MATERIALS AND METHODS

1. COV inhalation exposure system design

Crude oil vapor (COV) was generated using a computer-controlled syringe pump (Teledyne ISCO 500D; Lincoln, NE), which injected a flow of heated (85 F) oil into a collision atomizer (3076; TSI Inc.; Shoreview, MN). This stainless-steel atomizer used a constant 4 liter per minute (l/min) of clean dry air to create a fine mist of oil droplets. The air and oil droplet mixture exited the atomizer and was combined with 40 l/min of additional heated (85 F) clean air. This additional dilution air was humidified to achieve a constant 40% relative humidity inside the exposure chamber. The humidity was maintained constant during all exposures by custom software that made continuous adjustments to the ratio of air that passed through a heated water bath and the amount that bypassed it. All air flows were metered by using mass flow controllers (Alicat Scientific; Tucson, AZ). The atomizer's exhaust and dilution air combined then entered a 1 inch outside diameter 3-foot long PTFE tube. This tube acted as an evaporation and mixing column. This allowed time for droplets to produce vapors and for larger droplets to settle and drain through the atomizer into an air-tight catch tank. A two-stage HEPA filter was mounted on top of this column to remove all oil droplets, thus allowing only the vapors to pass. The HEPA filters were not heated and allowed for the air/vapor to cool to lab temperature (approximately 72 F) before entering the exposure chamber.

The total volatile organic compounds (TVOC) gas concentration was measured inside the exposure chamber with continuous samples by a flame ionizing detector (Model 10 FID; VIG Industries Inc.; Anaheim, CA), and adjustments were made automatically to the oil injection flow entering the atomizer via custom control software in order to maintain a constant TVOC level. The TVOC monitor was calibrated using benzene, toluene, ethylbenzene and xylene (BTEX) gas as a standard. Calibration BTEX gas tanks (Butler Gas Co.; Pittsburgh, PA) were obtained at TVOC levels of 30, 120, 240 and 480 ppm. The BTEX mixtures had equal concentrations of each compound. The calibration data is shown in Fig. 2. In addition to TVOCs, BTEX and other unidentified VOC were monitored during inhalation exposures (Model 8610C GC-FID; SRI Instruments; Torrance, CA). This was done to track changes in vapor composition over time. The methods used to analyze the samples collected by the GC-FID are described in section 7 below.

Exhaust air exited the exposure chamber through rails with several small holes located below the animal cages. More information about the custom exposure chamber is given in section 2. The exhaust air was then routed through HEPA and charcoal filters. A mass flow controller, with its downstream port connected to lab vacuum, controlled the amount of air leaving the exposure chamber based on the exposure chamber's pressure differential vs. ambient pressure within the laboratory. This pressure was measured with a pressure transducer (Model 264 ± 7.5″ H_2O; Setra Systems; Boxborough, MA). The custom exposure system software was set to maintain exposure chamber pressure at 0" H_2O at all times. A CO_2 probe (model GMP252; Vaisala; Helsinki, Finland) was used to monitor the CO_2 levels in the exhaust air, and a temperature and humidity probe (model HMP60; Vaisala) was mounted inside the exposure chamber.

2. Exposure chamber

A custom-built, air-tight exposure chamber was designed and fabricated for use with the COV inhalation exposure system. The chamber was 22 × 22 × 37 inch (W × D × H) in size. 16-Gauge stainless steel was used for all exposure chamber walls, with a clear polycarbonate ¼-inch thick front door. The transparent door allowed for visual monitoring of animals during exposures. A rubber gasket and several latches around the perimeter of the door were used to provide an air-tight seal. The COV entered the top center of the exposure chamber from a ½ inch OD stainless steel tube. This tube had a custom-built dispersion nozzle mounted to its outlet inside the exposure chamber located approximately 2 inches from the top wall. This nozzle assisted in uniform gas mixing within the chamber. The nozzle had eight ⅛-inch diameter holes equally spaced pointed downward at 45-degree angles. Air exited the exposure chamber through ⅛-inch diameter holes drilled into the bottom of exhaust rails near the bottom of the exposure chamber. These three exhaust rails were made from ⅜-inch OD stainless steel tubes.

The exposure chamber was designed to hold two removable animal cage racks. The stainless-steel racks had twelve individual partitions measuring 5 × 7 × 5 inch each, arranged in a 4 × 3 matrix. The cage wire bottom floor surface was of sufficient diameter to allow the animal to comfortably rest without bedding present. The lower cage rack would rest on the exhaust rails and the upper cage rack would rest on stainless steel angle bars. The angle bars were oriented to direct animal excretions into the upper excretion pan. This pan had large open spaces directly under the angle bars so fumes could readily flow from the upper portion of the exposure chamber to the lower section. A second, identical exposure chamber was built and used to expose control animals to filtered air under the same conditions.

3. COV inhalation exposure system software

The computer software controlling the exposure system was implemented in the LabVIEW (National Instruments; Austin, TX) programming environment. When data logging was enabled all settings and readings were saved to the computer every 2 sec. The software had an “Initiate exposure sequence” button located in the upper right section of the screen used by laboratory technicians to start an automated exposure run. When this button was pressed the system entered fully automatic control mode. While in automatic control mode the software would: 1) prompt the user for target exposure concentration and exposure duration, 2) check the amount of crude oil remaining in the syringe pump, then ask the user to add more if needed, 3) activate all heaters, 4) prompt the user to load the animals into the exposure chamber and shut the chamber door, 5) start saving all exposure readings and setting to a data file, 6) activate automatic control of exposure concentration, pressure, and humidity, 7) go into purge mode when desired exposure duration has been completed, and 8) prompt the attendant to remove animals from the chamber after concentration is reduced to 5 ppm or below.

4. Exposure system feedback control loops

The COV exposure system employed three different automated feedback loops which, when enabled, continuously controlled: 1) the animal exposure chamber TVOC concentration, 2) the animal exposure chamber pressure, and 3) the exposure chamber humidity. The exposure concentration was maintained at a constant value by adjusting the oil flow rate into the atomizer based on real-time TVOC measurements. The pressure in the animal exposure chamber was regulated by making corrections to the exhaust flow based on readings from a pressure transducer. The exposure chamber relative humidity was controlled by making adjustments to the amount of dilution air entering a heated water bath and the amount of air bypassing it.

A form of proportional-integral-derivative (PID) control algorithm (Nise, 1995) was implemented for each of the feedback control loops employed by the inhalation exposure system. The constants Pgain, Igain, and Dgain used by each PID control loop were determined using manual tuning methods. In brief, 1) the adjustment period was set to 1.75-times the system delay and Igain and Dgain were set to zero, 2) step responses were conducted at various values of Pgain until 25% overshoot and non-increasing oscillations were observed, 3) additional step responses were conducted while adjusting Dgain until overshoot was reduced to under 5% and steady state oscillations were eliminated, and 4) Igain was then adjusted to eliminate steady state error.

5. COV inhalation exposures

Rats were placed in the whole-body exposure chamber and exposed either to a single 6-h inhalation exposure to 300 ppm TVOCs (acute exposure) or to 300 ppm TVOCs lasting 28 d (6 h/d, 4 d/wk for 4 wk; sub-chronic exposure). Biological end points were measured at 1 and 28 (acute exposure) and 1, 28 and 90 d post-exposure (sub-acute exposure). During the 6-h exposures, the animals did not have access to food and water. In previous investigations involving exposures to other agents, animals manipulated in such a manner have not exhibited any observable body weight changes, behavioral, or adverse effects due to food/water deprivation. The total air flow entering the chamber was 44 l/min (eight air changes per h), and provided enough ventilation to maintain CO2 levels below 4,000 ppm and temperature from rising above ambient. Control animals, placed in separate chambers identical in design and functionality, breathed filtered air for the same periods.

6. Animals

All studies were conducted in facilities accredited by AAALAC International, were approved by the Institutional Animal Care and Use Committee and were in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (H1a: (SD)

CVF, approximate body weight of 200 – 275 g at arrival), were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). All animals were free of viral pathogens, parasites, mycoplasm, Heliobacter and cilia-associated respiratory bacillus. Animals were acclimated for 1 wk, housed in pairs in ventilated micro-isolator units supplied with HEPA-filtered laminar flow air (Thoren Caging Systems; Hazleton, PA), with 7090 Sani Chip and 7070C Diamond Dry combination (both from Harlan, now Envigo; Indianapolis, IN) for bedding, and provided tap water and 2918 irradiated Teklad Global 18% rodent diet (Harlan, now Envigo; Indianapolis, IN) ad libitum. Rats were housed under controlled light cycle (12 h light/12 h dark) and temperature (22 – 25 °C) conditions. Rats were housed in pairs except in studies in which animals were instrumented for telemetry studies.

7. Crude oil analysis

The crude oil used in this investigation was provided by BP Exploration and Production, Inc. The sample is reference material associated with the 2010 Deepwater Horizon spill from the Macondo Well in Mississippi Canyon Block 252 (MC 252), i.e., "surrogate oil," a sample of a Gulf of Mexico Sweet Louisiana Crude Oil that is physically, chemically and toxicologically similar to the Macondo Well crude oil from Mississippi Canyon Block 252. The oil was sent to Stratum Laboratories (Shenandoah, TX) for analysis, the results of which are depicted in Supplementary Fig. 1. It had an ºAPI gravity (60 ºF) of 34.2. Upon receipt the oil was distributed in aliquots in air-tight lined metal containers and stored at 68 ºF until used.

During each 6-h exposure run two samples were taken from inside the exposure chamber by a gas chromatograph flame ionizing detector (GC-FID) instrument (Model 8610C GC-FID; SRI Instruments, Torrance, CA). These two samples were taken at the 2 and 4 h time points. The GC-FID was set up with the temperature ramp that lasted 36 min total and provided good separation of peaks in the instrument's spectrum for COV. Four of these peaks on the chromatogram were identified as BTEX and calibrated to ppm levels using BTEX gas tanks (Butler Gas Co.; Pittsburgh, PA) of known concentrations (5, 20, and 80 ppm). A linear fit was used to find the relationship between the area of detected BTEX peaks and concentration in ppm.