FINAL REPORT

TIER 2 TESTING OF BIODIESEL EXHAUST EMISSIONS

Study Report Number FY98-056

Submitted to:

National Biodiesel Board (NBB)

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Submitted by:

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COMPLIANCE STATEMENT

This study was conducted according to U.S. Environmental Protection Administration Agency (EPA) testing requirements 40 CFR 79.53 (c) (1) Tier 2. Any areas of noncompliance are documented in the study records. No deviations existed that significantly affected the validity of the study.

Study Event	Date
Study Initiation: (Protocol signed by Study Director)	1-22-99
Experimental Start Date: (First date on which test atmosphere applied to test system)	2-22-99
Experimental Termination Date: (Last date on which data were collected directly from the study)	6-30-99

Charles H. Hobbs, DVM, DABT, DABVT	Study Completion Date
Sponsor Representative National Biodiesel Board	Date

LIST OF ACRONYMS

ACLAM	=	American College of Laboratory Animal Medicine
AD	=	Alveolar duct
AM	=	Alveolar macrophage
ANOVA	=	Analysis of variance
BEE-PSOF	=	Biodiesel Exhaust Emissions-Particulate Soluble Organic Fraction
BEE-SVEF	=	Biodiesel Exhaust Emissions-Semi-Volatile Extracted Fraction
DMBA	=	Dimethyl benzanthracene
DRI	=	Desert Research Institute
EDS	=	Engine Dynamometer Schedule
EPA	=	Environmental Protection Agency
GFAP	=	Glial fibrillary acidic protein
GLP	=	Good Laboratory Practices
GSD	=	Geometric standard deviation
H&E	=	Hematoxylin and eosin
Int.	=	Intermediate
LMJ	=	Lovelace multi-jet cascade impactor
LRRI	=	Lovelace Respiratory Research Institute
MMAD	=	Mass median aerodynamic diameter
MMDD	=	Mass median diffusion diameter
MN	=	Micronuclei
NAD	=	No abnormalities detected
NBB	=	National Biodiesel Board
NBF	=	Neutral-buffered formalin
NCE	=	Normochromatic erythrocytes
NO_x	=	Total oxides of nitrogen
PCE	=	Polychromatic erythrocytes
PFDB	=	Parallel flow diffusion battery
PSOF	=	Particulate soluble organic fraction
PUF	=	Polyurethane foam
SCE	=	Sister chromatid exchange
SNK	=	Student-Neuman-Kuels
SVEF	=	Semi-volatile extracted fraction
TB	=	Terminal bronchiole

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TIER 2 Testing of Biodiesel Exhaust Emissions

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QUALITY ASSURANCE STATEMENT

TIER 2 Testing of Biodiesel Exhaust Emissions

This study was inspected by the LRRI Quality Assurance Unit. Findings were discussed with study scientists at the time of inspection, and reports of findings were submitted to the study director and management as follows.

Study Phase	Inspection Date	Report Date
Protocol	2/9/99	2/9/99
Path/Tox Protocols	2/12, 19, 25/99	2/25/99
Study Inspections - Pre-study	1/26/99–2/21/99	2/25/99
Study Inspections - Inlife	2/22/99; 3/22–25/99; 5/13, 19-21/99	2/22; 3/25/99 5/21/99
Necropsy and Endpoint Measurements	5/26–28/99; 6/2–3/99; 6/8–11/99, 6/29/99	5/28/99 6/29/99
Histopathology/Pathology	9/1/99	9/1/99
Data Audits	9/7, 20; 10/11/99	10/11/99
Data Audit/Final Report	12/99; 1/00; 5/00	5/12/00
Final Report Review	5/18–22/00	5/22/00

The Quality Assurance Unit has reviewed this report and has determined that the report accurately reflects the raw data.

Dorothy L. Harris, MS, CRM, RQAP-GLP
Quality Assurance Manager

Date

TEST MATERIALS CHARACTERIZATION/STABILITY

The test substance was generated from test fuel supplied by the sponsor and is described in Section II, “Materials and Methods,” in this report. The development, characterization, and stability testing of the fuel were the responsibility of the sponsor.

ARCHIVAL STORAGE

LRRI will store all records and specimens resulting from this study for at least 10 years following completion of the study. After 10 years, the sponsor will be contacted to determine final disposition of all study materials.

SUMMARY

The Lovelace Respiratory Research Institute (LRRI) performed a 13-week subchronic inhalation study in F344 rats of the potential toxicity of biodiesel exhaust emissions. The study was designed to fulfill the requirements of Environmental Protection Agency (EPA)-mandated Tier 2 health effects testing. Groups of rats were exposed to diluted biodiesel exhaust emissions at targeted NO_x concentrations of 5, 25, or 50 ppm (low, intermediate, and high levels, respectively), while other rats served as air-exposed controls. Actual exposure concentrations achieved were within acceptable ranges. No effects of biodiesel-exhaust-emission exposure were observed in a variety of endpoints including mortality, toxicity as revealed by detailed clinical observations, feed consumption, toxicity to the eyes, neurohistopathology, formation of micronuclei (MN) in bone marrow cells, sister chromatid exchanges (SCEs), fertility, reproductive toxicity, and teratology. Endpoints in which effects were caused by biodiesel-exhaust-emission exposure, with minor changes not deemed as biologically significant, included group mean body weights, non-pulmonary organ weights at necropsy, clinical chemistry, and glial fibrillary acidic protein (GFAP) in the brain. Weak mutagenicity in a bacterial mutagenicity assay was observed from extracts of both particulate and semi-volatile fractions of biodiesel-exhaust-emission fractions. Relative to total body weights, lung weights were increased in female rats in the high-level group compared to controls (0.52 vs. 0.49% of total body weight), and histopathological evaluation of a number of tissues revealed exposure-induced changes only in the lungs. Findings included the presence of particles in macrophages and macrophage hyperplasia; these findings were judged to be a normal physiologic response to exposure and not a toxic reaction. Lesions included alveolar bronchiolarization, which was found only in rats in the high-level group, and alveolar histiocytosis, which was found in three of the four groups, but at slightly higher incidence in the high-level group. Based on these results, rats were adversely affected by exposure to high-level biodiesel exhaust emissions, the effect was greater in female rats than in males, and the no-adverse-effect-level for this study of inhaled biodiesel exhaust emissions was the intermediate level.

I. INTRODUCTION

This report describes a 13-week (90-day) subchronic test period of rats exposed whole-body to biodiesel exhaust emissions. The study was designed to fulfill the requirements of U.S. EPA-mandated Tier 2 health effects testing.

II. MATERIALS AND METHODS

A. Study Dates

Study Initiation:	January 22, 1999
Experimental Start:	February 22, 1999
Experimental Termination:	June 30, 1999

B. Protocol and Experimental Design

The study protocol, amendments, and deviation memoranda, as well as laboratory certifications and CVs of contributing LRRI personnel, are presented in Appendix A. The experimental design of the study, including number of animals per group and number of animals assigned to each endpoint, are shown in Tables 1 and 2.

C. Test Fuel Characterization

Two lots of biodiesel fuel were used during this project. One lot, A6-17-6, was used for the engine break-in phase of this study. The second lot, B12-11-6, was used for the animal exposures and to sample exhaust emissions for extraction in order to perform the mutagenicity testing. At protocol-specified times, samples of the fuel were taken and shipped to Williams Laboratory Services, Kansas City, KS, for analysis (see Appendix B).

D. Exposure Generation System

Diesel exhaust was generated using two 1998 Cummins engines operated following the EPA Heavy-Duty Engine Dynamometer Schedule (EDS) and burning the biodiesel fuel described above. A similar system and the approaches used are described in Mokler *et al.* (1984). The exhaust was diluted and delivered to the animal exposure chambers via a dilution/delivery system, and the dilution to individual exposure chambers was adjusted to match the target total oxides of nitrogen (NO_x) concentration. The NO_x was measured frequently during each exposure day using an NO_x analyzer. Other gas constituents were also measured daily. A complete description of the engine system, dilution/delivery system, sampling system, and characterization are detailed in Appendix C.

Table 1. Experimental design for Tier 2 study.

	Males					Females					
Group[a]	General Histo[b,c]	Special Histo[d]	GFAP, MN, SCE[b,e]	Recovery[f]	Total	General Histo[b,c]	Special Histo[d]	GFAP, MN, SCE[b,e]	Recovery[f]	Fertility, Repro Tox, Teratology[g]	Total
Control[h]	15	5	5		25	15	5	5		25	50
Low	15	5	5		25	15	5	5		25	50
Intermediate	15	5	5		25	15	5	5		25	50
High[h]	15	5	5	10	35	15	5	5	10	25	60
Acrylamide[i]		5			5		5				5
DMBA[j]			5		5			5			5
TOTAL					120						220

[a]Group denotes filtered air controls, low, intermediate or high concentration level, or positive control treatments.
[b]Blood collection for hematology and clinical chemistry before study start, at 30 days of exposure, and at study termination; all groups.
[c]General Histology Group: gross pathology; histopathology on all lesions, all lungs. The organ list given in Table 10 was examined in high-level and control groups, also tissues examined in intermediate- and low-level groups if findings in high-level group.
[d]Special Histology Group: whole-body *in situ* perfusion fixation; brain and peripheral nerve examined in high-level and control rats, also in intermediate and low levels if findings in high-level group; also lungs, reproductive organs; particle distribution analysis; general histopathological examination also as described in footnote (c).
[e]GFAP = glial fibrillary acidic protein assay. MN = micronucleus assay on bone marrow cells. SCE = sister chromatid exchange assay on peripheral blood lymphocytes.
[f]Recovery Group: recovery group rats exposed at the high level and sacrificed 28 days after cessation of exposures for general histology.
[g]Fertility/repro tox/teratology Group = female rats for fertility, reproductive toxicity, and teratology testing.
[h]Ophthalmology examination at study start and termination for control and high-level rats. Also done in intermediate and low concentration groups if effects are found in the high-level group.
[i]Repeated acrylamide treatment to provide positive controls for neurotoxicity assessment in special histology group rats.
[j]DMBA (dimethyl benzanthracene) treatment to provide positive controls for MN and SCE endpoints.

Table 2. Animal numbers and assignments for Tier 2 study.

Block A: General Histology and Recovery Groups; Fertility/Reproductive Toxicity Group Females		
Exposure	**Males[a]**	**Females**
Control	6278 A001-A015	6279 A016-A030 (Gen. Histo) 6279 A031-A055 (Fertility)
Low	6280 B001-B015	6281 B016-B030 (Gen. Histo) 6281 B031-B055 (Fertility)
Intermediate	6282 C001-C015	6283 C016-C030 (Gen. Histo) 6283 C031-C055 (Fertility)
High	6284 D001-D015 (Gen. Histo) 6284 D016-D025 (Recovery)	6285 D026-D040 (Gen. Histo) 6285 D041-D065 (Fertility) 6285 D066-D075 (Recovery)
Block B: Glial Fibrillary Acidic Protein, Micronucleus, and Sister Chromatid Exchange Group		
Exposure	**Males[a]**	**Females**
Control	6286 E001-E005	6287 E006-E010
Low	6288 F001-F005	6289 F006-F010
Intermediate	6290 G001-G005	6291 G006-G010
High	6292 H001-H005	6293 H006-H010
DMBA	6302 I001-I005	6303 I006-I010
Block C: Special Histology Group		
Exposure	**Males[a]**	**Females**
Control	6294 J001-J005	6295 J006-J010
Low	6296 K001-K005	6297 K006-K010
Intermediate	6298 L001-L005	6299 L006-L010
High	6300 M001-M005	6301 M006-M010
Acrylamide	6304 N001-N005	6305 N006-N010

[a]Male rats from Blocks A, B, and C used for breeding with Block A female rats.

E. Animals and Animal Husbandry

1. *Description*

This study was performed using CDF®(F344)/CrlBR male and female rats obtained from Charles River Laboratories (Raleigh, NC). Rats were approximately 5 to 7 weeks of age upon receipt. Animals were quarantined and acclimated to Hazelton H2000 whole-body exposure chambers for a minimum of 2 weeks before exposures began.

2. *Method of Identification*

During the pre-study phase, rats were identified by basket location. Approximately 7 days before exposures began, rats were weighed individually, then randomly assigned to exposure groups using the Path-Tox computer system (Xybion Medical Systems Corp., Cedar Knolls, NJ). After group assignments were made, rats were identified by tail tattoos.

3. *Housing*

Rats were housed in single cage units within H2000 whole-body exposure chambers throughout the study with three exceptions: 1) for the fertility/reproductive toxicity portion of the study, during mating periods, male and female pairs were housed overnight (i.e., during nonexposure hours) in polycarbonate shoebox-type cages with hardwood bedding; 2) for the MN, SCE, and neurotoxicity portions of the study, acrylamide and dimethyl benzanthracene (DMBA)-treated positive controls were housed singly in polycarbonate shoebox-type cages; and 3) during the 28-day post-exposure recovery period for the high-level animals in Block A, rats were housed in polycarbonate shoebox-type cages.

4. *Environment*

Rats were housed in either H2000 whole-body chambers or in polycarbonate shoebox-type cages located either in the exposure room or in a separate rodent housing building. Environmental conditions monitored in chambers included temperature, relative humidity, chamber flow, chamber pressure, and chamber oxygen percentage. Room temperatures were monitored in the exposure room. Room temperatures, relative humidity, and air changes were monitored in the separate rodent housing room (see Appendix D).

5. *Food*

Rats were fed Teklad Certified Rodent Diet (W) (Harlan Teklad, Madison, WI). Feed was available at all times except during exposures. Feed analysis is shown in Appendix E.

6. *Water*

Water was supplied via water lines and individual limit valves within each cage unit in the whole-body exposure chambers or in individual polycarbonate shoebox-type cages. Water was available at all times. Water purity analysis is shown in Appendix E.

F. Animal Exposures

Exposures were conducted for 6 hours per day, 5 days per week, with the exception that 7-day-per-week exposures were conducted when pregnant females were in the chambers. Exposures were conducted from February 22, 1999 through June 10, 1999. The start of exposure was staggered by blocks (A, B, and C). Fertility/reproductive toxicity group female animals were exposed for 60 to 72 days. Otherwise, all animals were exposed from 73 to 75 exposure days, and no scheduled exposure days were missed.

Exposure data are shown in Appendix F. The primary analyte for exposures was NO_x. Additional analytes included total particulate matter concentration, hydrocarbon vapors, and the gases CO, CO_2, and SO_2. For NO_x, both NO and NO_x, were measured, and the NO_2 concentration was calculated by difference.

G. Body Weights

All rats were weighed twice during the pre-study phase, twice weekly throughout the study after daily exposures ended, with the exception of the pregnant females, and at necropsy. With the exception of the terminal body weight at necropsy, body weights were collected in conjunction with detailed clinical observations using version 4.2.2 of the Xybion Path-Tox system (see Appendix G). Balance calibrations were documented during each body-weight collection session.

The Path-Tox system was used for a statistical analysis of possible exposure-related effects on body weight. For each body-weight acquisition session, summary by dose-group output tables were prepared with an analysis of variance (ANOVA) to examine data homogeneity (using Bartlett's test) and significance (using Dunnett's test with indications of group differences at $p \leq 0.05$ and at $p \leq 0.01$).

H. Mortality and Clinical Observations

All rats were visually inspected at least twice daily. Any animal with questionable health status was examined by the ACLAM Veterinarian and/or the Study Director. Decisions regarding euthanasia of moribund rats were made by the Study Director in consultation with the ACLAM Veterinarian. Any abnormal clinical signs were entered into the room log.

Detailed clinical observations were recorded twice weekly in conjunction with the body-weight collection sessions using the Path-Tox system (Appendix H).

I. Feed Consumption

Consumption of feed was measured throughout the course of the study (see Appendix I). As noted elsewhere, feeder troughs were removed from the whole-body exposure chambers during exposure hours, then returned to the chambers overnight. When the feeder troughs were returned to the chambers during the afternoon chamber service, the feeders were filled and weighed beforehand. The following morning before exposures, the feeder troughs were removed from the chambers and weighed. The difference between these weights (the morning feeder trough weight subtracted from the filled afternoon feeder trough weight) was taken as the quantity of feed consumed during that night.

As noted earlier, rats were weighed twice per week. Weighings were performed in the afternoons after exposure hours.

For the feed consumption analysis, one basket per chamber was selected for analysis. The basket selected was one in which male and female rats from the Block A fertility group were not included (to avoid the issue that these rats were removed for breeding overnight during a portion of the study). Both male and female rats were included in each exposure group. For the feed consumption analysis, the total weight of feed removed from the trough (i.e., the following morning's net weight) was divided by the weight of rats within the basket from that afternoon's weighing session to give grams of feed consumed per gram of rat. This was calculated for each weighing session for each group throughout the study (i.e., twice per week). The number of rats included throughout the majority of this analysis period was 17, 7, 7, and 20 for the control, low-level, intermediate-level, and high-level groups, respectively.

Mean values were calculated for each group over the course of the study, and potential differences between groups were examined using an ANOVA (Excel 97).

J. Ophthalmologic Evaluation

A veterinary ophthalmologist examined the eyes of all rats in Block A at the beginning of the study, and of the control and high-level rats at the end of the Block A exposures. The eyes were examined after full pupil dilation with 1% tropicamide by biomicroscopy and indirect ophthalmoscopy (see Appendix J).

K. Positive Control Study of Neuropathologic Lesions in Acrylamide-Treated Rats

1. *Procedures*

Five male and five female F344 rats were treated once daily with 50 mg acrylamide/kg body weight by intraperitoneal injection. Animals were weighed daily to establish the dose. The animals were injected (1 ml syringes with 26½ gauge needles; Becton Dickinson, Franklin Lakes, NJ) with freshly prepared acrylamide (Mallinckrodt Baker, Inc., Paris, KY) in physiologic saline (Butler Co., Columbus, OH) at approximately the same time of day of each treatment. The animals were observed twice daily for signs of toxicity. All rats developed marked weight loss and varying severity of hind-limb paresis and ataxia. The acrylamide dose was reduced to 30 mg/kg, then discontinued. During the treatment period, two male and two female rats died. Tissues from these animals were collected within 12–15 hours after death and fixed by immersion in 10% neutral-buffered formalin (NBF). The remaining rats were killed on the tenth day. Tissues from these animals were fixed by whole-body perfusion of 4% paraformaldehyde. Nervous tissues were harvested, trimmed, and transferred to 10% NBF the following day. The left tibial nerve from each animal (both those that died and those that were killed) was collected, placed in 10% NBF, processed, and teased free for microscopic examination.

2. *Results*

Neuropathologic lesions were found in the cerebellum of eight of the 10 animals (Table 3). Lesions varied in severity and distribution and consisted of granular layer cell necrosis and Purkinje cell necrosis in six of the eight affected, and perineuronal vacuolation. Individually necrotic Purkinje cells, when present, were few in number (<10 per section), widely scattered, and characterized by cell shrinkage, nuclear pyknosis or karyolysis, and hypereosinophilia. Necrotic granular layer cells were characterized by nuclear pyknosis and occasionally by karyorrhexis and were sometimes associated with necrotic Purkinje cells. In

more severely affected animals, multifocal and locally extensive regions of cerebellar folia contained numerous necrotic granular layer cells. In minimally affected animals, the necrotic granular layer cells were individualized and scattered in a few foci. Perineuronal vacuolation was noted in scattered foci of the Purkinje cell and granular layers and was sometimes associated with necrotic cells. Review of the teased tibial nerve preparations and sections of paraffin-embedded sciatic nerves and spinal cords from all 10 animals revealed no significant lesions.

Table 3. Cerebellar lesions found in acrylamide-treated rats.

		Cerebellar Lesions[a]	
Rat No.	Sex	Purkinje Cell Necrosis	Granular Layer Cell Necrosis
N001-6304	M	+	+3 to +4
N002-6304	M[b]	(–)	+2
N003-6304	M[b]	(–)	(–)
N004-6304	M	(–)	+
N005-6304	M	+	(–)
N006-6305	F	+	+2
N007-6305	F[b]	+	+2 to +3
N008-6305	F	+	+
N009-6305	F	+	(–)
N010-6305	F[b]	(–)	(–)

[a]Lesion severity: (–), absent; +, minimal; +2, mild; +3, moderate; +4, marked.
[b]Animal died prior to scheduled sacrifice.

3. *Conclusions*

The cerebellar lesions are consistent with previous reports of acrylamide-induced neuropathologic lesions where both granular layer cell degeneration (Abou-Donia *et al.*, 1993) and Purkinje cell necrosis (Abou-Donia *et al.*, 1993; Cavanagh and Nolan, 1982; Jortner and Ehrich, 1993) have been described. These results adequately fulfill the test regulation requirement (40 CFR, Part 79.66(e)(3)) that neuropathologic lesions be detected following the use of a positive-control substance. The lack of lesions in the peripheral nerves, the spinal cord, and the brainstem of clinically affected rats is consistent with previous reports. Clinical signs of ataxia and hind-limb paresis may occur in acrylamide-treated rats without significant axonal

degeneration being noted histologically (Abou-Donia *et al.*, 1993; Cavanagh and Nolan, 1982; Jortner and Ehrich, 1993; Lehning *et al.*, 1998).

L. General Histology Group

Necropsies were performed, gross observations made, and tissues/organs weighed as stated in the protocol. The histologic sections were examined as stated in the protocol. The tissue sections from all organs were examined from the control and the high-level concentrations for the General and Special Histology Groups. Since no compound-related lesions were found outside the lung, only lung tissue sections were examined for the low and intermediate groups.

After the control and high-level groups were examined, criteria were set for scoring the severity of the major lung lesions (alveolar macrophage [AM] hyperplasia, dust-laden AMs, and alveolar bronchiolarization) as well as the other lung lesions observed (chronic inflammation, centriacinar fibrosis, and alveolar histiocytosis) (Table 4). The severity of the lung lesions in all animals was scored in a blind fashion. The identity of the animal was unknown to the pathologist until after all slides were scored.

M. Special Histology Group

The rats in this group were anesthetized with an overdose of pentobarbital, their hearts cannulated, and tissues fixed by whole-body perfusion with 4% paraformaldehyde. The protocol-required tissues were embedded in paraffin and stained with hematoxylin and eosin. This procedure included obtaining weights of brains and special dissection of brains, spinal cords, and sciatic nerves. Nervous tissues from the high-level and control animals were evaluated by light microscopy. Tibial nerves from animals of all exposure groups were dissected free, and those from the high-level and control groups were processed, teased, mounted in plastic on slides, and evaluated by light microscopy. The lungs and reproductive tracts (testes, epididymes, prostates, and seminal vesicles or ovaries) were examined on all these animals.

N. Clinical Pathology

Clinical pathology endpoints were analyzed for 20 males and 20 females per exposure concentration. Animals came from the General Histology and GFAP/MN/SCE Groups. The analyses were performed three times: before exposures began, after 30 days on study, and at the end of exposure.

Table 4. Criteria for severity grading of lung lesions.

Diagnosis	Severity	Criteria
Dust-laden alveolar macrophages	NAD[a]	Essentially no particles in scant cytoplasm
	Minimal	A few black particles scattered in cytoplasm
	Mild	Increase in number of particles in cytoplasm (≤10); particles do not obscure nucleus of macrophage
	Moderate	Many particles (too many to count) in cytoplasm cover the nucleus; slightly enlarged cytoplasm
Alveolar macrophage hyperplasia	NAD	Few scattered AM in alveoli; difficult to find
	Minimal	Minimal increase in number of AM
	Mild	Mild increase in number of AM; easily found at high magnification; average 1/alveolus
Alveolar bronchiolarization	NAD	Normal epithelium lining, alveolar ducts and alveoli adjacent to terminal bronchioles
	Minimal	Minimal, proliferation of ciliated and Clara cells in alveolar ducts to produce alternating segments of newly lined alveolar duct walls and alveoli; adjacent alveoli may also be lined; a few first branch alveolar ducts involved
Chronic inflammation	NAD	Essentially no neutrophils and/or lymphocytes, monocytes and plasma cells present
	Minimal	A few scattered foci of neutrophils and/or lymphocytes, monocytes and plasma cells
Fibrosis, centriacinar	NAD	No evidence of accumulation of collagen
	Minimal	Minimal accumulation of collagen in the interstitium within the walls of terminal bronchioles, proximal alveolar ducts and/or associated alveoli
Alveolar histiocytosis	NAD	No evidence of AM aggregates
	Minimal	Minimal aggregates of AMs associated with a reaction in the alveolar septa

[a]NAD = No abnormalities detected.

Blood samples were obtained from each rat by retro-orbital bleeding at the pre-exposure sampling and the 30-day sampling. At the end of study, cardiac puncture at terminal sacrifice was used, as noted in Protocol Amendment 1 (Appendix A). The blood collection technique was changed to obtain larger samples in a cleaner fashion (no epithelial cell plugs and less chance of micro-fibrin clots) than could be obtained using the retro-orbital bleeding

technique. At earlier bleeding times, parameter values were more variable than desired. Such variability can occur with orbital bleeding, but there is no good alternative for bleeding a live rat (Neptun *et al.,* 1985). At necropsy, cardiac puncture is the preferred option.

Blood samples for clinical chemistry (1.5 ml) were collected into microtube serum separator tubes for centrifugation and separation of cell and serum fractions. The clinical chemistry analyses were performed with a Monarch 2000 with ion-specific electrodes (Instrumentation Laboratories, Lexington, MA). The specific tests and the method used are listed in Table 5. In some cases, not all analyses could be run because the sample volume was insufficient. Bile acids were not completed on many animals because of this factor.

Blood samples for hematology (1 ml) were collected into microtubes containing ethylenediaminetetraacetic acid. Hematology analyses were performed with a Baker 9110 Plus hematology analyzer (BioChem ImmunoSystems, Allentown, PA). Differential cell counts were performed manually. Methemoglobin was determined with an IL 682 CO-Oximeter (Instrumentation Laboratories, Lexington, MA). The specific analyses are listed in Table 6.

At one of the 30-day samplings, March 25, 1999, malfunction of the valving on the hematology analyzer required that the hematology samples be sent to an outside laboratory. They were sent to Ani Lytics, Inc., Gaithersburg, MD, a Good Laboratory Practices laboratory. Differential cell counts and methemoglobin were analyzed at LRRI.

1. *Statistical Analysis*

A repeated-measures ANOVA (SAS, SAS Institute, Cary, NC) was used to determine whether differences existed in the treatment groups. Of particular interest were the contrasts between the 30-day measurements and the baseline measurements, and the 13-week measurements and the baseline measurements. For each contrast, two main effects and one interaction effect were examined: a gender main effect, a dose main effect, and a gender × dose interaction effect. The effects of interest were the dose main effect and the gender × dose interaction effect. If the gender × dose interaction effect was found to be significant, an ANOVA was run with "difference from baseline" as the response and gender, dose and gender × dose interaction as the factors. If the dose effect was found to be significant (but not the gender × dose interaction), an ANOVA with "difference from baseline" was the response, and dose as the factor was run. For each individual ANOVA, least squares means were computed and used to make multiple comparisons among the four groups (control, low, intermediate, and high).

Table 5. Clinical chemistry analytical methods.

Test	Method
Albumin	Bromcresol green
Albumin/Globulin ratio	Calculated
Alanine aminotransferase	Wroblewski & LaDue, modified by Henry
Alkaline phosphatase	Bowers & McComb, optimized by Tietz
Aspartate aminotransferase	Karmen, modified by Henry
Bile acids	Enzymatic – Mashige
Total bilirubin	Jendrassik-Grof
Blood urea nitrogen	Urease
Blood urea nitrogen/Creatinine ratio	Calculated
Calcium	Bichromatic analysis – Substrate test
Chloride	Ion-specific electrode
Cholesterol	Bichromatic analysis – Allain
Creatinine	Substrate test – kinetic fixed time analysis
Creatine kinase	Oliver, modified by Rosalki
Gamma glutamyl transferase	International Federation of Clinical Chemistry Method
Glucose	Bichromatic analysis – Substrate test
Inorganic phosphorous	Bichromatic analysis – Substrate test
LDH (Lactate dehydrogenase)	Gay, McComb, Bowers
Potassium	Ion-specific electrode
Sodium	Ion-specific electrode
Sorbitol dehydrogenase	Asada & Galambos; also Wiesner
Total globulin	Calculated
Total protein	Modified Biuret
Triglycerides	Esders & Goodhue (modification)

Table 6. Hematology tests.

Test	Method
Red blood cell count	Baker 9100+ hematology analyzer
Hemoglobin	Baker 9100+ hematology analyzer
Hematocrit	Baker 9100+ hematology analyzer
Platelet count	Baker 9100+ hematology analyzer
White blood cell count	Baker 9100+ hematology analyzer
Differential white blood cell count	Manual
Methemoglobin	IL CO-Oximeter

O. Glial Fibrillary Acidic Protein Assay

In Block B animals of the study, GFAP was assayed on brain tissue at 13 weeks at terminal sacrifice. Five males and five females per group (controls, low-, intermediate-, and high-level of biodiesel exhaust) were analyzed.

Analyses were run in conjunction with a standard that had known quantities of GFAP and protein. Animals were sacrificed and their brains removed and weighed. Whole brain samples were placed in individually labeled vials over dry ice until processing. Processing included mechanical homogenization in hot 1% sodium dodecyl sulfate, then dilution in phosphate-buffered saline with 0.5% Triton X-100. Assays were performed using an enzyme-linked immunosorbent assay with optical density read on a plate reader and data recorded by computer.

Data were summarized by animal for GFAP, protein, and GFAP normalized by protein. Data from the groups were analyzed for normality with the Kolmogorov-Smirnov test and equal variance with Levene's median test (SigmaStat for Windows; Jandel Scientific; Corte Madera, CA). An ANOVA with exposure group and gender as independent variables was run on the dataset to determine whether groups differed significantly. Where significant differences were indicated ($p \leq 0.05$), the Student-Neuman-Keuls (SNK) pair-wise comparison test was used to isolate which group significantly differed from the others. Level of significance of difference was set at $p \leq 0.05$.

P. Micronucleus Assay

The MN test detects damage of the chromosome or mitotic apparatus of cells. Cells from the bone marrow, polychromatic erythrocytes (PCEs), are examined to visualize the MN, which may form under normal conditions. The assay is based on an increase in the frequency of micronucleated PCEs in the bone marrow of treated rats. Rats from Block B were designated for analysis of ratio of PCEs to normochromatic erythrocytes (NCEs) and for percentage of micronucleated PCEs at the post-exposure terminal sacrifice. Rats were sacrificed and femurs obtained. Femur ends were removed, then femurs were flushed with fetal bovine serum to collect bone marrow cells, and smears were prepared on glass slides. Immediately before examination, slides were stained with acridine orange (125 μg/ml) in Dulbecco's phosphate buffered saline. Slides were coverslipped, then examined using fluorescence light microscopy using a blue excitation filter, a chromatic splitter, and a barrier filter #50 to achieve a

green-yellow color of nuclear material. Slides were examined in a blinded fashion so that the identity and exposure group of the rats were not known to the microscopist. To determine the ratio of PCE/NCE, 200 erythrocytes were examined, classified as either PCEs or NCEs, then expressed as the ratio PCE/NCE. To determine the percentage of MN, 1,000 PCEs were examined for the presence of MN and the percentage calculated as (MN/1000) × 100.

Data were summarized by animal for the ratio of PCE/NCE and the percentage of PCE with MN. These data were analyzed for normality of distribution with the Kolmogorov-Smirnov test and equal variance with Levene's median test. An ANOVA with exposure group and gender as independent variables was run on the dataset to determine whether groups differed significantly. If gender or interaction between gender and exposure group in the ANOVA caused no effect, genders were pooled. Where significant differences were indicated ($p \leq 0.05$), the SNK pair-wise multiple comparison test was used to isolate which group significantly differed from the others. The level of significance of difference was set at $p \leq 0.05$.

Q. Sister Chromatid Exchange Assay

Rats from Block B were designated for analysis of SCE. Groups studied were controls, low-, intermediate-, or high-level biodiesel exhaust emission-exposed, and positive (DMBA-exposed) controls. Specific endpoints evaluated were percentage of metaphase cells observed, replicative index (equal to [(% 1^{st} division) + (2 × % 2^{nd} division) + (3 × % 3^{rd} division) metaphase cells] / 100), and number of SCEs per second-division metaphase cells.

Rats were sacrificed, and blood was obtained. Peripheral blood lymphocytes were isolated using a density gradient centrifugation with histopaque. Cells were washed in phosphate-buffered saline, then in a serum- and phytohemagglutinin-supplemented RPMI culture medium (complete medium), and plated at a concentration of 1×10^6 cells per well. Cells were incubated until the following day at which point the medium was replaced with complete medium containing bromodeoxyuridine but not phytohemagglutinin. Cells were incubated for an additional 24 hours at which point colchicine was added (0.25 µg/ml) for 4 hours. Cells were centrifuged onto frosted-end glass slides, given a 2-minute hypotonic shock, then fixed with 3:1 methanol:acetic acid for 10 minutes.

Cells were then stained in a fluorescence plus Giemsa stain after slides were exposed to light from a heat lamp for 2–3 hours and incubated at approximately 65ºC in buffer. Slides were evaluated for the number of metaphase cells present in 1,000 lymphocytes; the

number of first-, second-, and third-division metaphase cells in 100 total metaphase cells; and the number of SCEs in 25 second-division metaphase cells.

Data were summarized by animal for 1) the number of metaphase cells for 1,000 lymphocytes examined per animal, then a percentage calculated; 2) the replicative index by animal; and 3) the number of SCEs examined for up to 25 second-division cells per animal. A one-way ANOVA with exposure group as an independent variable was run on the dataset to determine whether groups differed significantly. Gender was not analyzed separately because in many cases there were fewer than 10 second-division metaphase cells per preparation, and gender was pooled to keep the group size sufficiently large (n = 3 to 5) to permit a statistical analysis. Where marginal differences ($0.10 \geq p > 0.05$) or significant differences ($p \leq 0.05$) were found in the ANOVA, an F-test was used to determine whether equality of variance could be assumed between data sets, then a t-test was used to perform multiple pair-wise comparisons. All statistical analyses were performed using Microsoft Excel (Excel 97), and the level of significance of difference for the pair-wise comparisons was set at $p \leq 0.05$.

R. Individual Animal Fertility

The stage of the estrous cycle of female rats within Block A of the study was used to determine the capability of the rats to become pregnant. Estrous cycle stage was determined before the rats were assigned to study groups, to cull unsuitable animals, and before mating began, to confirm that the rats were cycling properly.

To determine the stage of the estrous cycle, a vaginal lavage was performed using saline, then recovered fluid was placed onto a glass microscope slide, stained, and examined using light microscopy. The number and types of cells present were used to categorize the rats as being in the diestrous, proestrous, estrous, or metestrous stage of their cycle. During mating, the vaginal lavage specimen was examined for the presence of sperm as evidence of mating.

1. *Pre-study*

Beginning at approximately 8 weeks of age, female rats in Block A of the study received daily vaginal lavages for cytologic determination of the stage of the estrous cycle. Vaginal lavages were performed for 2 weeks before rats were assigned to study groups.

2. *Before and During Mating*

Daily vaginal lavages for cytologic determination of the stage of the estrous cycle were resumed for the female rats assigned to the fertility/reproductive toxicity portion of the study for 15 days prior to the beginning of mating. Mating was begun immediately following this 15th estrous cycle measurement. For mating, pairs of male and female rats were removed from exposure chambers following that day's exposure and placed into separate mating cages. The following morning, males were returned to the appropriate exposure chamber, then the females received a vaginal lavage and were returned to their exposure chamber. Once mating was confirmed, pairing of that pair of rats was discontinued.

For each female rat, gestational day zero was defined as the day that sperm was observed in that animal's vaginal lavage specimen. That animal was exposed through gestational day 15 and was then sacrificed followed by necropsy at gestational day 20.

S. Reproductive Toxicology and Teratology

This endpoint was evaluated by the subcontractor Pathology Associates International, Frederick, MD. Materials and methods used are described in their final report entitled "Teratology Report for Tier 2 Testing of Biodiesel Exhaust Emissions, LRRI Study Number FY98-056" included as Appendix S of this report.

T. *Salmonella Typhimurium* Reverse Mutation Assay

Biodiesel exhaust emissions were sampled over the course of 5 days, 6 hours per day, of engine operation as described in Appendix C. Samples were collected using a 20×20 inch filter assembly (to capture particulates) backed up with a polyurethane foam (PUF)/XAD-4 resin/PUF sandwich encased within glass sampling cylinders provided by Desert Research Institute (DRI). A separate filter and PUF/XAD-4/PUF cylinder were used for each of the 5 days of operation. Following collection, filters and glass sampling cylinders were placed into a low-temperature freezer (at approximately –80ºC). Filters and cylinders were shipped to DRI for separate extraction in dichloromethane. All five filters were extracted together to constitute the particulate soluble organic fraction (PSOF), and all five PUF/XAD-4/PUF samples were extracted together to constitute the semi-volatile extracted fraction (SVEF). Following extraction, the dichloromethane was gently evaporated, and the resulting PSOF and SVEF extracts were resuspended in dimethyl sulfoxide. Materials and methods are described in

Appendix T. Samples were then shipped to the subcontractor Chrysalis Preclinical Services, Olyphant, PA.

The mutation endpoint was evaluated by Chrysalis Preclinical Services. Materials and methods used are described in their protocols and in their final report given in Appendix T of this report.

III. RESULTS AND DISCUSSION

A. Test Fuel Characterization

Williams Laboratory Services, Kansas City, KS, provided copies of analyses of biodiesel fuel to LRRI (included as Appendix B of this report). Analyzed parameters were within the biodiesel specification, ASTM PS121, and within the acceptable limits as defined by the protocol used in this study.

B. Animals and Animal Husbandry

Animals were received in generally good health. In a few instances noted below, it was necessary to cull a few rats before assignment to study groups (e.g., rats with ocular or estrous cycle abnormalities). Mortality, body weights, and clinical observations are described in separate sections below. Results of feed and water analysis are given in Appendix E. No contaminants were detected in food or water at levels that would be expected to affect the results of the study.

Sentinel rats were evaluated serologically near the beginning and end of the study. Serum samples were evaluated for common rodent pathogens, and none tested positive (Appendix E; BioReliance, formerly Microbiological Associates, Rockville, MD).

C. Animal Exposures

As noted in the Materials and Methods section, chamber uniformity was measured before the study began. In addition, the chamber T_{90} time measured 14 minutes.

1. *NO_x Measurements*

Results of daily NO_x measurements are given in Appendix F. The overall average NO_x concentrations were 5, 26, and 51 ppm for the three levels which were 100, 104, and 102%, respectively, of the desired low, intermediate, and high concentrations of 5, 25, and 50 ppm NO_x. The coefficients of variation for the low, intermediate, and high levels were 20, 12, and 10%, respectively. As per 40 CFR §79.57(e)(2)(vi)(B) requirements, the daily average concentrations were within 10% of the target values 98%, 92%, and 91% of the total exposure days for the low, intermediate, and high levels. The data from daily measurements in each chamber are summarized as the averages and standard deviations in Table 7. The variability in

the data shown in Table 7 reflects correction made to keep daily averages within the required range.

Table 7. Summary of NO_x concentration data (ppm).[a]

	Control	Low Level	Intermediate Level	High Level
NO_x	1 ± <1	5 ± 1	26 ± 3	51 ± 5
NO	NQ[b]	5 ± 1	25 ± 3	49 ± 4
NO_2	1 ± 4	1 ± <1	1 ± <1	2 ± 1

[a]Values are mean plus or minus one standard deviation.
[b]Analyzer reading was below value that could be quantified.

2. *Gas Analyte Measurements*

CO, CO_2, SO_2, and hydrocarbon average concentrations for the three levels are summarized in Table 8. SO_2 and hydrocarbon readings were not taken during the first 14 exposure days because the analyzers were not available. Daily values are detailed in Appendix F.

Table 8. Summary of particulate and gas analyte concentration data.[a]

		Control	Low Level	Intermediate Level	High Level
CO, ppm		0.5 ± 0.5	2.2 ± 1.2	15.2 ± 4.8	36.8 ± 10.2
CO_2, % vol.		0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.1
SO_2, ppm		0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
Hydrocarbon, ppm		0.1 ± 0.2	0.1 ± 0.2	0.3 ± 1.4	0.5 ± 1.0
O_2, %		20.6 ± 0.2	20.5 ± 0.1	20.0 ± 0.2	19.3 ± 0.4
Particulate, mg/m^3	A.	0.017 ± 0.022[b]	0.04 ± 0.03	0.2 ± 0.1	0.5 ± 0.1
	B.	0.017 ± 0.022	0.1 ± 0.2	0.5 ± 1.8	1.1 ± 4.3

[a]Values are mean plus or minus one standard deviation.
[b]A. is the particulate concentration excluding the days when the fuel filter was plugged. B. is the particulate concentration with all days indicated. See text for more explanation.

3. *Total Particulate (Mass) Concentration*

During the study, there were three episodes when the fuel filter of the diesel engine became plugged. When this occurred, the particle concentrations in the chamber became progressively higher until the fuel filter was replaced. This condition was present on 5 days during the study. Value A. in Table 8 presents the mean particulate concentration with the values from these 5 days excluded; Value B. presents the mean particulate concentration with all days included. The first incident was on April 27–29, 1999. When this incident occurred, it took 3 days to determine the problem, and the filter was changed after the third exposure day. The other two instances when the fuel filter became plugged were on June 1 and June 7, 1999, and the fuel filter was changed after exposures ended on the day it was detected. These incidents and an explanation of why these particulate excursions did not materially affect the results of the study are included in Appendix F.

4. *Aerosol Size Distribution*

Size distribution of the particulate material in each exposure chamber was measured twice during the study (Appendix F and Table 9) using a seven-stage Lovelace multi-jet cascade impactor (LMJ; Newton *et al.*, 1977)/parallel flow diffusion battery (PFDB) serial sampling train (Cheng *et al.*, 1984; Barr *et al.*, 1989). Petroleum diesel exhaust particulates have been reported to have a bimodal size distribution when sampled with this system from inhalation exposure chambers (Barr *et al.*, 1989; Mauderly *et al.*, 1994). When sampling from the biodiesel exhaust exposure chambers, the LMJ impactor was operated in tandem with the PFDB, and the size of aerosol collected by the impactor was reported as mass median aerodynamic diameter (MMAD). The aerosol collected by the PFDB was the fraction of the aerosol that passed through the impactor. Its diameter was reported as mass median diffusion diameter (MMDD). As both fractions of the aerosol are typically log normally distributed, the geometric standard deviation was also reported. In the column of Table 9 entitled “Combined,” the fraction from the PFDB was treated as if it were collected by the final filter normally used in the LMJ impactor, and the data were analyzed and reported as MMAD.

Table 9. Particle size distribution of biodiesel exhaust particulates measured at two times during the study.

		Large Fraction Cascade Impactor			Small Fraction Parallel Flow Diffusion Battery			Combined[d]
Sample Period	Exposure	Percent of Total	MMAD[a] (μm)	GSD[b]	Percent of Total	MMDD[c] (μm)	GSD	MMAD (μm)
1	Low level	24.5	3.25	2.81	75.5	0.04	5.8	0.14
2	Low level	41.4	6.66	2.49	58.6	0.03	2.7	0.58
1	Intermediate level	29.1	2.41	2.27	70.9	0.16	4.0	0.31
2	Intermediate level	25.3	1.62	1.50	74.7	0.09	1.8	0.40
1	High level	55.5	4.88	2.92	44.5	0.18	5.3	1.15
2	High level	19.1	2.69	3.21	80.9	0.06	2.8	0.06

[a]MMAD = Mass median aerodynamic diameter.
[b]GSD = Geometric standard deviation.
[c]MMDD = Mass median diffusion diameter.
[d]In this case the MMAD was calculated assuming that the fraction collected by the PFDB was collected by the filter normally used with the LMJ impactor.

In two cases (low level, sample 2 and high level, sample 1) the MMAD of the large fraction was substantially higher than expected, and the percentage in the small fraction was substantially less than expected based on previous experience with petroleum diesel emissions (Mauderly *et al.*, 1994). The same is true when these values are compared to the other values from the other sampling times and chambers in this study. The data and collection techniques for the two values were carefully checked and no reason to eliminate these two data points was found. The reason for the larger size is unknown, but it is possible that during the relatively long sampling times that were required, a few relatively large particles with high mass such as food or dander particles from the animals may have been sampled and resulted in the high MMAD and lower percentage in the small fraction.

In any case, all of the aerosols in the exposure chambers had a bimodal distribution with a substantial portion in the submicron particle size as has been reported previously for diesel exhaust from older petroleum-fueled diesel engines (Cheng *et al.*, 1984; Barr *et al.*, 1989; Mauderly *et al.*, 1994). The MMAD calculated for the two fractions combined shows that the overall aerodynamic size distribution of the aerosols present in the chamber was small.

5. *Environmental Conditions*

Appendix D summarizes the following parameters: chamber temperature, relative humidity, chamber pressure, chamber exhaust flow, chamber oxygen levels, exposure room temperature, room lighting intensity, rodent housing room temperature, humidity, and room air flow changes. All parameters were determined to be acceptable during the study.

6. *Engine Cycle Analysis*

Data on speed, torque, and brake horsepower were analyzed for 1 randomly selected exposure day per month. Data are given in Appendix C. Each of 18 cycles per day was analyzed per 40 CFR 79.57. The cycles met the required tolerances on all 90 cycles except cycles 12 and 13 on February 24, 1999 (for torque r^2), and for cycle 18 on June 2, 1999 (for torque r^2). Thus, 87 of 90 cycles (97%) were within tolerances.

D. Body Weights

Appendix G includes the following: 1) graphs of male and female body weights for all three study blocks, 2) summaries of mean and standard deviations in body weight for each weigh session for both males and females in all three study blocks, and 3) detailed summaries of group, number of rats, mean body weight, and standard deviation, with level of statistical significance shown, for all male and female rats in the main toxicity study, Block A animals.

All groups of rats generally gained weight throughout the course of the study, as expected for normal growing rats. For Block A, the block in which detailed statistical evaluations were conducted, two statistically significant differences between the high-level exposure group and controls were observed for female rats only. On day 29, high-level females weighed less than controls at a $p < 0.01$ level, and on day 68, high-level females weighed less than controls at a $p < 0.05$ level. These differences were judged biologically unmeaningful for two reasons: 1) although statistically significant, the differences were minor; and 2) at all other time points, all exposure groups had body weights similar to controls.

E. Mortality and Clinical Observations

1. *Mortality*

With the exception of three rats having unscheduled deaths (sacrificed as moribund), all rats survived until their scheduled sacrifice. Two rats (A022 control, sacrificed at day 4 on study - General Histology Group and C034 intermediate level, sacrificed at day 16 on

study - Fertility Group) were sacrificed following caging-related traumas. A third rat (A042 control, sacrificed at day 12 on study - Fertility Group) was sacrificed after marked weight loss of undetermined origin. Causes of death were not associated with exposure to biodiesel exhaust emission.

2. *Detailed Clinical Observations*

Summaries of the individual animal clinical observations are included in Appendix H. This listing includes the nature of the abnormal observation and the date(s) observed.

The majority of the rats had normal clinical observations throughout the course of the study. Many abnormalities observed, such as missing tail tips and injuries to the paws/toes, were attributed to mechanical injury as basket units were removed from and placed into the whole-body inhalation chambers. Other abnormalities included bulging eyes and/or eye opacities. They were attributed to ocular injury due to the retro-orbital bleeding process. Two rats had oral cavity/dental problems. These abnormal clinical signs, which were scattered across all exposure groups, were not attributed to biodiesel exhaust emissions exposure.

F. Feed Consumption

Data describing feed consumption are given in Appendix I. Data include the following: 1) total weight of rats in measured baskets for each body-weighing session, 2) total weight of feed consumed overnight after a weighing session for measured baskets, 3) summarized values of grams of feed consumed per gram of rat by group, and 4) the ANOVA analysis of potential group differences.

Group mean values for feed consumption ranged between 0.062 to 0.066 grams of feed consumed per gram of rat per night. There was no apparent trend from control to high levels, and the ANOVA indicated no significant differences attributable to exposure group ($p = 0.542$).

Thus, exposure to biodiesel exhaust emission did not affect the feed consumption of rats in this study.

G. Ophthalmologic Evaluation

At both time points examined, baseline and after exposures, all rats had bilateral corneal dystrophy. This condition has been reported for F344 rats (Losco and Troup, 1988). At the baseline examination, two rats were found to have additional abnormalities, and these

animals were culled from the study. At the post-exposure examination, seven of 29 control rats and five of 50 rats in the high-level group (see Appendix J) showed ocular changes compared with their baseline evaluations. Several of the changes appeared to be complications of the retro-orbital bleeding procedure used for obtaining blood samples for clinical pathology. No exposure-related detrimental effects attributable to exposure were observed.

H. General Histology Group

1. *Sacrifice at End of 13-Week Exposure*

Individual animal absolute organ weight, percent organ to body weight, and statistical evaluations of potential differences between exposed and control groups are given in Appendix K. The liver absolute weights in the high-level group were significantly lower that those of controls at the $p < 0.05$ level for males (10.7 versus 11.6 grams, respectively) and $p < 0.01$ level for females (5.5 versus 6.2 grams, respectively). Relative liver weights normalized by the body weight of each rat were lower in female rats in both the high- and intermediate-level groups than controls ($p < 0.01$; 2.8, 3.0, and 3.1 percent of total body weight, respectively). Relative lung weights in female rats in the high-level group were greater than in controls ($p < 0.05$; 0.52 versus 0.49 percent of total body weight, respectively). In males, relative testes weights were greater in the high-level group than controls ($p < 0.05$; 0.91 versus 0.86 percent of total body weight, respectively). As no lesions were observed in liver and testes, these slight weight differences in liver and testes probably had no biological significance and could not be related to exposure to biodiesel exhaust.

Table 10 shows all the organs examined at histology. Examination of the tissue sections from the protocol-required organs and tissue in the control and high-level groups revealed exposure-related lesions only in the lung. Subsequently, only lung tissues were examined from the intermediate- and low-exposure groups. Table 11 lists the incidence and severity of lung lesions after 13 weeks of exposure. (See Appendix L for individual animal results.)

Table 10. Organs examined at histology.

	Group			
	General Histology		Special Histology	
Exposure	Number of Rats[a]	Organs	Number of Rats[a]	Organs
Control	29[b]	All	10	All
Low	30	Lungs	10	Lungs + reproductive tract
Intermediate	30	Lungs	10	Lungs + reproductive tract
High	30	All	10	All

[a]Includes equal numbers of males and females.
[b]One female sacrificed moribund after 4 days exposure.

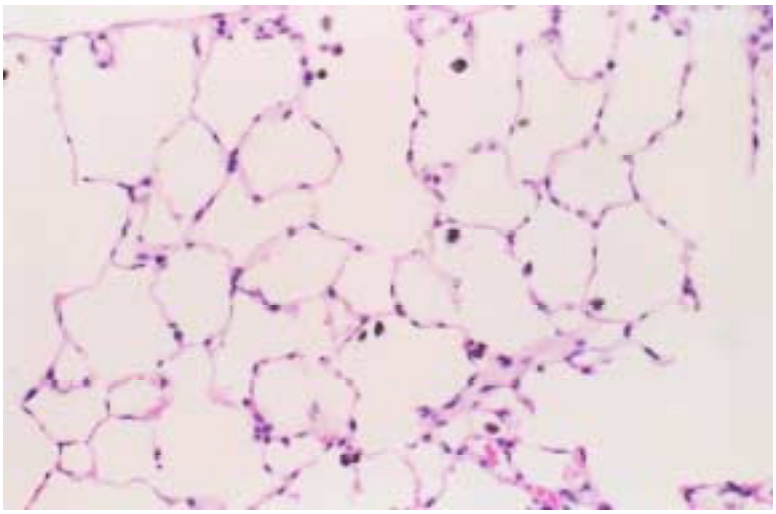
Table 11. Incidence and severity of lung lesions after 13 weeks of exposure.[a]

		Males				Females			
Diagnosis	Severity	Control	Low	Int.	High	Control	Low	Int.	High
Number examined:		20	20	20	20	19[b]	20	20	20
Dust-laden alveolar macrophages	NAD	20	5	–	–	17	5	–	–
	Minimal	–	14	3	–	2	14	4	–
	Mild	–	1	13	3	–	1	13	3
	Moderate	–	–	4	17	–	–	3	17
Alveolar macrophage hyperplasia	NAD	20	14	4	2	15	14	7	2
	Minimal	–	6	15	13	4	6	13	10
	Mild	–	–	1	5	–	–	–	8
Alveolar bronchiolarization	NAD	20	20	20	20	19	20	20	17
	Minimal	–	–	–	–	–	–	–	3
Chronic inflammation	NAD	19	20	20	20	19	20	20	20
	Minimal	1	–	–	–	–	–	–	–
Fibrosis, centriacinar	NAD	20	20	19	19	18	20	20	20
	Minimal	–	–	1	1	1	–	–	–
Alveolar histiocytosis	NAD	19	20	19	20	18	20	19	18
	Minimal	1	–	1	–	1	–	1	2

[a]Includes findings from General and Special Histology Groups.
[b]One female, A022, sacrificed moribund after 4 days exposure.
NAD = No abnormalities detected.
Int. = Intermediate.

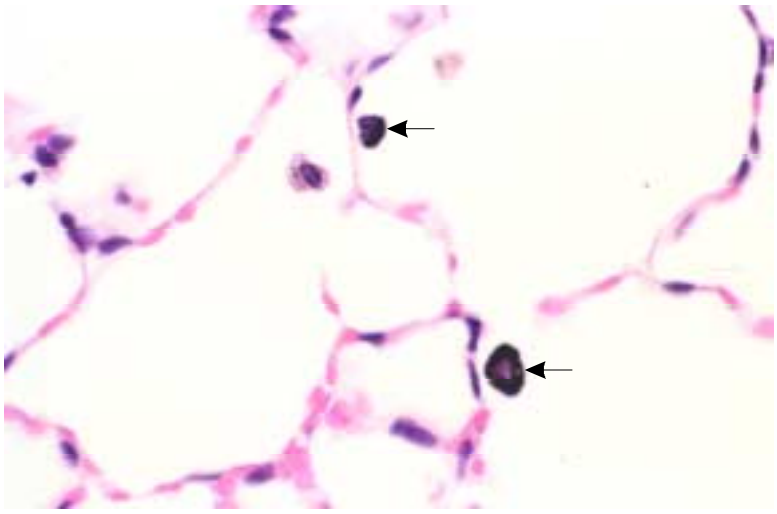
The lung findings in the high-level group were characterized by an increased number of AMs that contained multiple small spherical black particles, presumably carbon particles from biodiesel exhaust (Fig. 1). The increase in the number of AMs was not great and did not exceed an average of about one macrophage per alveolus. The macrophages were most often uniformly distributed about the alveoli of the lung and only infrequently in clumps. Most of the AMs in the high-level rats contained many black particles that obscured the cellular details and nucleus of the macrophage (Fig. 2). However, the cytoplasm of these macrophages was only slightly enlarged. Particles were rarely found free in the alveoli. Degenerative or disintegrated macrophages were rarely seen, and neutrophils were not associated with the AMs.

Alveolar bronchiolarization was a lesion minimal in severity and seen only in a few airways in each of three high-level female rats. This lesion was characterized by lining of alveolar ducts (ADs) and alveoli adjacent to terminal bronchioles (TBs) by ciliated and Clara cells (Fig. 3). The new lining cells produced short segments resembling respiratory bronchioles with alternating newly lined AD walls and alveoli (Dungworth *et al.*, 1992). The reaction represents a subtle response to injury in the ADs and alveoli adjacent to the TB.



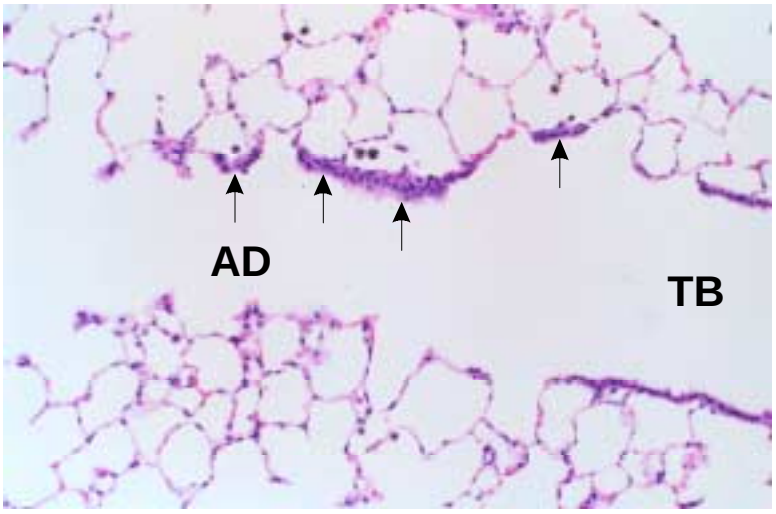
4080-4

Figure 1. An increased number of AMs containing black particles was characteristic of the lung lesions in the rats exposed to the high concentration (142X magnification, H&E stain).



4080-5

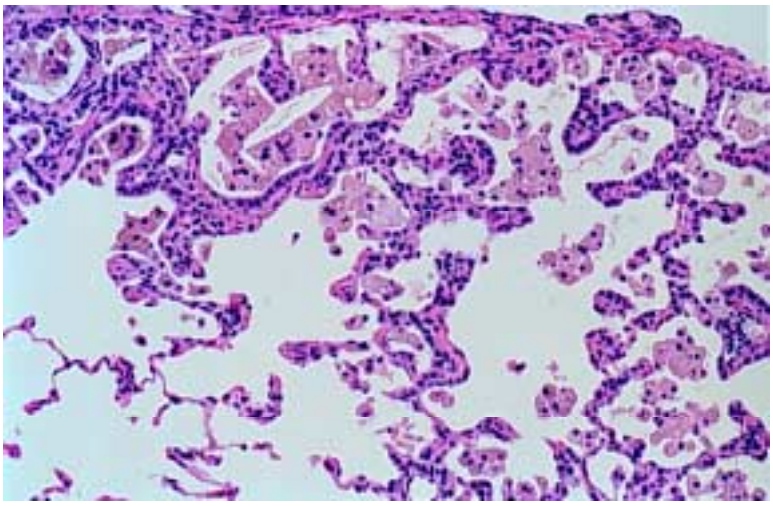
Figure 2. AMs in the rats in the highest exposure concentration contained many black particles that obscured the cellular details and nucleus of the cell (arrows) (570X magnification, H&E stain).



4080-12

Figure 3. Alveolar bronchiolarization (arrows), lining of ADs and alveoli adjacent to TBs with ciliated and Clara cells, was seen in a few airways of rats in the highest exposure concentration (142X magnification, H&E stain).

In two rats, aggregates of AMs were associated with a reaction in the alveolar septa, a lesion termed *alveolar histiocytosis* (Fig. 4). However, alveolar histiocytosis was also found in control rats, albeit, without particles in the macrophages. Other lung lesions, chronic inflammation, and centriacinar fibrosis were infrequent and could not be related with certainty to the biodiesel exposures.

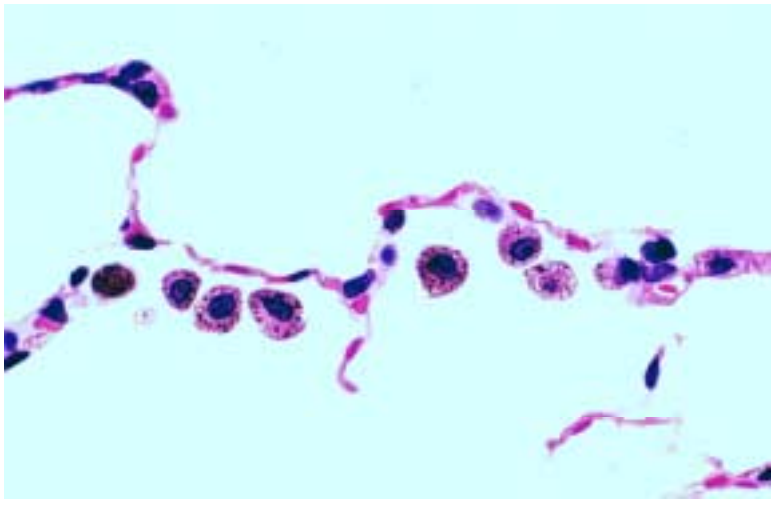


4080-16

Figure 4. In a few rats, aggregates of AMs were associated with a reaction in the alveolar septa; a lesion termed alveolar histiocytosis (285X magnification, H&E stain).

Lung findings in the low and intermediate groups were characterized by lesser increases in numbers of AMs that contained fewer black particles (Table 11). In the low-level group, only a few particles were present in the cytoplasm of the AMs (Fig. 5).

A few lesions were commonly found in non-respiratory tract tissues, but could not be related to exposure. These were calculi and mineralization in the renal tubules. No lesion was seen in the testicles that could be related to biodiesel exhaust exposure. No effect was noted on spermatogenesis.



4080-19

Figure 5. AMs in the rats in the lowest exposure concentration contained few black particles in the cytoplasm (570X magnification, H&E stain).

2. *Sacrifice After 13 Weeks of Exposure and 28 Days Recovery*

Ten male and 10 female rats in the high-level group were sacrificed after 13 weeks in exposure chambers and 28 days in polycarbonate caging. The lung lesions were characterized by increased numbers of AMs containing black particles. However, the severity of these lesions was less than that in rats killed at the end of exposure (Table 12). The reduction in AM hyperplasia was notable. One feature noted, but not apparent in the scoring, was the general reduction in the cytoplasm of the AMs in the recovery rats. This reduction indicates a return of the macrophages toward a less active state.

Table 12. Comparison of lung lesions after 13 weeks of high-level exposure and 13 weeks of high-level exposure with 28 days recovery.[a]

		Males		Females	
Diagnosis	Severity	13 Weeks[a]	13 Weeks + 28 Days Recovery	13 Weeks[a]	13 Weeks + 28 Days Recovery
Number examined:		20	10	20	10
Dust-laden alveolar macrophages	NAD	–	–	–	–
	Minimal	–	1	–	–
	Mild	3	5	3	3
	Moderate	17	4	17	7
Alveolar macrophage hyperplasia	NAD	2	4	2	2
	Minimal	13	6	10	8
	Mild	5	–	8	–
Alveolar bronchiolarization	NAD	20	10	17	9
	Minimal	–	–	3	1
Alveolar histiocytosis	NAD	20	10	18	8
	Minimal	–	–	2	2

[a]Includes findings from General and Special Histology Groups.
NAD = No abnormalities detected.

I. Special Histology Group

There were no differences in either absolute or relative brain weights between any exposure group and controls (Appendix K). The histopathology examination conducted on the

Special Histology Group rats was the same as the one conducted on the General Histology Group with two exceptions (Appendix L). The nervous system was given a special neurotoxicology examination, and the reproductive tracts were examined from rats in all exposure groups. The whole-body perfusion with fixative in these rats allowed for better comparisons of particle distribution. The histology findings in the lung are recorded with the findings of the General Histology Group (Table 11).

1. *Neurotoxicology*

No lesions were evident in the paraffin-embedded nervous tissues or the plastic-mounted, teased tibial nerves of the high-level, exhaust-exposed and filtered air control groups of rats. The highest level of biodiesel exhaust did not cause any histologic evidence of nervous tissue toxicity. Based on these findings, evaluation of central and peripheral nervous tissues from the intermediate- and low-level exhaust exposure groups was unnecessary.

2. *Reproductive Tract Pathology*

The reproductive tract was examined from the high-level and control groups in the General Histology Group, from all exposure groups in the Special Histology Group, and from rats with gross lesions or that failed to reproduce in the Fertility Group. No dose-related lesions were noted in the reproductive tracts of rats in the General or Special Histology Groups (Appendix L).

3. *Particle Distribution*

Few particles were found in the lungs. Those particles observed were found nearly exclusively in the cytoplasm of AMs. These particle-laden macrophages were scattered in alveoli throughout the lung with a slight concentration in alveoli adjacent to the pleura or large conducting airways (Fig. 6). Occasional particle-laden macrophages could be seen in the bronchial lumens, adventitia surrounding the large airways, and lymphoid tissue surrounding the large airways (Fig. 7). Particles were not found in the interstitium in the alveolated areas of the lung nor in the epithelium lining the airways. These findings indicate that the particles were located primarily in AMs, an expected finding because of the role of macrophages in the normal removal of particles from the lung.

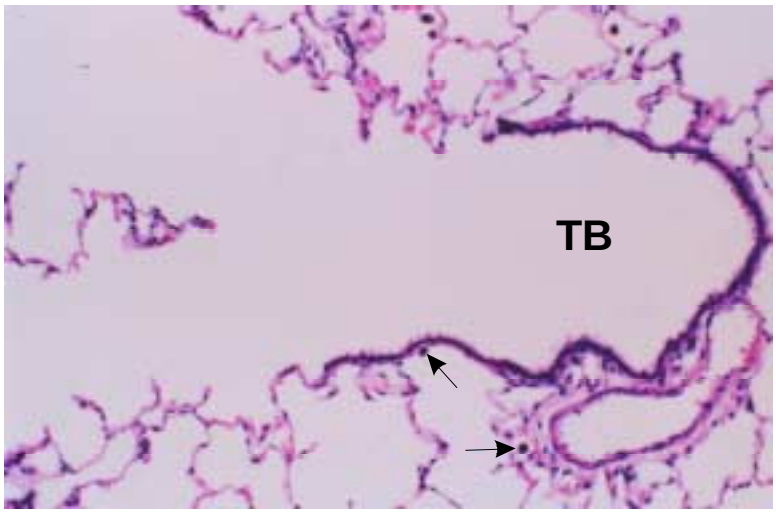


Figure 6. Particle-laden macrophages (arrows) were scattered in alveoli throughout the lung with a slight concentration in alveoli adjacent to conducting airways (TB) (142X magnification, H&E stain).

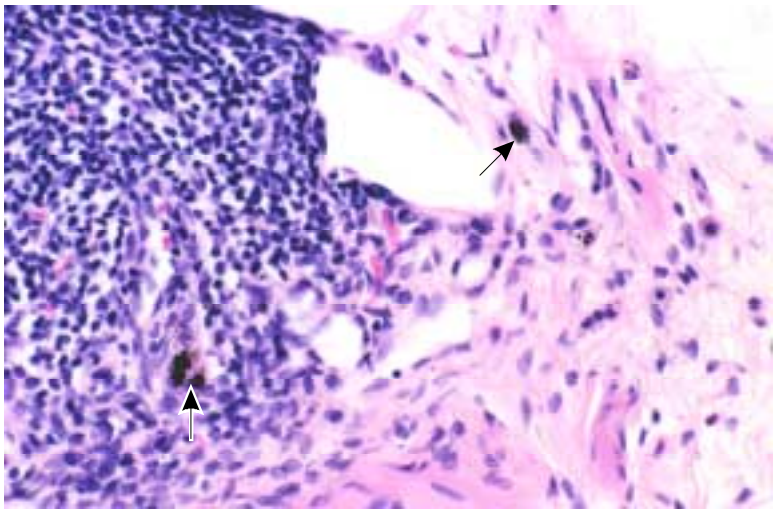


Figure 7. Occasional particle-laden macrophages could be seen in lymphoid tissues surrounding large airways (arrows) (285X magnification, H&E stain).

J. Histology Discussion

Histologic findings associated with the biodiesel exhaust exposure were limited to the lung. Alveolar bronchiolarization, a lesion indicative of tissue response to injury, was noted in three of the 20 females exposed to the high concentration, but none was seen in the males. In addition, alveolar histiocytosis increased equivocally in the high-level group. In the intermediate

group, alveolar bronchiolarization was not found, and the alveolar histiocytosis was not increased compared to controls.

All exposure concentrations showed an increase in numbers of AMs and accumulations of black-dust pigments in AMs, changes that were more prominent in the high-level group (Table 11). None of these changes, however, was scored as more than mild in severity. Dust-laden macrophages and increases in AMs were present in the intermediate-level group, as they were to a lesser degree in the low-level group. These findings were not accompanied by an influx of neutrophils and were judged to be normal physiologic responses to particles inhaled and deposited within the lungs.

Thus, based on histologic findings, the no-adverse-effect-level for this study of inhaled biodiesel exhaust was the intermediate level.

K. Clinical Chemistry

Individual animal data and mean values by exposure group for each sampling point for each analyte are shown in Appendix M. Not all analyses could be run on every animal because of insufficient sample size. Analysis of total bile acids was significantly affected; however, other analyses of liver function were not affected. The lack of some clinical chemistry analyses did not affect the overall evaluation of the animal's state of health.

The result of the initial repeated-measure ANOVA is shown in Table 13, which shows all analytes with significant differences. For those analytes with a significant difference related to dose or with a significant dose × gender interaction, additional repeated ANOVAs were performed. The difference of each individual value from its baseline value was calculated and the significance of the difference determined.

Alkaline phosphatase and blood urea nitrogen serum concentrations were both decreased with time and increasing exposure level in both males and females. A decrease in alkaline phosphatase has been reported in other studies of exposed rats (Maejima and Nagase, 1989), but there is no good explanation for this change. Total protein and albumin were also decreased with increasing exposure level in both males and females in this study. In other studies where similar decreases have been seen, the changes were related to liver dysfunction (Maejima and Nagase, 1989), which was not seen in the current study. Glucose values were increased in the current study in both males and females. The reason for these changes is unknown, but they are not judged to have biological significance.

Table 13. Clinical chemistry data analysis.
(Repeated measures analysis of variance contrasts)
(30 days vs. baseline, 13 weeks vs. baseline)

	Baseline-30 days			Baseline-90 days		
	Gender	Dose	Gender × Dose	Gender	Dose	Gender × Dose
Albumin	0.7056	< 0.05	0.0898	< 0.05	< 0.05	0.7913
Alkaline phosphatase	< 0.05	< 0.05	0.2884	< 0.05	< 0.05	0.0733
Alanine transferase	0.2017	0.0650	< 0.05	< 0.05	0.6398	0.9888
Albumin/globulin ratio	0.5359	0.0656	< 0.05	< 0.05	0.4723	< 0.05
Cholesterol	0.9745	< 0.05	0.4959	< 0.05	0.7076	0.1249
Chloride	< 0.05	< 0.05	0.9677	< 0.05	0.1112	0.6538
Gamma glutamyl transferase	0.0958	0.1162	< 0.05	0.3039	0.4340	0.2384
Globulin	0.2016	0.6647	0.1446	< 0.05	0.1181	< 0.05
Glucose	0.9085	< 0.05	0.4313	0.0720	< 0.05	0.1396
Sodium	< 0.05	< 0.05	0.5749	< 0.05	0.1189	0.2506
Phosphorus	0.2183	< 0.05	0.1275	< 0.05	0.3240	0.6102
Sorbitol dehydrogenase	0.8537	< 0.05	0.9507	< 0.05	0.7749	0.4859
Total protein	0.2305	< 0.05	0.2728	< 0.05	< 0.05	0.1097
Urea nitrogen	0.9185	0.1301	0.7375	0.3982	< 0.05	< 0.05

In conclusion, none of the clinical chemistry findings in the current study has biological significance. The significant differences observed in this study were not associated with any lesions in target organs.

L. Hematology

No biologically significant differences were noted in the hematology data (Appendix N). The white blood cell counts significantly decreased in rats from all exposure groups over the 13-week-exposure period. Reduction in lymphocyte and monocyte numbers accounted for the reduction in total white blood cells. The finding of reduced lymphocyte numbers is consistent with previous reports in aging Sprague Dawley rats (Wolford *et al.*, 1987).

In conclusion, no biologically significant differences were observed in the hematology endpoints. This finding is consistent with findings in other studies of the potential

toxicity of diesel exhaust emissions where few hematologic changes have been observed in studies of longer duration.

M. Glial Fibrillary Acidic Protein Assay

Summary results by group for levels of GFAP, protein, and GFAP normalized by protein are presented in Table 14. Appendix O contains individual animal data on body weights and brain weights; individual animal data on GFAP, protein, and GFAP normalized by protein; and statistical analyses of the significance of differences between control rats and rats in each of the three exposure groups.

Table 14. Summary data for glial fibrillary acidic protein assay.

	GFAP (μg/ml)		Protein (mg/ml)		GFAP/protein (μg GFAP/ mg protein)	
	Mean	SD	Mean	SD	Mean	SD
Control	20.9	2.2	9.0	0.3	2.3	0.2
Low	17.4[a]	3.9	9.1	0.4	1.9[a]	0.4
Intermediate	19.3	2.2	9.0	0.5	2.2	0.3
High	22.9	2.3	8.9	0.4	2.6	0.3

[a]Significantly less than controls ($p < 0.05$).

All Block B animals survived to terminal sacrifice and had no clinical signs of neurotoxicity. For both male and female rats, there were no significant differences in mean group body weights for the last in-life weight collection between controls and any of the three exposure groups. Similarly, for both male and female rats, there were no significant differences in mean group brain weights at necropsy between controls and any of the three exposure groups.

There were no significant differences at the $p < 0.05$ level between any of the three exposure groups and controls for levels of protein. For GFAP concentrations in the brain homogenates, there was no effect of gender. For the sexes combined, mean GFAP concentration in the low-level group was significantly less than that for controls (17.4 vs. 20.9 μg/ml, respectively; $p < 0.05$). Levels of GFAP appeared greater for the high-level group compared with controls (22.9 vs. 20.9, respectively), although this was only of marginal statistical

significance ($0.05 < p < 0.10$). These differences corresponded with a statistically significant decrease of normalized GFAP for the low-level group compared to controls (1.9 vs. 2.3 μg GFAP/mg protein, respectively; $p < 0.05$), and an apparent although not significant increase of normalized GFAP for the high-level group compared to controls (2.6 vs. 2.3 μg GFAP/mg protein, respectively; $0.05 < p < 0.10$).

Our data showing a decrease of GFAP in the low-level group compared to controls are not consistent with toxicity, because toxic responses are expected to be accompanied by an increase in GFAP. Our data also showed a slight increase in GFAP in the high-level group compared to controls, but this increase was not statistically significant. Given the 1) variability between groups and between animals within groups, 2) absence of any differences in either body weights or brain weights between controls and any of the three exposure groups, 3) absence of any brain pathology in Block A animals (see Section I), and 4) absence of any clinical signs of neurotoxicity or brain toxicity (see Section E), we conclude the between-group differences observed in both GFAP levels and protein-normalized GFAP levels are of no biological relevance.

N. Micronucleus Assay

Summary data are presented in Table 15. Individual animal data, including animal identities, gender, number of erythrocytes examined (200), number of polychromatic erythrocytes (PCEs) counted, ratio of PCEs to normochromatic erythrocytes (NCEs), number of PCEs examined (1,000), number of MN counted per 1,000 PCEs, and percentage of PCEs having MN, are shown in Appendix P.

All animals survived to terminal sacrifice and had erythrocytes evaluated for the PCE/NCE and percentage of PCEs with MN endpoints.

For the ratio of PCE/NCE, the ANOVA indicated an interaction between gender and exposure group. Therefore, genders were analyzed separately for the exposure group comparisons. The ANOVA indicated a significant effect of exposure group on PCE/NCE ratio. Therefore, multiple pair-wise comparisons were examined. For males, no exposure group (low-, intermediate-, or high-level biodiesel exhaust, or DMBA-positive control) was significantly different from controls. For females, low-, intermediate-, and high-level biodiesel exhaust groups were not significantly different from controls, whereas the DMBA-positive controls were significantly different from controls.

Table 15. Summary data for micronucleus evaluation from bone marrow cells from biodiesel study rats.

		Ratio of PCEs/NCEs				Percentage of Micronucleated PCEs			
		By Gender		Genders Pooled		By Gender		Genders Pooled	
Group	Gender	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	M	0.34	0.09			0.36	0.32		
Control	F	0.47	0.14	0.4	0.13	0.18	0.08	0.27	0.24
Low	M	0.34	0.08			0.38	0.26		
Low	F	0.51	0.09	0.43	0.12	0.32	0.15	0.35	0.20
Int.	M	0.51	0.09			0.36	0.19		
Int.	F	0.4	0.08	0.46	0.1	0.44	0.27	0.40	0.23
High	M	0.41	0.09			0.38	0.08		
High	F	0.34	0.11	0.38	0.1	0.32	0.18	0.35	0.14
DMBA	M	0.21	0.03			1.90	0.64		
DMBA	F	0.21[a]	0.07	0.21[a]	0.05	1.56	1.51	1.73[b]	1.11

[a]Significantly different ($p < 0.05$) from appropriate negative controls.
[b]Significantly different ($p < 0.005$) from appropriate negative controls; gender not separately tested (see text).

Since there was no significant effect of sex in the ANOVA of the ratio of PCE/NCE (only an interaction between sex and exposure group), it was also useful to examine the multiple pair-wise comparison of exposure groups with genders pooled. This evaluation demonstrated that 1) low-, intermediate-, and high-level biodiesel exhaust groups were not significantly different from controls, but DMBA-positive controls were significantly different from controls; and 2) controls, and low-, intermediate-, and high-level biodiesel exhaust groups were all significantly different from DMBA-positive controls.

For the analysis of the percentage of PCEs with MN, the initial ANOVA indicated that normality of distribution and equality of variance between groups could not be assumed. For that reason, a rank transformation was performed and the analysis repeated. This was successful in meeting the normality and equality of variance assumptions. The ANOVA indicated neither a significant effect of sex nor an interaction between sex and exposure group. Therefore, sexes were pooled for the exposure group comparison. Neither the low-,

intermediate-, nor high-level biodiesel exhaust exposure groups were significantly different from controls, but the DMBA-positive controls were significantly different from controls, low-, intermediate-, and high-level biodiesel exhaust exposure groups.

In no instance were any of the biodiesel exhaust exposure groups significantly different from the negative control group; however, in most cases, the negative controls and the low-, intermediate-, and high-level biodiesel exhaust exposure groups were significantly different from the DMBA-positive controls. This evaluation demonstrates that 1) biodiesel exhaust exposure did not affect either an alteration of PCE/NCE ratio or the induction of MN in PCEs, and 2) DMBA functioned as an appropriate positive control for this assay.

O. Sister Chromatid Exchange Assay

Summary data are presented in Table 16. Individual animal data for percentage of metaphase cells, replicative index, and number of SCEs per second-division metaphase cell are shown in Appendix Q.

All animals survived to terminal sacrifice. In some cases, due to concurrent use of blood samples for clinical pathology or to insufficient cell recovery, samples were insufficient to obtain enough cells for analysis. Animals for which this occurred are noted in Appendix Q. In other cases, the metaphase cell spreads were rounded and difficult to accurately classify. In these cases, less than 100 metaphase cells could be evaluated for classification into first-, second-, or third-division metaphase. Finally, not all individual animals with interpretable spreads yielded 25 second-division cells. Because of this, the normal criterion that data from these cells be used was revised to include rats with, at minimum, 10 second-division cells. Data and statistics were summarized on rats having all three of the following criteria: 1) 1,000 evaluable peripheral blood lymphocytes, 2) 100 evaluable metaphase cells, and 3) at least 10 second-division cells evaluable for the number of SCEs. Genders were pooled because 1) a gender-related effect of exposure was not expected, and 2) pooling increased the sample size per group to provide a more meaningful statistical analysis. For the negative-control, low-level, intermediate-level, high-level, and positive-control groups, respectively, 3, 5, 5, 5, and 4 rats were evaluable using these criteria.

Table 16. Summary data for sister chromatid exchange assay.

	Percent Metaphase			Replicative Index[a]			SCEs per 2nd Div. Cell		
Group	Mean	SD	N	Mean	SD	N	Mean	SD	N
Control	4.17	2.11	3	1.35	0.08	3	3.37[b]	0.67	3
Low	6.44	3.55	5	1.40	0.18	5	3.36[b]	0.99	5
Int.	8.22	8.12	5	1.42	0.21	5	3.10[b]	0.88	5
High	5.98	5.13	5	1.51	0.15	5	3.52[b]	0.92	5
Pos. control	2.28	1.13	4	1.28	0.07	4	5.03[c]	1.10	4

[a]Replicative index = [(% 1st div) + (2 × % 2nd div) + (3 × % 3rd div) metaphases] / 100.
[b]Significantly different ($p < 0.05$) from DMBA-treated positive controls (one-tailed t-test).
[c]Significantly different ($p < 0.05$) from either negative controls or biodiesel exhaust-exposed groups (one-tailed test).

Using a one-way ANOVA, between-group differences were not indicated in either the percentage of lymphocyte cells in metaphase ($p = 0.500$) nor in the replicative index ($p = 0.308$). For the number of SCEs per second-division cell, a marginally significant ($p = 0.059$) between-group effect was indicated. Because of this marginal difference, multiple pair-wise comparisons were investigated; pair-wise examinations were performed for negative controls versus the low, intermediate, high levels, and positive controls; and between the positive controls and the low, intermediate, and high levels.

These pair-wise comparisons were preceded by F-tests for equality of variance assumptions. For each of the seven pair-wise comparisons, variances could be assumed equal, and therefore t-tests assuming equal variances were used. To increase the likelihood of observing any effect of exposure, and because toxic responses in the SCE assay are evidenced by an increase in the number of SCEs per second-division metaphase cell, one-tailed p values were examined for significant differences between group means. In comparison to the negative controls, the positive DMBA-treated controls were significantly greater ($p = 0.035$), whereas no differences occurred between negative controls and either the low, intermediate, or high levels ($p = 0.496$, 0.335, or 0.406, respectively). On the other hand, group mean values were significantly greater for the positive controls compared to either the low, intermediate, or high levels ($p = 0.024$, 0.011, or 0.030, respectively).

Thus, no effects were caused by either biodiesel exposure nor DMBA treatment on the percentage of peripheral blood lymphocytes in metaphase nor in the replicative index. In contrast, DMBA induced a statistically significant increase in the number of SCEs when compared to either the negative controls or the biodiesel exhaust-exposed animals. This indicates that DMBA served as an adequate positive control for this study, and no effect was caused by any level of biodiesel exhaust exposure on the formation of SCEs in peripheral blood lymphocytes.

P. Individual Animal Fertility

Individual animal fertility pre-study and before and during mating procedures are shown in Appendix R.

1. *Pre-study*

Seven rats were culled from possible assignment to the study because of definite estrous cycle abnormalities. An additional eight animals had questionable estrous cycles, and five had possible cycle abnormalities. None of these rats having questionable or possible estrous cycle abnormalities was assigned to the fertility/reproductive portion of the study.

2. *Before and During Mating*

No striking abnormalities were observed in the cycles of any of the rats during the 15-day period of pre-mating vaginal cytologic evaluation. Mating was begun immediately following this 15^{th} lavage.

Throughout the first 9 days of mating, 21 females did not have evidence of mating. At this point, each male of the pair was replaced by another male proven to be fertile (i.e., had impregnated another female). After 14 days of mating, nine females did not have evidence of mating. Five of these rats, however, had become acyclic, and pregnancy was suspected. Gestational day zero was estimated and the rats scheduled for sacrifice on gestational day 20. Of these five, one actually delivered pups the day before her scheduled sacrifice, three were successfully sacrificed on gestational day 20, and the fifth was sacrificed before gestational day 20. The remaining four rats were sacrificed and found not pregnant.

The reproductive tracts of the four non-gravid rats were histologically examined (Appendix L). No abnormal pathology was observed in these animals.

Q. Reproductive Toxicology and Teratology

This endpoint was evaluated by the subcontractor Pathology Associates International, Frederick, MD.

Pregnancy rates were 21/22, 18/23, 22/23, and 25/25 for the control, low-, intermediate-, and high-level groups; no treatment-related differences were observed. No statistically significant or biologically meaningful differences were observed between control and exposed groups for the cesarean section parameters evaluated, including the number of corpora lutea; implantations; viable and nonviable fetuses; early, late, and total resorptions; fetal sex ratios; and fetal weights. Similarly, no treatment-related fetal malformations or variations were observed in any exposed group compared with controls. In conclusion, exposure of pregnant rats was not fetotoxic or teratogenic.

Detailed results, including individual and group mean data, are described in the final report entitled "Teratology Report for Tier 2 Testing of Biodiesel Exhaust Emissions, LRRI Study Number FY98-056" included as Appendix S of this report.

R. Mutagenicity Testing

1. *Samples for Extraction for Mutagenicity Testing*

A quantity of 490 and 1702 mg of organic material was extracted from the particulate filters and PUF/XAD-4/PUF sandwiches, respectively. The extraction of the samples was done by Desert Research Institute (see Appendix T). A total of 1098 mg of particles was collected on the filters. Thus, about 45 percent of the particles was extractable organics.

2. *Salmonella Typhimurium Reverse Mutation Assay*

This endpoint was evaluated by Chrysalis Preclinical Services (Olyphant, PA) on PSOF and SVEF of biodiesel exhaust emissions.

Both PSOF and SVEF fractions produced toxicity in all tester strains in the toxicity pre-screen used to select doses for the mutagenicity assay. For PSOF, positive mutagenicity was observed in all tester strains without S9 and in four of the five strains with S9. Comparing the relative mutagenicity of the PSOF in the various strains produced a rank order of: TA100 ±S9 (2.4 rev/μg·plate^{-1}) > TA98 ±S9 (1.0–1.5 rev/μg·plate^{-1}) > TA1537 ±S9 (0.72–0.75 rev/μg·plate^{-1}) > TA98/1,8-DNP$_6$ ±S9 (0.31–0.64 rev/μg·plate^{-1}) > TA1535 ±S9 (0.061–0.076

rev/μg·plate^{-1}). Thus, a substantial portion, but not all, of the mutagenicity observed in tester strain TA98 can be attributed to the presence of nitroaromatics in the sample.

For SVEF, positive mutagenicity was observed in four of the five tester strains with or without S9. Comparing the relative mutagenicity of the SVEF in the various strain produced a rank order of: TA100 ±S9 (0.25–0.26 rev/μg·plate^{-1}) > TA98 ±S9 (0.060–0.092 rev/μg·plate^{-1}) > TA1535 +S9 (0.025 rev/μg·plate^{-1}) > TA98/1,8-DNP$_6$ –S9 = TA1537 +S9 (0.020 rev/μg·plate^{-1}) > TA1537 –S9 (0.017 rev/μg·plate^{-1}) > TA1535 –S9 (0.67 rev/μg·plate^{-1}). Thus, a substantial portion of the mutagenicity observed in tester strain TA98 in the absence of S9, and all of the mutagenicity observed in tester strain TA98 in the presence of S9, can be attributed to the presence of nitroaromatics in the sample.

Detailed results of the mutagenicity testing are described in the two reports entitled "Ames/*Salmonella* Plate Incorporation Assay on Biodiesel Exhaust Emissions-Particulate Soluble Organic Fraction (BEE-PSOF)" and "Ames/*Salmonella* Plate Incorporation Assay on Biodiesel Exhaust Emissions-Semi-Volatile Extracted Fraction (BEE-SVEF)" included as Appendix T of this report.

IV. CONCLUSIONS

This final report summarizes the materials and methods used, and the results obtained in LRRI study FY98-056. Additional supplemental data, including the experimental protocol, protocol amendments, and protocol and SOP deviations, are found in the Appendices and in the archived Study File.

F344 rats were exposed to diluted biodiesel exhaust emissions at targeted NO_x concentrations of 5, 25, or 50 ppm, while other rats served as air-exposed controls. Actual exposure concentrations achieved were within acceptable ranges.

No pronounced toxicity resulted from the subchronic exposure of rats to biodiesel exhaust emissions at any concentration. Neither mortality nor abnormal clinical observations were attributed to exposure to biodiesel exhaust. Similarly, there were no adverse ocular responses due to biodiesel exposure, nor was feed consumption affected by exposure. Serological samples from sentinel rats before and after the study demonstrated that the animals were free from infections by common rodent pathogens. During 2 days of the study (study days 29 and 68), the body weights of Block A high-level female, but not male, rats were less than body weights of control female rats. Although statistically significant, the decrease was minor,

was not observed at other timepoints, and therefore was not judged to be a toxic response to exposure.

Groups of rats in the Special Histology Group were free from any histologic evidence of neurotoxicity, and the ability of our neurohistopathology techniques was demonstrated by identifying neurotoxic responses of rats treated with acrylamide.

Rats in the General Histology Group of the study were examined for both organ weights and histopathology. Relative to total body weights, lungs of female rats in the high-level group weighed more than lungs from control group females. In addition, minimal to mild dark gray mottling of the surface of the lungs was observed at gross necropsy with increasing exposure level. These responses are probably related to lung histology findings. In addition, other non-pulmonary organ weight differences were observed. These included lower absolute liver weights for both male and female rats in the high-level group compared with controls, lower body weight-normalized liver weights in females in the high-level group compared to controls, and higher body weight-normalized testes weights in the high-level group compared to controls.

Only a few occasional lesions were found within non-pulmonary tissues in rats in this study, and the lesions found were not associated with biodiesel exhaust exposure. Several histopathologic findings were present in the lungs of rats in this study. Centriacinar fibrosis and chronic inflammation were seen infrequently but were not related to the level of exposure. Rats in all biodiesel-exposed groups had accumulations of particles, presumably carbonaceous biodiesel exhaust particles. Particles were located within AMs, and AM hyperplasia was also observed. These findings were generally related to the level of exposure, with both the incidence and severity of dust-laden macrophages and macrophage hyperplasia increased with increasing exposure. These findings were not accompanied by an influx of neutrophils and were judged to be normal physiologic responses to particles inhaled and deposited within the lungs.

Two types of lung lesions were observed in this study: alveolar bronchiolarization was observed in four of the 30 female rats exposed to high-level exhaust (three at the 13-week timepoint, and one after the 28-day recovery period); alveolar histiocytosis was observed in two of 19 control rats (one male and one female), two of 20 intermediate-level rats (one male and one female), and in four of 30 high-level rats (all females; two at the final sacrifice and two after the 28-day recovery period). Thus, although not striking, the presence of these lesions in the high-level group, with the accompanying increase in lung weight in the females, indicates an adverse effect of exposure of female rats to high-level exhaust.

A number of clinical chemistry and hematology parameters were examined pre-study, at 30 days on study, and at the 13-week study termination. Although some of these parameters were changed in biodiesel exhaust-exposed rats compared with controls, there was no pathologic responses in associated organs (e.g., liver), and these changes were judged to have no biological significance.

Brains of selected rats were examined at the 13-week study termination for brain weight, protein, and GFAP. Compared with controls, differences included decreased GFAP in low-level rats and a equivocal increase in high-level rats. These differences were judged to have no biological relevance, because the differences were not consistent compared with controls, brain weights were not affected, no neurotoxic effects were clinically indicated, and no pathology was associated with the brain in the high-level group.

A micronucleus assay was performed on bone marrow-derived erythrocytes, and a SCE assay was performed on peripheral blood lymphocytes at the 13-week study termination. In both cases, no differences were found between biodiesel exhaust-exposed animals and controls. In addition, for both assays, DMBA-treated positive controls responded in a statistically significant manner, indicating the ability of our laboratory to measure changes in these endpoints.

Estrous cycling, fertility, and potential reproductive toxicity and teratology were examined in a designated group of rats. Cycling and fertility were not affected by exposure to biodiesel exhaust emissions. Histologic evaluation of the reproductive tracts of females that failed to become pregnant did not reveal any abnormalities. Similarly, no teratologic effect of exposure was caused on fetuses of pregnant dams.

Samples of both particulate and semi-volatile fractions of biodiesel exhaust emissions were evaluated in a bacterial mutagenicity assay. Some observed mutagenic activity was attributed in part to the presence of nitroaromatic compounds. The extracts were expected to have mutagenic activity.

In summary, mutagenicity was observed from both particulate and semi-volatile extracts. In the intact animal, the only biologically significant effect of exposure to biodiesel exhaust emissions was found in the lungs. Four of 30 female rats in the high-level group had alveolar bronchiolarization, and four of 30 rats had alveolar histiocytosis. These changes were accompanied by a slight, yet statistically significant, increase in lung weight in high-level female rats compared to controls. Neither alveolar bronchiolarization nor significantly increased lung weights was found in the intermediate-level animals, and the incidence of alveolar histiocytosis

was identical to that in controls. Based on these results, rats were adversely affected by exposure to high-level biodiesel exhaust emissions, the effect was greater in female rats than in males, and the no-adverse-effect-level for this study of inhaled biodiesel exhaust emission was the intermediate-exposure level.

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