

Characterization and Quantification of Microenvironmental Contaminants in Isolator Cages with a Variety of Contact Beddings

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Abstract | Microenvironmental contaminants were measured within isolator-type cages housing DBA/1J mice on 8 contact beddings. Each cage contained 850 cm³ of bedding and 5 mice randomized by body weight. Seven cages with and 2 to 4 cages without mice were evaluated per bedding. Macroenvironmental conditions were defined and controlled. Macro- and microenvironmental temperatures, relative humidity, and carbon dioxide and ammonia concentrations were determined daily during each of 3 seven-day test periods. An air sampling pump and detector tubes were used to measure hydrogen gas, 2-butanol, acetone, ethanol, carbon monoxide, acetic acid, hydrogen sulfide, sulfur dioxide, and formaldehyde on the final day of each test period. In addition, gas chromatographic analysis was used on the seventh day to detect additional volatile alcohols and ketones. Ammonia concentrations ranged from 0 to 410 ppm, depending on the bedding type and day of measurement. On the basis of the mean microenvironmental ammonia concentration in the cages with mice, the beddings were ranked from highest to lowest in ammonia generated: aspen shavings, pine shavings, reclaimed wood pulp bedding, virgin pulp loose bedding, hardwood chip bedding, recycled paper bedding, virgin cellulose pelleted bedding, and corn cob bedding. The temperature, relative humidity, and carbon dioxide concentration were similar between beddings. No other contaminants were detected except acetic acid (mean = 0.86 ppm) in the cages with and without mice containing corn cob bedding. Sulfur dioxide (mean = 0.42 ppm) was only detected in cages with mice and corn cob bedding. In summary, the concentration of ammonia generated varied significantly in cages containing mice and different contact beddings.

Isolator caging systems provide individual animal microenvironments and aid in the development and maintenance of specific-pathogen-free rodents for use in research. These caging systems are cost effective and allow containment at the cage level without expensive equipment. Although the filter tops may protect rodents from contamination with adventitious microbes (1–5), intracage airflow may be restricted, thus allowing the accumulation of prohibitively high concentrations of waste gases and humidity (6–11). High humidity prevents fecal and urinary desiccation and provides the optimal milieu for bacterial proliferation and subsequent ammonia production. Ammonia has been detected at amounts documented to impact physiologic systems (10, 12–21). Temperature, relative humidity, carbon dioxide, and ammonia are routinely evaluated microenvironmental parameters. There are potentially numerous fermentation gases produced by bacterial flora in excrement that may accumulate within the cage. These products may reach concentrations that alter physiologic and immunologic function (15–18, 20, 21). The effects of the microenvironment on laboratory animals and on experimental results are, however, controversial, which has made it increasingly difficult to establish comprehensive environmental guidelines for housing rodents.

Contact beddings should provide a physiologically stable and stress-free environment for the animals. The selection of contact beddings should be based on optimal characteristics. Ideally, beddings should be chemically and biologically inert, highly absorptive, nontoxic, dust-free, compatible with the research study, and inexpensive.

The purpose of this study was to characterize and quantify microenvironmental contaminants in isolator cages and evaluate the effects of eight commercially available contact beddings on microenvironmental conditions.

Materials and Methods

Overall study design: Eight contact beddings were evaluated to determine their effect on microenvironmental conditions in isolator

cages housing five mice of uniform body weight. Micro- and macroenvironmental temperature, relative humidity, and carbon dioxide and ammonia concentrations were determined on each day of a 7-day test period. On the seventh day hydrogen gas, 2-butanol, ethanol, acetone, carbon monoxide, acetic acid, hydrogen sulfide, sulfur dioxide, and formaldehyde concentrations were determined. In addition on the seventh day, mass air samples were obtained from each cage for gas chromatographic analyses for volatile gases and ketones. Selection of evaluated contaminants was based on the expected fermentation products generated by the gastrointestinal tract flora of the mouse. The analyses were conducted over 7-day test periods. The study was run in triplicate so that seven cages containing mice per bedding were evaluated. Additionally, 2 to 4 cages per bedding without mice were included to analyze for the presence of contaminants in the head space that may be off-gassed from the beddings. A total of three of the cages with mice and two of the cages without mice containing corncob bedding were further analyzed to confirm the presence of acetic acid and sulfur dioxide in the head space, using National Institute of Occupational Safety and Health (NIOSH)-approved methods #1603 and #6004, respectively.

Animals: Two-hundred female DBA/1J mice (*Mus musculus*) (Jackson Laboratory, Bar Harbor, Maine) were studied. Mice were specific-pathogen-free and had a conventional (undefined) gastrointestinal tract flora. Mice were randomized according to biomass and were rerandomized for each test period. Five mice were housed per experimental cage. Total biomass (mean \pm SEM, 68.8 \pm 1.46 g) per cage was below the standards for housing density recommended by the *Guide* (21). Statistical analysis verified that there was no significant difference among biomass means between cages. Mice were fed a commercial diet (Prolab 3000, Agway, Inc., Syracuse, N.Y.) and provided distilled water in water bottles ad libitum.

Macroenvironment: The animals were housed in a holding room measuring 13.50 x 13.50 x 7.92 feet in an AAALAC-accredited animal facility incorporating a clean/dirty corridor system. The HVAC system was a constant volume, terminal reheat type with direct steam humidification. Only animals and caging under study were housed in the holding room. Ventilation provided fifteen changes of 100% fresh air per h. Differential pressures provided directional airflow so

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that air flowed from the clean corridor into the holding room and out to the dirty corridor. There was a 12-h light/dark cycle. The daily temperature ($71.17 \pm 0.21^\circ\text{F}$) and relative humidity ($48.86 \pm 0.18\%$) were determined by a continuously operating hygrothermograph (Hygro-Thermograph, Belfort Instrument Co., Baltimore, Md.).

Caging and beddings: Modified isolator-type cages consisted of a polycarbonate shoebox cage of dimensions $11.50 \times 7.25 \times 5.00$ in. Each cage was equipped with a stainless steel wire-bar lid that held a 16-ounce water bottle and pelleted feed. Isolator tops (Micro-BARRIER, standard height, #MBT7115HT, Allentown Caging Equipment Co., Allentown, N.J.) with polyester filter media (Reemay #2033, The Intertech Group Inc., Old Hickory, Tenn.) were used on all cages. Isolator tops were not removed for the duration of each 7-day test period. Filters were replaced before each test period. Cage modifications, for measurement purposes, included a 1-in-diameter port located on the vertical surface of the isolator top and a 0.5-in-diameter port located on the vertical surface of the cage (Figure 1). The vertical port on the isolator top was closed with nonporous tape when not in use. The port in the shoebox cage was sealed with a silicone rubber diaphragm when not in use. All cages were set up with the water bottle sipper-tube facing away from the measurement ports.

Each cage contained 850 cm^3 of autoclaved contact bedding. The beddings evaluated included aspen shavings (Aspen shavings, Northeastern Products Corp., Warrensburg, N.Y.), virgin pulp loose bedding (Alpha-Dri, Shepherd Specialty Products, Kalamazoo, Mich.), recycled paper bedding (Recycled paper gray granules, A & W Products Inc., Cincinnati, Ohio), corncob bedding (Bed O'Cobs, combination size, The Anderson Lab Division, Maumee, Ohio), reclaimed wood pulp bedding (Carefresh, Absorbptive Corp., Bellingham, Wash.), virgin cellulose (Cellu-Dri, Shepherd Specialty Products), pine shavings (Pine shavings laboratory grade, Northeastern Products Corp.), and hardwood chip bedding (Sani-Chips, R. J. Murphy Forest Products Co., Rochelle Park, N.J.).

Cages were placed on a six-shelf rack. Each shelf measured 65.75×15.25 in with 10.50 in of vertical space between each shelf. The cages were randomly assigned per shelf with equal distance between the cages. Precise positions were designated to ensure equal and repeatable spacing. The rack was placed parallel to and 12 in from the wall of the holding room. Air velocity was measured at 1 linear ft/min

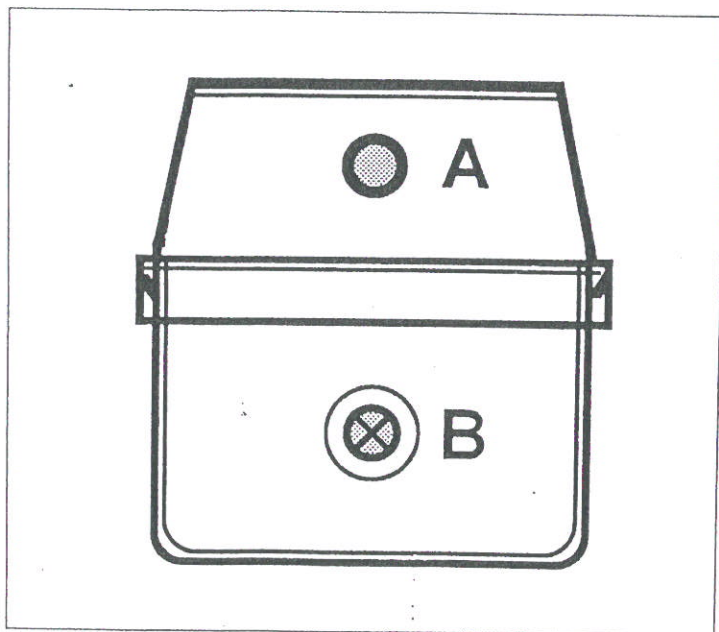


FIG. 1. Modified isolator cage. (A) 1-inch-diameter port in isolator top; (B) 0.5-inch-diameter port in cage.

across each cage and shelf of the rack, 1 inch above and parallel to the isolator top of each cage with a multipurpose environmental monitor (MPM 500e, Solomat Partners, L.P., Stamford, Conn.).

Evaluation of micro- and macroenvironmental parameters: Microenvironmental (intracage) temperature (T) and relative humidity (RH) were measured in the cages with mice through the modified port in the isolator top and were measured at the level of the wire-bar lid at the center point of the cage. All other cage-sampling procedures were performed through the port in the cage at the level of the mice. Macro- and microenvironmental temperature and relative humidity were measured daily with the multipurpose environmental monitor calibrated and certified ($T \pm 0.35^\circ\text{F}$ and $\text{RH} \pm 2\%$ at all ranges) as specified by the National Institute of Standards and Technology. Carbon dioxide concentration was measured in the cages containing mice and the room, using a portable carbon dioxide meter (Portable CO_2 Indicator #RI-111; Gastech Inc., Newark, Calif.) that was calibrated with known concentration sample gases (CO_2 Calibration Kit; Gastech Inc.) prior to commencement of the study. Macro- and microenvironmental ammonia concentrations were obtained daily, using an air-sampling pump (Toxic Gas Detector Sampling Pump #8014-400A, Matheson Gas Products, Secaucus, N.J.) and ammonia detector tubes (Length-of-stain Detector tubes #8014-105Sc and #8014-105Sb, Matheson Gas Products). These tubes detected ammonia concentrations between 5 and 260 or between 50 and 900 parts per million (ppm), respectively. Tube error, as provided by the manufacturer, was 5 to 10% depending on the concentration of the gas.

In addition, macroenvironmental temperature and relative humidity were monitored continuously, using a recording thermograph and hygrometer calibrated to the manufacturer's specifications by methods traceable to the National Institute of Standards and Technology.

On the seventh day of each test run, concentrations of 2-butanol, ethanol, acetone, hydrogen, carbon monoxide, acetic acid, hydrogen sulfide, sulfur dioxide, and formaldehyde were measured, using the air-sampling pump and detector tubes for each compound in cages with and without mice in the room. The detector tubes (Length-of-stain Detector tubes, Matheson Products), scale and tube error were as follows: 2-butanol, 10 to 300 ppm, 10 to 15% (Detector tube #8014-189U); ethanol, 0.05 to 5.0%, 5-10% (#8014-104SA); acetone, 40 to 800 ppm, 5 to 10% (#8014-102SD); hydrogen gas, 0.05 to 8.0%, 10% (#8014-137U); carbon monoxide, 5 to 1,000 ppm, 5 to 10% (#8014-106SA); acetic acid, 1 to 50 ppm, 10 to 15% (#8014-216S); hydrogen sulfide, 0.75 to 37.5 ppm, 5 to 10% (#8014-102SB); sulfur dioxide, 1 to 60 ppm, 5 to 10% (#8014-103SD); and formaldehyde, 20 to 1,500 ppm, 10% (#8014-171SA).

Air samples for gas chromatography were also taken on the seventh day from 4 cages with mice and 2 cages without mice containing each bedding; measurement was by use of flame ionization detection (Detector HP5890 Series II, Hewlett Packard, Kennett Square, Pa.) of organic vapors. The detector was calibrated with standard solutions of analytes in carbon disulfide. Three liters of air were drawn from each cage, using an air-sampling pump (Model HFS513A, Gilian Instrument Corp., West Caldwell, N.J.) through activated charcoal tubes (standard size, LOT #226-01; SKC, Inc., Eighty-Four, Pa.), and an additional three liters of air were obtained through silica gel tubes (standard size, LOT #226-10, SKC, Inc.), both at 0.15 L/min. The sample tubes were desorbed in 2 ml of carbon disulfide. Quantitative analysis (NIOSH method #1300) was conducted for butanol, ethanol, and acetone on each sample tube (22). In addition, semi-quantitative analysis (NIOSH method #1300) was performed for methyl ethyl ketone (MEK) and methyl isobutyl ketone (MIBK) (22). The limit of detection of each analyte was 5 ug per sample. Thus, the effective minimal detection limit for each analyte was as follows: 0.6 ppm butanol; 0.9 ppm ethanol; 0.7 ppm acetone; 0.6 ppm MEK and 0.4 ppm MIBK.

Positive results for acetic acid in cages with and without mice (corn-

cob bedding) and for sulfur dioxide in cages with mice (corn cob bedding) by detection tube was confirmed by a commercial laboratory (ESA Laboratories, Chelmsford, Mass.). For acetic acid, 3.25 L of air was sampled, using the air-sampling pumps, at 0.61 L/min through a cellulose and KOH filter preceded by a cellulose ester membrane from three cages with mice, two cages without mice, and the room. Sulfur dioxide analyses were performed from 3.45 L of air sampled at 0.11 L/min through a charcoal filter from three cages with mice, two cages without mice, and the room. The analyses were performed by using NIOSH-approved methods (acetic acid method #1603; sulfur dioxide method #6004) (22).

Statistical analysis: Differences among contact beddings for each measured parameter were evaluated, using a two-way analysis of covariance, with day as a repeated measure (23). Macroenvironmental measurements were used as a covariant for each evaluated microenvironmental parameter with the exception of ammonia. These results were confirmed by using the Kruskal-Wallis nonparametric test (24, 25) for ammonia and the Newman-Keuls test (23) for the other parameters. The effect of relative humidity, temperature, and carbon dioxide on the variation of ammonia concentration in the cages with mice was determined, using a multivariate analysis of variance (23). A probability of 0.05 or less was considered significant. Values are expressed as mean \pm SEM, unless otherwise indicated.

Results

Temperature, relative humidity, and carbon dioxide concentration (7-day mean \pm SEM) in cages with mice for each bedding are provided in Table 1.

Temperature: There were no significant differences in temperature detected among cages with mice and various beddings throughout the 7-day observation periods.

Relative humidity: Intracage relative humidities detected were not significantly different among cages with different beddings throughout the 7-day observation periods. Mean relative humidity for each bedding increased significantly each day throughout the 7-day test period.

Carbon dioxide: There were no significant variations detected in carbon dioxide concentration among cages with various beddings throughout the 7-day observation periods.

Ammonia: Microenvironmental ammonia concentration ranged from 0 to 410 ppm, depending on the bedding type and day of measurement. The mean daily ammonia concentrations for each bedding type are detailed in Figure 2. The cages containing aspen shavings had a significantly higher 7-day mean ammonia concentration, compared with other beddings. Additionally, the cages containing reclaimed wood pulp and pine shavings had a significantly higher 7-day mean ammonia concentration, compared with other beddings, with the exception of aspen shavings. Cages containing virgin pulp loose bedding had a significantly higher 7-day mean ammonia concentration, compared to the cages with virgin cellulose and corn cob bedding.

TABLE 1. Temperature, relative humidity, and carbon dioxide levels in the cages with mice and the room (mean \pm SE).

Bedding type	Temp (°F)	Humidity (%)	CO ₂ (ppm)
Aspen shavings	74.97 \pm 0.28	73.65 \pm 0.84	4,422 \pm 112
Virgin pulp	74.99 \pm 0.35	72.07 \pm 0.79	4,279 \pm 137
Recycled paper	75.27 \pm 0.31	72.94 \pm 0.89	4,049 \pm 155
Corn cob bedding	74.69 \pm 0.27	71.99 \pm 0.86	3,999 \pm 103
Reclaimed wood pulp	75.21 \pm 0.32	73.33 \pm 0.78	4,095 \pm 104
Virgin cellulose	74.88 \pm 0.32	70.67 \pm 0.85	4,064 \pm 142
Pine shavings	75.07 \pm 0.30	73.08 \pm 0.87	4,077 \pm 118
Hardwood chips	75.09 \pm 0.29	72.99 \pm 0.87	4,468 \pm 130
Macroenvironment	71.17 \pm 0.21	48.86 \pm 0.18	369 \pm 15

Ammonia was first detected in cages containing aspen and pine shavings on day 2. On day 3, ammonia was first detected in cages containing reclaimed wood pulp. Cages with virgin loose pulp and hardwood chip beddings had detectable ammonia beginning on day 4, followed by the cages with recycled paper bedding on day 6. The cages containing virgin cellulose bedding had no ammonia production until day 7, whereas the cages with corn cob bedding never had detectable ammonia concentration.

There was a significant difference in ammonia concentration in cages with different beddings and the day of evaluation, indicating that ammonia production differed among the beddings over time. Using a multivariate analysis of variance with ammonia as the dependent variable and relative humidity, carbon dioxide, and temperature as the independent variables, relative humidity was shown to have twice as much influence on ammonia as temperature, with carbon dioxide having very little, if any influence on ammonia production. In addition, the independent variables were shown to account for 23.9% of the variation in ammonia production ($P < 0.0001$). The remaining 76.1% of the variation was due to the beddings themselves or additional unknown parameters.

Acetic acid: Acetic acid concentration in cages containing corn cob bedding, with and without mice, ranged from 0.5 to 1 ppm, with a mean of 0.86 ppm by use of the detector tube method. The acetic acid values confirmed by the commercial lab ranged from 0.32 to 0.96 ppm, with a mean value of 0.51 ppm. There was no significant difference between acetic acid determined by detector tubes versus the commercial laboratory, nor between cages with and without mice.

Sulfur dioxide: Sulfur dioxide values in the cages containing corn cob bedding, with mice, ranged from 0.25 to 0.5 ppm, with a mean value of 0.42 ppm by use of the detector tube method. The commercial laboratory values ranged from 1.11 to 1.87 ppm, with a mean value of 1.49 ppm in the test cages. Sulfur dioxide was not detected by use of either method in the cages without mice. There was significantly higher sulfur dioxide concentration, as determined by the commercial laboratory versus the detector tubes ($P < 0.02$). By use of both methods, there was a significantly higher sulfur dioxide concentration in the cages with mice, compared with cages without mice.

No additional macro- or microenvironmental contaminants were detected in the cages with or without mice.

Discussion

These results indicate that the type of contact bedding used in isolator-type cages can have a marked effect on the accumulation of intracage ammonia. The corn cob bedding had no accumulation of ammonia over the 7-day test period. The 7-day mean ammonia concentrations in the corn cob and virgin cellulose beddings were significantly lower, compared with that in the other beddings. The independent variables (relative humidity, temperature, and carbon dioxide) in the multivariate analysis only contributed 23.9% to the variation in ammonia production. The major contributor to variations in ammonia production most likely was the beddings themselves. It may be beneficial to choose a bedding that limits ammonia production to decrease the number of cage changes required per week, thus improving the animals' environment and decreasing the overall cost of husbandry.

Ammonia is a severe irritant, which affects the mucous membranes of the eyes and respiratory tract, and the skin. Ammonia concentration in cages with particular beddings was similar to that documented to impact physiologic systems (10, 12-21). Microenvironmental ammonia concentration in isolator-type cages often exceeded acceptable standards for human exposure (26). Because of toxicosis resulting from prolonged exposure to ammonia, the Occupational Safety and Health Administration (OSHA) sets limits for ammonia exposure as a time-weighted average (TWA) at 50 ppm during an 8-h workday