



NAVAL MEDICAL RESEARCH UNIT DAYTON

Sensory Irritant Potential of Amyris C15 Bio-Based Jet Fuel

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STATEMENTS

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The study protocol was reviewed and approved by the Wright Patterson AFB Institutional Animal Care and Use Committee in compliance with all applicable federal regulations governing the protection of animals and research.

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ABBREVIATIONS, ACROYNMS, AND SYMBOLS

AFRL	Air Force Research Laboratory
Amyris	Amyris C15 jet fuel
ASTM	American Society for Testing and Materials
Avg	average
EPA	Environmental Protection Agency
JP-8	Jet Propellant 8
USAF	United States Air Force
HEFA	hydroprocessed esters and fatty acids
°F	degrees Fahrenheit
FTIR	Fourier Transform Infrared Spectrophotometer
FT SPK	Fischer Tropsch
g	grams
H ₂ O	water
L	liters
μm	micrometers
m ³	cubic meters
mg	milligrams
min	minutes
mL	milliliters
mm	millimeters
NAMRU-D	Naval Medical Research Unit Dayton
NOEU	nose-only exposure unit
OPS	Optical Particle Sizer
POSF	AFRL/RQTF fuel batch number
%RH	percent relative humidity
r ²	coefficient of determination
RD ₅₀	50 percent (%) depression of respiratory rate
SD	standard deviation
SPK	synthetic parrafinic kerosene

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1.0 Summary

JP-8, a petroleum-based jet fuel, is currently the primary fuel used in U.S. Air Force aircraft. Because the United States Government is interested in lowering dependence on crude oil for military use, alternative fuels have been developed that would be either combined with or used in place of JP-8 for military operations. This study investigated the sensory irritation potential from inhalation of Amyris C15 aerosols and vapors.

Amyris C15 (Amyris) is an alternative fuel that is produced by a direct sugar to hydrocarbon process and consists primarily of the alkane farnesane. Sensory irritation is the most common harmful effect of inhaled airborne chemicals, and irritancy can be quantitatively measured in mice by reflex inhibition of respiratory rate. The concentration of a chemical that induces a 50% respiratory depression is termed the RD₅₀ value and describes the sensory irritancy potential of that chemical. The testing method that was used followed the American Society for Testing and Materials guideline: Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals. Separate cohorts of Swiss-Webster mice were exposed nose-only to five concentrations of an Amyris aerosol/vapor mixture for 30 minutes. Respiratory rates were measured with a body plethysmograph during the exposure and 10 minutes post-exposure and compared to a pre-exposure baseline. Exposure to Amyris did not induce a respiratory rate depression of at least 50% at any of the concentrations tested. It was concluded that Amyris was not a sensory irritant at similar concentrations to which JP-8 was found to induce sensory irritation in the current study, as well as in previous literature.

2.0 Introduction

The United States Government has had interest in developing alternative, bio-based jet fuels that could be used as “drop in” substitutes for the current jet fuel used by the United States Air Force (USAF), Jet Propellant 8 (JP-8). These fuels are defined as “drop in” fuels, because they could be either combined with or used in place of JP-8 for fueling military aircraft without any modifications necessary to the aircraft themselves (U.S. GAO, 2015). Although the alternative fuels may have the same functional properties as JP-8 for providing energy to military aircraft, they are compositionally different from JP-8 and therefore need to be evaluated for distinct health effects. Prior toxicity testing has been performed on bio-based jet fuels such as hydroprocessed ester and fatty acid (HEFA) and Fischer Tropsch synthetic paraffinic kerosene (FT SPK), which are currently approved for use (Sterner *et al.*, 2013 and Mattie *et al.*, 2018).

Amyris C15 (Amyris) is an alternative jet fuel that is produced by a direct sugar to hydrocarbon (DSHC) process, in which the fuel is generated by fermentation of sugar by engineered microorganisms (Lew and Biddle, 2014). Amyris contains more than 97% farnesane (trimethyl dodecane) by mass. Farnesane has undergone limited toxicity testing because it is sold in Europe as a diesel fuel. Acute health effects were assessed via a short-term inhalation exposure of Sprague-Dawley to 2190 mg/m³ farnesane for four hours following the EPA Health Effects Test Guidelines, OPPTS 870.1300 (U.S.

EPA, 1998), which includes a 14-day observation period following exposure. The only observed effects immediately following exposure were closed eyes, piloerection, and coating of the fur with test material. Coated fur and piloerection were observed on the day following exposure. Piloerection in one animal persisted into day 3 of the observation period (European Chemicals Agency, 2017).

Sensory irritation is the most common harmful effect of inhaled airborne chemicals, and irritancy can be quantitatively measured in mice by reflex inhibition of respiratory rate. Alarie (1973) demonstrated a concentration-response for breathing rate depression in mice related to irritation of the respiratory system, called an RD₅₀ value. The RD₅₀ is the concentration of an inhaled substance that causes breathing rate depression to reach 50% of the baseline value. Alarie (1981) validated the relevance of the mouse-derived RD₅₀ value to airborne chemical sensory irritation in humans by establishing a correlation of RD₅₀ values with existing Threshold Limit Values. The calculated concentration at which the respiratory rate decreased 50% (RD₅₀) was 2,876 mg/m³ for JP-8 (Whitman and Hinz, 2001). Hydroprocessed ester and fatty acid (HEFA) and synthetic paraffinic kerosene (SPK) alternative jet fuels have been tested using the RD₅₀ assay and found to be less irritating than JP-8 (Stern *et al.*, 2013; Mattie *et al.*, 2018). The sensory irritant potential of Amyris has not been evaluated.

This study was designed to assess the sensory irritation potential of Amyris alternative jet fuel by establishing an RD₅₀ value from a series of 30-minute mouse inhalation exposures to multiple concentrations of Amyris. The testing method followed the ASTM International (formerly the American Society of Testing and Materials) guideline E981-04 “Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals” (ASTM, 2004).

3.0 Methods

3.1 Animal Husbandry

A total of 40 male Swiss-Webster [CrI:CFW(SW)] mice (*Mus musculus*) were purchased from Charles River Laboratories (Wilmington, MA) at approximately 3-4 weeks old and 16 to 18 g body weight. Mice were acclimated to the facility for seven days after arrival at the vivarium. Food and water were available *ad libitum* while mice were housed in the vivarium prior to exposure; neither were available during exposure. Animals were weighed upon receipt and again on the day of exposure.

3.2 Chemicals

Mice were exposed to either JP-8 or Amyris for 30 minutes. JP-8 was a blend of Jet-A with JP-8 additives obtained from the Air Force Research Laboratory Aerospace Systems Directorate: Turbine Engine Division, Fuels & Energy Branch (AFRL/RQTF)

and designated as POSF log book number 4658 by the fuels branch. The liquid sample was received by NAMRU-D approximately six months prior to exposure. Amyris Biotechnologies manufactured the biofuel, labeled as distilled farnesane, which was acquired by the AFRL/RQTF. A liquid sample (POSF log book number 10324) was received by NAMRU-D from the AFRL/RQTF approximately one month prior to exposure. Fuels were stored in amber glass jars at room temperature until use.

3.3 Exposure System

The generation system for the JP-8 and Amyris exposures (Figure 1) used a nebulizer (Spraying Systems Co., fluid cap model 1650 and air cap model 67147, Wheaton, IL) to create test substance concentrations in the generator tube from compressed air and pressurized liquid. The compressed air supply was prepared in-house and set to 15 L/min during exposures. The concentration of the atmosphere was determined by the amount of liquid material delivered into the nebulizer, which was controlled by a variable speed via a peristaltic pump (FMI pump, Fluid Metering Inc, model #RH00 with #QG50-1, Syosset, NY) for JP-8 exposures and a syringe pump (Model Pump 11 Elite, Harvard Apparatus, Holliston, MA) for Amyris exposures. A bypass valve was placed downstream of the generator tube to alternate delivery of either aerosolized fuel or clean air to the exposure system.

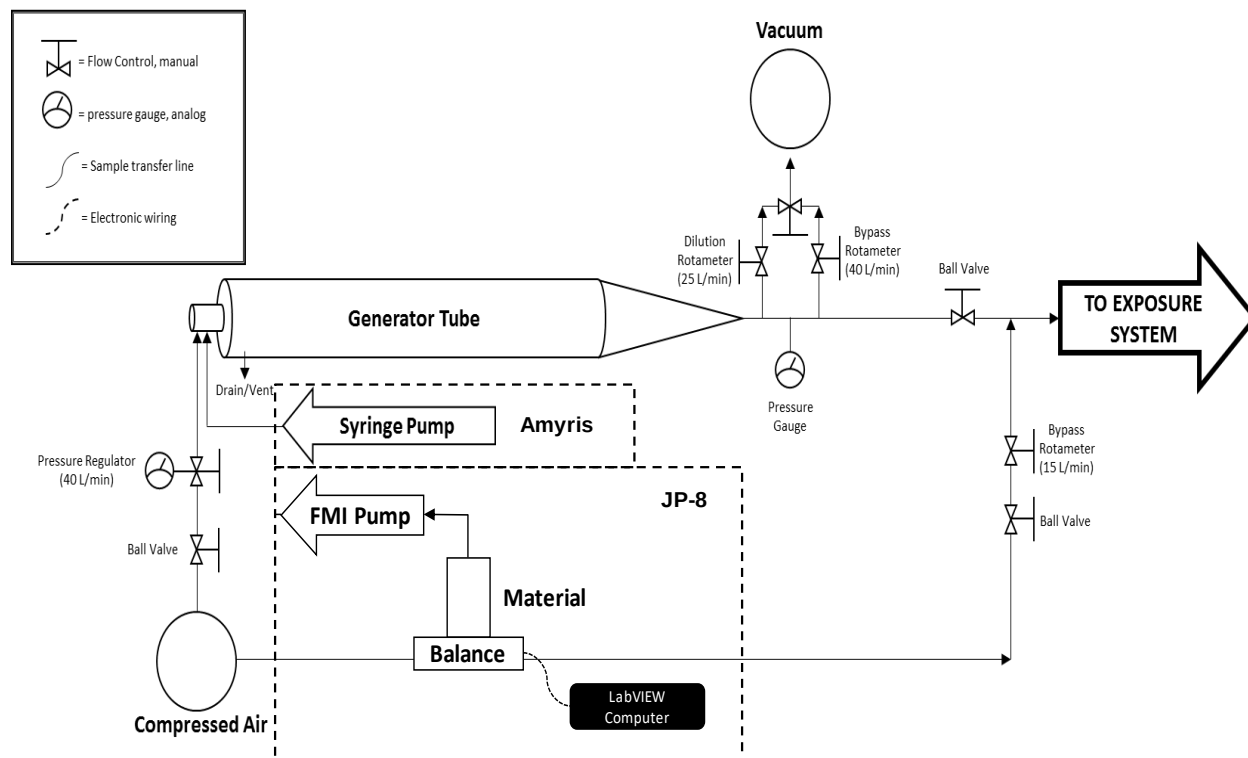


Figure 1. Jet Fuel Exposure Atmosphere Generation System

A schematic of the rodent exposure and respiratory rate measurement system is provided in Figure 2. Animals were exposed using a 52-port nose-only exposure unit (NOEU) made by Lab Products (Seaford, DE). The NOEU operated as a push-pull system where the air supply was under positive pressure and the exhaust pulled negative pressure. The supply was set at the target flow rate and the exhaust was adjusted to maintain a static pressure in the range of 0.00 to - 0.30 inches of water. The exposure atmosphere flowed from the generation system at a total flow rate of approximately 15 L/minute through the central, inner plenum and out through the delivery nozzles into the breathing zone of each animal at approximately 0.288 L/minute per open port.

During the exposures, animals were restrained in nose-only plethysmographs (Data Sciences International, New Brighton, MN). The plethysmographs consisted of a head chamber and a body chamber that were sealed off from each other by a latex dam that collared the neck of the mouse and was held in place by sandwiching between two flat, hard plastic sheets. The head chamber was attached to the NOEU, allowing the nose of the mouse to be exposed to the atmosphere being delivered from the port of the NOEU. The body chamber was used for respiratory rate measurement, which is described in Section 3.6.

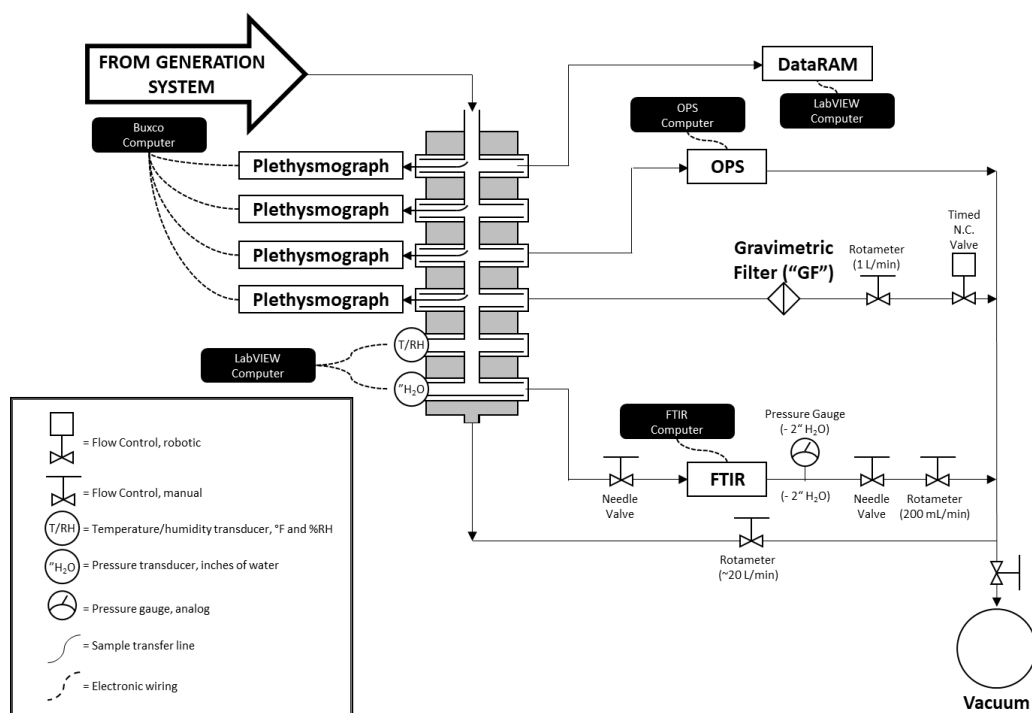


Figure 2. Rodent Exposure and Respiratory Rate Measurement System

Vapor concentrations were measured by a Fourier Transform Infrared Spectrophotometer (FTIR, Thermo Scientific model Nicolette IS10, Waltham MA) after passing through a 47 mm filter to remove aerosol particles. The FTIR signal was

processed on a computer that was networked to LabVIEW (National Instruments LabVIEW Software v.12.0, National Instruments Corporation, Austin, TX). A data acquisition device (NI 9207) recorded the conditions every 20 seconds in a NI cDAQ-9178 8-slot NI CompactDAQ USB chassis (National Instruments Corporation, Austin, TX). Temperature, relative humidity (Rotronics Instruments Inc. model HF532WB6XD1XX & model HC2-S, Hauppauge, NY) and static pressure (Building Automation Products Inc. model ZPS-05-SR09-EZ-ST-D, Gays Mills, WI) were collected at two of the NOEU ports and logged by the LabVIEW computer.

Aerosol concentrations were obtained by collection of aerosol from an exposure port on 47 mm thick fiberglass filters in a stainless steel filter holder (In-Tox Products, Moriarty, NM) for 3 to 20 minutes followed by gravimetric analysis. The total collection time was adjusted for each exposure to obtain a measurable amount of aerosol on the filter without overloading. A cascade impactor (In-Tox Products, Moriarty, NM) with seven stages that have cutoffs ranging from 0.5 to 8.3 μm was used to collect aerosol samples on 0.003" thick Mylar discs, cut to 1.5" diameter. The cascade impactor sampled from an NOEU port for gravimetric analysis during each exposure. Samples were collected once per exposure for 7-14 minutes. An optical particle sizer (OPS, TSI Inc. model 3330, Shoreview, MN) sampled aerosol from an NOEU port for 1 minute and calculated the aerosol size distribution three times during each exposure.

3.4 FTIR Instrument Calibration

FTIR spectrophotometer was used for the analysis of vapor concentrations of JP-8 and Amyris. Characterization of the FTIR began by analyzing a qualitative bag of each fuel vapor in air and determining the characteristic wavenumber for each fuel from the spectrum that was produced.

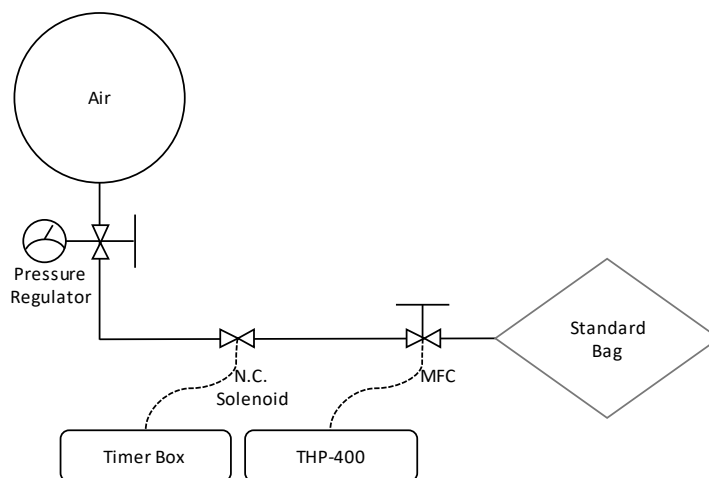


Figure 3. Standard Bag Preparation System

A standard curve for detection of fuel vapor concentration by the FTIR was obtained by standard bag methodology (Figure 3). Known amounts of liquid fuel and known amounts of compressed air were injected into polyvinylidene fluoride gas bags (SKC, SamplePro PVDF Sample Bag, City, State). Liquid sample was transferred by various sizes of gas-tight syringes. The air was transferred by mass-flow controller (MFC, HFC-202, 0 - 1000 mL/min, Teledyne-Hastings Instruments, Hampton, VA) controlled by a power pod (PowerPod; THP-400, Teledyne-Hastings Instruments, Hampton, VA) which was calibrated with a primary standard flow meter (BIOS; Model: BIOS Defender 30 - 30000 mL/min, MesaLabs, Lakewood, CO). After injection of the compressed air and liquid fuel, the bags were warmed with a heat gun (Leister Technologies, model Ghibli-M, Kaegiswil, Switzerland) on low setting approximately three feet from the bag for approximately 60 seconds.

A standard curve was developed for the short- and long-path gas cells on the FTIR for JP-8 and only the short-path gas cell on the FTIR for Amyris. The short path gas cell was used to measure target JP-8 vapor concentrations of $>1642 \text{ mg/m}^3$ on the FTIR, while the long-path gas cell was used to measure target JP-8 concentrations of $<1642 \text{ mg/m}^3$. The short-path gas cell was used for all target Amyris concentrations. Pre-exposure testing determined the optimal range of settings of the FMI pump and syringe pump necessary to obtain the approximate range of anticipated target concentrations for JP-8 and Amyris exposures, respectively.

3.5 Exposure Design

Five exposure concentrations were tested for each jet fuel, JP-8 and Amyris; four mice were tested at each concentration. For each fuel, $2,000 \text{ mg/m}^3$ was used as the starting concentration. This concentration was selected based on the starting point used in prior RD_{50} studies of alternative jet fuels (Stern et al., 2013; Hinz et al., 2012) and also corresponds a concentration slightly lower than the RD_{50} for JP-8 in the literature ($2,876 \text{ mg/m}^3$, Whitman and Hinz, 2001). The subsequent concentrations were chosen to attempt to obtain a concentration-response curve with at least one of the average respiratory changes being greater than a 50% decrease.

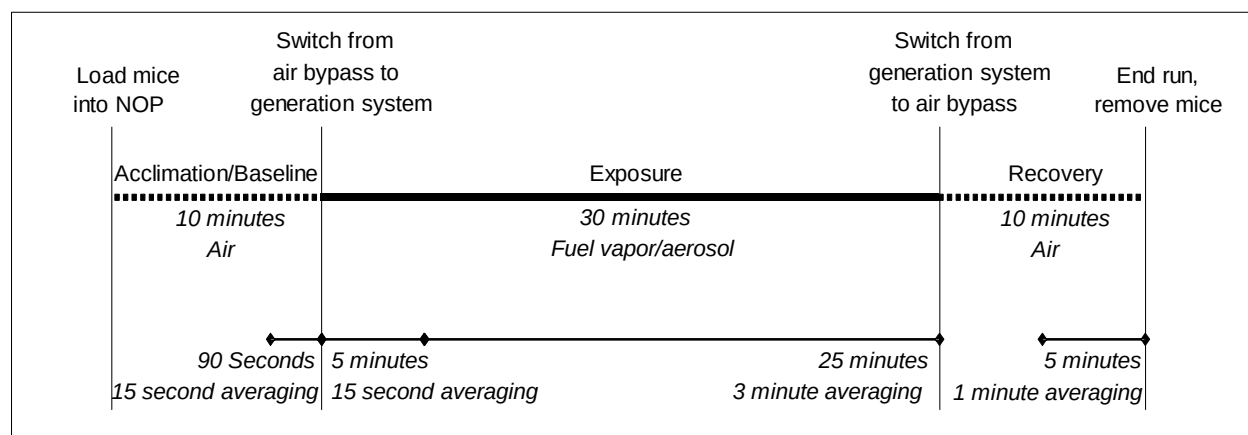


Figure 4. Timeline for JP-8 or Amyris Exposures

A maximum of two exposures were performed on each day of the study, with both Amyris and JP-8 exposure being completed over a period of three days. Because of the rapid growth of the mice during the acclimation period, larger animals were selected for the earlier exposures so that the weight of the animals would not surpass the recommended range of 22-28 g in the ASTM guidelines. By selecting the larger animals for the earlier exposures, the smaller mice were allowed more time to gain weight for the later exposures.

On the day of the study, the animals were transported from the vivarium to the study room approximately one hour prior to the beginning of the exposure. After being loaded into the exposure tubes and being placed on the NOEU, the animals were allowed an 8.5 minute acclimation period before the 1.5 minute baseline recordings (Figure 4). All animals breathed clean air from the bypass flow during acclimation and baseline. At the end of the baseline period, the jet fuel flow was switched on and the mice were exposed for 30 minutes to either Amyris or JP-8 (Figure 4). Upon completion of the JP-8 exposure period, the bypass flow was switched back on and mice breathed clean air during the 10-minute recovery period (Figure 4). JP-8 concentrations were monitored with the FTIR to ensure rapid washout of JP-8 from the exposure system. After completion of the Amyris exposure, the mice were removed from the exposure tower and placed on the counter during the 10 minute recovery period because it took much longer for Amyris concentrations within the system to drop to background levels. Amyris concentrations in room air in proximity to the animals were monitored with the FTIR during the recovery period.

Animals were returned to the vivarium for euthanasia immediately following the recovery period. Mice were euthanized by inhalation of carbon dioxide in a closed chamber to produce unconsciousness, followed by cervical dislocation. A complete macroscopic gross necropsy was performed on all animals, and included observations of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia.

3.6 Breathing Rate Measurement

Flow transducers (Flow Measurement Transducers, DataSciences International, New Brighton, MN) with a sampling rate of one sample/second were used to detect changes in pressure within the body chamber of each nose-only plethysmograph that corresponded to the motion of the chest wall of each mouse. The time series of these pressure changes produced the respiratory waveform for each mouse. The waveforms were collected and stored, and the respiratory rates were calculated from the respiratory waveforms using Buxco Finepointe software (DataSciences International, New Brighton, MN). Respirations were measured during the entire acclimation, exposure, and recovery periods, but only the periods designated in Figure 4 were used for analysis.

3.7 Data Analysis

Following the ASTM guidelines, the baseline respiratory rate for each mouse was calculated as the average of six 15-second intervals immediately preceding the test agent exposure period. The respiratory rate for each 15-second interval of the first five

minutes of exposure and at three-minute intervals for the remainder of the 30-minute exposure period was calculated. Data for all files was processed by visually identifying and removing segments of high frequency, low periodicity noise that typically corresponded to movement of the animal within the plethysmograph tubes.

For each mouse, the average respiratory rate during the first five minutes and subsequent 25 minutes of exposure was calculated, with the 25-minute average being used for the development of the concentration-response curve. Respiratory rates were calculated at one-minute intervals for the 10-minute post-exposure period and the mean of the last five minutes of the recovery period represented the recovery response for each mouse.

The RD_{50} was calculated by developing a concentration-response curve using the average respiratory rate decreases at each of the five exposure concentrations as the dependent variable, and the common logarithm of the exposure concentration as the independent variable for each jet fuel. The data sets were used to prepare the concentration-response regression to calculate an RD_{50} value (the concentration required to reduce the respiratory rate by 50%) and 95% confidence limits. Data fit was assessed using the method of least squares (Armitage, 1971; ASTM, 2004).

4.0 Results

4.1 Animals

Mice that underwent JP-8 exposures weighed an average of 16.7 g on the day of arrival at the vivarium, with a weight range of 15.5 g to 18.6 g. Because of the rapid growth of the mice, the larger mice were selected for the earlier exposures, and the smaller mice were selected for the later exposures. On the day of exposure, the mice weighed an average of 28.0 g, with a range of 25.5 g to 31.5 g.

Animal #6 that was intended to be exposed to the 1109 mg/m³ concentration was found deceased during pre-exposure loading of the animals into the nose-only plethysmographs. Necropsy by the attending veterinarian revealed that the mouse had a cervical dislocation. The injury may have resulted from imprecise positioning of the animal in the nose-only plethysmograph or excessive struggling by the mouse upon confinement or both.

Animal #4 was removed after approximately 20 minutes following the start of exposure to the 511 mg/m³ concentration because the mouse appeared to be unresponsive. Clinical observations of the animal were normal following removal of the animal from the tube; however, no further respiratory data were obtained from the animal. While the unresponsive state may have been an effect of exposure, it is possible that airway constriction resulted from the mouse's movements in the tube. Animal #4 weighed the most among the mice exposed to JP-8; this may have increased the likelihood of some

type of airway constriction occurring. All other clinical observations of the animals during JP-8 exposure and all observations at necropsy were normal.

The mice that were exposed to Amyris weighed an average of 17.6 g on the day of arrival at the Vivarium, with a range of 15.9 g to 20.0 g. Similar to the JP-8 exposures, because of the rapid growth of the mice, the larger mice were selected for the earlier exposures, and the smaller mice were selected for the later exposures. On the day of exposure, the mice weighed an average of 28.9 g, with a range of 26.8g to 33.1g.

All clinical observations of animals during Amyris exposures and all observations at necropsy were normal.

4.2 Environmental Conditions

The environmental conditions during the exposure are given in Table 1. The average temperature, relative humidity and static pressure during the time periods that the animals were on the exposure tower for each concentration are listed as the mean $\pm$ the standard deviation of all measurements. The ambient temperature was intentionally increased for the 3719 mg/m³ exposure with the intent of driving more of the Amyris into the vapor phase in the exposure tower. The 1551 mg/m³ Amyris exposure was run on the same day, so there was a similar temperature elevation during that exposure.

Table 1. Environmental Conditions during Exposure

Test Substance	JP-8				
Exposure Concentration (mg/m ³)	511	1109	1861	3069	5028
Temperature (°C)	20.4 $\pm$ 0.10	22.3 $\pm$ 0.03	21.8 $\pm$ 0.08	20.5 $\pm$ 0.05	20.9 $\pm$ 0.09
Relative Humidity (%)	3.7 $\pm$ 0.09	5.0 $\pm$ 0.09	4.7 $\pm$ 0.11	3.0 $\pm$ 0.07	3.2 $\pm$ 0.31
Static Pressure (in H ₂ O)	-0.4 $\pm$ 0.5	-0.2 $\pm$ 0.16	-0.2 $\pm$ 0.13	-0.2 $\pm$ 0.10	-0.2 $\pm$ 0.11
Test Substance	Amyris				
Exposure Concentration (mg/m ³)	545	1551	1858	2756	3719
Temperature (°C)	22.6 $\pm$ 0.08	25.4 $\pm$ 0.10	22.1 $\pm$ 0.07	20.5 $\pm$ 0.09	25.3 $\pm$ 0.11
Relative Humidity (%)	4.2 $\pm$ 0.56	2.2 $\pm$ 0.37	5.3 $\pm$ 0.61	4.6 $\pm$ 0.65	4.2 $\pm$ 0.48
Static Pressure (in H ₂ O)	-0.2 $\pm$ 0.10	-0.2 $\pm$ 0.07	-0.3 $\pm$ 0.10	-0.3 $\pm$ 0.15	-0.2 $\pm$ 0.08

4.3 Exposure Atmosphere

Exposure concentrations and aerosol size data are given in Table 2. JP-8 and Amyris concentrations are listed for each of the five exposures performed for each substance. Total concentration represents the sum of the aerosol and vapor concentrations. The FTIR data for the exposure were averaged from the samples represented by the black dots in Appendices B and C. Because of the delay of stabilization of the concentrations within the gas cells in the FTIR, only the data points from the plateau of the concentration time series were used to define the exposure average. The plateau in concentration was defined as the first/last data points where the concentration changed less than 1% from the previous/following data point. Similarly, because of this delay, the

start and end of the plateau is offset from the 0- and 30-minute time points, which represent the beginning and ending time points of fuel exposure in Appendices B and C, respectively. The exception was the 545 mg/m³ Amyris exposure, for which there was no plateau and the data points from the initial increase in concentration to final decrease in concentration were used to calculate the average concentration. The optical monitoring of aerosol, which has a faster response time than the FTIR, confirmed that the delay in change of the chemical concentrations on the FTIR was due to the slow FTIR response time. There were sharp changes in the chemical concentration at the 0- and 30- minute time points on the optical instrument that would not have been observed had the actual concentration in the system been slow to reach the target level.

For both substances, aerosol was a larger proportion of the exposure atmosphere as the total concentration increased. Aerosol size was consistent across all concentrations of JP-8 that were tested, but varied with concentration for Amyris. The highest Amyris concentration tested was the maximum obtainable by the generation system due to the relatively low vapor pressure of Amyris compared to JP-8. Cascade impactor data were not acquired during the Amyris exposure.

Table 2. Characterization of Exposure Atmosphere for JP-8 and Amyris

Test Substance	JP-8				
Exposure Concentration					
Total Concentration (mg/m ³)	511	1109	1861	3069	5028
Mean Vapor Concentration (mg/m ³)	504 ± 19	1109 ± 39	1815 ± 24	2723 ± 72	4095 ± 178
Aerosol Concentration (mg/m ³)	7	0	46	346	933
% Aerosol	1	0	2	11	19
Aerosol Size - Optical Particle Sizer					
Mass Median Diameter (µm)	2.2	2.0	2.3	2.7	2.7
Geometric Standard Deviation	1.6	1.6	1.6	1.6	1.6
Aerosol Size - Cascade Impactor					
Median Particle Size (µm)	ND	2.0	0.5	2.3	2.7
Geometric Standard Deviation	ND	1.4	4.5	2.0	2.2
Test Substance	Amyris				
Exposure Concentration					
Total Concentration (mg/m ³)	545	1571	1858	2756	3719
Mean Vapor Concentration (mg/m ³)	536 ± 288	1551 ± 65	1758 ± 62	1473 ± 76	2399 ± 79
Aerosol Concentration (mg/m ³)	9	20	100	1283	1320
% Aerosol	2	1	5	47	35
Aerosol Size - Optical Particle Sizer					
Mass Median Diameter (µm)	0.7	0.9	1.6	2.2	2.4
Geometric Standard Deviation	0.7	0.8	1.5	2.1	2.1

4.4 Animal Response Data for JP-8 Exposure

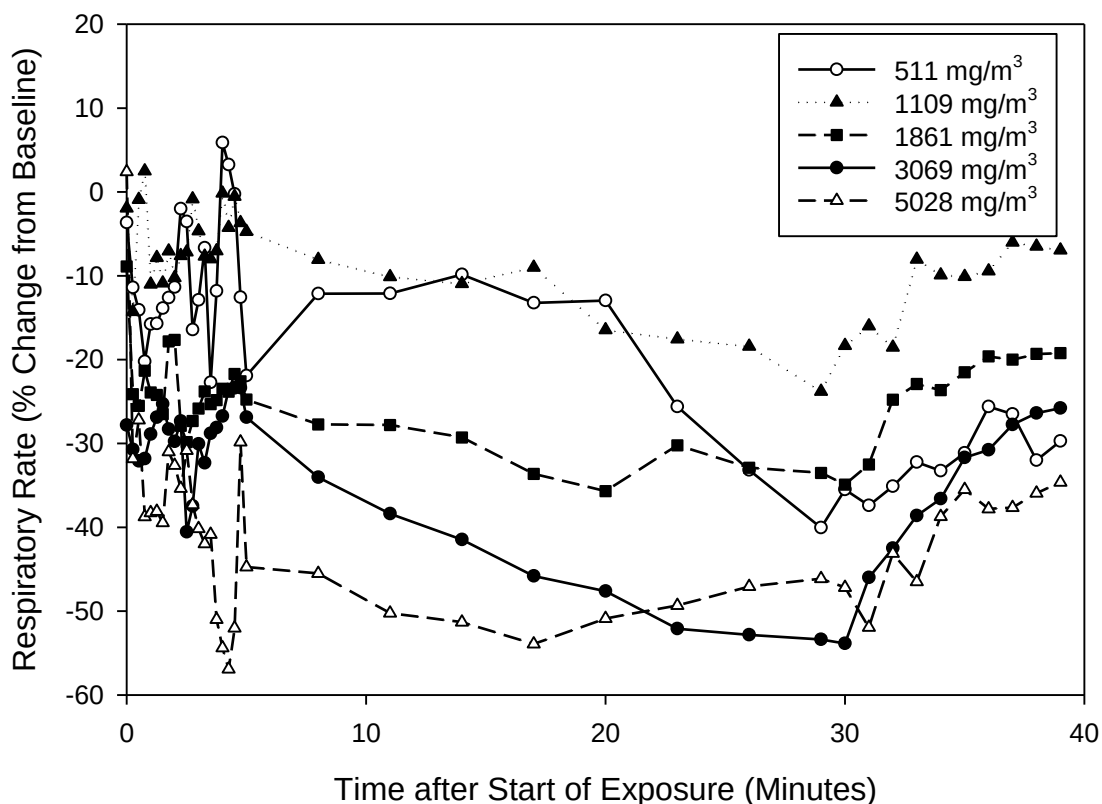


Figure 5. Average Respiratory Rate Response to JP-8 Exposures

In general, the average respiratory rate of each exposure group trended lower with increasing concentrations of JP-8 (Figure 5). After an initial rapid decline in the first few minutes of exposure, the respiratory rate decrease slowed and plateaued within the 30 minutes of exposure for four of the five groups. The exception was the 511 mg/m³ exposure group, for which the respiratory rate began a secondary decline to an approximately 40% decrease following an initial plateau at approximately 12% . Although all five exposure groups demonstrated a recovery trend following the cessation of the JP-8 exposure, only the recovery of the 1109 mg/m³ exposure group approached a return to the pre-exposure baseline.

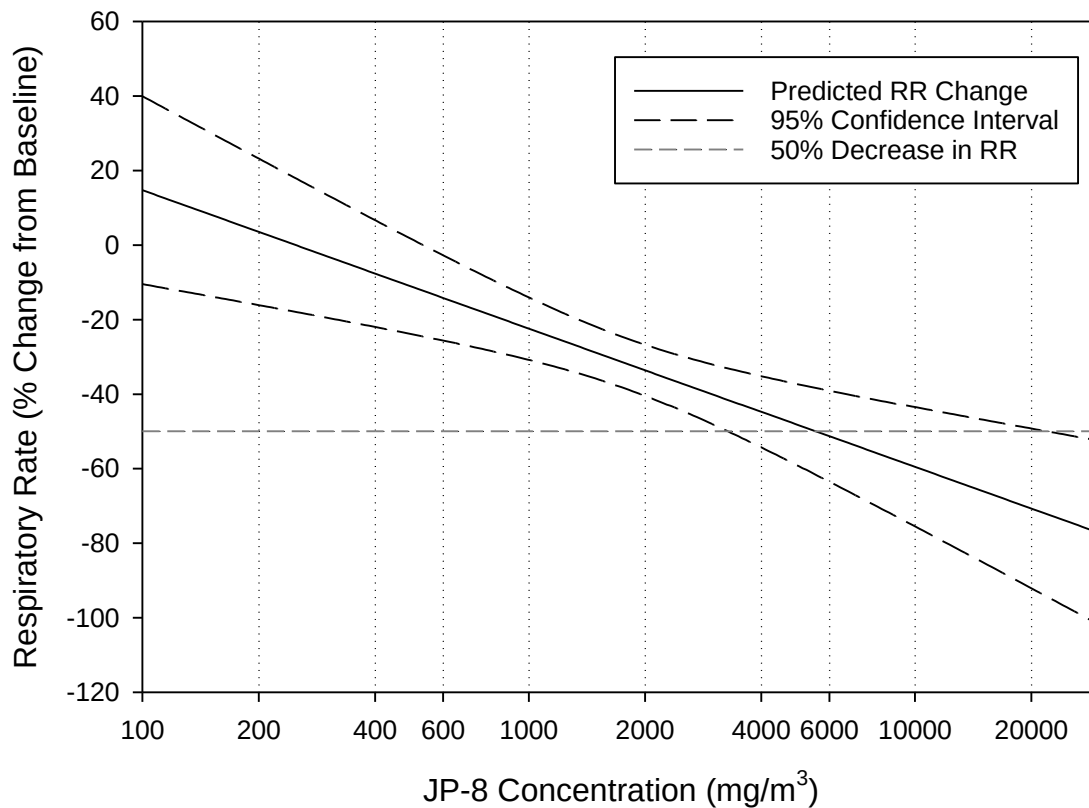


Figure 6. Predicted Respiratory Rate Response to JP-8 Exposure

The predicted respiratory rate response to JP-8 exposure was determined to have a slope of -37 ± 9 and a y-intercept of 89 ± 30 . The predicted regression is plotted in Figure 6. The RD_{50} concentration calculated from this regression was 5537 mg/m^3 with a 95% confidence interval of 3263- 21576 mg/m^3 .

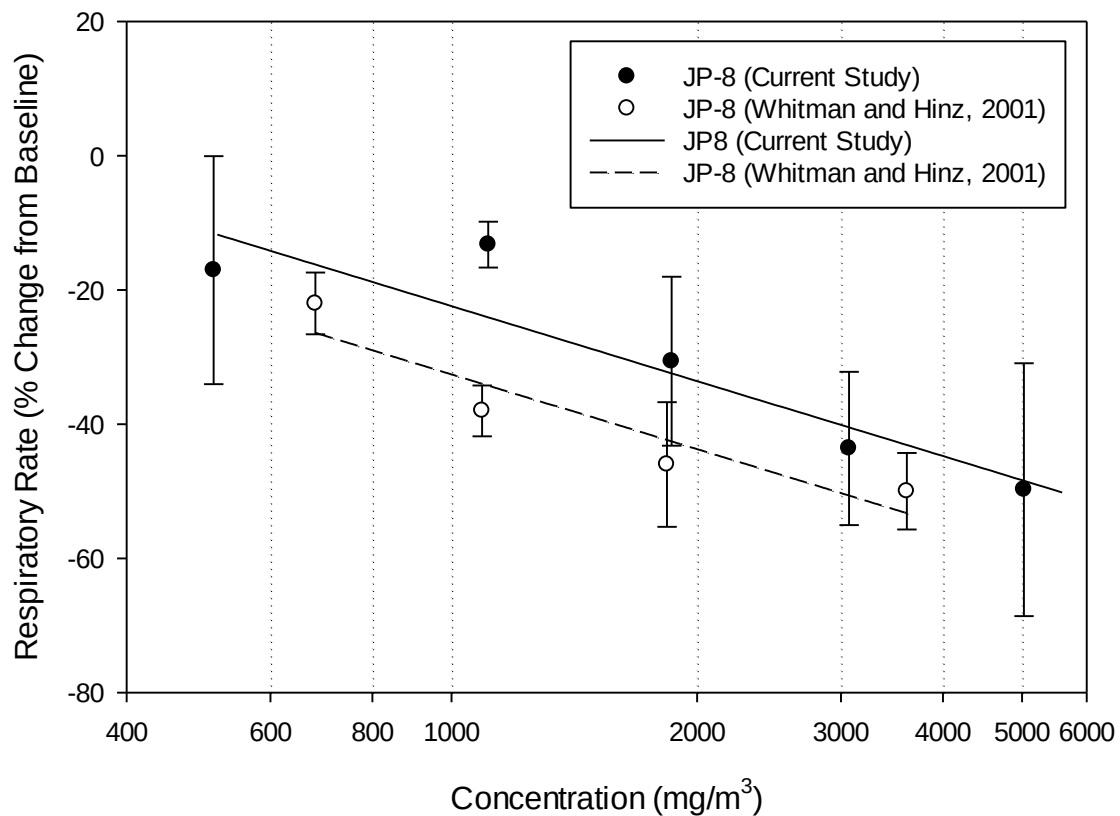


Figure 7. Concentration-Response Curve for Current Study Compared to Whitman and Hinz, 2001.

The comparison of the regression line of the present study to that of Whitman and Hinz, 2001 is displayed in Figure 7. The regression line in the present study is right-shifted from the Whitman and Hinz respiratory rate responses to JP-8 exposure, with the Whitman and Hinz study also obtaining a lower RD_{50} of value of 2876 mg/m³.

4.5 Animal Response Data for Amyris Exposure

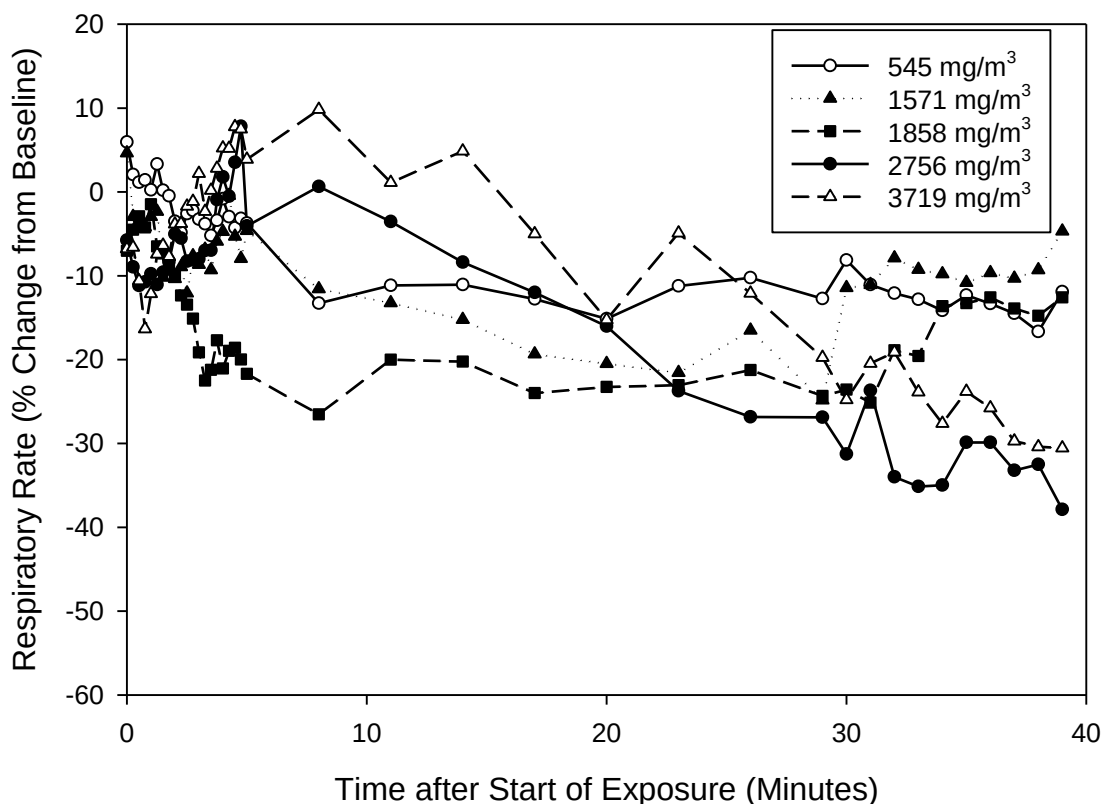


Figure 8. Average Respiratory Rate Response to Amyris Exposures

The average respiratory rate of lowest three exposure groups (545, 1571, and 1858 mg/m³) all followed the same pattern as the responses to JP-8 exposure, with a plateau in respiratory rate decrease after an initial variability at the onset of exposure (Figure 8). Similarly, there was a slight recovery following the end of exposure. However, at higher concentrations (2756 mg/m³ and 3719 mg/m³), the average respiratory rate did not decrease for the first ten minutes after the start of exposure, but once it did, it continued to decrease even through the recovery period. None of the respiratory rate decreases reached greater than 40% below baseline for any of the exposure groups.

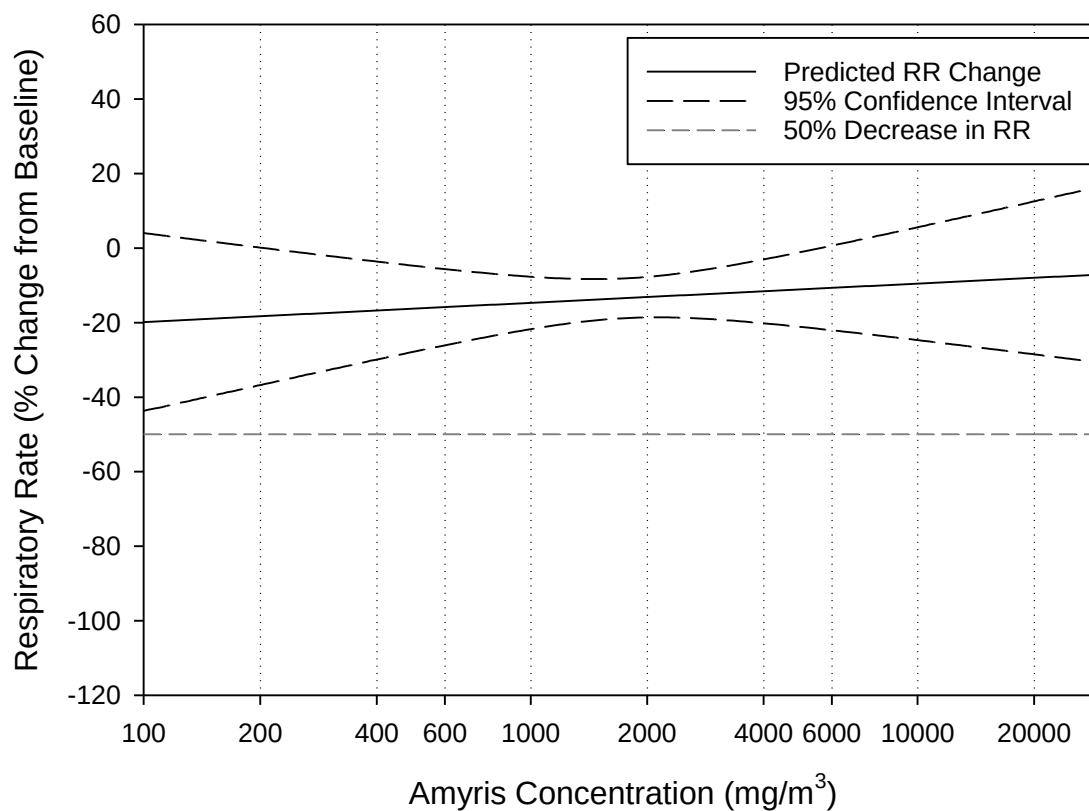


Figure 9. Predicted Respiratory Rate Response to Amyris Exposure

The predicted regression line (Figure 9) for the respiratory rate response to Amyris exposure was determined to have a slope of 5 ± 9 and a y-intercept of -30 ± -29 . Therefore, the respiratory rate only minimally changed with exposure to increasing concentrations of Amyris.

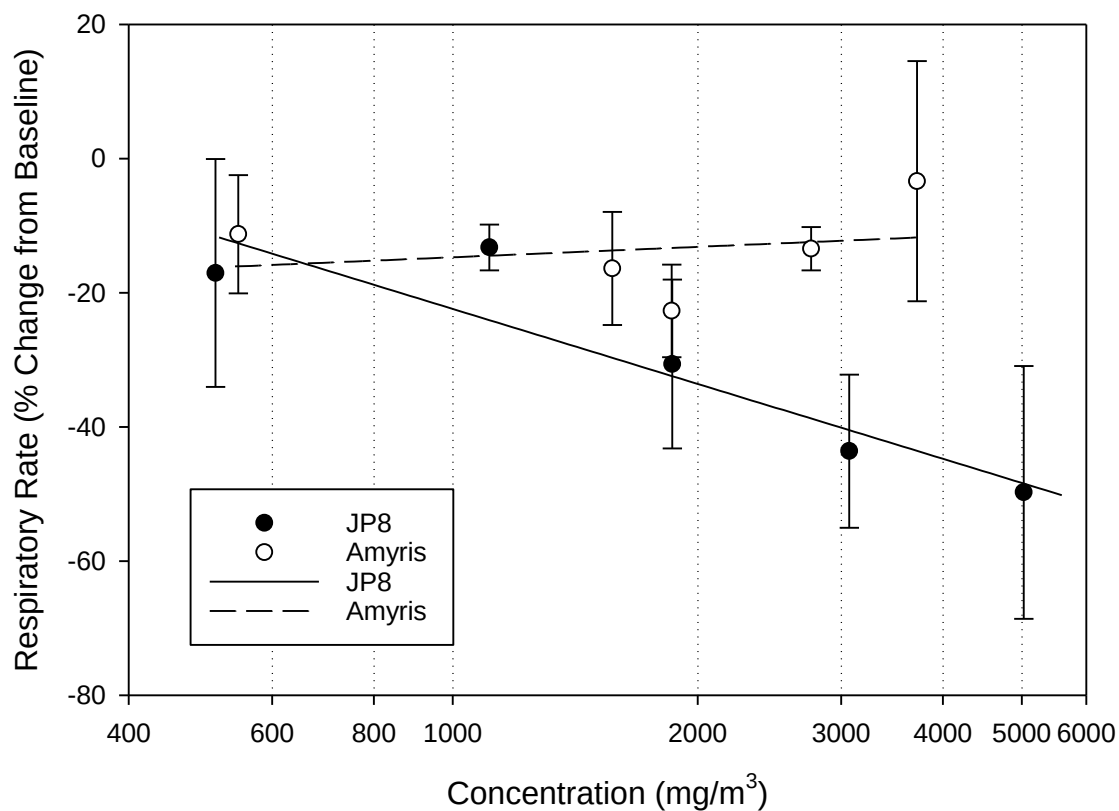


Figure 10. Concentration-Response Curve for JP-8 and Amyris Exposures

The r^2 value for the Amyris concentration response curve was 0.02 and the p-value was 0.57, indicating that there is not a significant linear relationship between the common logarithm of Amyris exposure concentration and respiratory rate. Additionally, attempting to calculate an RD_{50} concentration would result in an unrealistic negative concentration. Therefore, an RD_{50} value was not obtained for Amyris exposure. A comparison of the predicted regression lines for respiratory rate response to JP-8 and Amyris exposure is displayed in Figure 10.

Table 3. Parameters of Predicted Concentration-response Curves

Test Substance	JP-8	Amyris
Slope	-37 ± 9	5 ± 9
Intercept	89 ± 30	-30 ± 29
r ²	0.49	0.02
p-value	<0.001	0.57
RD ₅₀ (mg/m ³)	5537	ND
RD ₅₀ 95% Confidence Interval (mg/m ³)	3263-21576	ND

Characteristics of the concentration-response curves calculated for JP-8 and Amyris, and the RD₅₀ value and 95% confidence interval calculated for JP-8 are given in Table 3. The RD₅₀ value for JP-8 was higher than the previously published value (Whitman and Hinz, 2001) with a larger 95% confidence interval.

5.0 Discussion

The objective of the study was to determine the sensory irritation potential of Amyris C15 by determining its RD₅₀ value. While ASTM sensory irritancy testing guidelines were followed, none of the Amyris concentrations tested produced an average respiratory depression greater than 40% at any time point during or after the exposure. There was also no concentration-response relationship between Amyris concentration and respiratory rate change. Consequently, it was concluded that Amyris was not a sensory irritant at the concentrations tested in the study. The highest concentration of Amyris tested (3719 mg/m³ total concentration) was believed to be the maximum concentration obtainable with the exposure system due to the relatively low vapor pressure of Amyris compared to JP-8 and other bio-derived jet fuels. Higher concentrations of vapor may have been able to be obtained by a heating-based generation system, such as the heated glass “counter current” generator used by Whitman and Hinz to generate vapor test atmospheres. However, the sprayer based generation system better reflects real-world exposures.

Although it did not appear that Amyris was a sensory irritant, the respiratory changes associated with the two highest exposure concentrations, 2756 mg/m³ and 3719 mg/m³, could be indicative of pulmonary-level effects. The relatively slow onset of respiratory rate decrease and absence of recovery post-exposure are both consistent with respiratory rate changes observed with exposures to irritants in mice through a tracheal cannula that bypassed the trigeminal nerve in the upper respiratory tract (Gagnaire, *et al.*, 1989; Weyel and Schaffer, 1985; Alarie, *et al.*, 1987). The response could be related to pulmonary irritancy or could be associated with the development of pulmonary inflammation.

Although the vapor concentrations of the 2756 mg/m³ and the 3719 mg/m³ exposures were approximately 0.8-1.3 times greater than the 1858 mg/m³ concentration, the aerosol concentrations of these exposures were approximately 12- 13 times higher than with the 1858 mg/m³ exposure. Therefore, the apparent pulmonary level effects may be related to the high concentrations of aerosol exposure and potentially support further experimentation to determine what, if any, pulmonary toxicity results from exposure to high concentrations of Amyris aerosol.

The sensory irritation assay methods used to test Amyris in the study were validated for our lab by exposing Swiss-Webster mice to JP-8 to verify that a concentration-response curve and RD₅₀ value could be obtained with a known sensory irritant. An RD₅₀ value of 5537 mg/m³ was obtained, which supports the conclusion that the lack of response observed with the Amyris exposure was a result of the lack of sensory irritancy and not a consequence of methodology.

The RD₅₀ value obtained in the present study was approximately double the published RD₅₀ value for JP-8 of 2876 mg/m³ (Whitman and Hinz, 2001). Additionally, the 95% confidence interval was much wider in the present study, compared to the 95% confidence limits of 2107 to 3925 mg/m³ in the Whitman and Hinz study. Whitman and Hinz used JP-8 POSF 3404 (3509 with additives). JP-8 is not a single fuel produced by one manufacturer with a rigid ingredient list. Instead, JP-8 is a kerosene-cut petroleum fuel that conforms to a military performance specification (MIL-DTL-83133) and the content varies with crude source, refinery and purchase. The difference between the two studies could be a result of variation in the JP-8 fuels tested.

Additionally, the results could be influenced by study differences including animal use practices and data analysis techniques. The ASTM guideline recommends a weight range of 22 to 28 g, however because of the lengthy acclimation period required by the WPAFB vivarium to ensure colony health, animals used were up to three grams larger than the recommended range. This deviation from the guideline may have increased within-group variability in the respiratory rate responses that led to a large confidence interval. Additionally, with the overall larger animals in the present study, the lower apparent response could have been due to a lower delivered dose at comparable exposure concentrations.

There were also differences in the method in which the average respiratory rate response for each animal was calculated. In the present study, the respiratory rate change was calculated as the average of the last 25 minutes of the exposure, which was deemed appropriate because of the typical plateau in the data following the decrease in respiratory rate over the first five minutes. In the Whitman and Hinz study, the respiratory rate change was calculated as the “lowest representative rate during exposure”; however, there were no published criteria for determining the lowest representative rate.

Using the average of the last 25 minutes of exposure was advantageous over determining the lowest representative rate because it was an objective measure of determining the respiratory rate response for each animal. Additionally, it made the JP-8 response data more comparable to the Amyris data for which there wasn't always a clearly defined plateau in respiratory rate decrease. However, using the average of the last 25 minutes likely resulted in a lower average percent change for each animal because, although the average generally represented a plateau in the respiratory rate decrease, it did not select a specific nadir in respiratory rate to average that would be free from dilution of periods where a decrease may still be occurring. Therefore, the higher RD₅₀ value for JP-8 in the present study compared to Whitman and Hinz (2001) could be partially explained by the method for determining the average respiratory rate response for each animal.

6.0 Conclusion

Amyris C15 was not a sensory irritant at similar concentrations to which JP-8 was found to induce sensory irritation in the current study, as well as in previous literature.

7.0 References

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Appendix A. Animal Body Weight Data

Table A1. Individual and Average Body Weights on Day of Receipt of Animals and on Day of Exposure

JP-8 (mg/m ³)	ID	Weight (g) At Receipt	Average (g) At Receipt	Weight (g) At Exposure	Average (g) At Exposure
511	4	18.3	17.1 ± 0.8	31.5	30.5 ± 1.1
	10	16.6		29.9	
	12	16.7		31.4	
	19	16.8		29.2	
1109	7	17.4	17.2 ± 1.2	28.4	27.6 ± 1.1
	15	15.9		26.3	
	17	18.3		28.1	
1861	3	15.8	16.7 ± 0.7	27.0	27.3 ± 0.6
	13	17.3		27.8	
	14	16.8		27.8	
	16	17		26.7	
3069	1	17.6	17.0 ± 1.4	25.7	26.8 ± 3.1
	2	16.2		25.5	
	5	15.5		24.5	
	11	18.6		31.4	
5028	8	16.1	16.7 ± 0.6	26.7	27.6 ± 1.4
	9	17.4		26.3	
	18	16.8		28.3	
	20	16.3		29.2	
Amyris (mg/m ³)	ID	Weight (g) At Receipt	Average (g) At Receipt	Weight (g) At Exposure	Average (g) At Exposure
545	30	18.2	18.4 ± 0.8	28.5	31.3 ± 1.8
	33	20.0		30.6	
	38	16.5		28.2	
	39	18.8		29.0	
1571	21	18.5	18.4 ± 1.5	27.2	29.1 ± 1.1
	24	16.6		26.8	
	27	17.5		26.8	
	29	16.6		27.3	
1858	23	17.6	16.9 ± 0.7	29.2	29.9 ± 0.8
	32	18.3		30.6	
	34	19.5		33.1	
	40	18.4		32.5	
2756	25	16.5	16.9 ± 1.1	29.1	28.3 ± 0.3
	31	17.5		28.3	
	35	17.4		29.9	
	37	16.1		28.2	
3719	22	15.9	17.3 ± 0.9	28.3	27.0 ± 0.3
	26	16.5		28.6	
	28	18.5		28.0	
	36	16.8		28.5	

Appendix B. Time Series of FTIR Measurements During JP-8 Exposures

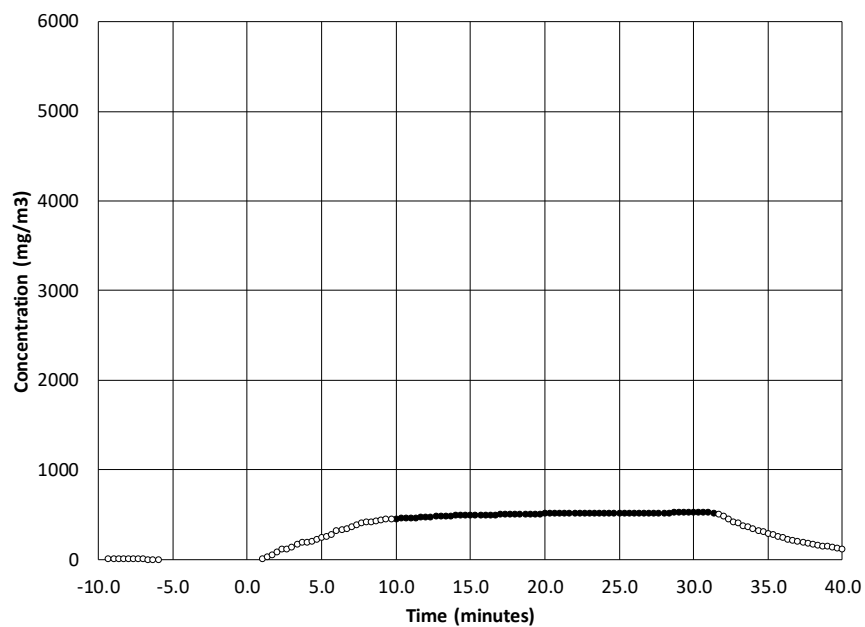


Figure B1. Time Series Measurements of JP-8 Vapor Concentration During 511 mg/m³ Exposure

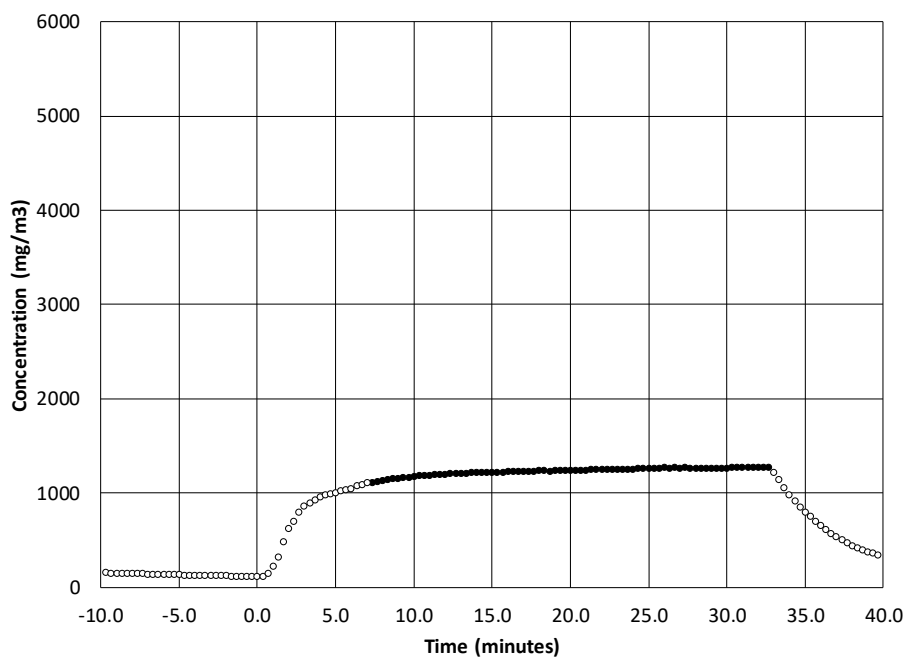


Figure B2. Time Series Measurements of JP-8 Vapor Concentration During 1109 mg/m³ Exposure

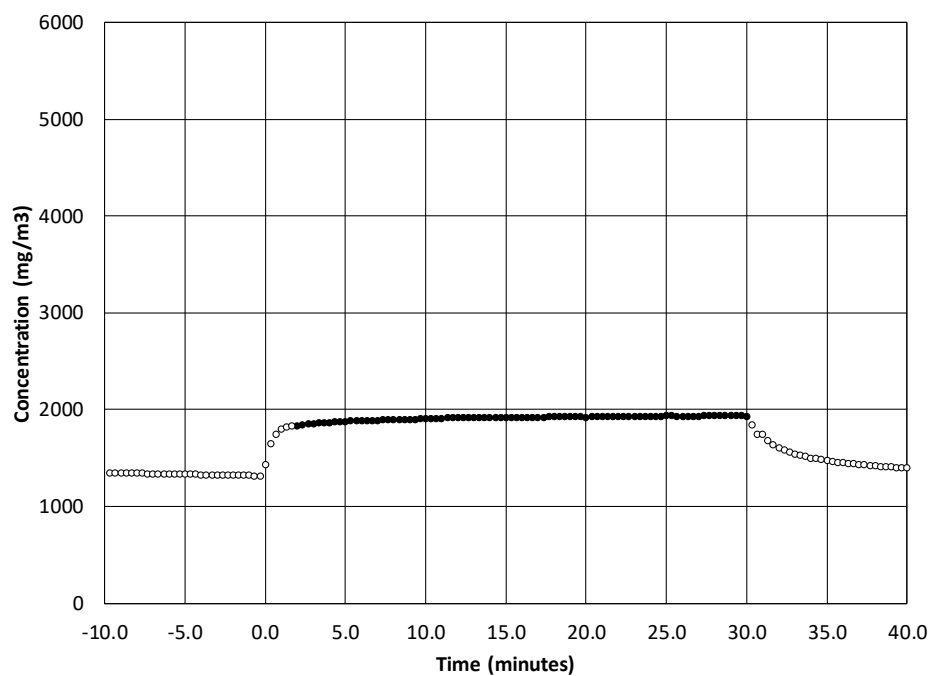


Figure B3. Time Series Measurements of JP-8 Vapor Concentration During 1861 mg/m³ Exposure

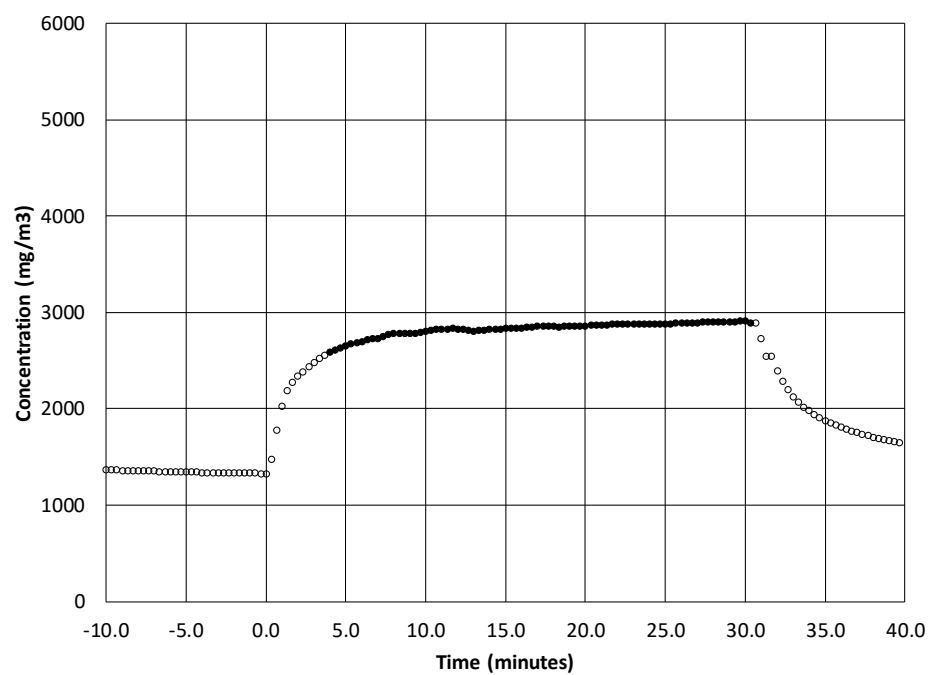


Figure B4. Time Series Measurements of JP-8 Vapor Concentration During 3069 mg/m³ Exposure

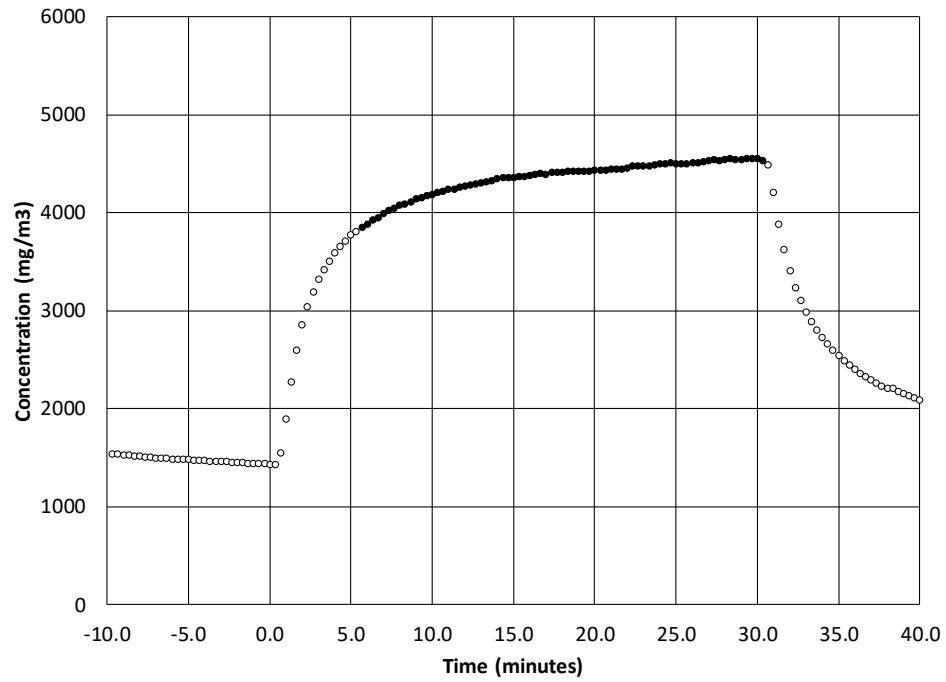


Figure B5. Time Series Measurements of JP-8 Vapor Concentration During 5028 mg/m³ Exposure

Appendix C. Time Series of FTIR Measurements During Amyris Exposures

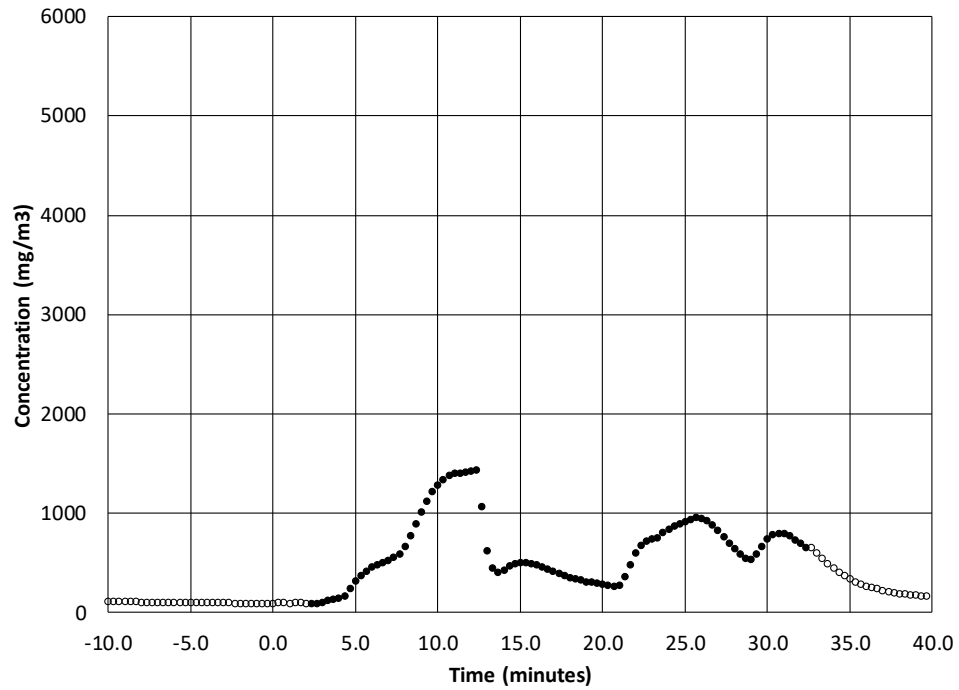


Figure C1. Time Series Measurements of Amyris Vapor Concentration During 545 mg/m³ Exposure

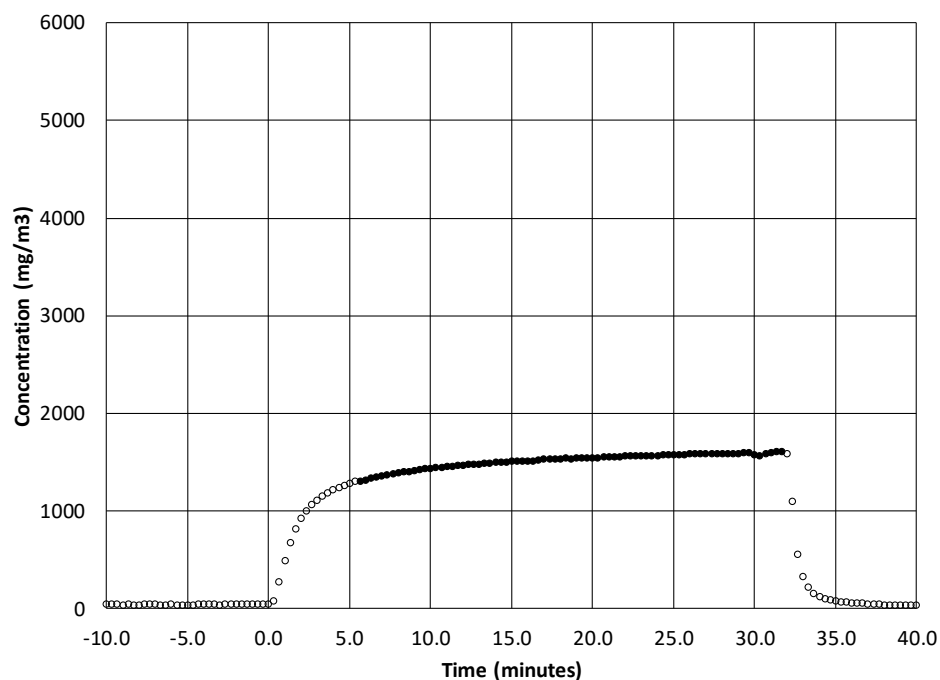


Figure C2. Time Series Measurements of Amyris Vapor Concentration During 1571 mg/m³ Exposure

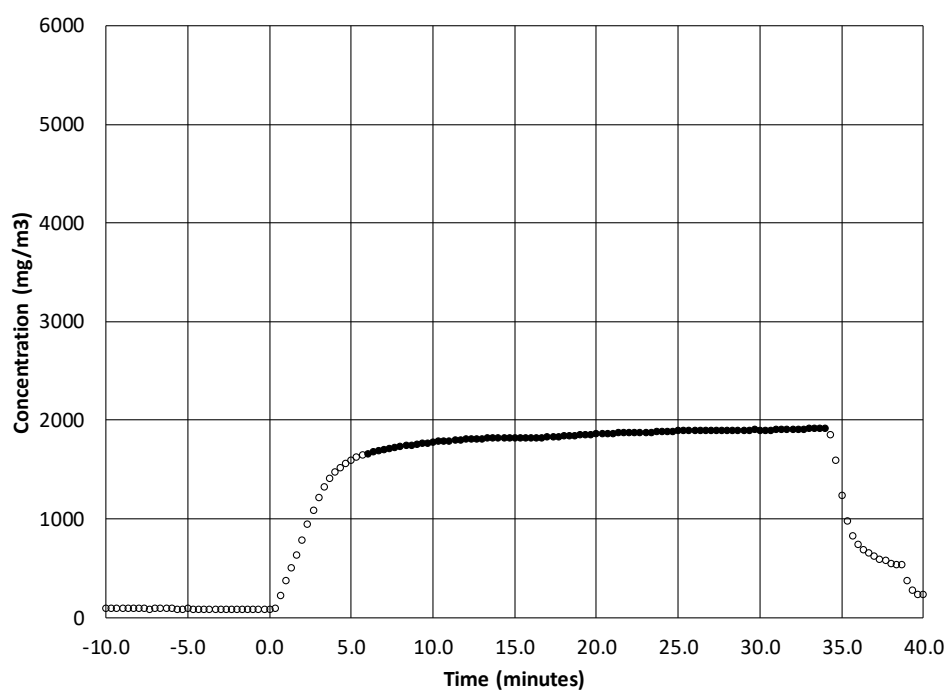


Figure C3. Time Series Measurements of Amyris Vapor Concentration During 1858 mg/m³ Exposure

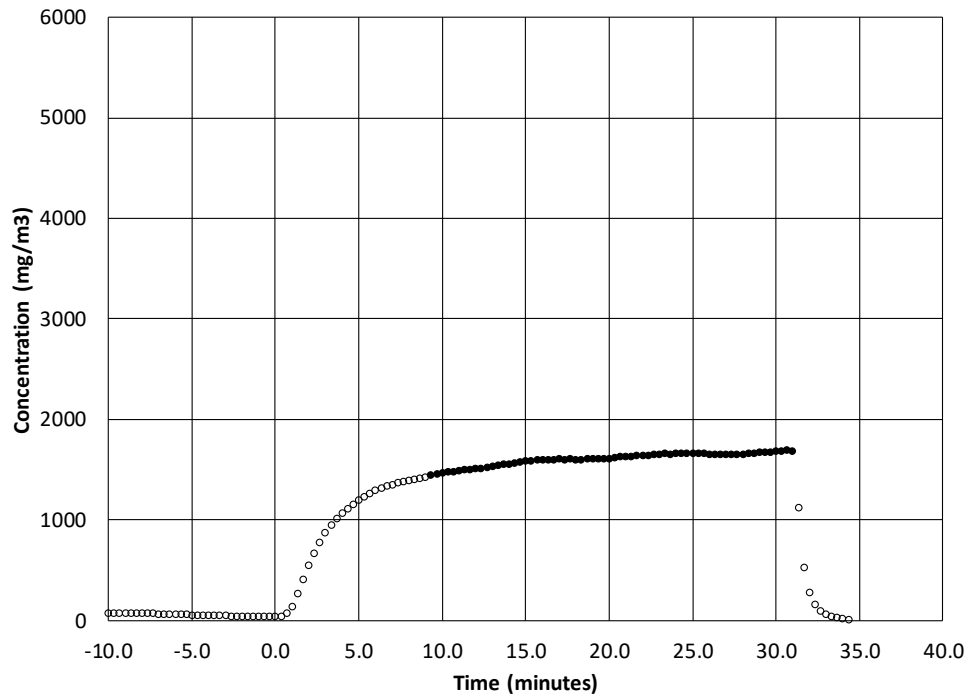


Figure C4. Time Series Measurements of Amyris Vapor Concentration During 2756 mg/m³ Exposure

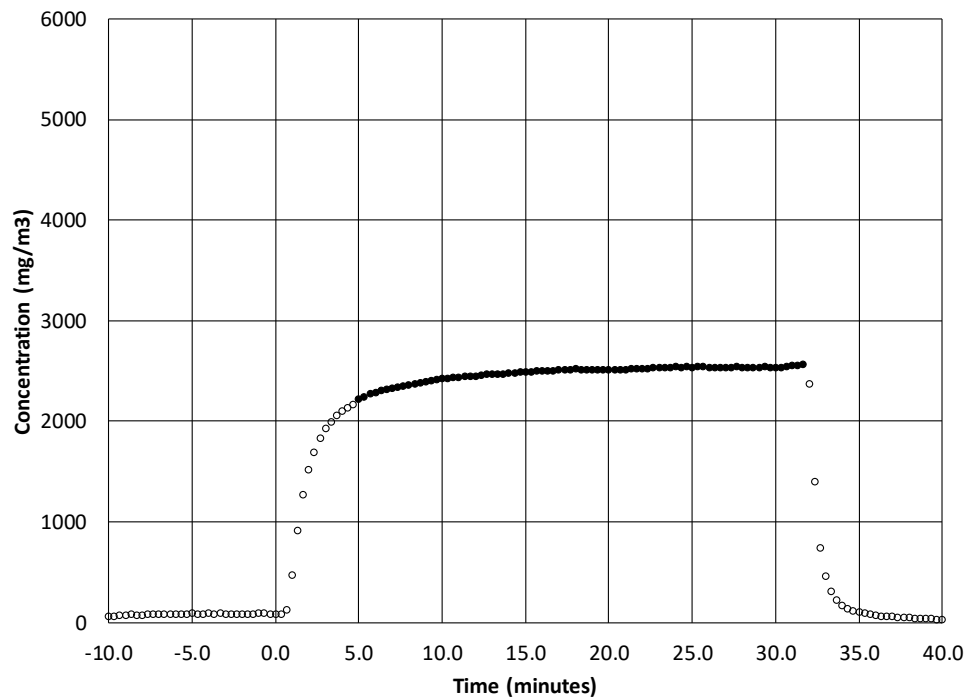


Figure C5. Time Series Measurements of Amyris Vapor Concentration During 3719 mg/m³ Exposure

Appendix D. Individual Respiratory Rate Responses to JP-8 Exposure

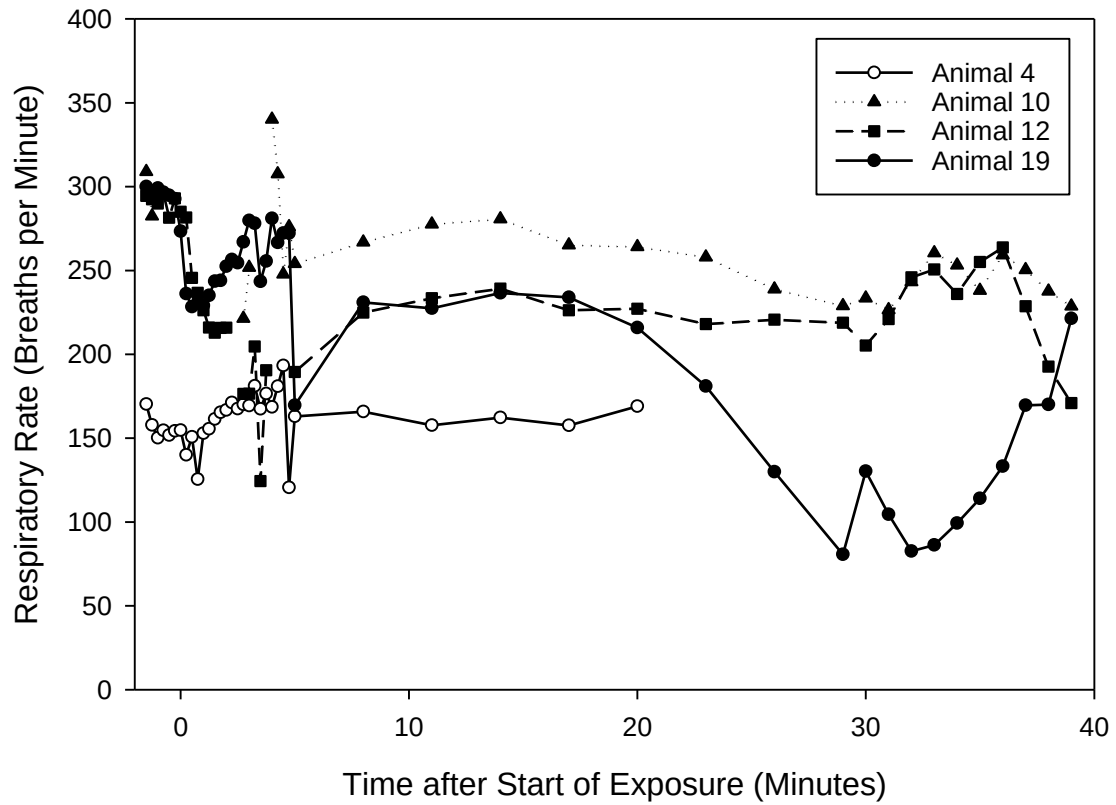


Figure D1. Average Respiratory Rate Response to 511 mg/m³ JP-8 Exposure

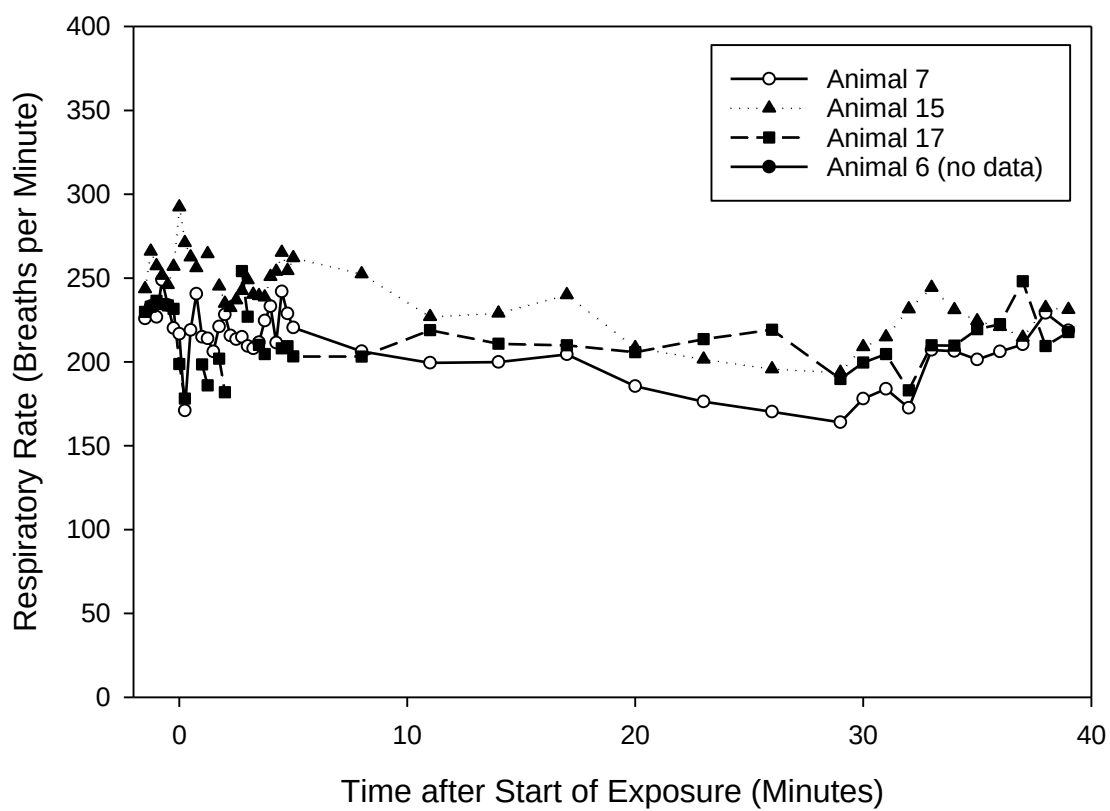


Figure D2. Average Respiratory Rate Response to 1109 mg/m³ JP-8 Exposure

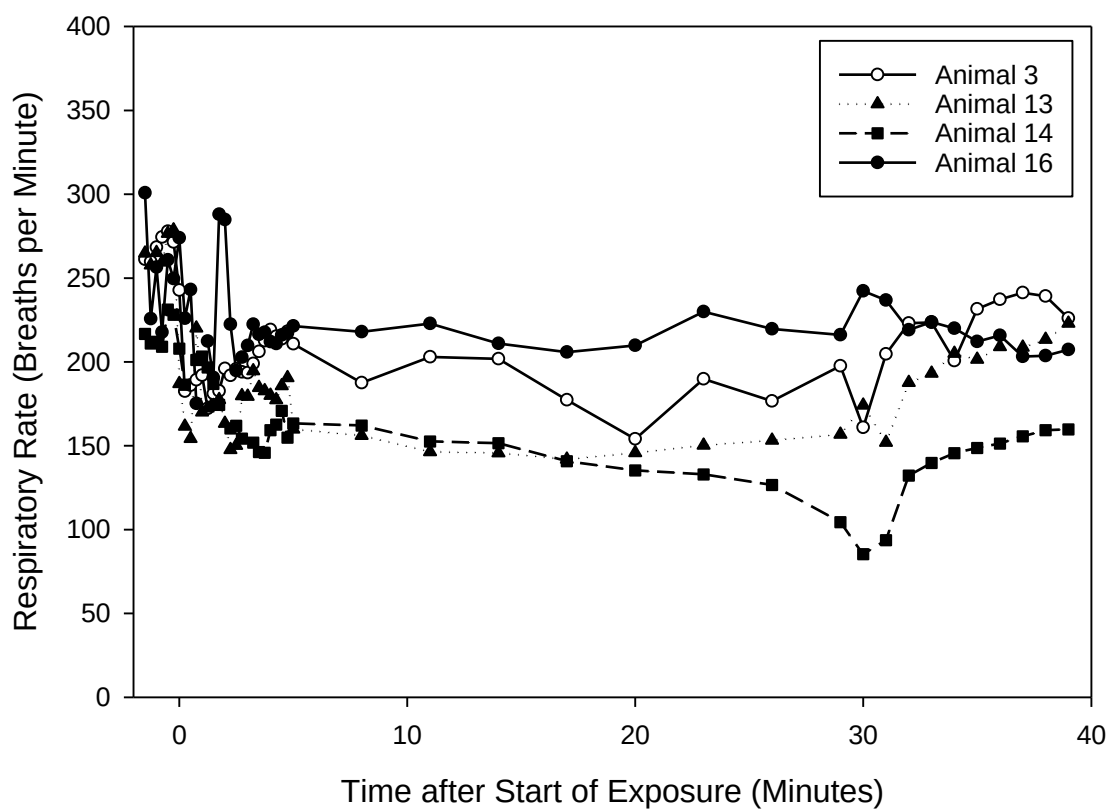


Figure D3. Average Respiratory Rate Response to 1861 mg/m³ JP-8 Exposure

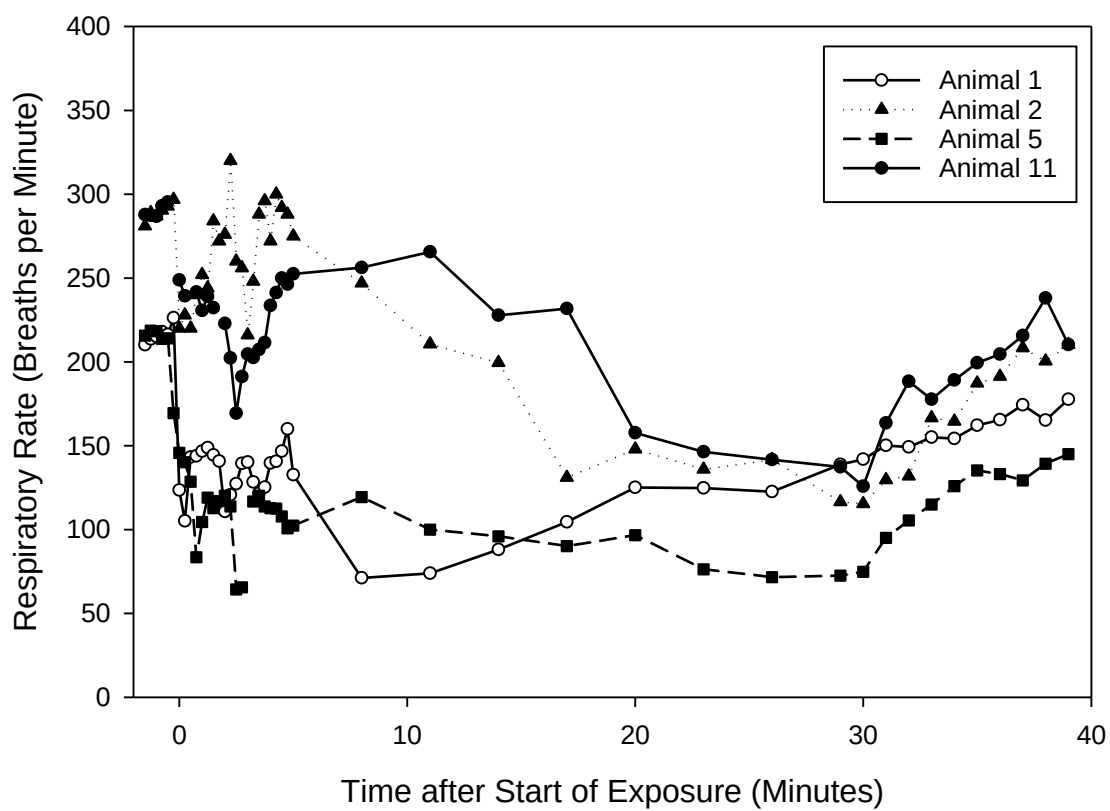


Figure D4. Average Respiratory Rate Response to 3069 mg/m³ JP-8 Exposure

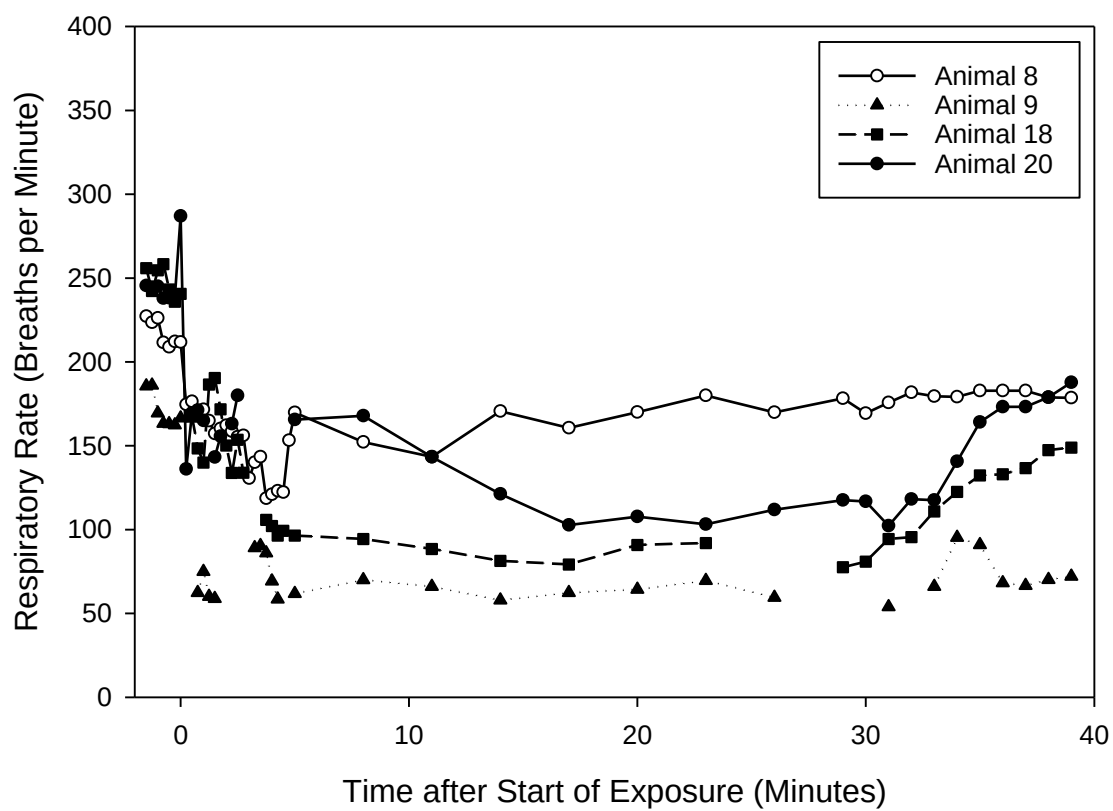


Figure D5. Average Respiratory Rate Response to 5028 mg/m³ JP-8 Exposure

Table D1. Individual Respiratory Rate Averages for JP-8 Exposure

JP-8 (mg/m ³)	ID	Respiratory Rate (breaths per minute, Avg±SD)				Respiratory Rate (% of baseline, Avg±SD)		
		Baseline 1.5 mins	Exposure		Recovery Last 5 mins	Exposure		Recovery Last 5 mins
			1st 5 mins	Last 25 mins		1st 5 mins	Last 25 mins	
511	4	156±7	162±18	162±5	ND	4±12	4±3	ND
	10	296±19	274±44	259±17	243±12	-7±15	-12±6	-18±4
	12	291±5	215±42	221±14	222±40	-26±14	-24±5	-24±14
	19	296±3	255±18	189±55	162±41	-14±6	-36±19	-45±14
1109	6	ND	ND	ND	ND	ND	ND	ND
	7	231±10	217±15	191±19	213±11	-6±7	-17±8	-8±5
	15	254±8	252±15	223±25	225±27	-1±6	-12±10	-11±3
	17	233±2	205±21	208±9	224±15	-12±9	-11±4	-4±6
1861	3	269±7	199±17	189±17	235±6	-26±6	-30±7	-13±2
	13	267±9	177±17	151±6	211±8	-33±6	-44±2	-21±3
	14	218±9	172±21	141±19	155±5	-21±10	-35±9	-29±2
	16	252±29	223±30	217±7	208±5	-12±12	-14±3	-17±2
3069	1	217±5	135±14	109±26	169±7	-38±6	-50±12	-22±3
	2	290±5	264±30	178±57	200±10	-9±10	-38±20	-31±3
	5	208±19	111±21	92±16	136±6	-47±10	-56±8	-35±3
	11	290±4	223±23	202±55	214±15	-23±8	-30±19	-26±5
5028	8	218±8	154±23	166±12	181±2	-30±11	-24±6	-17±1
	9	172±11	82±32	64±4	74±10	-53±19	-63±3	-57±6
	18	248±9	148±40	88±7	140±8	-40±16	-65±3	-44±3
	20	242±4	175±44	127±26	176±9	-28±18	-48±11	-27±4

Appendix E. Individual Respiratory Rate Responses to Amyris Exposure

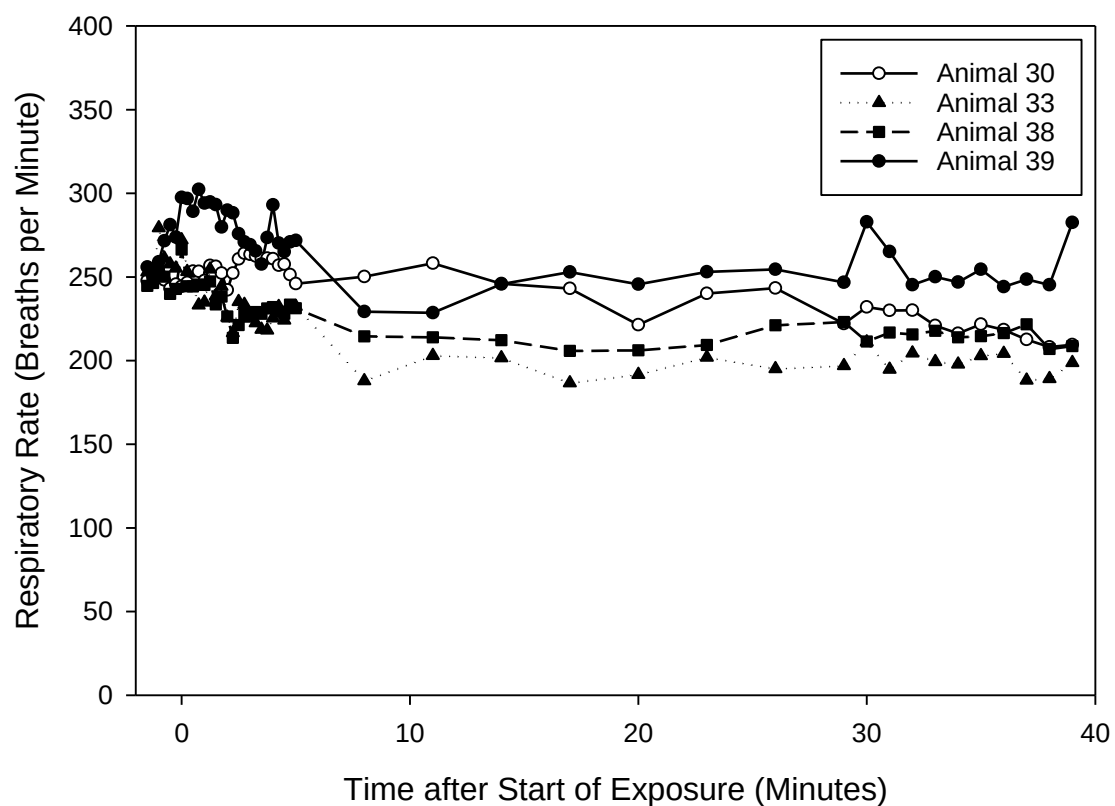


Figure E1. Average Respiratory Rate Response to 545 mg/m³ Amyris Exposure

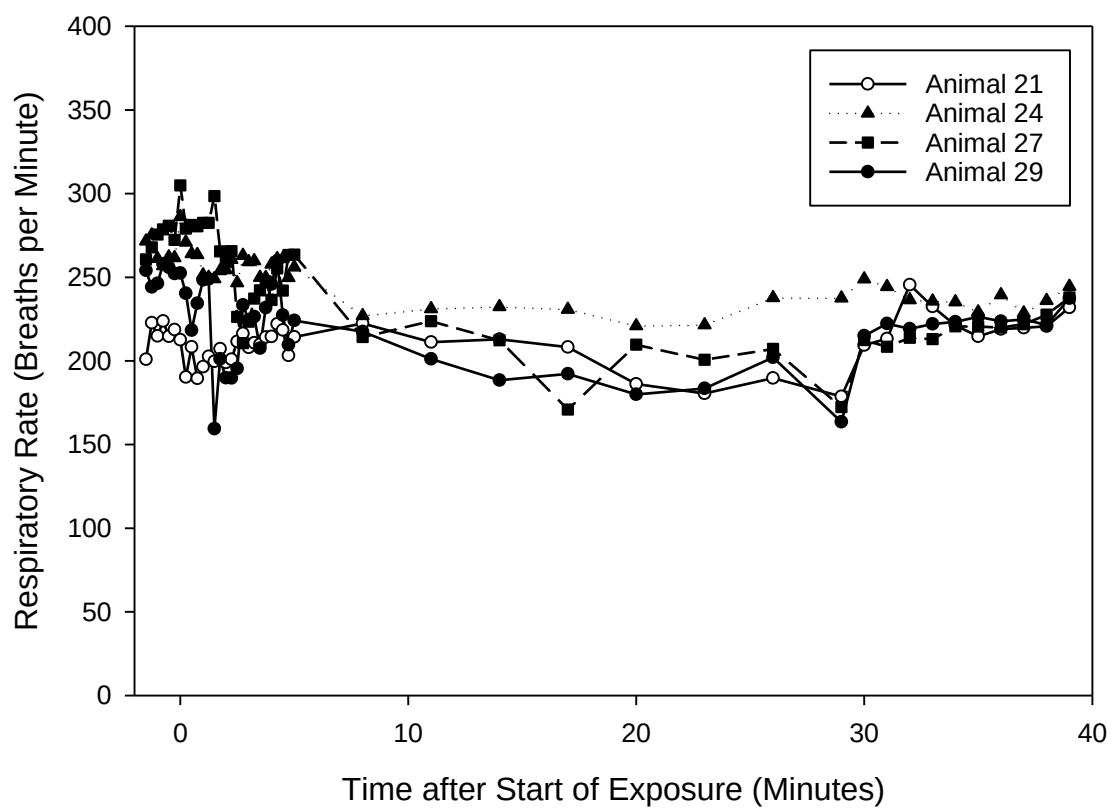


Figure E2. Average Respiratory Rate Response to 1571 mg/m³ Amyris Exposure

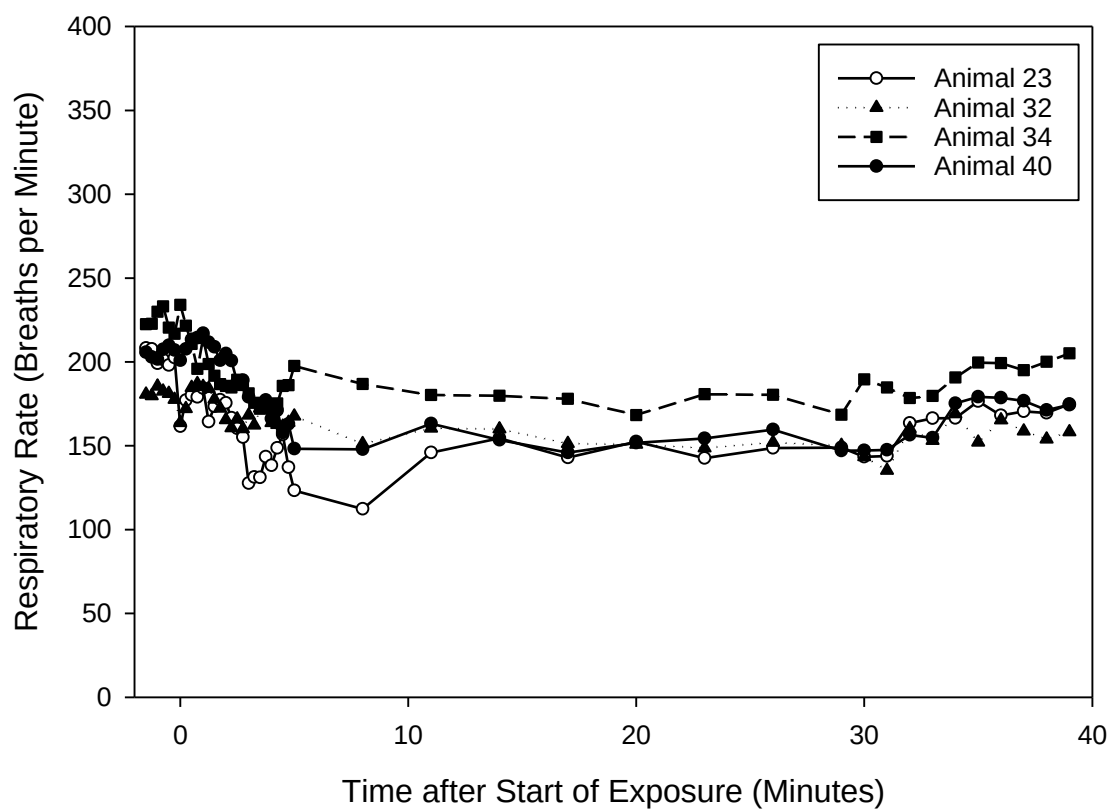


Figure E3. Average Respiratory Rate Response to 1858 mg/m³ Amyris Exposure

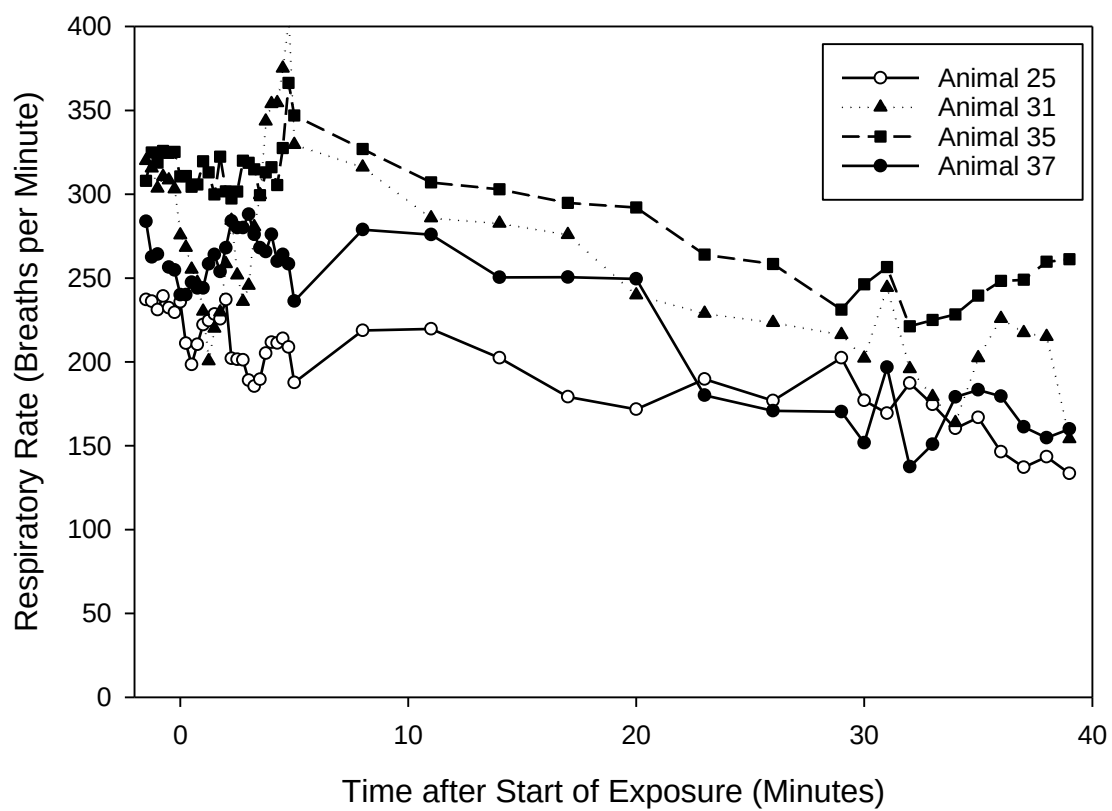


Figure E4. Average Respiratory Rate Response to 2756 mg/m³ Amyris Exposure

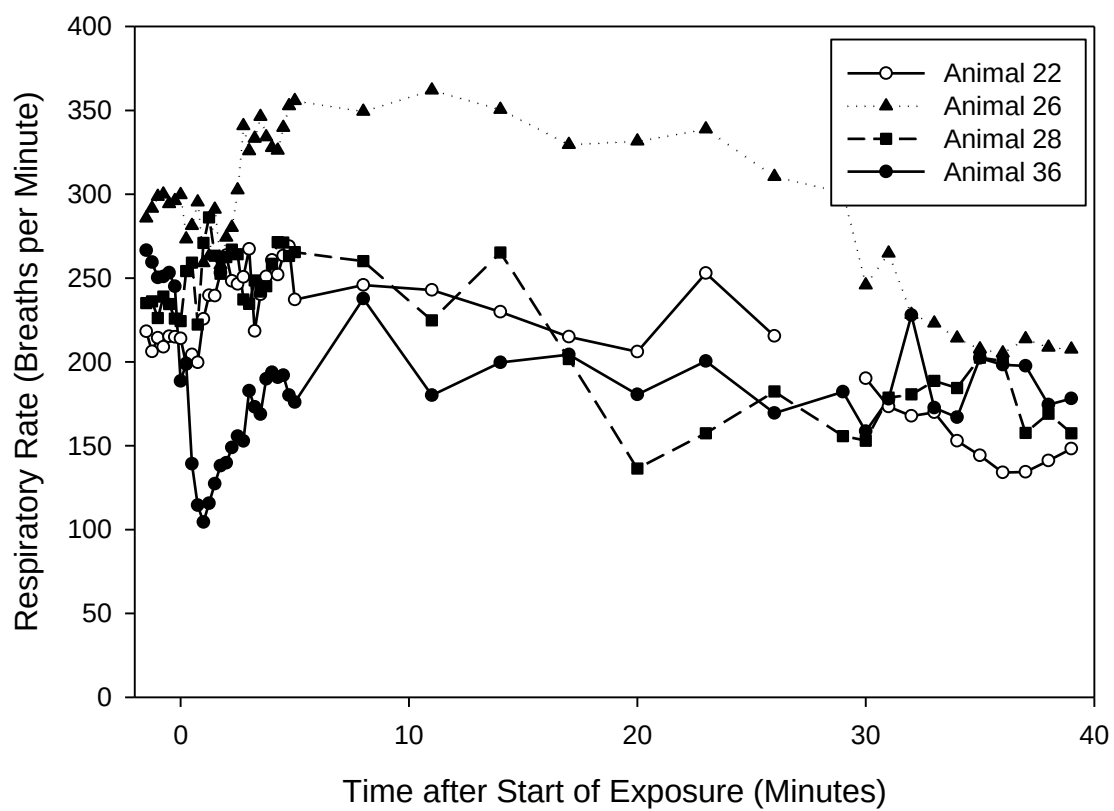


Figure E5. Average Respiratory Rate Response to 3719 mg/m³ Amyris Exposure

Table E1. Individual Respiratory Rate Averages for Amyris Exposure

Amyris (mg/m ³)	ID	Respiratory Rate (breaths per minute, Avg±SD)				Respiratory Rate (% of baseline, Avg±SD)		
		Baseline	Exposure		Recovery	Exposure		Recovery
		1.5 mins	1st 5 mins	Last 25 mins	Last 5 mins	1st 5 mins	Last 25 mins	Last 5 mins
545	30	248±3	255±6	241±12	214±6	3±3	-3±5	-14±2
	33	259±11	234±14	200±14	197±8	-10±5	-23±5	-24±3
	38	246±5	235±12	215±8	214±6	-5±5	-13±3	-13±2
	39	265±12	282±13	248±13	255±16	6±5	-7±5	-4±6
1571	21	216±8	207±9	201±17	221±6	-4±4	-7±8	2±3
	24	265±7	258±9	233±11	236±7	-3±4	-12±4	-11±3
	27	273±7	259±26	208±28	226±8	-5±9	-24±10	-17±3
	29	252±5	222±26	195±19	227±6	-12±10	-23±7	-10±2
1858	23	203±4	158±19	141±14	172±4	-22±9	-30±7	-15±2
	32	181±3	170±9	155±6	158±5	-6±5	-15±4	-13±3
	34	224±6	191±17	180±9	200±4	-15±8	-20±4	-11±2
	40	206±3	193±19	152±6	176±3	-6±9	-26±3	-14±2
2756	25	234±4	211±15	194±18	145±13	-10±6	-17±8	-38±6
	31	310±7	281±57	267±41	203±29	-9±18	-14±13	-35±9
	35	321±7	314±15	292±36	252±9	-2±5	-9±11	-22±3
	37	264±12	263±15	229±44	168±13	-1±6	-13±17	-37±5
3719	22	213±4	240±23	231±17	140±6	13±11	8±8	-34±3
	26	294±5	305±32	337±21	209±3	4±11	14±7	-29±1
	28	233±5	255±17	206±51	178±23	10±7	-12±22	-24±10
	36	254±8	160±30	192±21	190±13	-37±12	-24±8	-25±5