

In Vitro Genotoxicity of Gasoline and Diesel Engine Vehicle Exhaust Particulate and Semi-Volatile Organic Compound Materials

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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

the US Department of Energy –
Office of Energy Efficiency and Renewable Energy

Engine Emission Samples

gasoline autos and light-duty diesel vehicles - chassis dynamometer tested

(SWRI)

exhaust particulate was filter collected, acetone wash recovered;

SVOC was sorbent resin-collected, acetone extraction recovered

(DOE-NREL, LRRI, DRI)

Samples

Acetone extracts/ Tween 80 suspensions of

- D8 (gasoline exhaust particulate)
- D9 (diesel exhaust particulate)
- SVOC 8 (semi-volatile organic compounds from gasoline exhaust)
- SVOC 9 (semi-volatile organic compounds from diesel exhaust)
- NIST (standard diesel exhaust particulate) 1650a

In vitro genotoxicity assays

Mutagenicity -

“Ames Test” : *Salmonella Typh.* reverse mutation

Chromosomal damage -

micronucleus induction in V 79 mammalian cells

DNA damage -

single-cell gel electrophoresis for single and double DNA strand breaks in V 79 cells

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

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

- Test for back-mutation reversion 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

Ames test protocol

- Positive controls: 2-aminoanthracene (2AA) for indirect (+S9 treated samples); 1-nitropyrene for direct (-S9)
- NIST SRM (standard reference material) 1650a diesel soot reference sample (1980's technology automotive diesel)
- Positive response criteria:
 - test sample # revertant colonies/plate = at least twice negative control
 - dose/response evident

Ames test protocol

- Micro-suspension:
10 ul sample + 65 ul S9 mixture or saline
+ 25 ul *S. typh.* culture @ 4×10^9 cells/ml
- Pre-incubation:
30 min @ 37C
- Add 2.5 ml top agar @ 45C
- Incubate 48h (YG1029)
or 66h (YG1024) @ 37C

Ames test protocol

Experiments for each sample:

- 5 doses for each sample
- Doses spanned range to toxicity
- Tween 80 negative control
- 2 plates read for each sample/dose
- 3 colony counter readings for each plate
- 2 replicate experiments

Mutagenicity of Particulate Fractions

Average number of revertant colonies per plate

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

Mutagenicity of SVOC Fractions

Average number of revertant colonies per plate

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

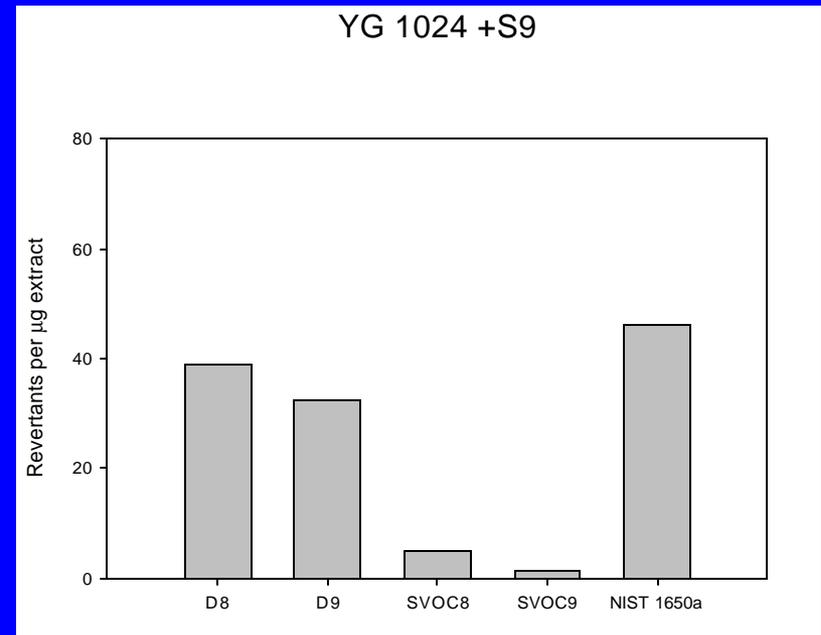
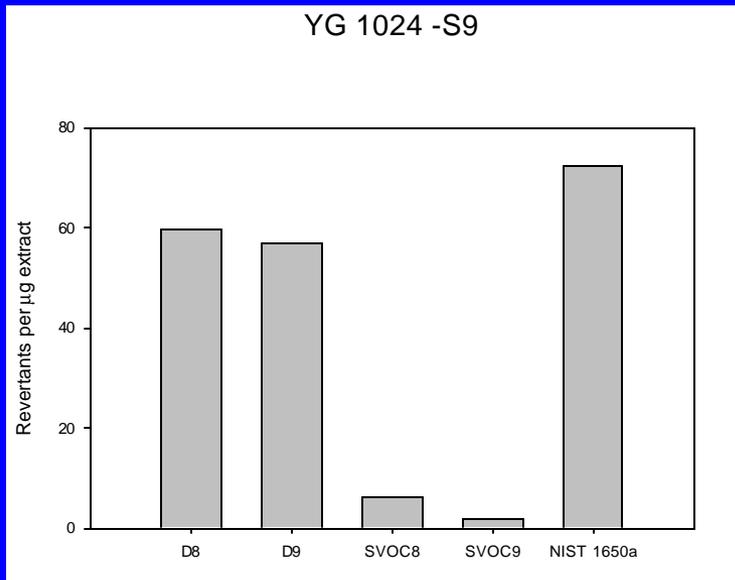
Mutagenicity of NIST SRM 1650a

Average number of revertant colonies per plate

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

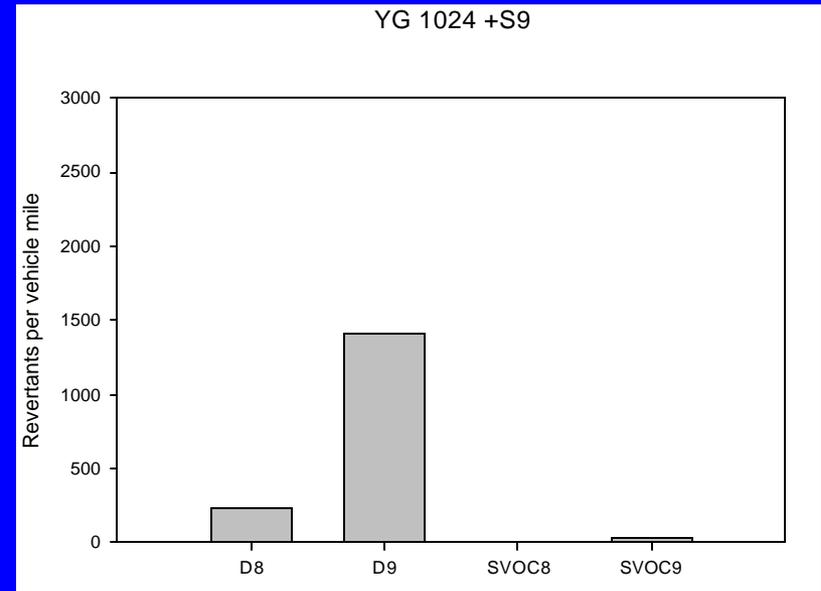
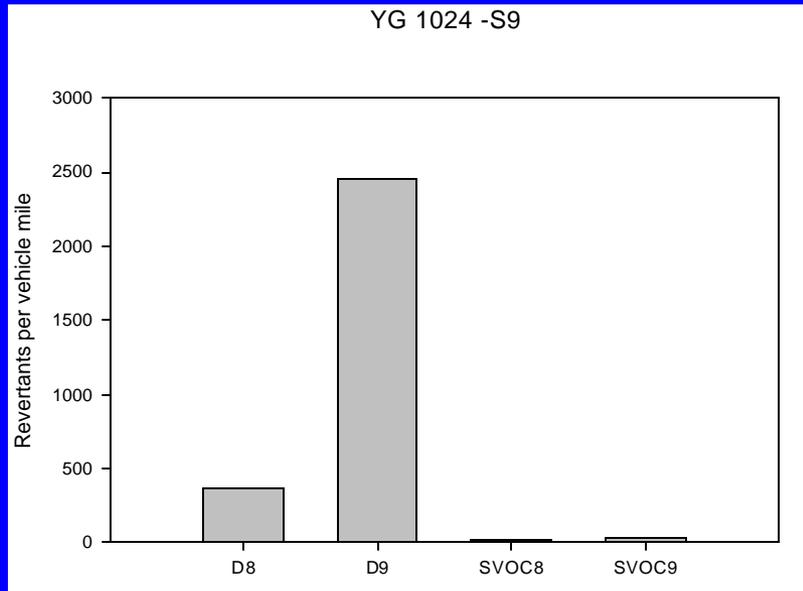
YG1024 +/- S9

Revertants per microgram extract



YG1024 +/- S9

Revertants per mile



Ames assay data interpretation

- Calculate average number of revertant colonies and standard deviation for each sample-dose:
3 readings/plate; 2 plates/sample-dose; 2 experiments
- Lack-of-fit test for linear dose-response repeatedly applied to each sample:
high concentration data deleted until adequate linear trend for at least 3 doses
- Calculate weighted least-squares estimate of linear slope of colony number versus dose for each sample/ tester strain +/- S9 =
calculate Revertants/mass of extract
- convert to Revertants/mile

YG 1024 Mutagenicity

		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	—

YG1029 Mutagenicity

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	-

Results of Mutagenicity Assays

- D8, D9, SVOC8, SVOC9, NIST
all positive for both tester strains, +/- S9 activation
- On a mass basis,
particle extract mutagenic activities were approximately equal for D8, D9, NIST
- On a mileage basis,
particle extract mutagenic activities for D9 (diesel) were on the order of
3- to 10-fold greater than for D8 (gasoline)

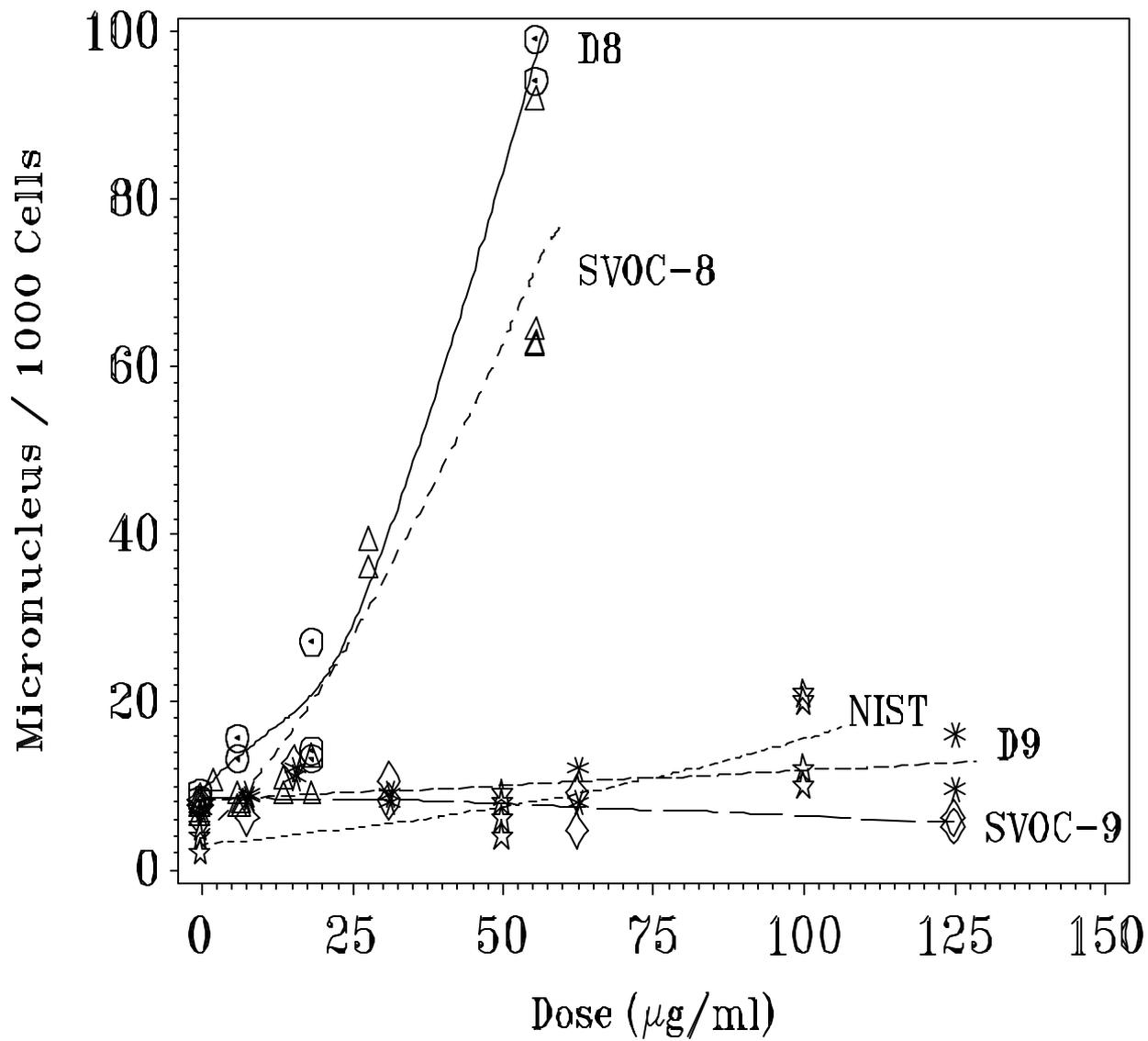
Particle extract activities generally were an order of magnitude greater than SVOC activities

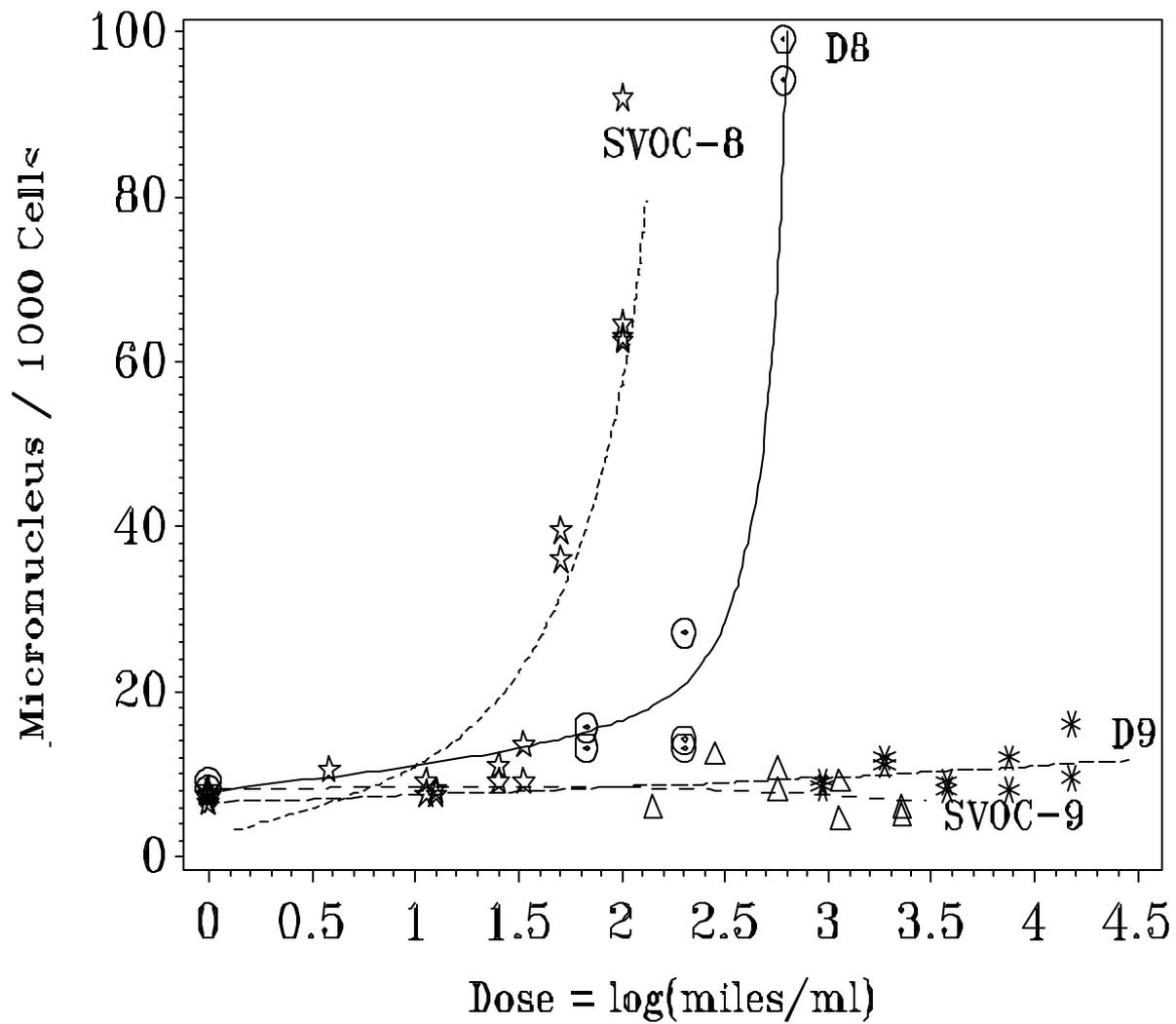
- On a mass basis, SVOC 8 was on the order of 3- fold greater than SVOC 9
- On a mileage basis, SVOC 9 was on the order of 3- fold greater than SVOC 8

Chromosomal Damage Assay

Induction of Micronuclei in 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 cytopsin on microscope slides; stained
- 3000 cells scored for micronuclei for each sample
- Experiment repeated



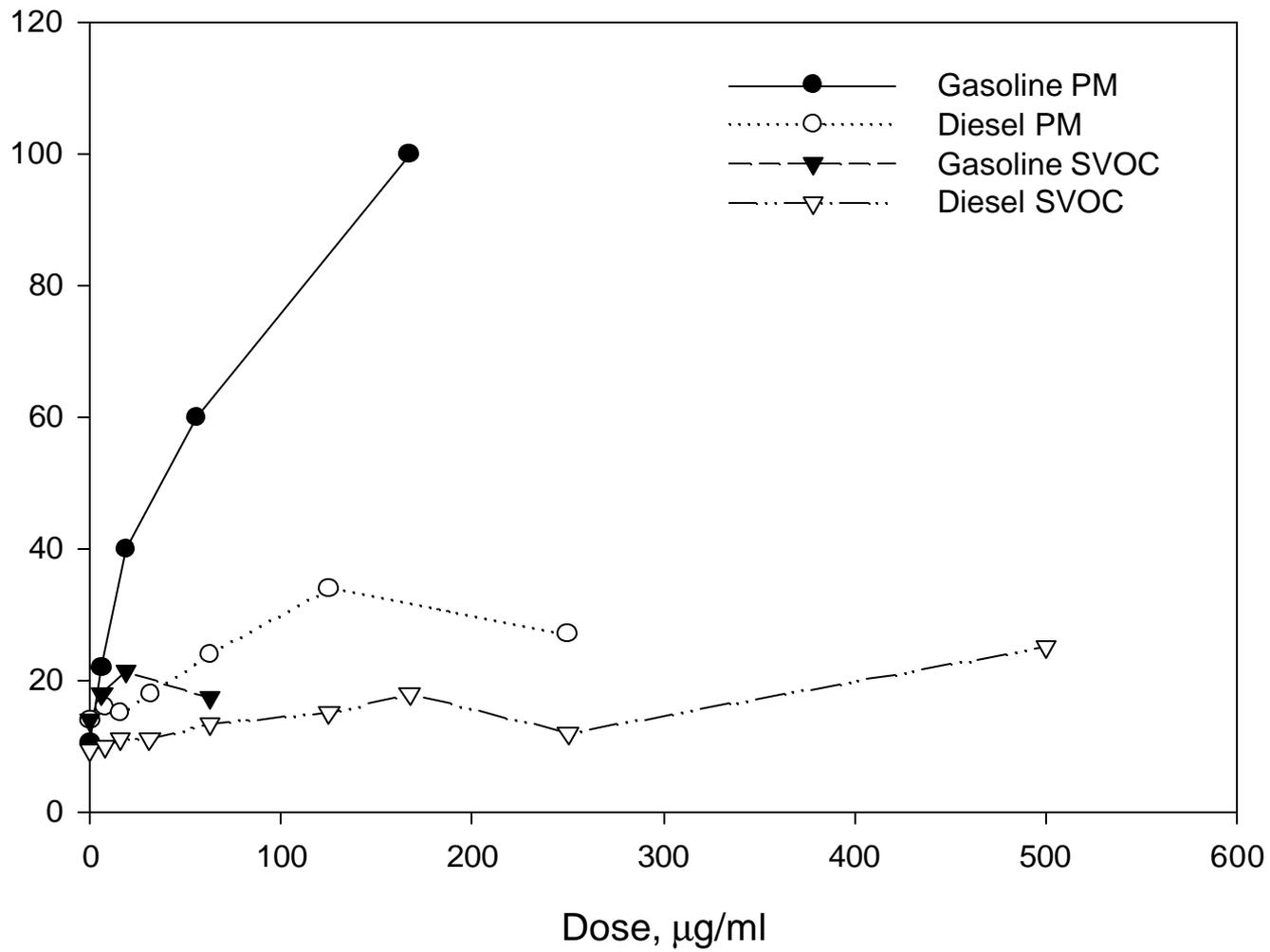


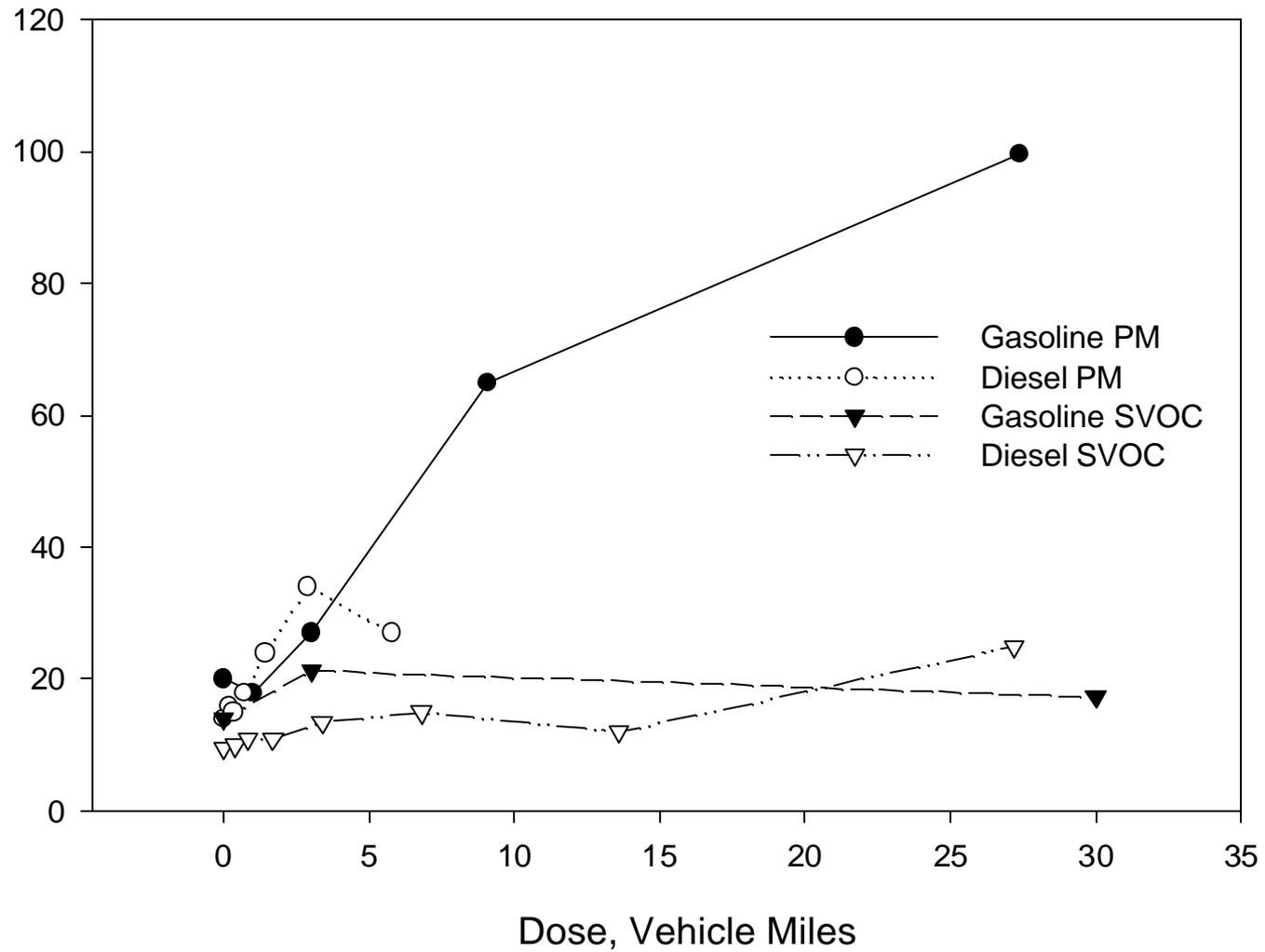
chromosomal damage assay micronucleus induction in V 79 Cells results

- Samples D8 and SVOC-8 positive for micronucleus induction:
indicative of chromosomal breaks and/or spindle damage
- D9, SVOC9, NIST, weak, near background

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 in % damaged cells





Results of DNA damage assays

- D8 positive for DNA damage (Single-cell gel electrophoresis assay)
- SVOC-8 positive at low doses; toxicity at higher doses
- D9 positive at low doses:
lower than D8 per microgram, higher than D8 per mile;
toxicity at higher levels
- SVOC-9 weak

Assay Summary/Interpretation

- Diesel and Gasoline engine exhaust particulate extracts
 - comparably mutagenic/ mass
 - 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

Qualitative Summary

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

Caveats:

In NIOSH studies of other diesel exhaust particulate:

- Some particulate extracts expressed
 - bacterial cell mutagenicity
 - mammalian cell DNA damage
 - mammalian cell chromosomal damage
- Mutagenic activity of diesel particulate extract was observed to be a function of engine operating conditions, e.g., rpm and loading
- Some particulate dispersions in a lung surfactant expressed mutagenic, DNA damage, and chromosomal damage

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

Literature includes some NIOSH / DOE collaborative studies

- US Dept. of Energy/METC-90/6110 DE90000480 (1990).
- U.S.Dept. Energy/METC-91/6122; DE91002091 (1991).
- M McMillian et al., SAE Fuels and Lubricants Conference and Exhibition, 2002.

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 not.
- Question: is the organic genotoxicant material in particulate exhaust soot biologically-available in vivo?

NIOSH Findings: bioavailability of soot genotoxicants in lung fluids

Some diesel exhaust whole particulate material
dispersed in lung surfactants

(not solvent extracted)

induce in vitro genetic damage:

- Mutation in bacterial cells
- DNA damage in mammalian cells
- Chromosomal/ clastogenic damage in mammalian cells

Diesel Exhaust Particulate Mutagenicity in lung surfactants

- Some whole diesel exhaust particulate samples (non-extracted) when dispersed in pulmonary surfactant components of lung lining fluid are mutagenic in the Ames assay:

-- *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

Diesel Exhaust Particulate genotoxicity in lung surfactants

- Some whole diesel exhaust particulate samples
 - which are not extracted with organic solvent
 - but which are dispersed in major components of pulmonary surfactant

are active in mammalian cell assay systems
for DNA or chromosomal or spindle damage:

-- *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)

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

Conclusions and directions

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