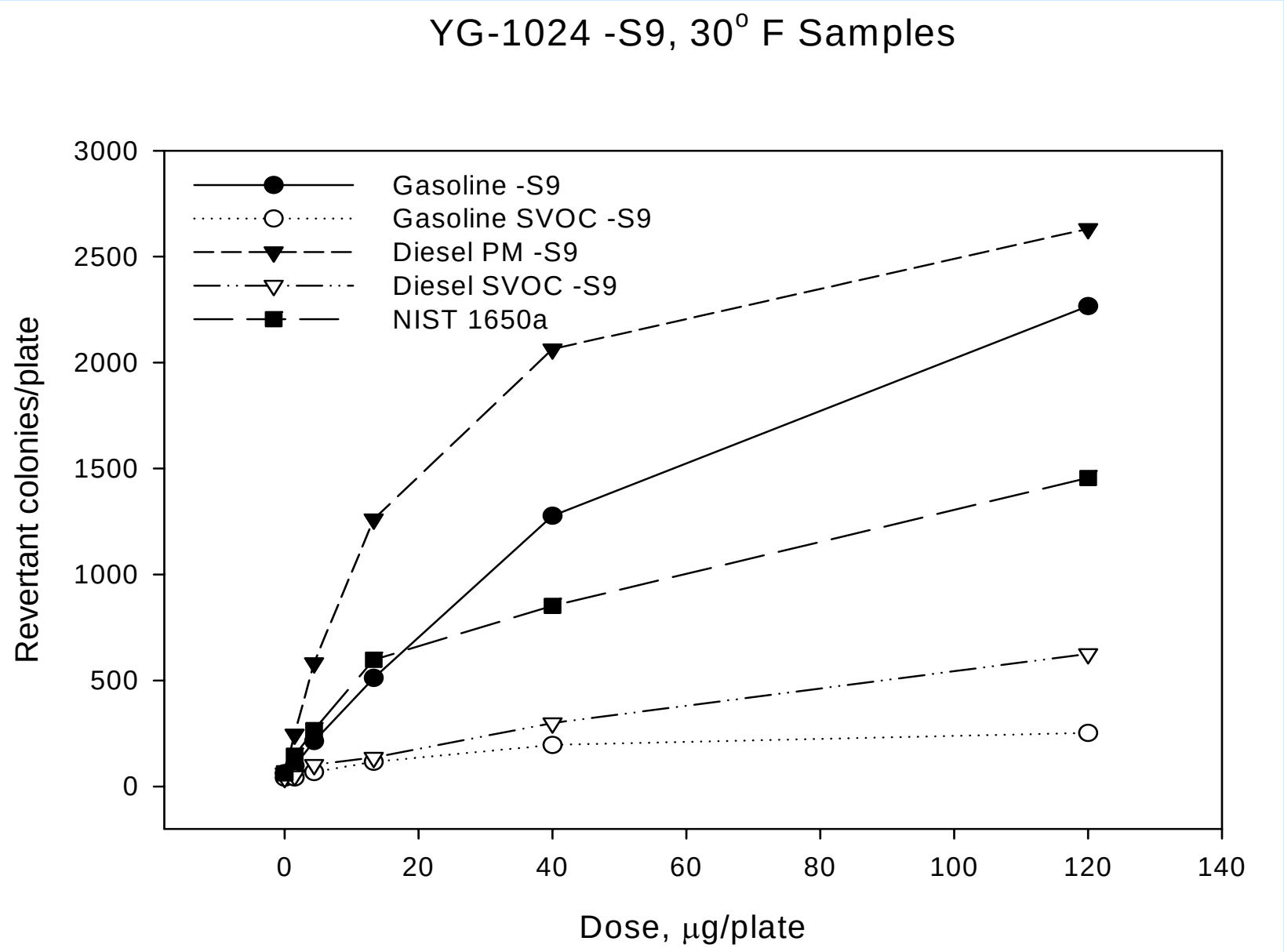
- attempt 5 doses for each sample
- doses spanned range to toxicity
- 2 plates read for each sample/dose
- 3 colony counter readings for each plate, 1 recorded
- 2 replicate experiments

Mutagenicity (Gene Mutation) of Particulate Fractions 30°F operation[a]

Sample	Concentration (µg/plate)	YG1024 -S9	YG1024 +S9	YG1029 -S9	YG1029 +S9
Tween 80	700	62±16	66±11	99±6	97±24
2-AA	0.05	143±100	1863±1746	109±8	2656±127
1-NP	0.5	1382±322	2519±1152	835±113	2214±206
D6	1.48	97±9	132±35	110±9	140±6
D6	4.44	214±64	240±116	159±15	247±8
D6	13.3	512±138	592±229	295±27	499±49
D6	40.0	1277±188	1166±424	570±7	903±55
D6	120.0	2266±143	1890±516	1149±119	1255±29
D7	1.48	246±19	248±107	131±22	247±17
D7	4.44	582±86	680±337	229±10	637±64
D7	13.3	1261±319	1558±751	491±74	1365±171
D7	40.0	2063±740	2248±1321	1349±206	2219±77
D7	120.0	2631±951	2488±1323	2143±328	2543±290

[a] Average number of revertant colonies per plate

Mutagenicity (Gene Mutation) of diesel and gasoline exhaust



Mutagenicity (Gene Mutation) slope estimates (revertants/ug extract) 30°F

	YG1024		YG1029	
	-S9	+S9	-S9	+S9
D6 Gas PM	33.8	39.5	14.7	30
D6 Gas SVOC	5.6	2.5	6.8	2.8
D7 Diesel PM	90.1	164	29.5	95
D7 Diesel SVOC	7.1	9.2	8.5	13.8
NIST	54	48.1	22.3	51.5

Mutagenicity (Gene Mutation) slope estimates: (revertants/ug) or (revertants/mile) and

ug-to-miles normalization factors

	YG1024-S9		YG 1024+S9		YG1029-S9		YG1029+S9		Nor
	Rev/ug	kRev/mile	Rev/ug	kRev/mile	Rev/ug	kRev/mile	Rev/ug	kRev/mile	
Gas PM	**33.8**	**574.6**	**39.5**	**671.5**	**14.7**	**250**	**30**	**510**	**17**
Gas SVOC	**5.6**	**37**	**2.5**	**16.5**	**6.8**	**44.9**	**2.8**	**18.5**	**6.6**
Diesel PM	**90.1**	**14506**	**164**	**26404**	**29.5**	**4750**	**95**	**15295**	**161**
Dsl. SVOC	**7.1**	**339.7**	**9.2**	**440.2**	**8.5**	**406.7**	**13.8**	**660.3**	**47.9**
NIST	**54**	**NA**	**48.1**	**NA**	**22.3**	**NA**	**51.5**	**NA**	**NA**

Results of Mutagenicity (Gene Mutation) Assays

All samples, D6, D7, SVOC6, SVOC7, NIST, were positive for both tester strains, +/- S9 activation

- Particle extract mutagenic activities, on a mass basis:
  D7 (diesel) > NIST > D6 (gasoline)
  D7 approximately 2X to 3X the activity of D6

- Particle extract mutagenic activities, on a mileage basis:
  D7 (diesel) were on the order of 10- to 30-fold greater than for D6 (gasoline)

Particle extract activities generally were an order of magnitude greater than SVOC activities

- On a mass basis, SVOC 7 activity was about 1X to 3X that of SVOC 6

- On a mileage basis, SVOC 7 was on the order 10X to 30X greater than SVOC 6

Pro-mutagens

- YG1024 → frameshift mutations
- YG1029 → base pair substitutions and frameshift mutations

- D6 / YG1024 → little change in activity with +S9 activation
- D6 / YG1029 → ca. 50% increase +S9
- D7 /YG1024 → little change +S9
- D7 / YG1029 → ca. 100% increase +S9

- Indicates pro-mutagens in D6, D7 for base-pair substitution mutations

- SVOC7 / YG1029 → small increase +S9

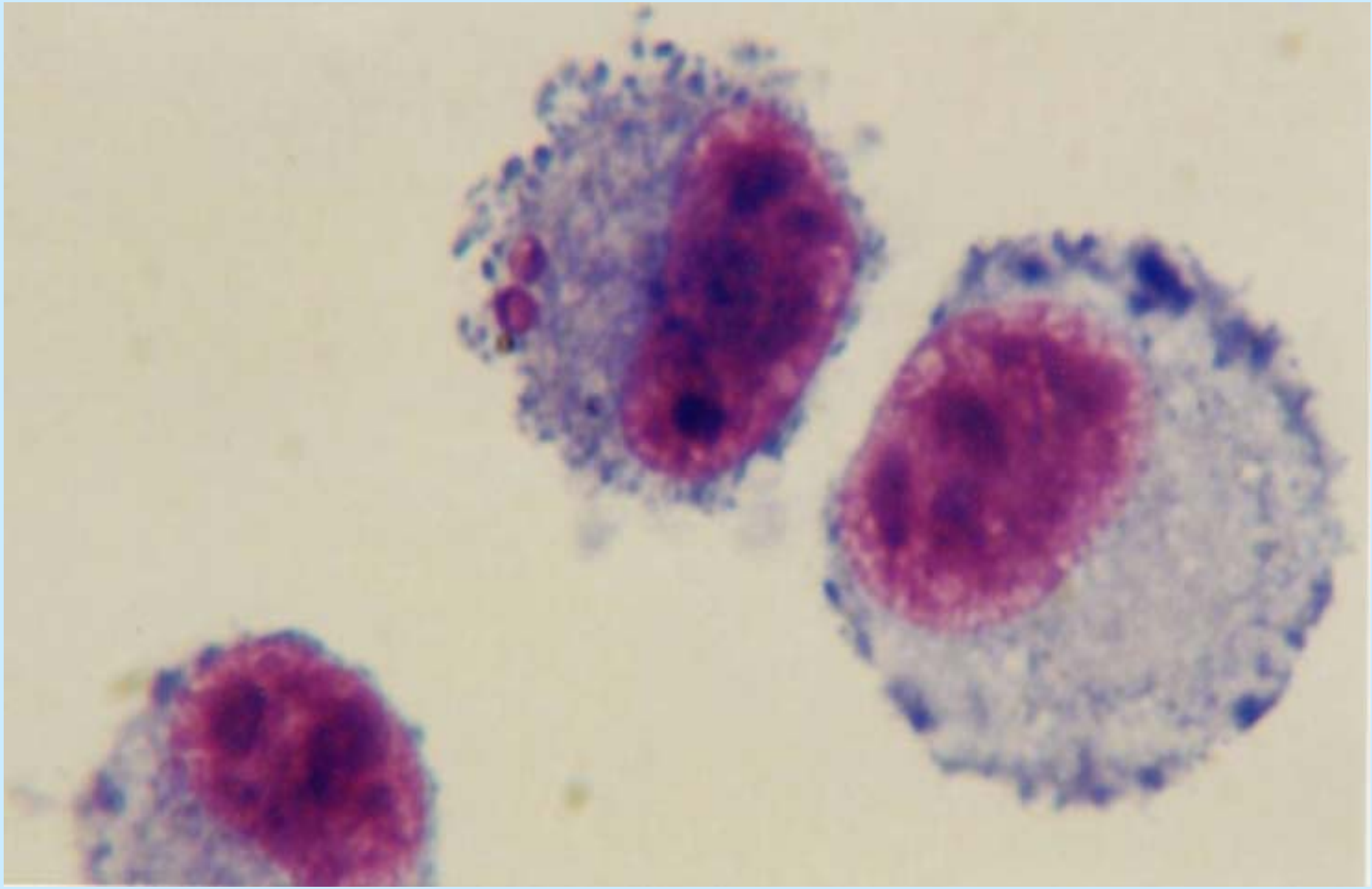
- NIST / YG1029 → ca. 75% increase +S9

Chromosomal Damage Assay

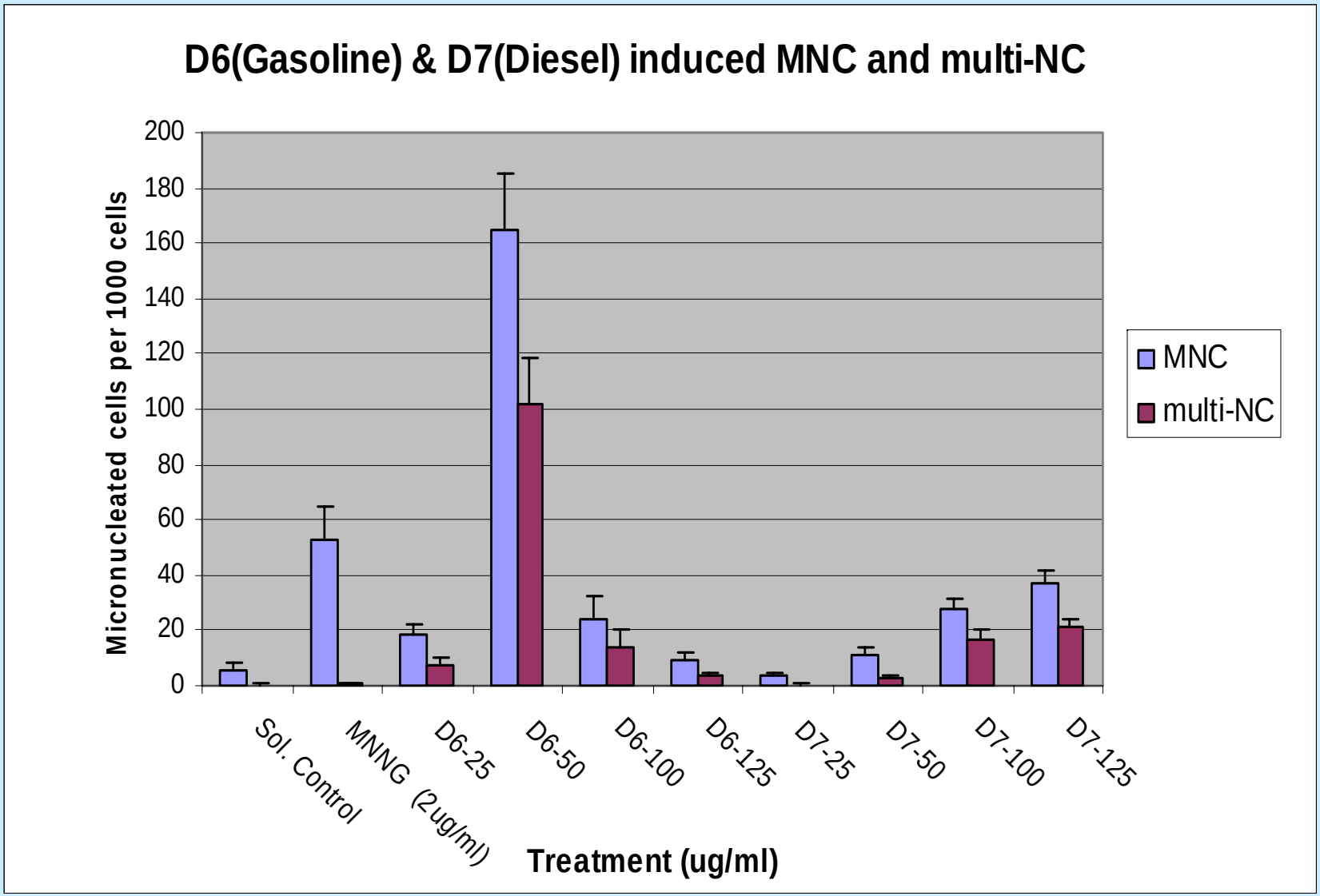
Induction of Micronucleated V79 mammalian cells *in vitro*

- Cells incubated in complete medium
- Cells challenged 24h
- Cells challenged at 4 or more sample concentrations, up to evident cellular toxicity
- Cells harvested, fixed, prepared by cytospin on microscope slides, stained
- 4 slides read for each concentration
- Experiment repeated
- 3000 cells total scored for each concentration

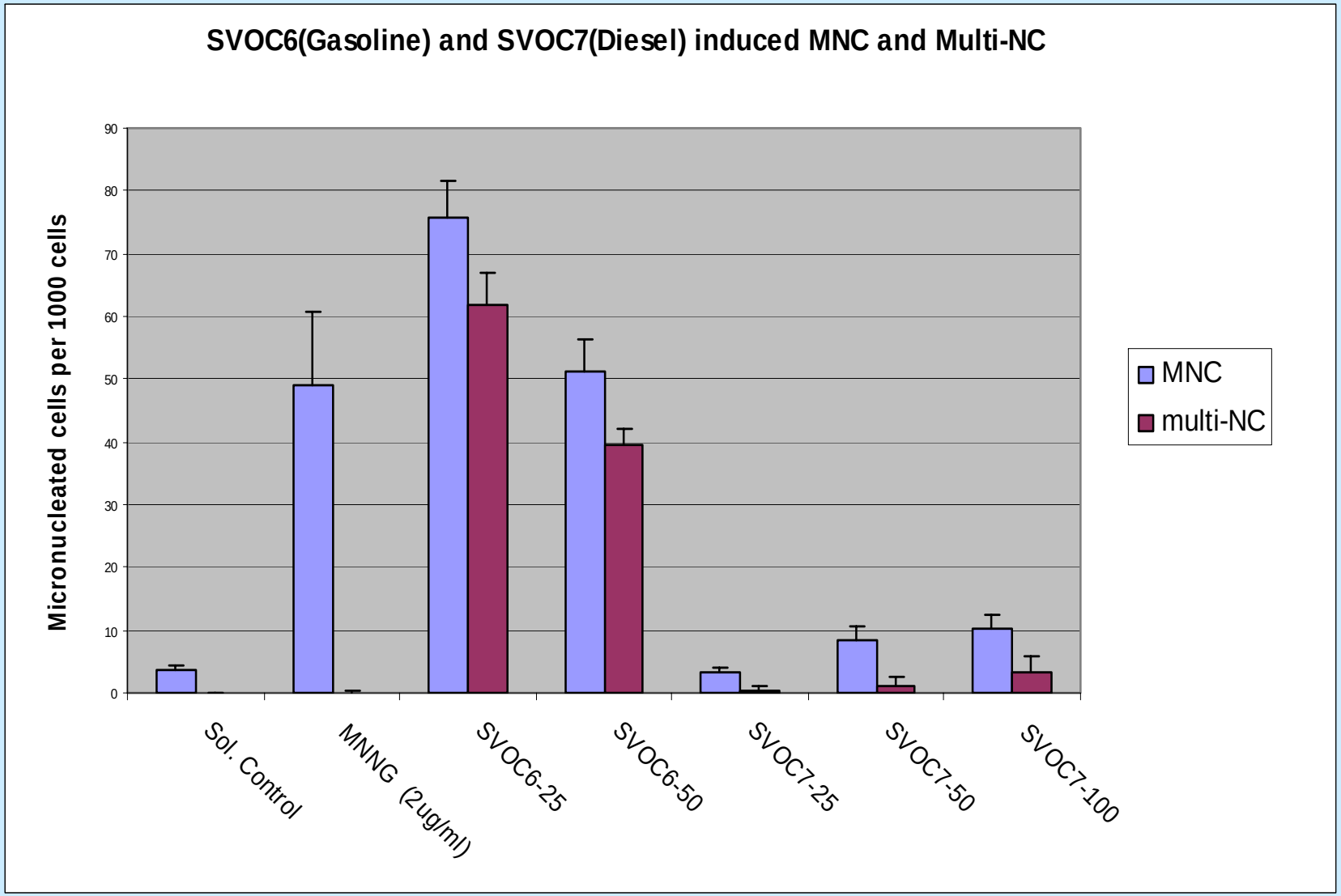
Micronucleus induction in V79 cells



Micronucleus Induction - Chromosomal damage



Micronucleus Induction - Chromosomal damage



Chromosomal damage assay micronucleus induction in V79 Cells

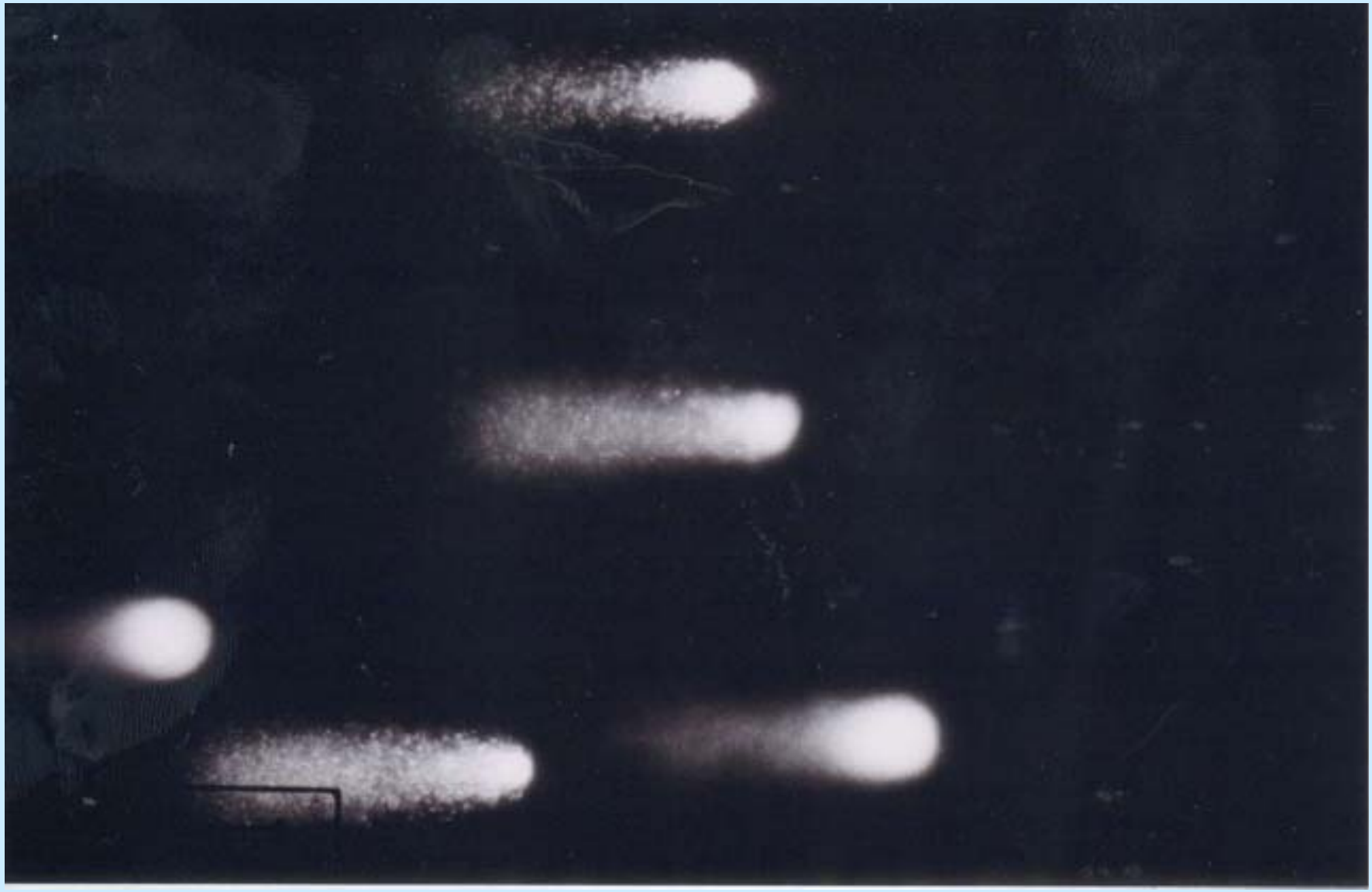
D6 PM and SVOC 6 strongly active for micronucleus induction at intermediate doses; toxicity interference at high doses, indicative of chromosomal breaks and/or spindle damage

D7 PM and SVOC 7 relatively weakly active at higher doses

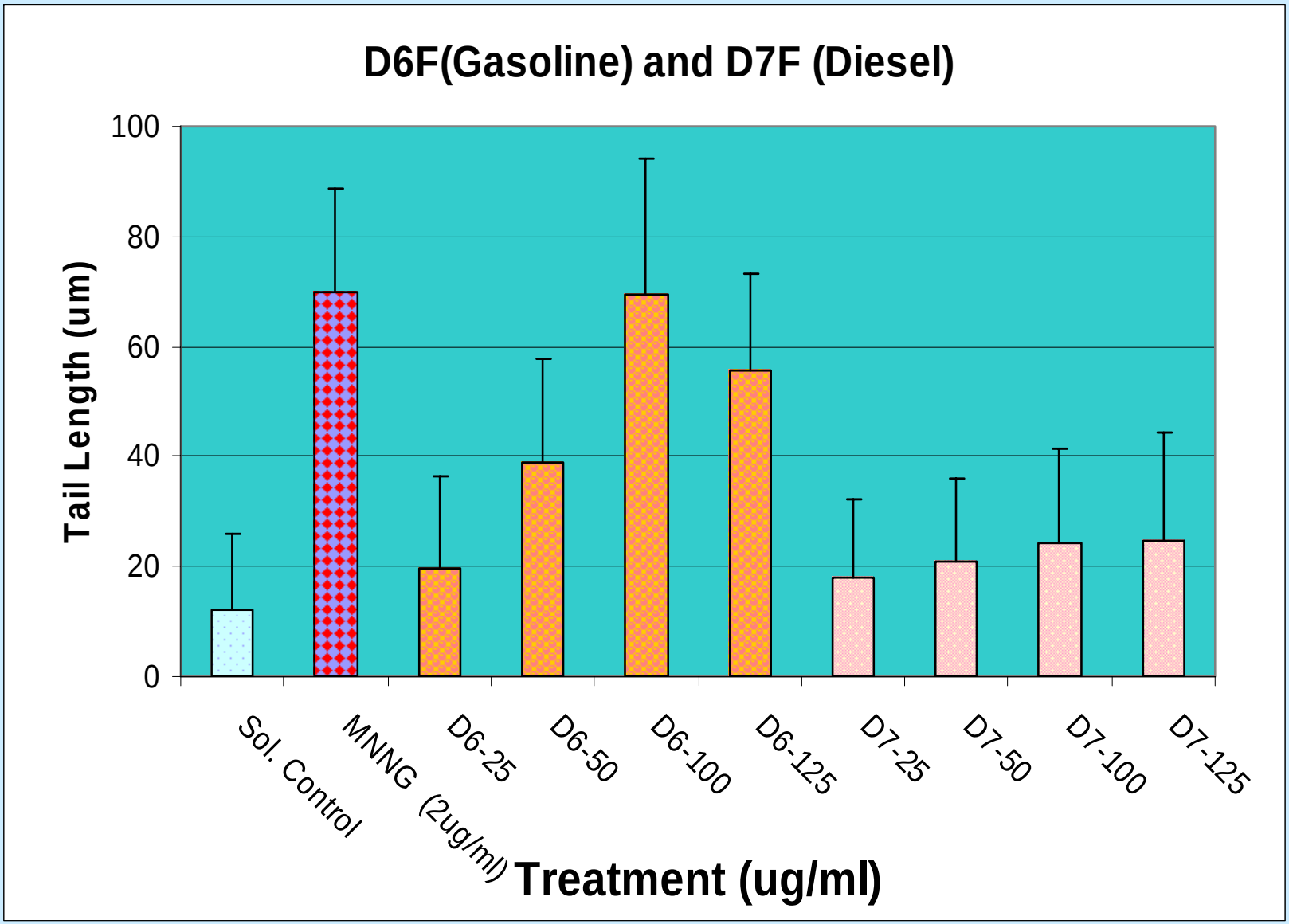
DNA Damage Assay

- Single-cell gel electrophoresis (SCGE) assay (the “Comet” assay)
- Assay for single-and double-stranded DNA breaks in V79 cells: electrophoretic migration patterns for DNA fragments
- Results expressed as electrophoretic pattern tail length
- 2 Experiments
- 100 cells total read at each concentration

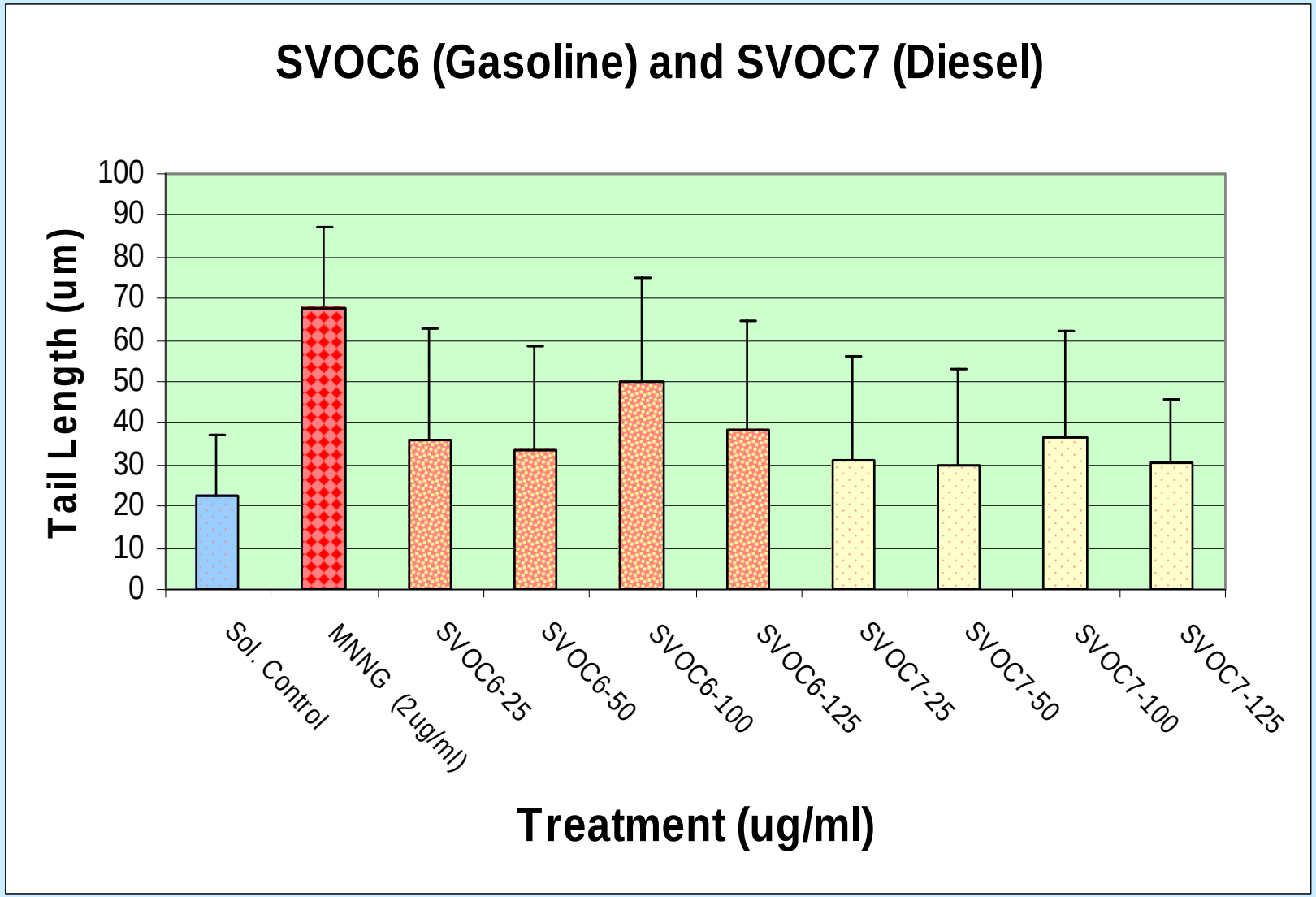
Single cell gel electrophoresis (*Comet*) assay for DNA damage in V79 cells



DNA Damage by Single Cell Gel Electrophoresis



DNA Damage by Single Cell Gel Electrophoresis



Results of DNA damage assays

- D6 PM extract positive for DNA damage at all but the lowest dose (Single-cell gel electrophoresis assay) with some toxicity effect at highest dose
- SVOC 6 weakly active
- D7 PM weakly active at highest doses, inactive at lowest doses
- SVOC 7 weakly active

Assay Summary/Interpretation
30°F operation

Gene Mutation:

Diesel 2X to 3X > gasoline/ug extract

Diesel > NIST > gasoline

Diesel 10X to 30X > gasoline/mile

Assay Summary/Interpretation
30°F operation

Mammalian cell genotoxicity:

- Gasoline PM **strongly active for DNA damage and for chromosomal damage**
- Diesel PM weakly active for DNA damage and for chromosomal damage
- Gasoline SVOC weakly active for DNA damage; **strongly active for chromosomal damage**
- Diesel SVOC inactive or weakly active for DNA damage or chromosomal damage

Qualitative Summary
30°F operation

Sample	Gene mutation	DNA Damage	Chromosomal Damage
Diesel PM	+	weak	weak
Gasoline PM	+	+	+
Diesel SVOC	weak	weak	weak
Gasoline SVOC	weak	weak	+

Qualitative Summary
72°F operation

Sample	Gene mutation	DNA Damage	Chromosomal Damage
Diesel PM	+	(+) toxic	-
Gasoline PM	+	+	+
Diesel SVOC	weak	-	-
Gasoline SVOC	weak	(+) toxic	+

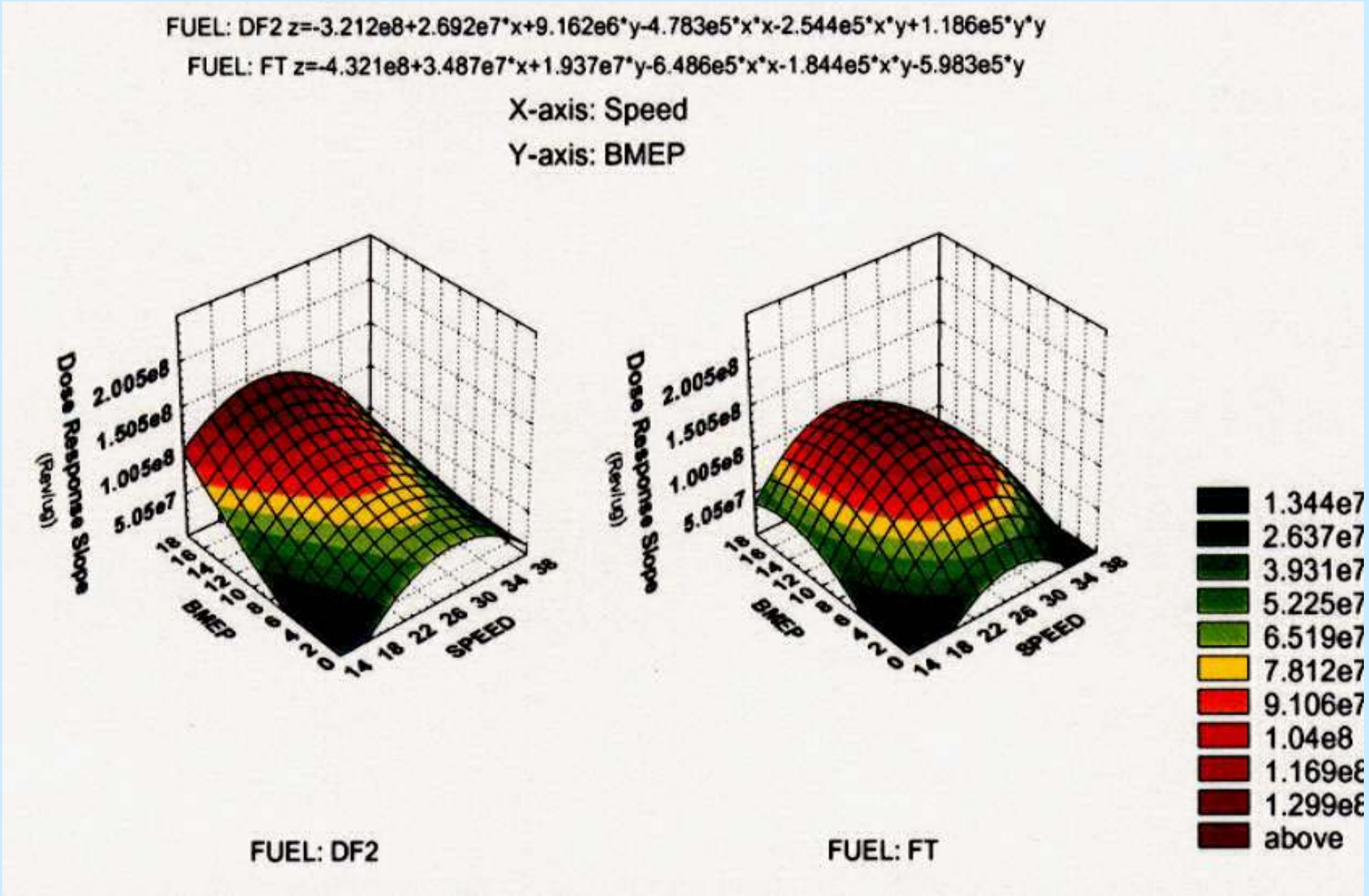
Engine Operating Parameters Affect the *In Vitro* Genotoxicity of Diesel Exhaust Particulate Extract

Some NIOSH / DOE collaborative studies

- US Dept. of Energy/METC-90/6110 DE90000480 (1990)
- U.S.Dept. Energy/METC-91/6122; DE91002091 (1991)
- McMillian MH,et al. Society of Automotive Engineers Technical Paper 2002-01-1699, pp. 1-18. (2002).

Quadratic response surface:
Mutagenicity (gene mutation) vs. engine speed and load

McMillian MH,et al. Society of Automotive Engineers Technical Paper 2002-01-1699, pp. 1-18. (2002).



Are data on the genotoxic activities of organic solvent extracts of exhaust particulate physiologically meaningful?

Background Finding by DOE, EPA, Industry

- *Organic solvent extracts* of Diesel Exhaust Particulate cause in vitro damage to genetic material.
- *Lung lining fluid – pulmonary surfactant- extracts* of Diesel Exhaust Particulate do <u>not</u> cause significant damage in vitro.
- Question:
  is the organic genotoxicant material in particulate exhaust soot biologically-available in the lung?

NIOSH Findings:
bioavailability of soot genotoxicants in lung fluids

Some diesel exhaust whole particulate material dispersed in lung surfactants induce in vitro genetic damage:

Mutation in bacterial cells

J. Tox. Env. Health, 21:163-171 (1987)

Environmental Hygiene II, pp. 7-10; Springer-Verlag, Berlin (1990), ISBN 0-387-52725-4

J. Environ. Sci. Health, A28:505-523 (1993)

ref. in : IARC Monograph 46 (1989)

DNA or Chromosomal damage in mammalian cells

Mutation Res. 260:233-238 (1991)

Mutation Res. 279:55-60 (1992)

Ann. Occ. Hyg. 38:345-349 (1994)

Air. Poll. Health Eff. Lab., Report 99-01, U. Cal. Irvine, pp. 611-616 (1999)

Lung surfactant-mediated genotoxicity of gasoline or advanced fuel engine exhausts has not yet been measured

Conclusions and Directions

- Diesel and Gasoline engine exhaust particulate can contain genotoxic compounds
- DEP genotoxicant content is affected by fuel,
  engine operating condition
- DEP can express genotoxic activity *in vitro*
  under conditions modeling soot deposition in the lung

Conclusions and Directions

- Biologically-available genotoxicant activity of engine exhaust particulate can be assayed in short-term tests in a physiologically-plausible manner
- Correlation can be sought with fuel, engine design and operation parameters
- To help evaluate and guide the development of fuels, engine design, and emission controls

Acknowledgments

- Joe Mauderly, LRRI
- JeanClare Seagrave, LRRI
- Doug Lawson, NREL
- Mridul Gautam, WVU
- Mike McMillian, DOE-NETL
- Mike Gurevich, DOE-OHVT
- Jim Wegrzyn, DOE-BNL
- Frank Stodolsky, DOE-ANL
- Jim Eberhardt, DOE – OFCVT
- SwRI
- DRI

CDC
CENTERS FOR DISEASE CONTROL AND PREVENTION

NIOSH

Back-up slides

Vehicles sampled

- Gasoline engine group:
- 1982 Nissan Maxima 190,203 miles
- 1993 Mercury Sable 70,786 miles
- 1994 GMC 1500 pickup truck 68,325
- 1995 Ford Explorer 76,733 miles
- 1996 Mazda Millenia 35,162 miles

- Diesel Engine Group:
- 1998 Mercedes Benz E300 47,762 miles
- 1999 Dodge 2500 pickup truck 37,242 miles
- 2000 Volkswagen Beetle TDI 7,495 miles

Samples

Acetone extracts/ Tween 80 suspensions of

- D6 (gasoline exhaust particulate)
- D7 (diesel exhaust particulate)
- SVOC 6 (semi-volatile organic compounds from gasoline exhaust)
- SVOC 7 (semi-volatile organic compounds from diesel exhaust)
- NIST (National Institute of Standards and Technology) standard diesel exhaust particulate 1650a (1980's technology automotive diesel particulate)

Sample preparation for assays

Particulate samples

Filter-collected; washed from filters with acetone , delivered to NIOSH

SVOC

Sorbent-resin collected; extracted with acetone, delivered to NIOSH

At NIOSH:

Samples were filtered;

liquid fraction evaporated under N_2 ;

residue ultra-sonicated in Tween 80/water to homogeneous suspension

Standard Reference Material NIST SRM 1650a, extracted with acetone at NIOSH/ into Tween 80

Salmonella test protocol

- Micro-suspension:
  10 ul sample + 65 ul S9 mixture or saline
  + 25 ul *S. typh.* culture @ $4X10^9$ cells/ml

- Pre-incubation:
  30 min @ 37C

- Add 2.5 ml top agar (+ trace histidine & biotin) @ 45C

- Incubate 48h (YG1029)
  or 66h (YG1024) @ 37C

Mutagenicity (Gene Mutation) of SVOC Fractions 30°F operation[a]

Sample	Concentration (µg/plate)	YG1024 -S9	YG1024 +S9	YG1029 -S9	YG1029 +S9
Tween 80	700	62±16	66±11	99±6	97±24
SVOC 6	1.5	42±14	49±9	102±30	106±31
SVOC 6	4.4	67±45	52±14	120±34	107±27
SVOC 6	13.3	116±51	75±17	174±19	134±48
SVOC 6	40	195±45	201±119	353±35	278±157
SVOC 6	120	252±38	351±34	236±34	489±226
SVOC 7	1.5	49±16	57±14	117±18	115±19
SVOC 7	4.4	102±43	79±31	129±28	156±29
SVOC 7	13.3	136±48	165±81	197±55	281±162
SVOC 7	40	299±88	405±240	344±79	447±210
SVOC 7	120	625±89	766±205	730±145	854±435

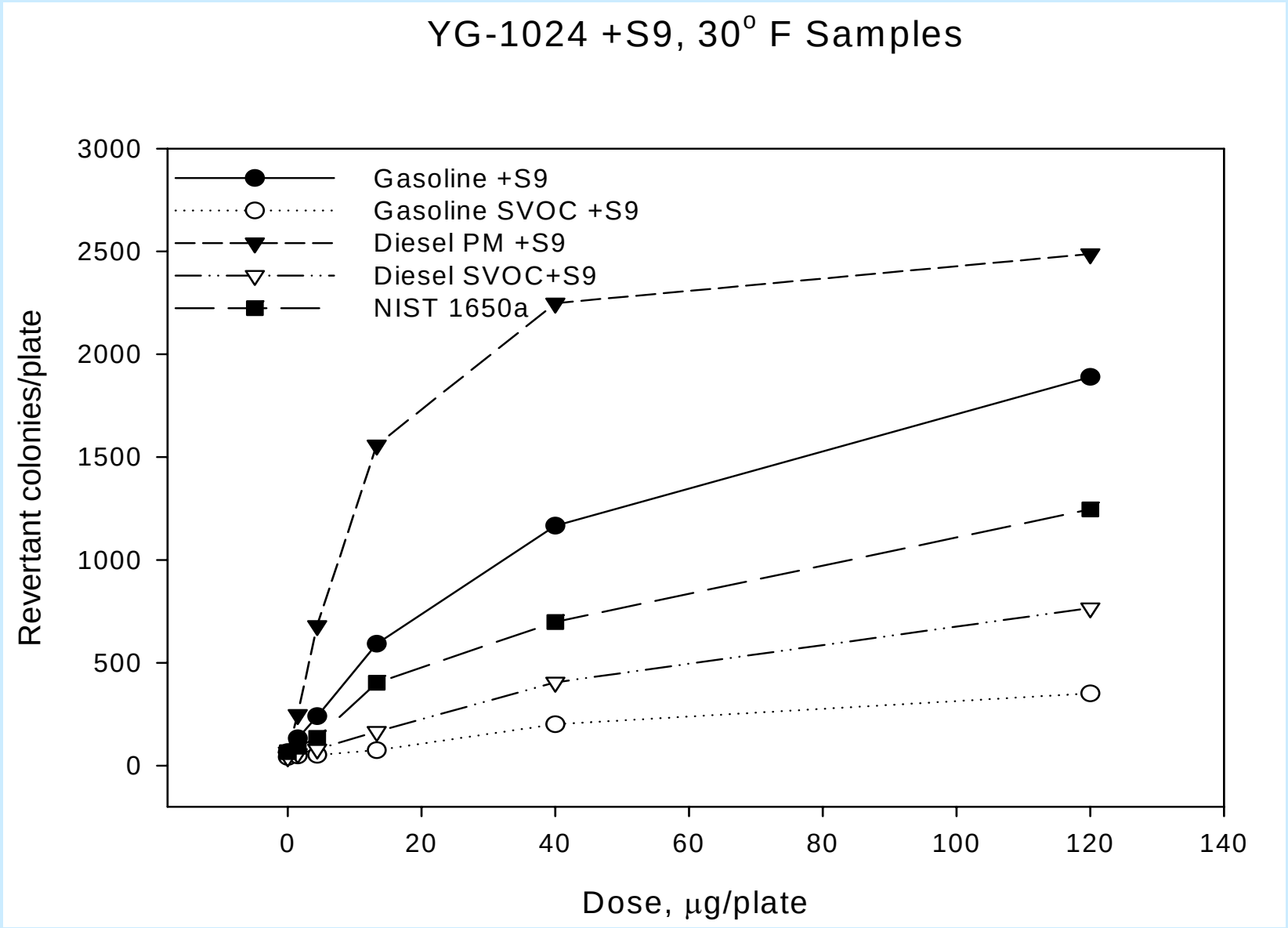
[a] Average number of revertant colonies per plate

Mutagenicity (Gene Mutation) of NIST SRM 1650a tested with 30°F operation samples[a]

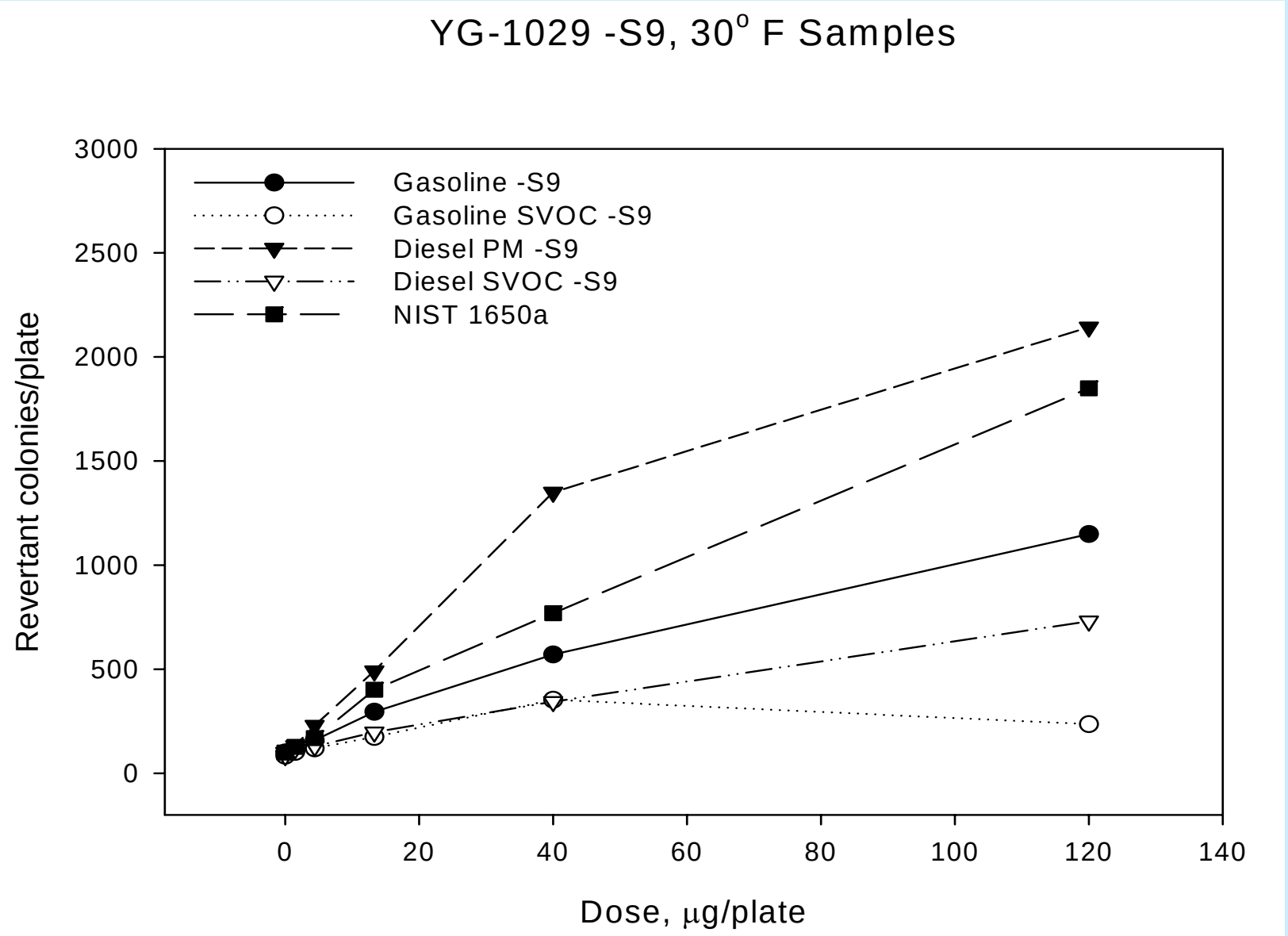
Sample	Concentration (µg/plate)	YG1024 -S9	YG1024 +S9	YG1029 -S9	YG1029 +S9
Tween 80	700	62±16	66±11	99±6	97±24
NIST	1.48	186±53	138±55	120±12	141±24
NIST	4.4	353±103	250±134	159±24	251±21
NIST	13.3	776±206	706±351	395±25	782±116
NIST	40	1360±606	523±968	929±200	1705±234
NIST	120	2470±1173	2324±1246	2220±430	2270±74

[a]Average number of revertant colonies per plate

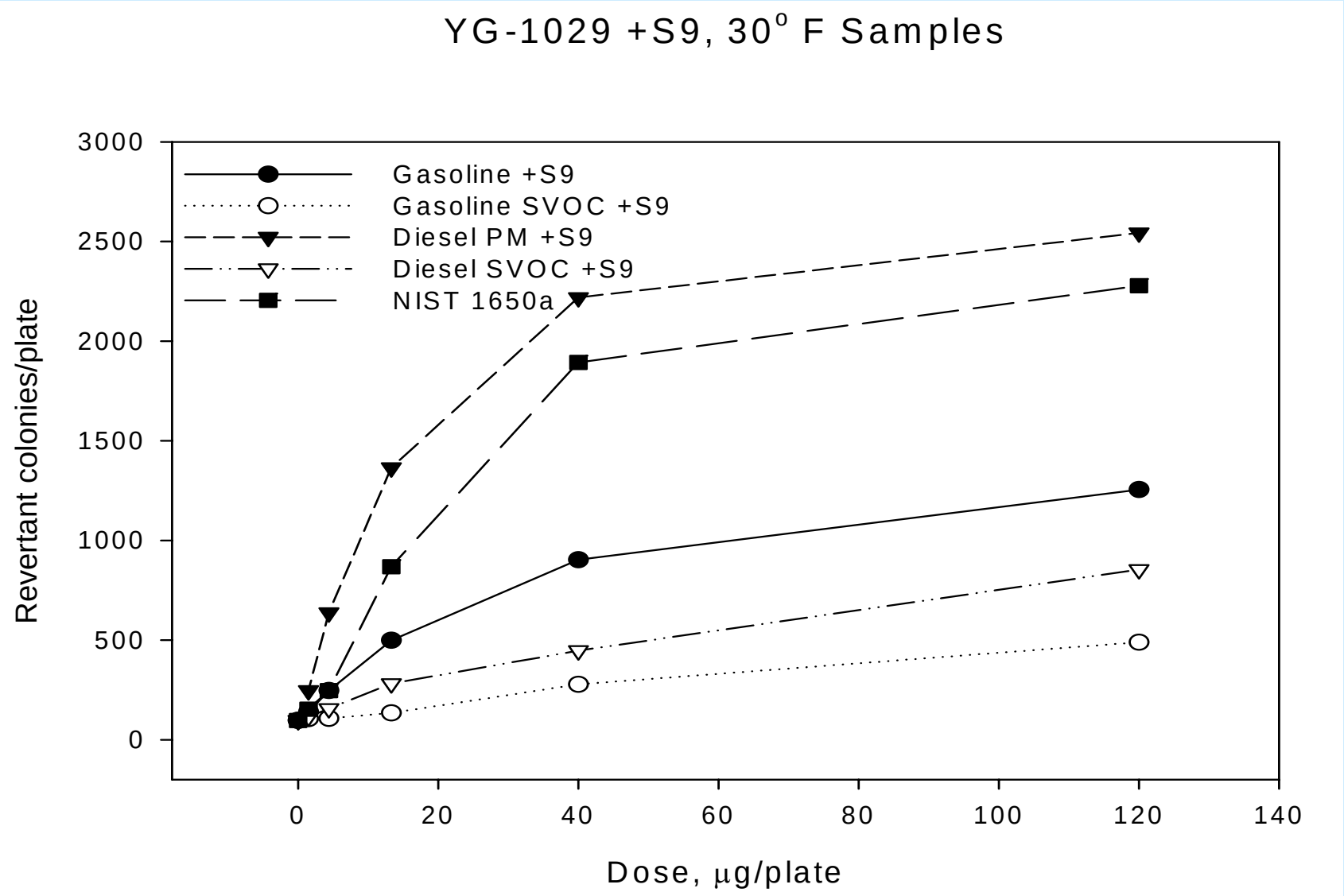
Mutagenicity (Gene Mutation) of diesel and gasoline exhaust



Mutagenicity (Gene Mutation) of diesel and gasoline exhaust



Mutagenicity (Gene Mutation) of diesel and gasoline exhaust



Mutagenicity of Particulate Fractions 72°F operation

Average number of revertant colonies per plate

	Sample	Concentration (µg/plate)	YG1024 -S9	YG1024 +S9	YG1029 -S9	YG1029 +S9
			Colony Number	colony number	colony number	colony number
–	Tween 80	0	38±5	49±8	115±18	106±21
–	D8	1.48	197±50	90±21	159±4	148±14
–	D8	4.44	355±115	211±42	224±22	204±14
–	D8	13.3	667±190	590±59	375±34	461±32
–	D8	40.0	1011±189	1171±188	583±27	853±48
–	D8	120.0	1298±49	1373±95	907±63	1030±96
–	D9	1.48	141±35	113±15	155±7	185±34
–	D9	4.44	273±58	209±23	191±7	323±17
–	D9	13.3	459±107	548±75	299±33	882±53
–	D9	40.0	797±85	1238±163	552±21	1485±119
–	D9	120.0	719±88*	1625±41	725±363*	1256±222*

Mutagenicity of SVOC Fractions 72°F operation

Average number of revertant colonies per plate

Sample	Concentration (µg/plate)	YG1024 -S9	YG1024 +S9	YG1029 -S9	YG1029 +S9
		Colony Number	colony number	colony number	colony number
Tween 80	0	44±3	50±7	101±14	105±16
SVOC#8	4.44	76±23	67±10	130±17	134±16
SVOC#8	13.3	122±19	109±15	181±9	170±8
SVOC#8	40.0	149±23	265±41	234±23	315±22
SVOC#8	120.0	165±14	390±32	264±44*	509±4
SVOC#8	360.0	262±63**	257±19*	379±29**	525±24*
SVOC#9	13.3	73±10	65±8	110±13	127±6
SVOC#9	40.0	118±5	102±6	153±10	184±28
SVOC#9	120.0	188±16	245±21	177±9	303±38
SVOC#9	360.0	362±15	459±27	331±23	447±22
SVOC#9	1080.0	635±64	755±52	533±12	562±24

Mutagenicity of NIST SRM 1650a tested with 72°F operation samples

Sample	Concentration (µg/plate)	Average number of revertant colonies per plate: YG1024 -S9 Colony Number	YG1024 +S9 colony number	YG1029 -S9 colony number	YG1029 +S9 colony number
• TWEEN 80	0	62±8	65±5	99±3	97±12
• NIST	1.48	186±6	138±27	120±6	141±12
• NIST	4.4	353±5	250±69	159±12	256±12
• NIST	13.	776±10	706±175	395±13	782±58
• NIST	40.	1360±30	1523±623	929±100	1705±117
• NIST	120.	2470±58	2324±623	2220±215	2270±37

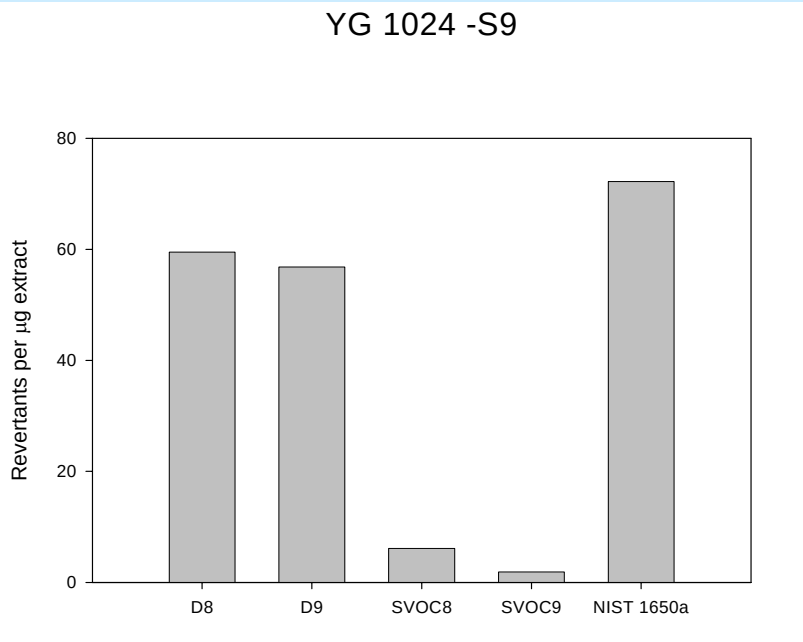
YG1029 Mutagenicity
72°F operation

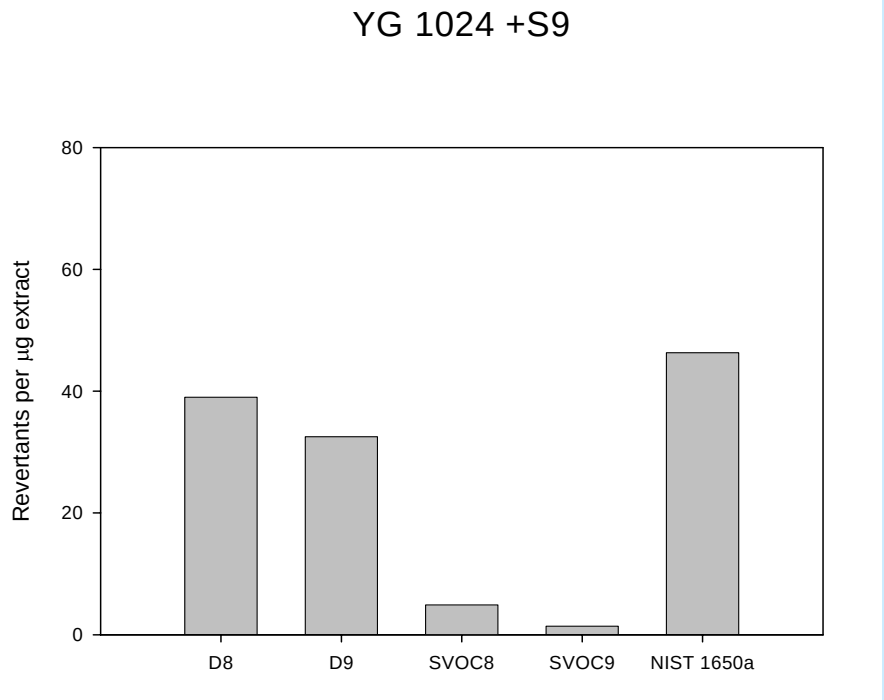
Factor Combination	Group	Slope Estimate (Revertants/ug extract)	Slope Estimate (Revertants x 10^3/mile)
YG1029 - S9	D8	19.4	118
	D9	13.1	566
	SVOC-8	5.7	10.5
	SVOC-9	1.0	18.4
	NIST	21.8	-
YG1029 + S9	D8	17.7	108
	D9	43.3	1871
	SVOC-8	5.2	9.6
	SVOC-9	1.8	33.1
	NIST	35.3	-

YG 1024 Mutagenicity
72°F operation

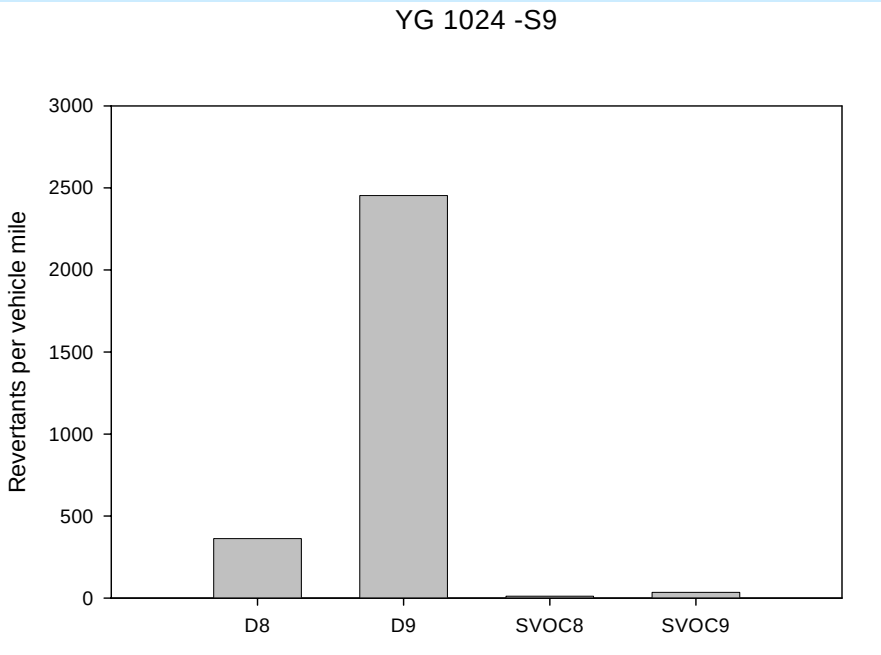
		Revertants/ug extract	Revertants x1000/mile
YG1024 - S9	D8 (gasoline)	59.5	363
	D9 (diesel)	56.8	2454
	SVOC-8	6.1	11.3
	SVOC-9	1.9	35
	NIST (SRM 1650a)	72.2	___
YG1024 + S9	D8	39.0	238
	D9	32.5	1404
	SVOC-8	4.9	9.1
	SVOC-9	1.4	25.8
	NIST	46.3	_

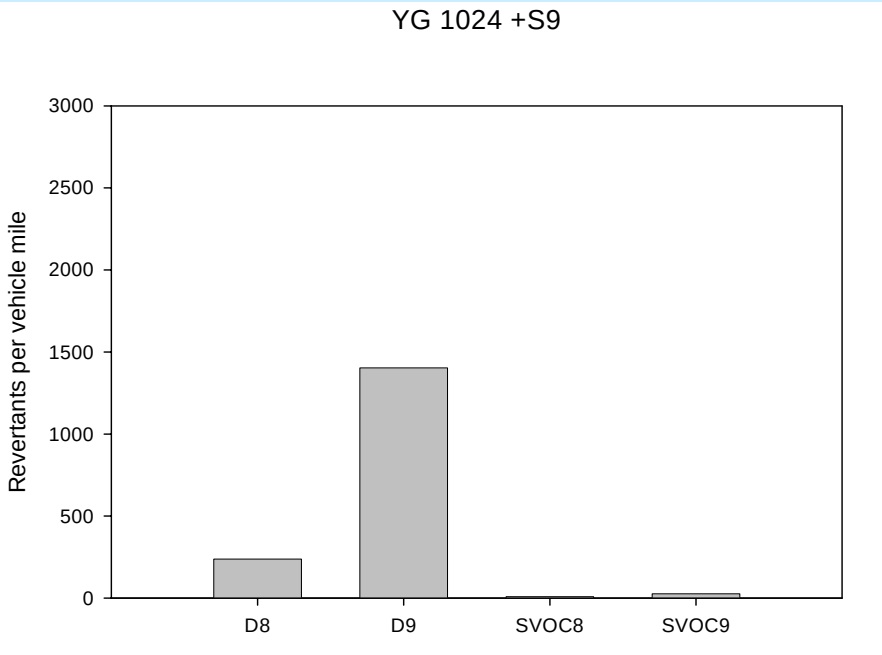
YG1024 +/- S9
Revertants per microgram extract
72°F operation





YG1024 +/- S9
Revertants per mile
72°F operation

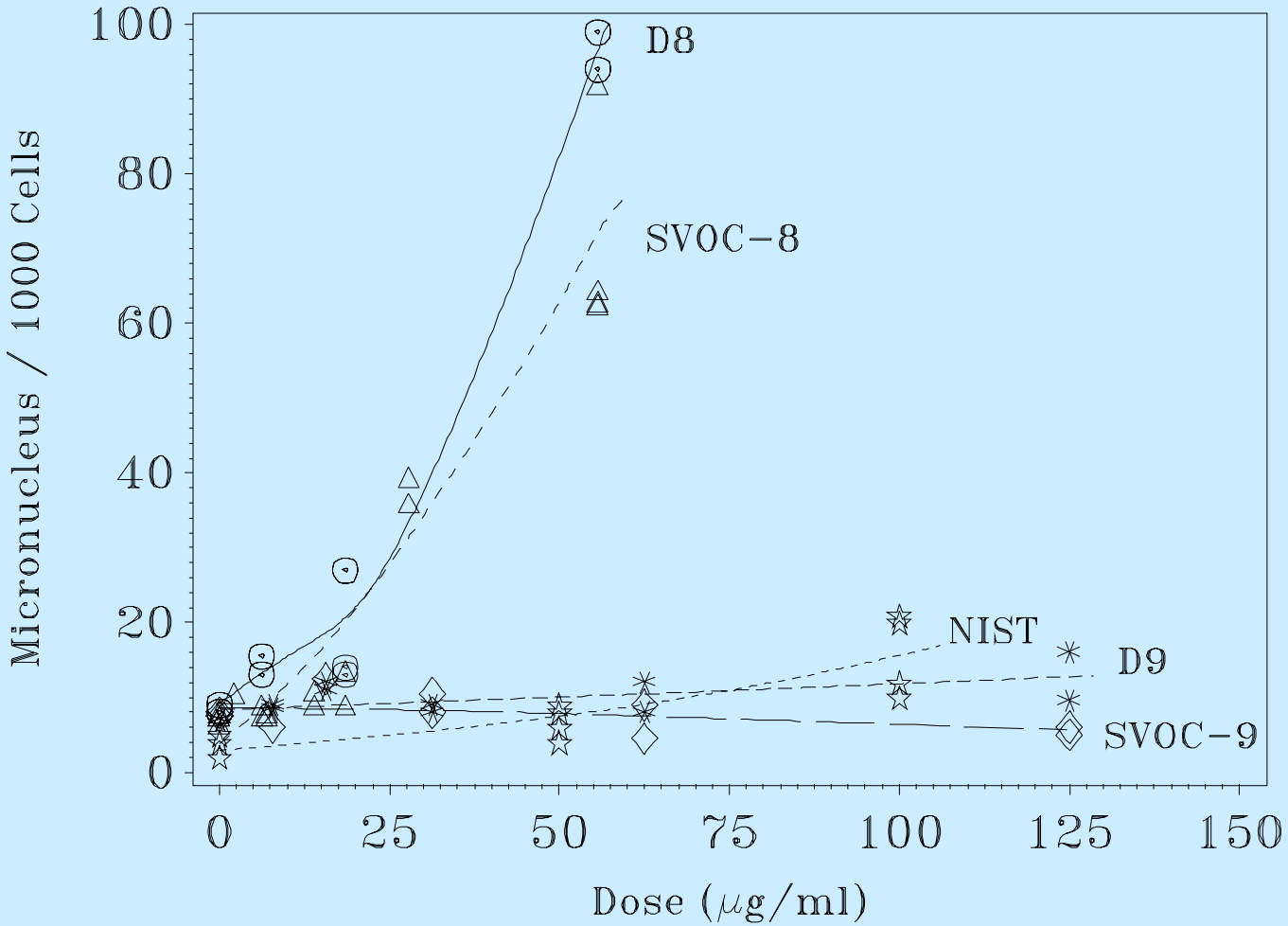




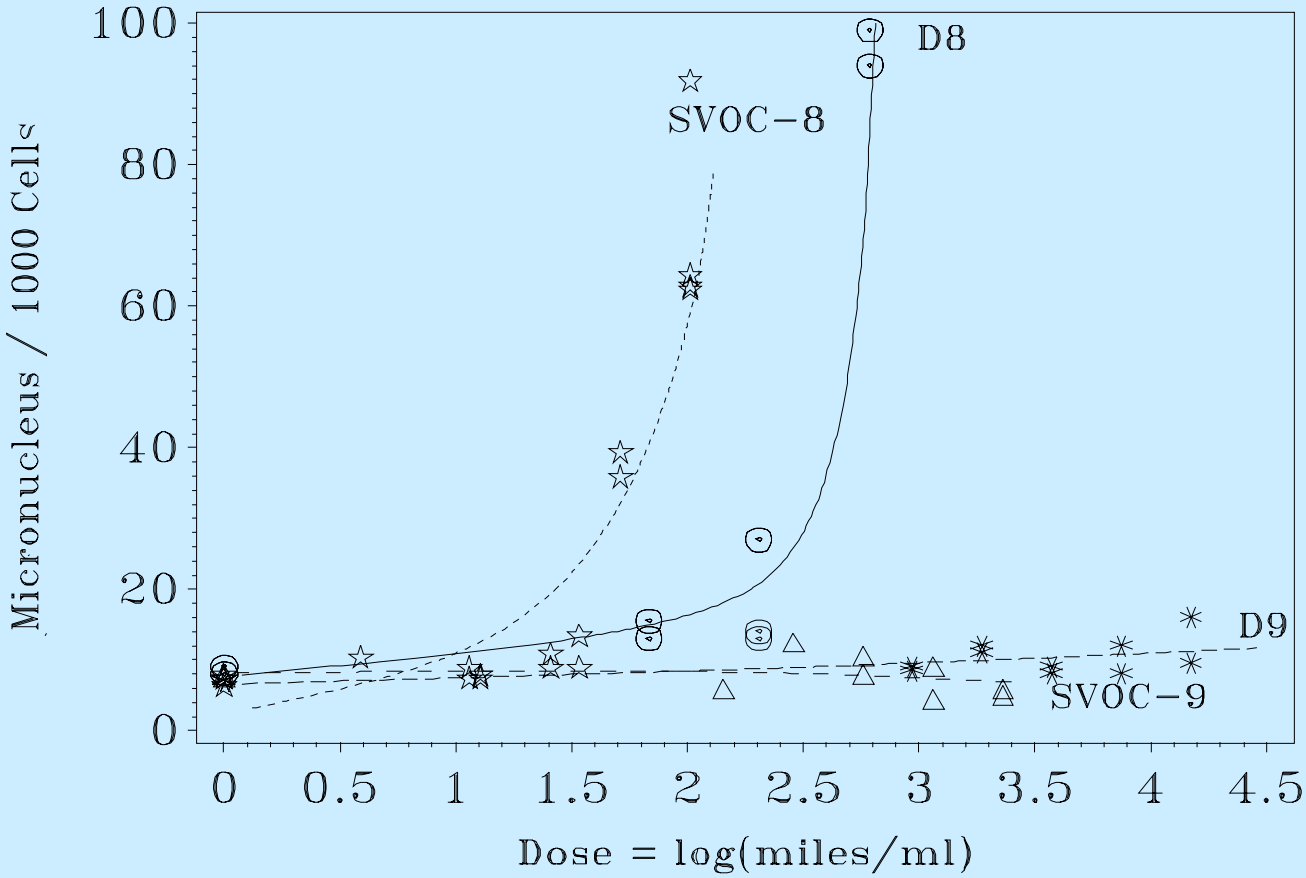
Mutagenicity slope estimates (revertants/ug extract) 72°F

	YG1024		YG1029	
	-S9	+S9	-S9	+S9
D6 Gas PM	59.5	39.0	19.4	17.7
D6 Gas SVOC	6.1	4.9	5.7	5.2
D7 Diesel PM	56.8	32.5	13.1	43.3
D7 Diesel SVOC	1.9	1.4	1.0	1.8
NIST	72.2	46.3	21.8	33.1

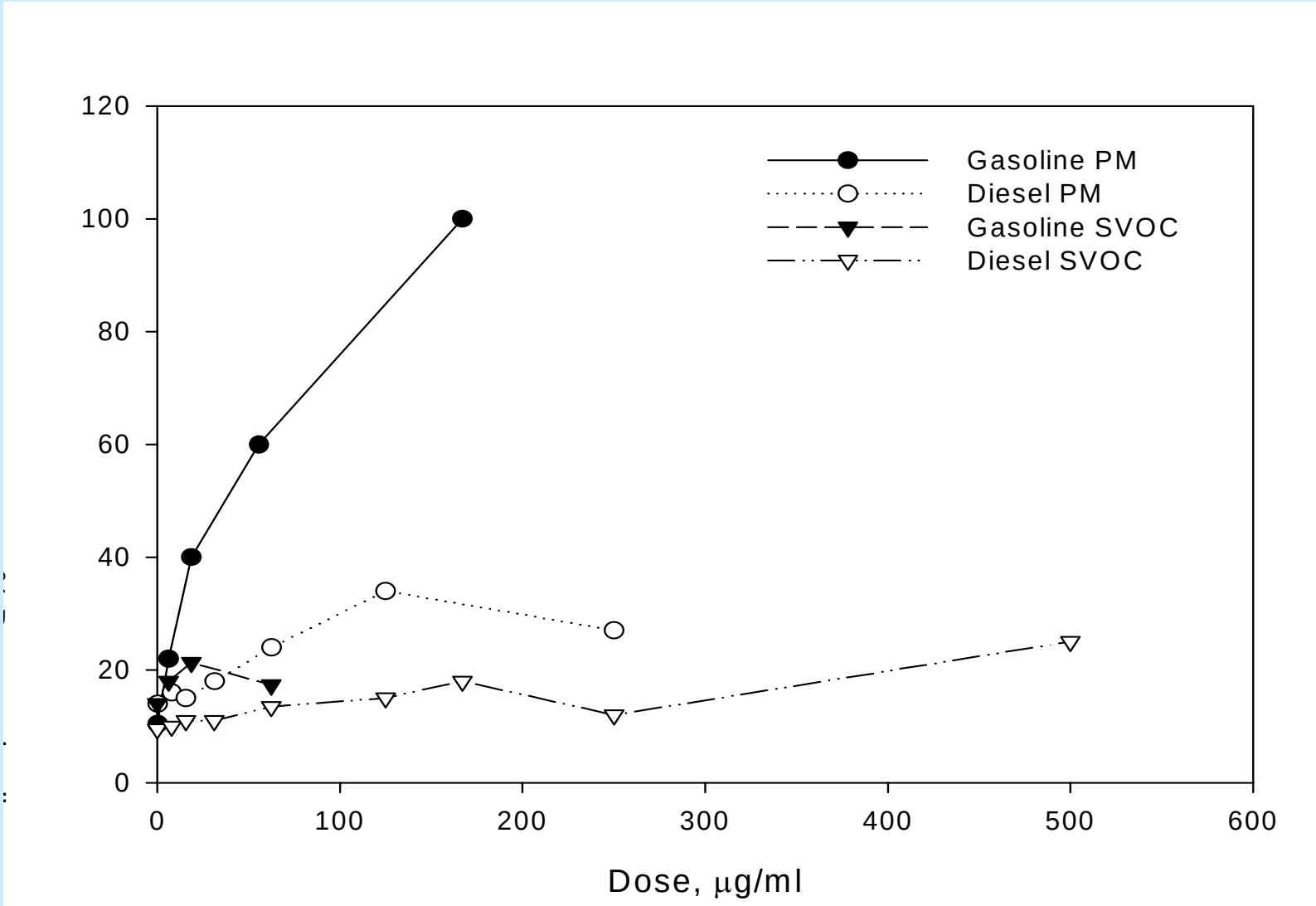
Micronucleus induction 72°F operation



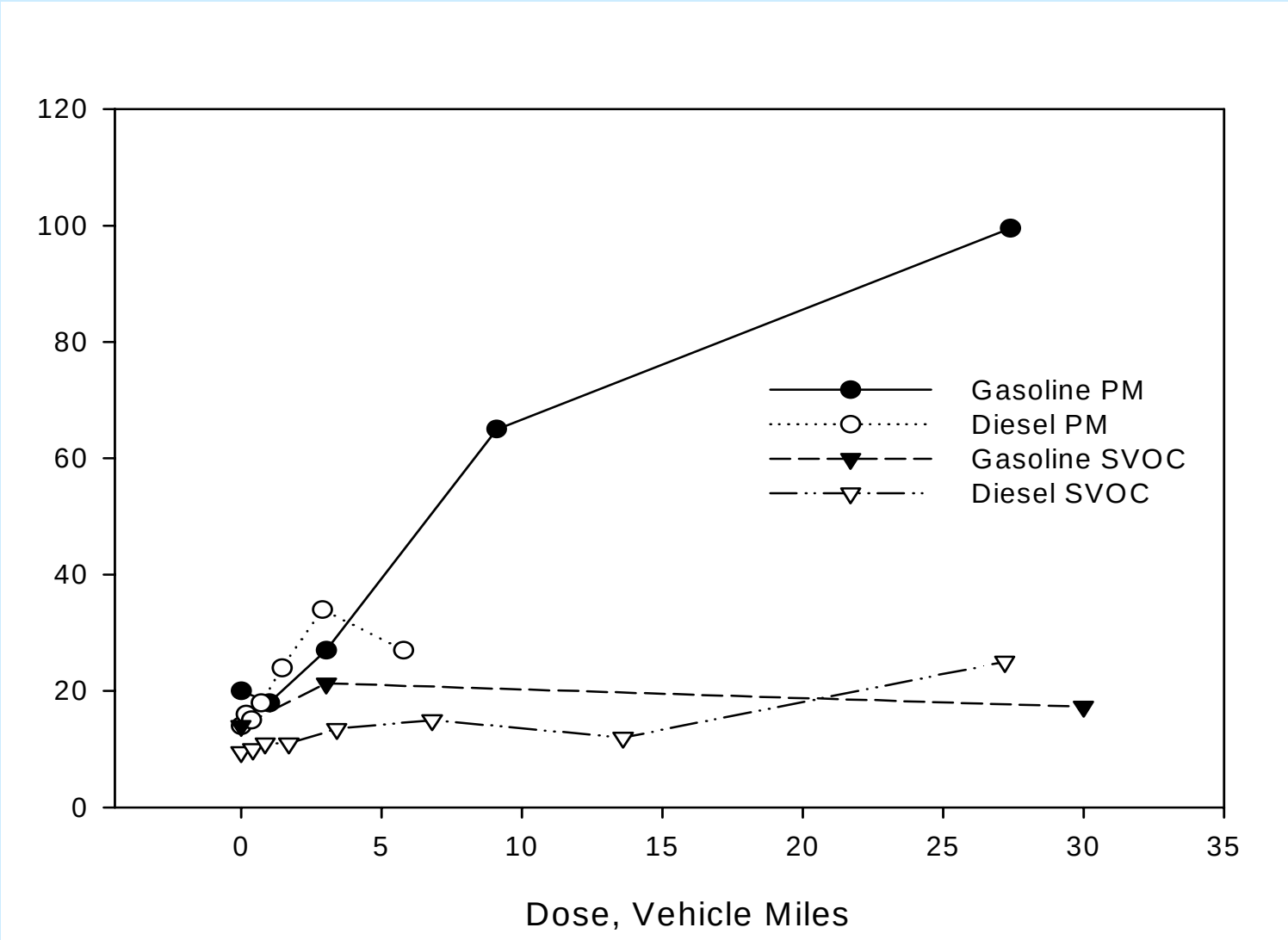
Micronucleus induction 72°F operation



DNA damage (SCGE) activity (% damaged cells) of D8 or D9 72°F operation



DNA damage (SCGE) activity (% damaged cells) of D8 or D9 72°F operation



Assay Summary/Interpretation
72°F operation

Gasoline and Diesel exhaust particulate extract mutagenicity:
- comparably mutagenic/ mass
- NIST > Gasoline > Diesel per mass of extract
- diesel exhaust greater mutagenic activity/mile

Gasoline exhaust particulate extract
- active for DNA damage
- active for chromosomal damage

Diesel exhaust particulate extract
- inactive or weakly active for chromosomal damage

Diesel exhaust particulate extract
- active at low doses for DNA damage;
- cell toxicity interference with assay at higher dose

SVOC extracts generally not as active as companion particulate extracts

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