Polycyclic Aromatic Hydrocarbons in Urban Air and Their Carcinogenicity and Endocrine Disrupting Activity

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Introduction In recent years, suspended particulate matter (SPM), which is primary derived from emissions of automobiles, especially diesel-powered cars, has been considered as a major air pollutant in Japanese urban areas. A principal concern regarding exposure to polluted air has been cancer risk for lung and other organs. Recently, endocrine disrupting activity of polluted air has also attracted much attention since dysfunction of the male and female reproductive systems was found in mice and rats exposed to diesel emissions. SPM and DEP contain a number of polycyclic aromatic hydrocarbons (PAHs) and nitropolycyclic aromatic hydrocarbons (NPAHs) such as benzo[*a*]pyrene (BaP) and 1,8-dinitropyrene (1,8-DNP). It is probable that carcinogenic and endocrine disrupting effects of SPM and DEP are due in part to PAHs and/or NPAHs. In this paper, we describes the determination method, .atmospheric behavior, mutagenicity and endocrine disrupting activity of PAHs, NPAHs and extracts of DEP.

Determination method Many studies using HPLC with fluorescence detection or GC/MS have been reported for the determination of PAHs. On the other hand, NPAHs have been little studied in spite of the strong direct-acting mutagenicity, mainly because their atmospheric concentrations are much lower than those of PAHs. PAHs are sensitive to fluorescence detection. Moreover, NPAHs are chemically reduced to their corresponding amino-derivatives which are sensitive to not only fluorescence but also peroxyoxalate chemiluminescence detection. We developed a highly sensitive HPLC method for NPAHs with fluorescence and chemiluminescence detection. The detection limits were at sub femtomole levels, which are two orders of magnitude lower than those by HPLC with fluorescence detection or GC/MS. Utilizing this method, we determined several NPAHs such as 1,3-, 1,6- and 1,8-DNPs and 1-nitropyrene (NP) in a sub milligram of automobile exhaust and airborne particulates. By introducing a Pt/Rh reducer column and a switching valve in to the HPLC system, both PAHs and NPAHs in particulates have been determined simultaneously after simple clean-up treatments.

Atmospheric behaviors Airborne and automobile exhaust particulates were collected. When airborne particulates were collected simultaneously at downtown and suburban sites in several Japanese cities, the mean atmospheric concentrations were lower at the suburban sites. The difference in the PAH concentrations in particulates was smaller between the two sites in spite of the larger difference of particulate concentration. However, the difference in NPAH concentrations in particulates between the two sites was greater, suggesting that the NPAHs were

less stable. The concentrations of 1-NP and 1,3-, 1,6- and 1,8-DNPs were much higher in automobile exhaust particulates than in airborne particulates. Analytical results suggested that main contributors of these compounds in urban air were diesel engine vehicles. However, several NPAHs such as 2-nitrofluoranthene (2-NFR) and 2-NP were not observed in DEP but in airborne particulates and showed different diurnal concentrations. From these results, the atmospheric formation of 2-NFR and 2-NP was considered. However, airborne particulate samples collected in Vladivostok showed different chromatographic patterns, suggesting that other large contributors such as power plant and domestic heating which consume coals were also considered.

Mutagenicity When airborne particulates were collected by using an Andersen high-volume air sampler, the NPAH concentrations were highest in the finest particulate fraction (< 1.1 um) in which DEP were main components. If the effect of coexisting compounds is assumed to be negligible, more than 1/3 of the direct-acting mutagenicity of airborne particulates could be attributed to this fraction in the Ames test using the *Salmonella typhimurium* strain. When the DEP extracts were separated into five fractions by silica-gel column chromatography with hexane, hexane/dichloromethane, dichloromethane and ethanol, the strong direct-acting mutagenicity was observed in the dichloromethane fraction (almost 2/3) and in the ethanol fraction. More than 1/2 of the activity in the former fraction was attributed to only four NPAHs, 1-NP and 1,3-, 1,6- and 1,8-DNPs.

Male Fischer 344 rats were exposed to 0.3 mg/m^3 of DEP for 12 h per day for 4 weeks; this dose corresponded to the mean SPM concentration in Tokyo. The levels of mRNA of P450 1B1 in lungs and livers were significantly increased after the exposure. Organic-solvent extracts from DEP (DEPE) induced *umu* gene expression in *Salmonella typhimurium* TA1535/pSK1002 and were further activated by human recombinant P450 1B1. Using an *O*-acetyltransferase overexpressing *Salmonella* strain, genotoxic activation of 1-nitropyrene and DEPE by lung, liver and kidney microsomes was increased by exposure of 0.3 mg/m^3 of DEP. These results suggest that a daily contaminant level of DEP can induce P450 1B1 in rats and that the induced P450 1B1 may catalyze the genotoxic activation of DEP.

Endocrine disrupting activity Three DEP samples were collected from three diesel-powered vehicles. The DEPE samples showed the antiestrogenic effect in the estrogen-responsive MCF-7 cells transiently transfected with ERE-driven luciferase expression plasmid. The antiandrogenic effect was reduced by the co-treatment with α-naphthoflavone, an aryl hydrocarbon receptor (AhR). The antiestrogenic effect was stronger on a DEPE with higher concentrations of PAHs acting as AhR agonists. These results suggest that a part of the antiestrogenic effect of DEP is attributed to PAHs through AhR-mediated process. We also found that several hydroxylated metabolites of PAHs such as 3-hydroxybenzo[*a*]pyrene bound to estrogen receptors. This process would also contribute to the disrupting activity of DEP.

Determination of Carcinogenic and Endocrine Disrupting Chemicals in Airborne Particulates

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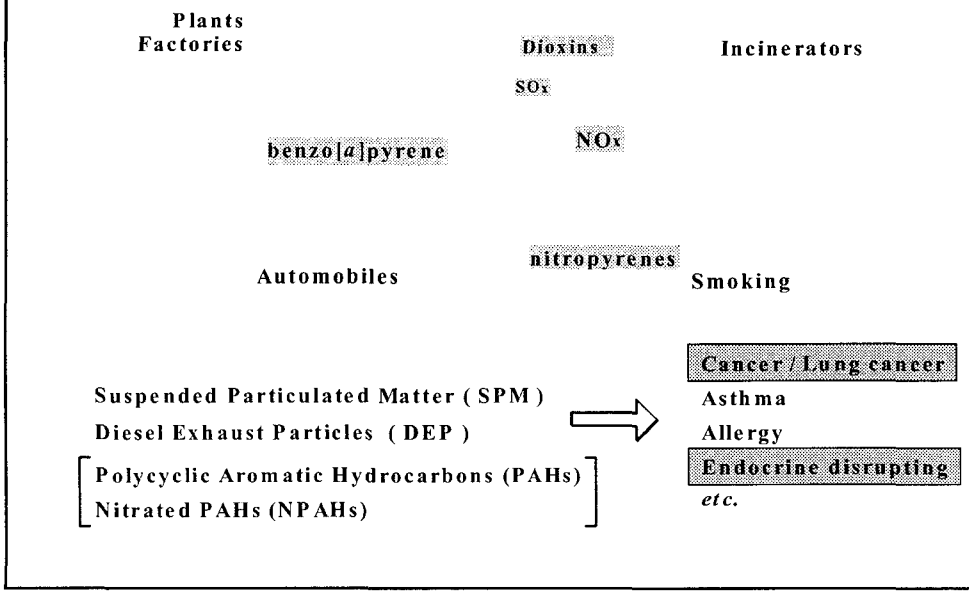
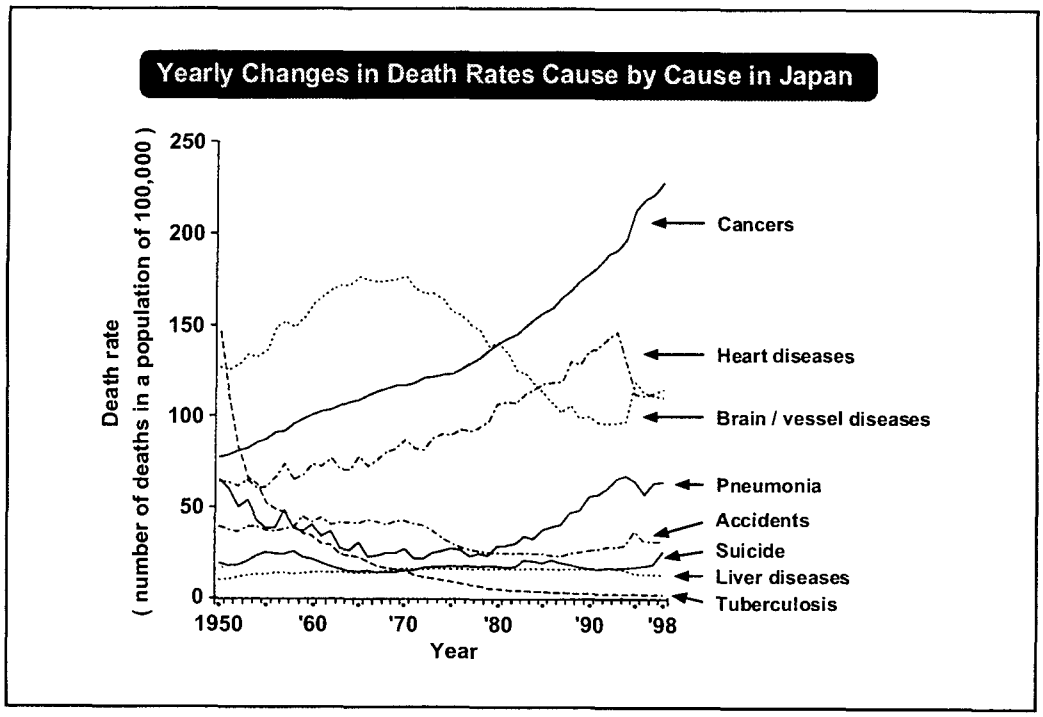
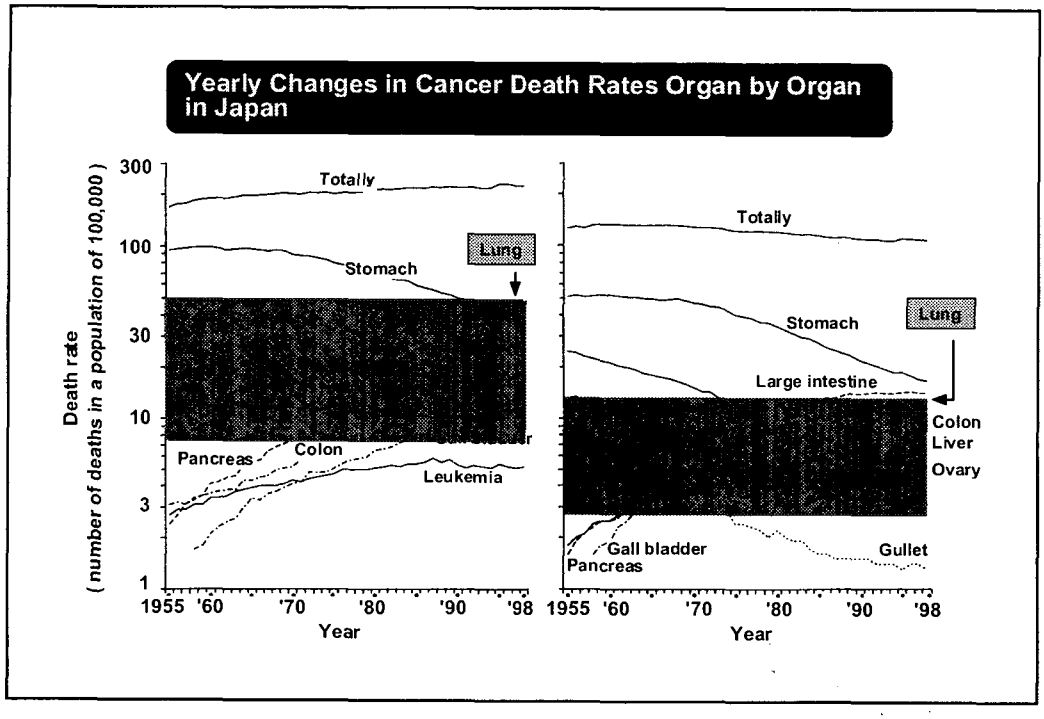



Yearly Changes in Death Rates Cause by Cause in Japan
Death rate
(number of deaths in a population of 100,000)
250
200
150
100
50
0
1950
'60
'70
'80
'90
'98
Year
Cancers
Heart diseases
Brain / vessel diseases
Pneumonia
Accidents
Suicide
Liver diseases
Tuberculosis


Yearly Changes in Cancer Death Rates Organ by Organ in Japan
Death rate
(number of deaths in a population of 100,000)
300
100
30
10
3
1
1955
'60
'70
'80
'90
'98
Year
Totally
Stomach
Lung
Pancreas
Colon
Leukemia
Totally
Stomach
Lung
Large intestine
Colon
Liver
Ovary
Gall bladder
Pancreas
Gullet

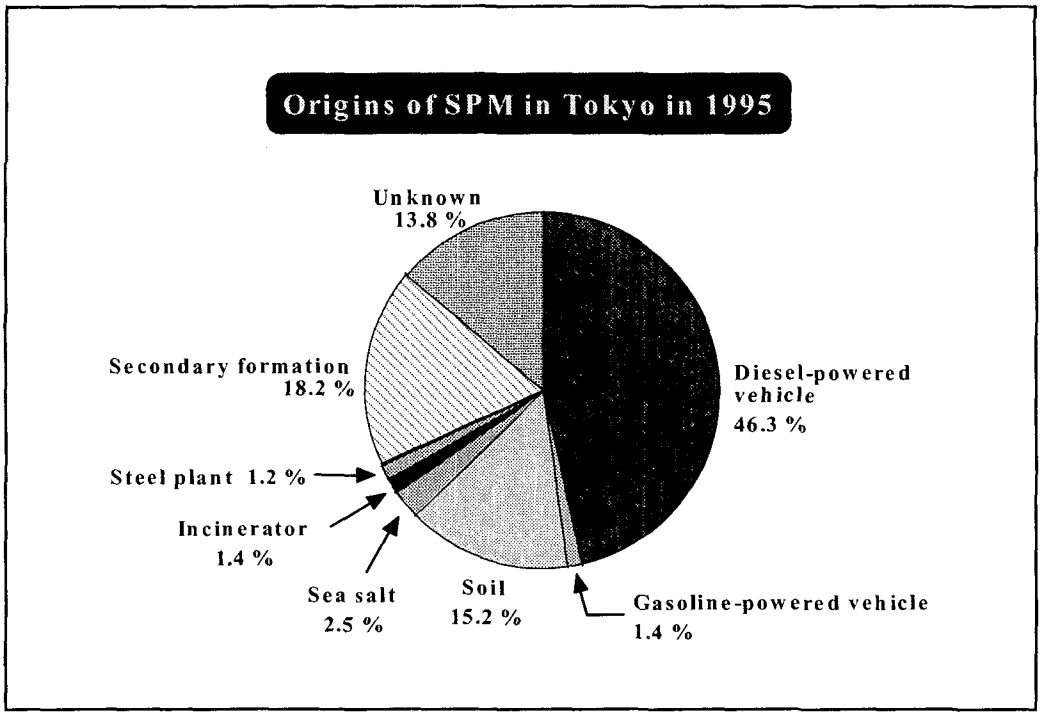
Evaluations of the Carcinogenicity of Some PAHs

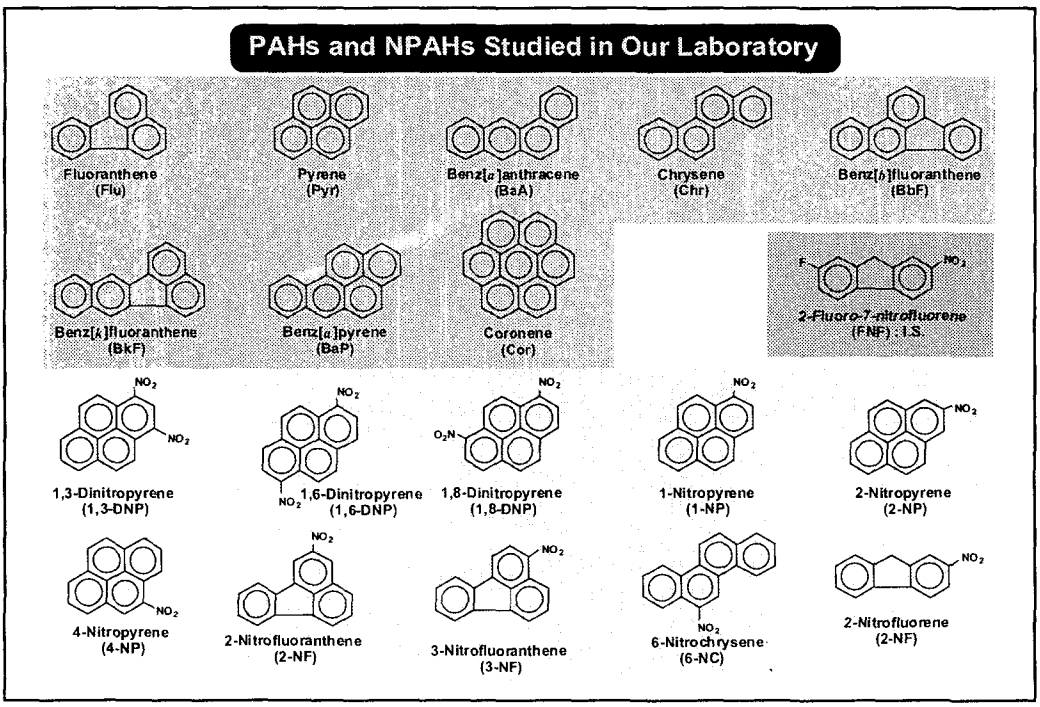
Compound	IARC	US EPA
Anthracene	3	D
Benzo[*a*]anthracene	2A	B2
Benzo[*b*]fluoranthene	2B	B2
Benzo[*j*]fluoranthene	2B	B2
Benzo[*k*]fluoranthene	2B	B2
Benzo[*g h i*]perylene	3	D
Benzo[*a*]pyrene	2A	B2
Benzo[*e*]pyrene	3	C
Chrysene	3	B2
Fluoranthene	3	D
Fluorene	3	D
Phenanthrene	3	D

Mutagenicities of Several Carcinogens by the Ames Test Using the *S. typhimurium* TA98 and TA100 Strains

	Revertants / μg	
Chemical	TA98	TA100
1,8-Dinitropyrene	940,000	274,000
MeIQ + S9 mix[a]	661,000	30,000
IQ + S9 mix[a]	433,000	7,000
MeIQx + S9 mix[a]	145,000	14,000
Trp-P-2 + S9 mix[a]	104,000	1,800
Glu-P-1 + S9 mix[b]	49,000	3,200
Trp-P-1 + S9 mix[a]	39,000	1,700
4-NQO + S9 mix[a]	970	9,900
Benzo[*a*]pyrene + S9 mix[a]	320	660
Nitrosodiethylamine + S9 mix[c]	0.02	0.15

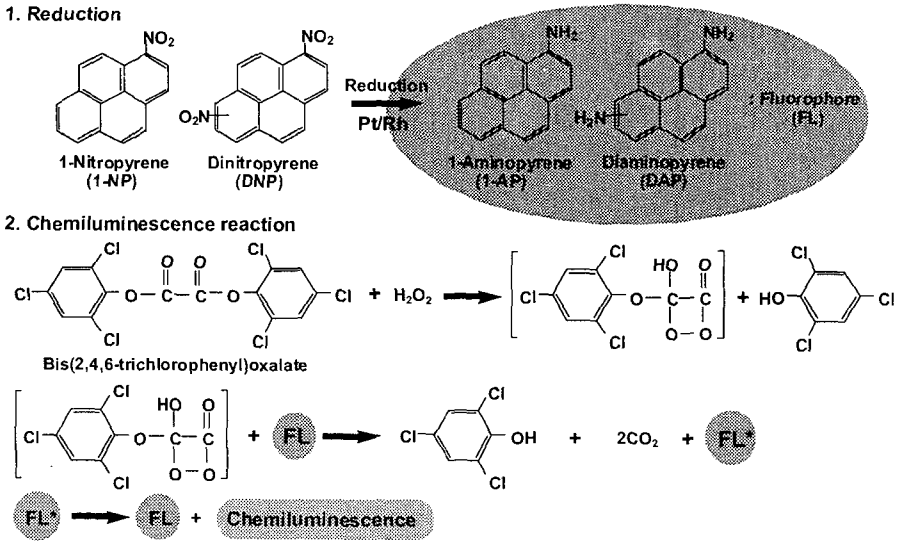
S9 mix / plate (μL): a, 10; b, 30; c, 150. (Wakabayashi et al., (1982.))

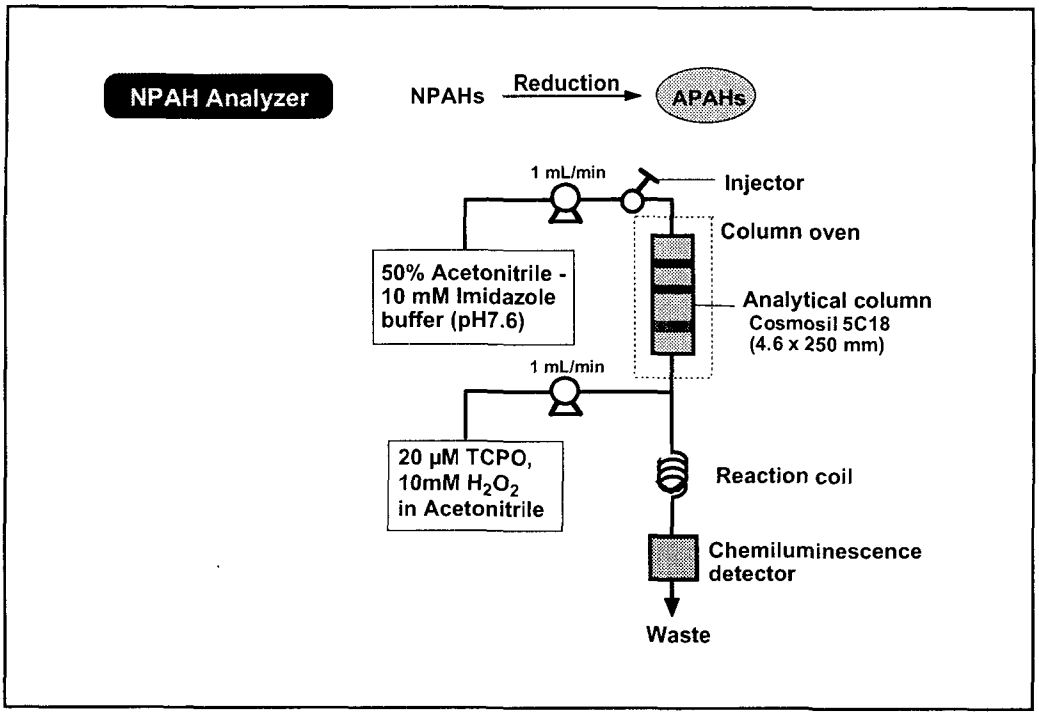

Origins of SPM in Tokyo in 1995
Unknown
13.8 %
Secondary formation
18.2 %
Diesel-powered
vehicle
46.3 %
Steel plant 1.2 %
Incinerator
1.4 %
Sea salt
2.5 %
Soil
15.2 %
Gasoline-powered vehicle
1.4 %

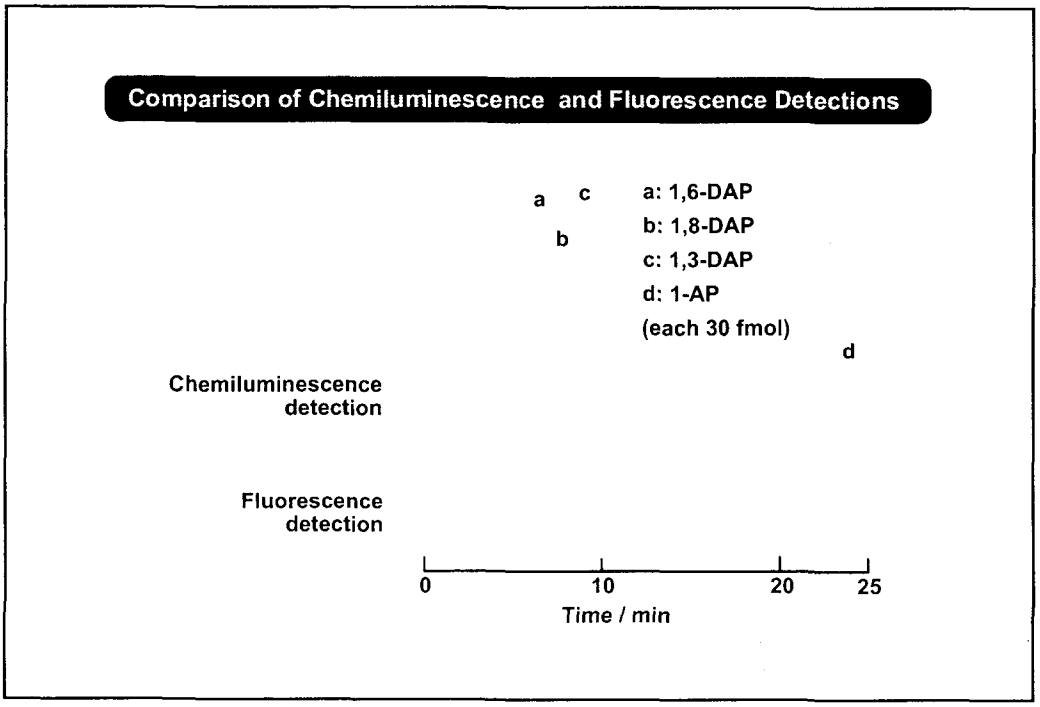

PAHs and NPAHs Studied in Our Laboratory
Fluoranthene
(Flu)
Pyrene
(Pyr)
Benz[a]anthracene
(BaA)
Chrysene
(Chr)
Benz[b]fluoranthene
(BbF)
Benz[k]fluoranthene
(BkF)
Benz[a]pyrene
(BaP)
Coronene
(Cor)
2-Fluoro-7-nitrofluorene
(FNF) : I.S.
1,3-Dinitropyrene
(1,3-DNP)
1,6-Dinitropyrene
(1,6-DNP)
1,8-Dinitropyrene
(1,8-DNP)
1-Nitropyrene
(1-NP)
2-Nitropyrene
(2-NP)
4-Nitropyrene
(4-NP)
2-Nitrofluoranthene
(2-NF)
3-Nitrofluoranthene
(3-NF)
6-Nitrochrysene
(6-NC)
2-Nitrofluorene
(2-NF)

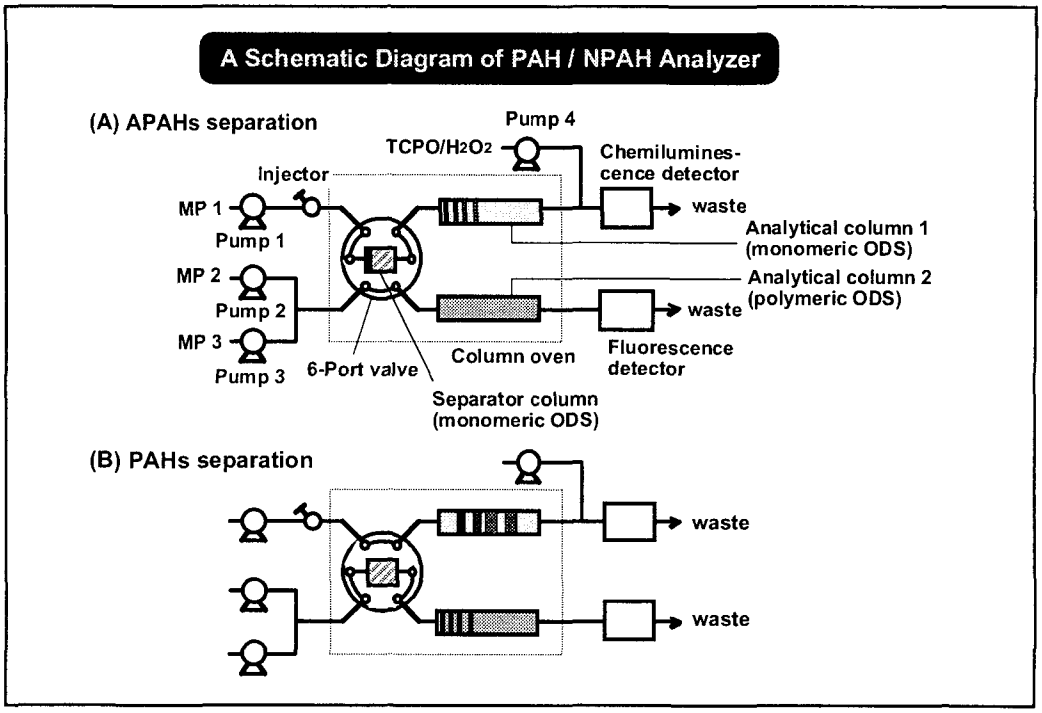
Determination Method for PAHs and NPAHs in SPM and DEP

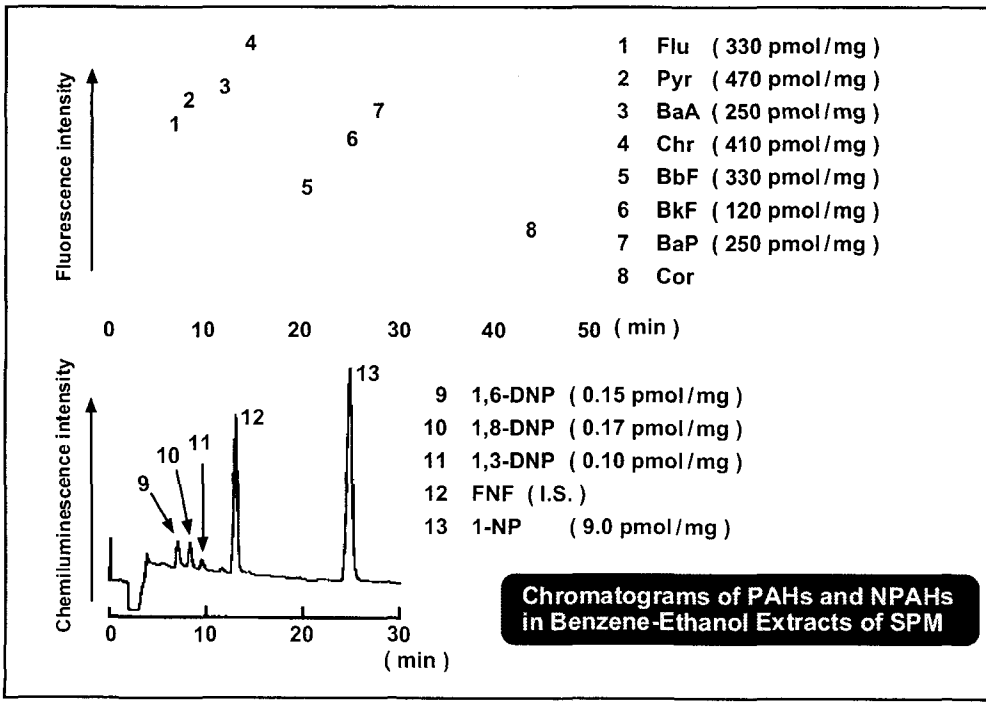
Chemiluminescence Detection Mechanism for NPAHs

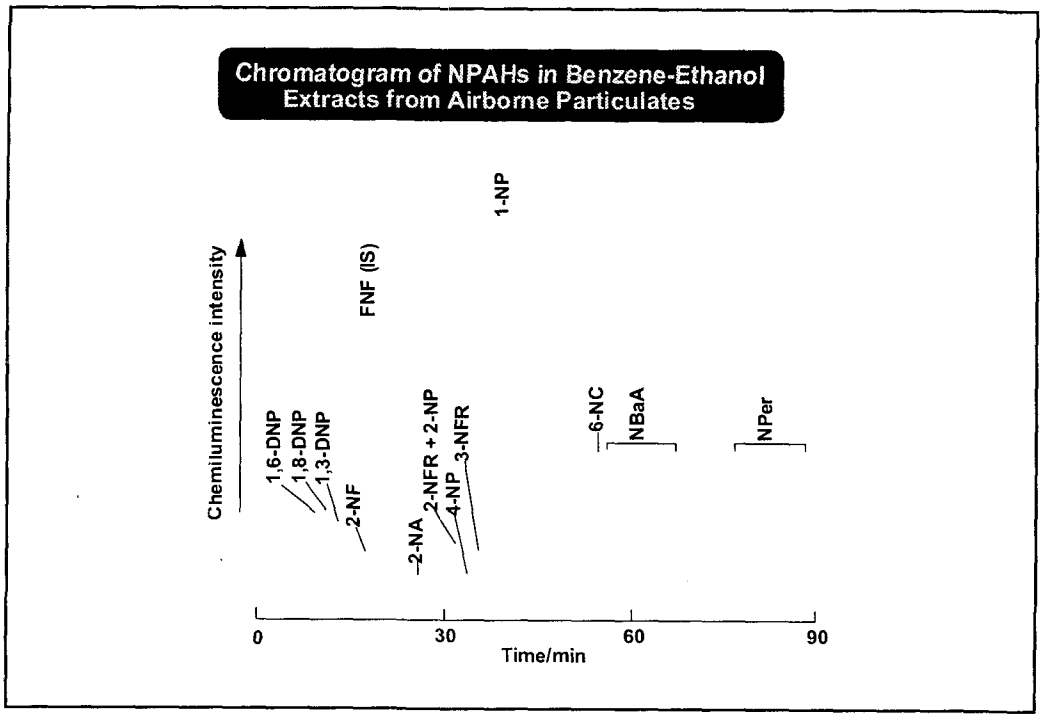


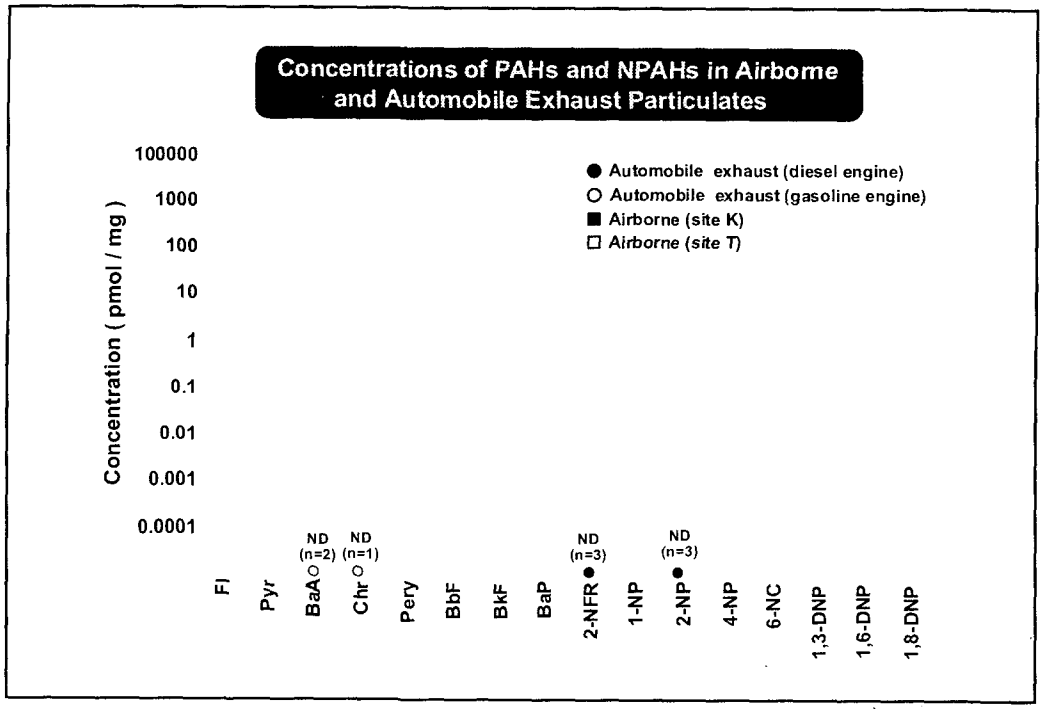

NPAH Analyzer
NPAHs
Reduction
APAHs
1 mL/min
Injector
Column oven
50% Acetonitrile -
10 mM Imidazole
buffer (pH7.6)
Analytical column
Cosmosil 5C18
(4.6 x 250 mm)
1 mL/min
20 μM TCPO,
10mM H_2O_2
in Acetonitrile
Reaction coil
Chemiluminescence
detector
Waste

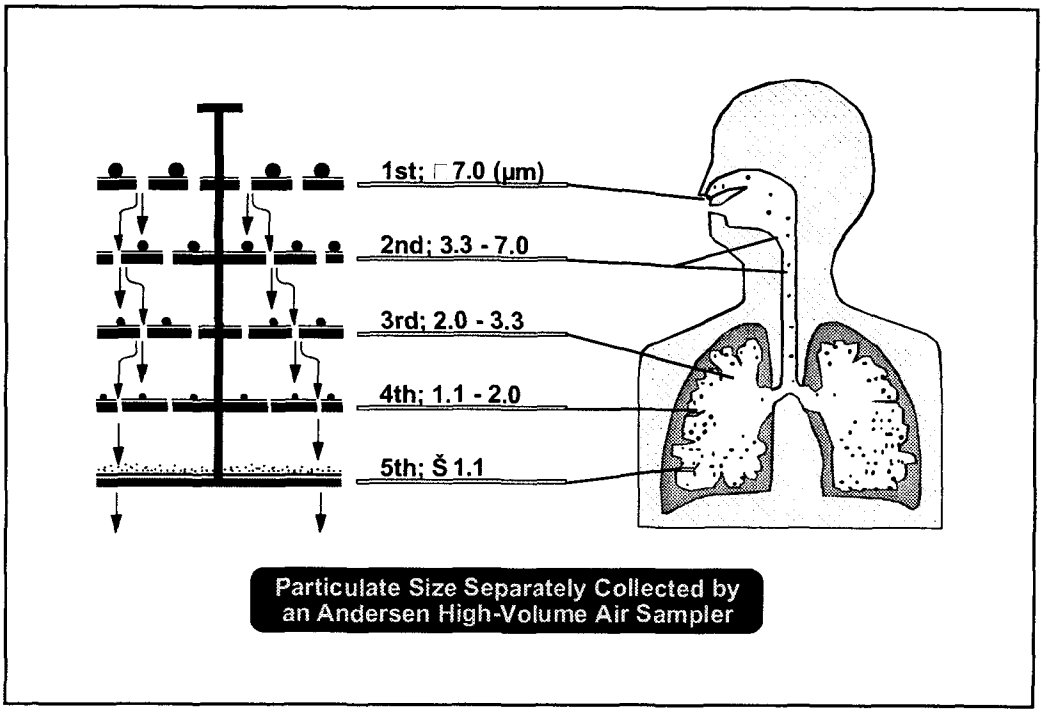

Comparison of Chemiluminescence and Fluorescence Detections
a
c
b
d
a: 1,6-DAP
b: 1,8-DAP
c: 1,3-DAP
d: 1-AP
(each 30 fmol)
Chemiluminescence
detection
Fluorescence
detection
0
10
20
25
Time / min


A Schematic Diagram of PAH / NPAH Analyzer
(A) APAHs separation
Pump 4
TCPO/H2O2
Chemilumines-cence detector
Injector
MP 1
Pump 1
waste
Analytical column 1 (monomeric ODS)
MP 2
Pump 2
Analytical column 2 (polymeric ODS)
waste
MP 3
Pump 3
6-Port valve
Column oven
Fluorescence detector
Separator column (monomeric ODS)
(B) PAHs separation
waste
waste

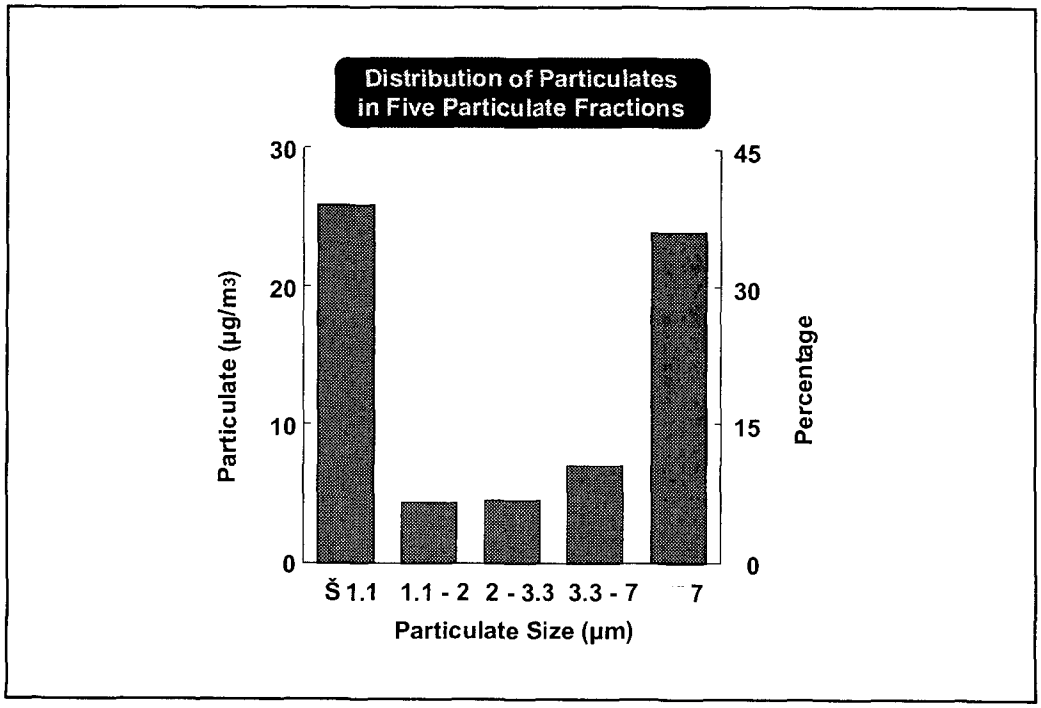

Fluorescence intensity
1 Flu (330 pmol/mg)
2 Pyr (470 pmol/mg)
3 BaA (250 pmol/mg)
4 Chr (410 pmol/mg)
5 BbF (330 pmol/mg)
6 BkF (120 pmol/mg)
7 BaP (250 pmol/mg)
8 Cor
0 10 20 30 40 50 (min)
Chemiluminescence intensity
9 1,6-DNP (0.15 pmol/mg)
10 1,8-DNP (0.17 pmol/mg)
11 1,3-DNP (0.10 pmol/mg)
12 FNF (I.S.)
13 1-NP (9.0 pmol/mg)
0 10 20 30
(min)
Chromatograms of PAHs and NPAHs in Benzene-Ethanol Extracts of SPM

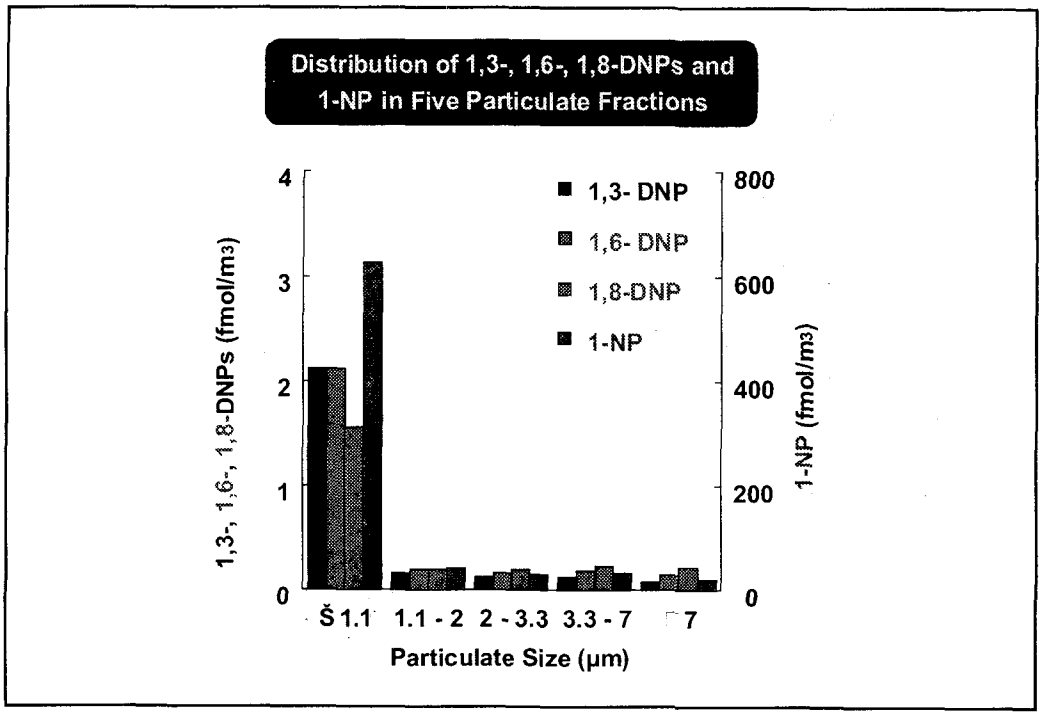

Chromatogram of NPAHs in Benzene-Ethanol Extracts from Airborne Particulates
Chemiluminescence intensity
1,6-DNP
1,8-DNP
1,3-DNP
2-NF
FNF (IS)
2-NA
2-NFR + 2-NP
4-NP
3-NFR
1-NP
6-NC
NBaA
NPer
0
30
60
90
Time/min

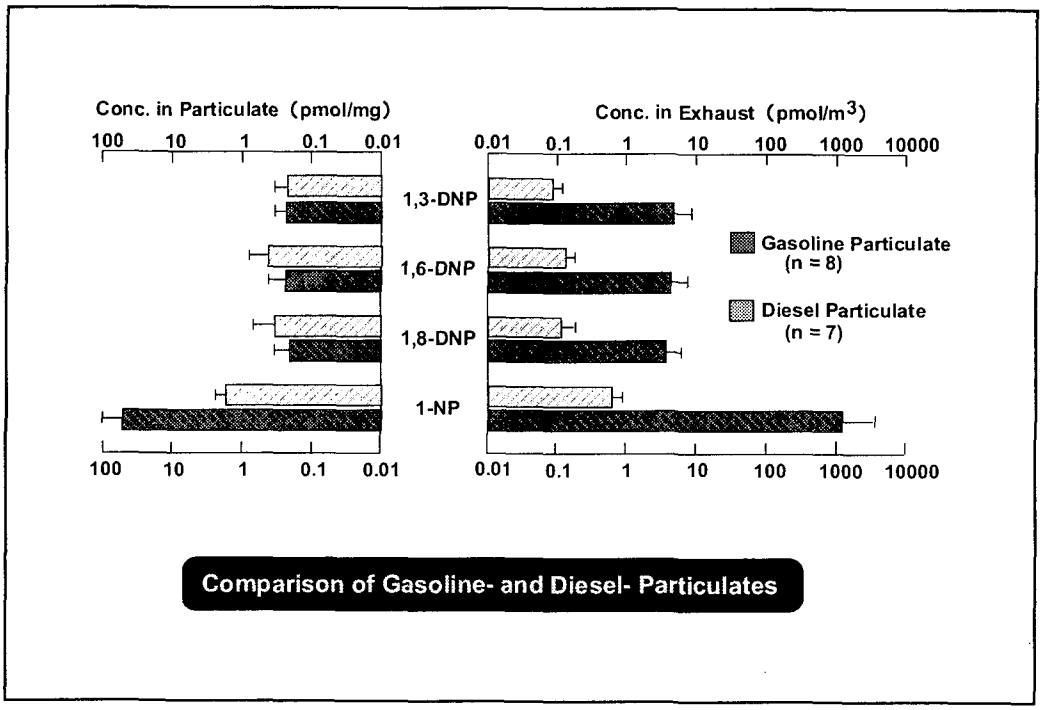

Concentrations of PAHs and NPAHs in Airborne and Automobile Exhaust Particulates
Concentration (pmol / mg)
100000
1000
100
10
1
0.1
0.01
0.001
0.0001
Automobile exhaust (diesel engine)
Automobile exhaust (gasoline engine)
Airborne (site K)
Airborne (site T)
ND (n=2)
ND (n=1)
ND (n=3)
ND (n=3)
Fl
Pyr
BaA
Chr
Pery
BbF
BkF
BaP
2-NFR
1-NP
2-NP
4-NP
6-NC
1,3-DNP
1,6-DNP
1,8-DNP

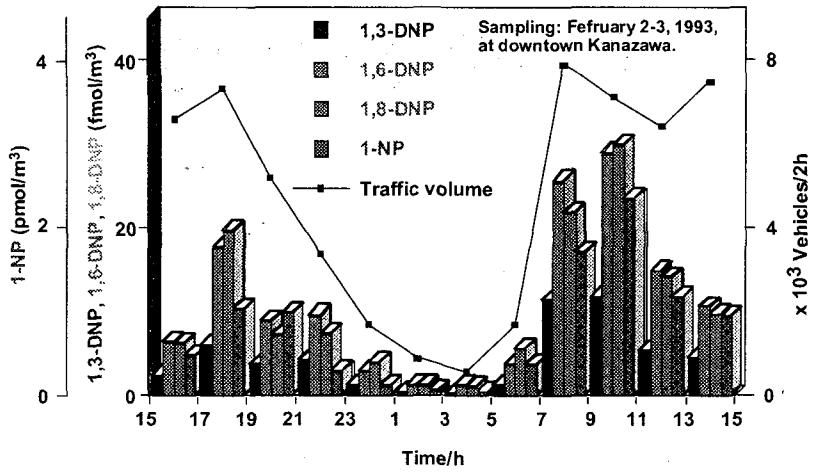


Particulate Size Separately Collected by an Andersen High-Volume Air Sampler




Distribution of 1,3-, 1,6-, 1,8-DNPs and 1-NP in Five Particulate Fractions
1,3- DNP
1,6- DNP
1,8-DNP
1-NP
1,3-, 1,6-, 1,8-DNPs (fmol/m3)
1-NP (fmol/m3)
0
1
2
3
4
0
200
400
600
800
Š1.1
1.1 - 2
2 - 3.3
3.3 - 7
7
Particulate Size (µm)


Conc. in Particulate (pmol/mg)
100 10 1 0.1 0.01
Conc. in Exhaust (pmol/m3)
0.01 0.1 1 10 100 1000 10000
1,3-DNP
1,6-DNP
1,8-DNP
1-NP
Gasoline Particulate (n = 8)
Diesel Particulate (n = 7)
Comparison of Gasoline- and Diesel- Particulates

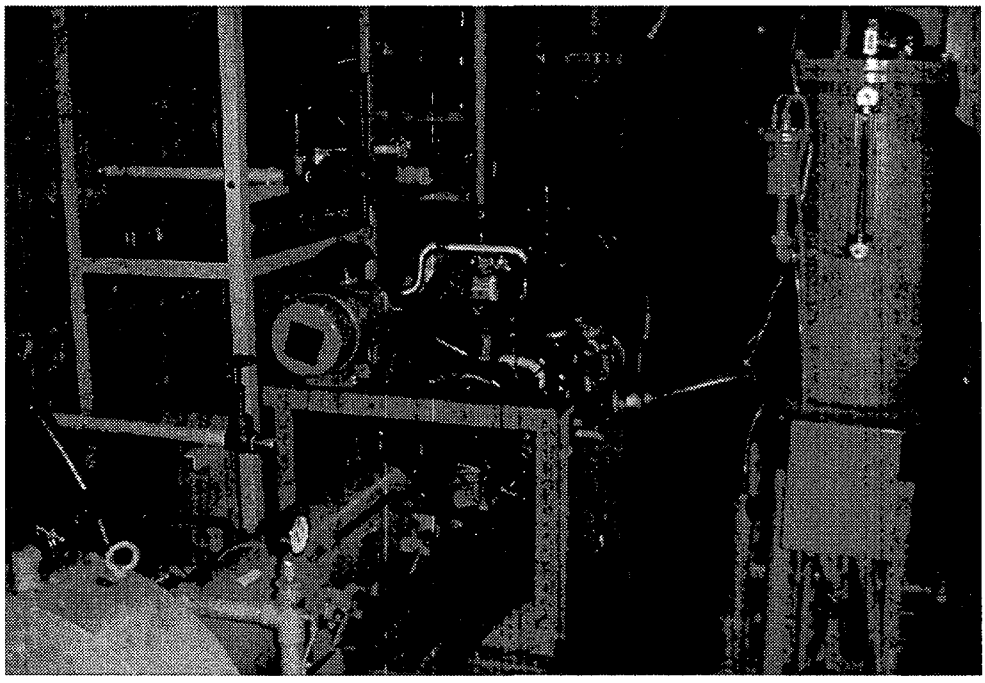


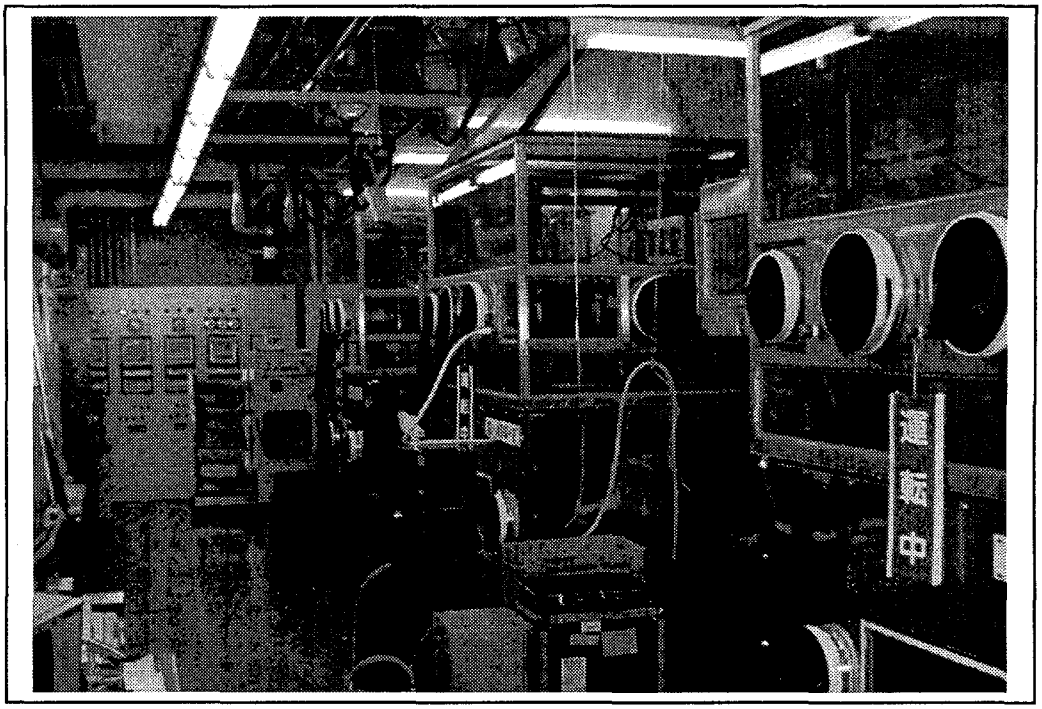
Diurnal Variations of 1,3-, 1,6-, 1,8-DNPs and 1-NP in Urban Air

Carcinogenecity / Mutagenicity

DEP Exposure Experiment

Animal :	**male Fischer 344 rats (4 weeks old)**
Exposure :	**12 hr (night time) per day** **4 weeks**
DEP conc. :	**0, 0.3, or 3.0 mg / m^3**
mRNA level :	**RT-PCR**
Genetoxicity :	***umu* gene expression test** **using *S. typhimurium* TA1635 / pSK1002**





Relative mRNA Expression Levels of P450 Family 1 Enzymes in Lungs, Livers, and Kidneys of DEP-Exposed Rats

Organ	DEP dose (mg/m^3)	P450 mRNA level		
		1A1	1A2	1B1
Lung	0	100 ± 8	100 ± 31	100 ± 6
	0.3	116 ± 4	79 ± 30	114 ± 5
	3	228 ± 31	93 ± 35	116 ± 6
Liver	0	100 ± 6	100 ± 16	100 ± 8
	0.3	122 ± 32	121 ± 9	143 ± 21
	3	111 ± 3	131 ± 21	114 ± 4
Kidney	0	100 ± 12	Not detected	100 ± 11
	0.3	92 ± 15	Not detected	100 ± 16
	3	96 ± 6	Not detected	96 ± 10

mRNA levels were corrected with β-actin mRNA levels and are expressed as percentage of control (untreated group).

Mean ± S.D. for 3 rats.

Significantly different from control ($p < 0.05$).

Genotoxic Activities of Chemicals in the Presence of Lung, Liver, and Kidney Microsomes from DEP-Exposed Rats

Organ	DEP dose (mg/m^3)	Genotoxic activity (*umu* units/min/mg protein)			
		DEPE	1-NP	Trp-P-1	MeIQ
Lung	0	51 ± 2	54 ± 6	27 ± 1	22 ± 1
	0.3	124 ± 1	227 ± 5	37 ± 1	22 ± 3
	3	147 ± 14	154 ± 5	54 ± 1	25 ± 1
Liver	0	105 ± 26	550 ± 34	510 ± 12	1421 ± 88
	0.3	153 ± 31	815 ± 26	546 ± 42	1370 ± 168
	3	173 ± 13	914 ± 36	562 ± 48	1603 ± 74
kidney	0	13 ± 2	144 ± 10	51 ± 4	10 ± 2
	0.3	17 ± 1	147 ± 14	58 ± 5	10 ± 1
	3	19 ± 2	165 ± 11	50 ± 5	8 ± 1

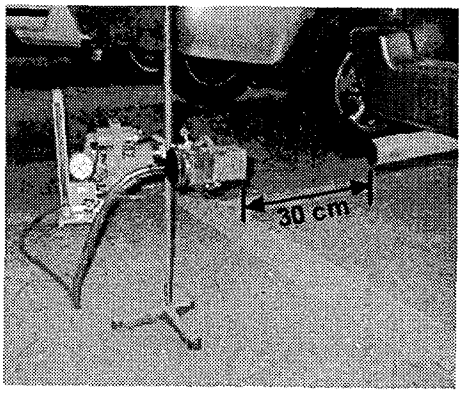
DEPE (5 μg/mL), 1-NP (0.3 μM), Trp-P-1 (5 μM) and MeIQ (5 mM) were incubated with liver microsoms (10 μg/mL) and lung and kidney microsomes (30 mg/mL) in the presence of *S. typhimurium* NM2009.

Mean ± S.D. for 5 rats.

Significantly different from control ($p < 0.05$).

Endocrine Disrupting Effect

DEP Collection and Sample Preparation



Automobile : car, bus, truck

DEP collection

Pallflex T60A20 glass-fiber filter
(55 mm ϕ)

30 L / min (low-volume air sampler)

DEP ca. 5 mg (5 filters)

↓ + benzene / EtOH (3 : 1)
160 mL
ultrasonicated for 30 min

benzene / EtOH fraction

↓ filtered
evaporated to dryness

residue

↓ + EtOH

DEPE sample (2 mg / mL)

DC (Car)
DB (Bus)
DT (Truck)

Assay of Estrogenic and Antiestrogenic Effects of DEPE Samples

Luciferase reporter gene assay

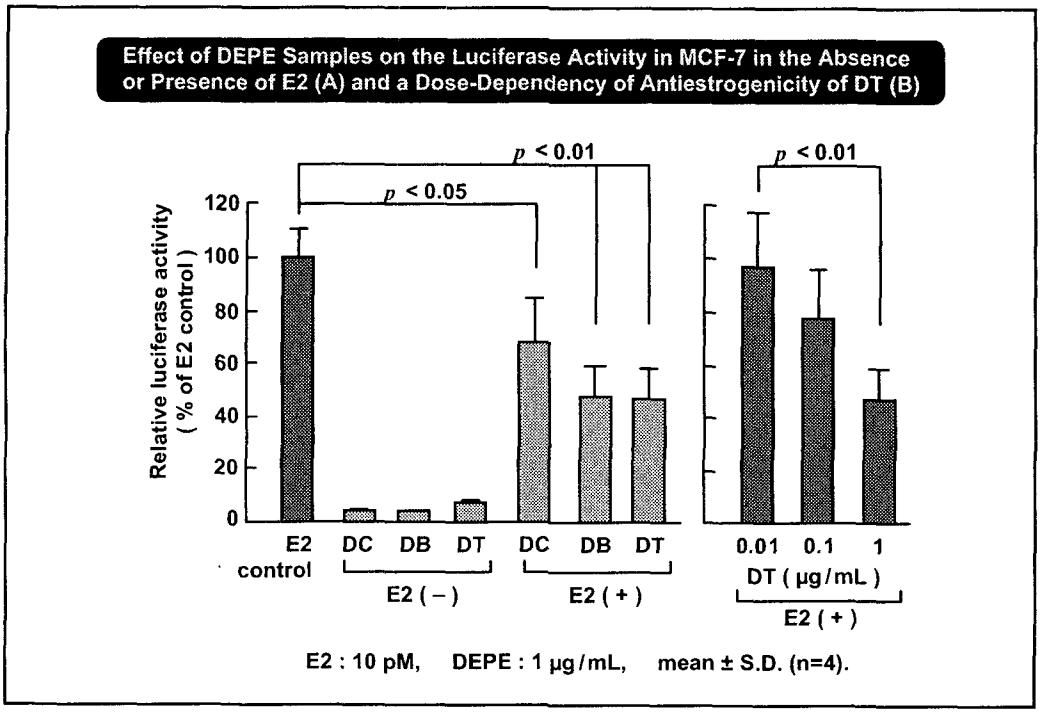
- Human breast cancer MCF-7 cells were transiently transfected with ERE-driven luciferase expression plasmid vector.
- The cells were then treated with E2, DEPE sample, α-NF or SKF-525A alone or in combinations for 36 hr.
- The cells were lysed and measured for luciferase activity.

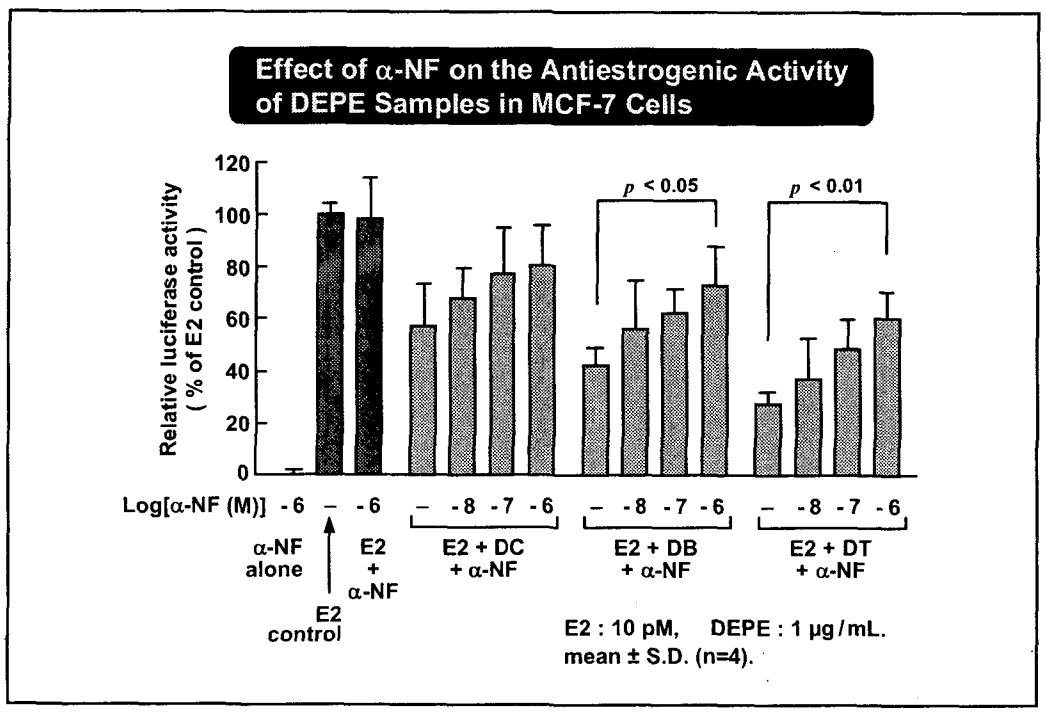
Chemicals

E2 : 17β-estradiol, an estrogen.
α-NF : α-naphthoflavone, an aryl hydrocarbon receptor antagonist.
SKF : SKF-525A, an inhibitor of xenobiotic metabolizing enzymes.

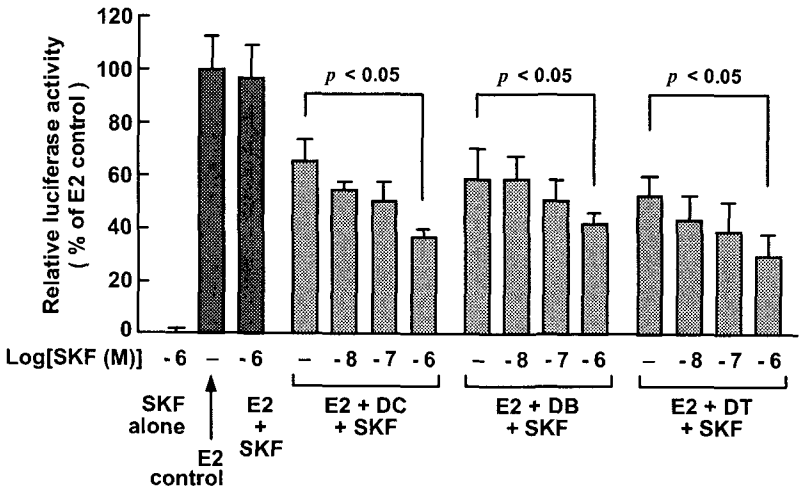
Concentrations for cell treatment

E2 : 10 pM, α-NF : 1 μM, SKF : 1 μM.


Effect of DEPE Samples on the Luciferase Activity in MCF-7 in the Absence or Presence of E2 (A) and a Dose-Dependency of Antiestrogenicity of DT (B)
p < 0.01
p < 0.05
p < 0.01
Relative luciferase activity (% of E2 control)
120
100
80
60
40
20
0
E2 control
DC
DB
DT
DC
DB
DT
0.01
0.1
1
E2 (–)
E2 (+)
DT (μg/mL)
E2 (+)
E2 : 10 pM, DEPE : 1 μg/mL, mean ± S.D. (n=4).


Effect of α-NF on the Antiestrogenic Activity of DEPE Samples in MCF-7 Cells
Relative luciferase activity (% of E2 control)
120
100
80
60
40
20
0
p < 0.05
p < 0.01
Log[α-NF (M)] - 6 – - 6 – - 8 - 7 - 6 – - 8 - 7 - 6 – - 8 - 7 - 6
α-NF alone
E2 control
E2 + α-NF
E2 + DC + α-NF
E2 + DB + α-NF
E2 + DT + α-NF
E2 : 10 pM, DEPE : 1 μg/mL.
mean ± S.D. (n=4).

Effect of SKF on the Antiestrogenic Activity of DEPE Samples in MCF-7 Cells



E2 : 10 pM, DEPE : 1 μg/mL, mean ± S.D. (n=4).

Concentrations of Several PAHs Found in the DEPE Samples

Compound	Concentration (pmol/mg DEPE) DC	DB	DT
Benz[*a*]anthracene	10.5	66.7	53.9
Chrysene	65.8	101	127
Benzo[*b*]fluoranthene	9.5	61.5	46.4
Benzo[*k*]fluoranthene	2.0	20.6	16.3
Benzo[*a*]pyrene	6.3	58.7	68.3
Benzo[*ghi*]perylene	N.D.*	57.6	33.0
Total (6 PAHs)	94	366	345

* Not detected.