Appendix: Protocols for Characterizing Aerosol Emission from Heater Cooler Devices

PROTOCOLS

P1. FDA Bubbler for Use as Positive Control

For aerosol emission testing the FDA recommends including both positive and negative controls. Device manufacturers can choose to use their own devices that are known to aerosolize (when the water tanks are contaminated) as positive control. However, when unsure about the device to use as positive control, device manufacturers can also use the FDA bubbler design. This bubbler is built to recreate the bubbling process in heater cooler water tanks that have been the primary mode of aerosolization of Nontuberculous mycobacterium from heater coolers.[1]

A schematic of the FDA bubbler setup as well as the actual bubbler is shown in Figure S1 below.

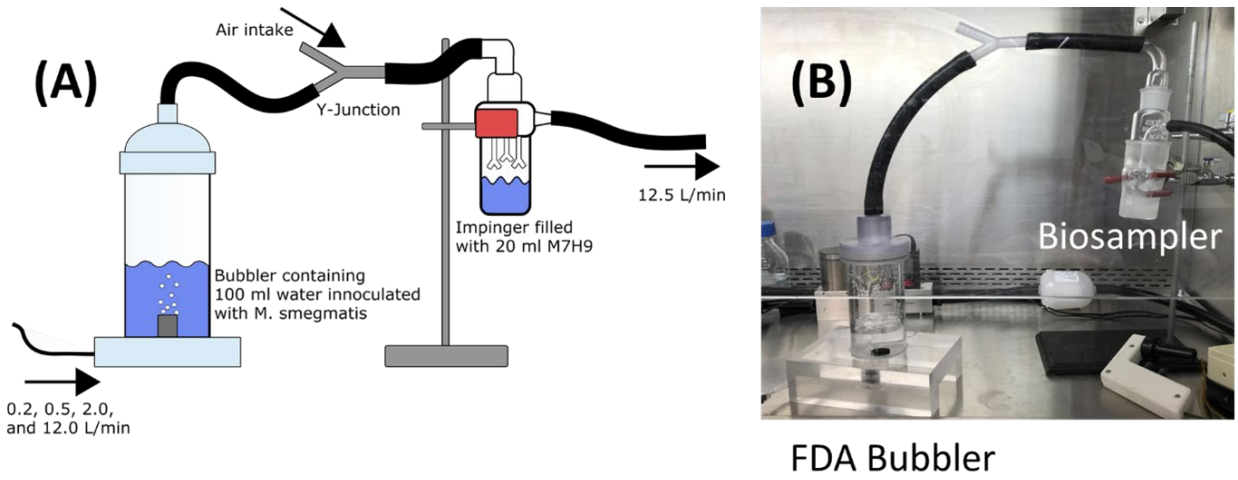

Figure S1 (A) Schematic of the FDA bubbler including the SKC biosampler. (B) Actual test setup as observed inside a biosafety cabinet.

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The bubbler was carefully constructed after going through some design iterations. The design iteration process is discussed in more details elsewhere.[2] The key considerations when designing the bubbler tested constructed and testing by the FDA is:

a. The height and diameter of the bubbler should be approximately 140 mm and 50 mm, respectively.
b. The aquarium aerator can be used to generate bubbles and it should be placed centrally.
c. Filtered air will need to be connected to the aerator through the bottom, and the air should be controlled with a mass flow controller. The mass flow controller should be able to deliver flow rates < 10 L/minute with high accuracy.
d. The top outlet of the bubbler should also be centrally located and can be of inner diameter around 10 mm.
e. At the top of the chamber, a conductive silicon tubing (TSI Inc. Shoreview, MN) should transport the aerosolized NTM to a SKC Biosampler (SKC Inc., PN 225-9595), which needs to be operated at a fixed flow rate of 12.5 L/minute.
f. The bubbler should preferably be operated at < 1 L/minute. Hence the offset of the flow rate (SKC biosampler pulling at 12.5 L/minute) needs to be accomplished by using a Y-connector after the outlet to allow supplementary air to dilute the aerosols emitting from the bubbler before reaching the SKC biosampler.

P2. Preparation of the bacteria before aerosolization

a. Fresh cultures for both M. chimaera and *M. smegmatis* (if desired for early feasibility experiments) can be prepared by diluting stationary phase cells 1:100 and subculturing to an optical density (OD550) of ~1.5.
b. After reaching an OD550 of ~1.5, cultures should be centrifuged at 5,000 RPM for 20 minutes at room temperature.
c. The supernatant should be decanted, and cells should be washed twice before suspending to an OD550 of 1.0 in sterile deionized (DI) water.
d. Cultures should then be exactly calculated by performing 10-fold serial dilutions and plating 100 µl aliquots on MB 7H10 agar plates. Typically, in our experience, *M. smegmatis* correlates ~2 × 108 colony forming units (CFU)/ml for OD550 of 1.0. and M. chimaera correlates ~4 x 108 CFU/ml for OD550 of 1.0.
e. These bacterial suspensions can be diluted with autoclaved tap water to create desirable final concentrations to be used in the bubbler. Typically, we recommend preparing inoculum concentrations of 100 – 107 CFU/mL that can also allow for a serial dilution study to understand the performance of the bubbler at a specific air flow rate.

P3. Operating the bubbler

a. The FDA bubbler should be filled to 100 mL with inoculum.
b. If the inoculum will be prepared to the desired concentration in the bubbler, the bubbler should be filled with autoclaved tap water of the correct volume and the corresponding volume of concentrated inoculum. For example: the OD550 ~1.0 solution was determined to be 2 x 108

CFU/ml. To make a 100 ml of 107 CFU/ml bacterial inoculum, add 95 ml of autoclaved tap water to the bubbler, then 5 ml of OD550 ~1.0 should be added and mixed to the solution.

c. We recommend starting experiments using an inoculum concentration of 105 CFU/mL. As indicated in P2 above, concentrations from 100-107 CFU/ml can be used for testing to determine the optimum concentration and flow rate to act as a positive control.
d. The flow rate should be gradually increased using the mass flow controller from 0.2 L/minute in increments of 0.2 L/minute up to 1.0 L/minute.
e. User can choose a final flow rate to perform their positive control test. The final flow rate would ultimately be dictated by the response obtained from the test set-up and can depend on a variety of factors including the aerosolization potential of strain of NTM used, the tubing lengths, the aerator design, the design of the bubbler etc.

P4. Protocol for Sampling

a. The SKC biosampler should be filled with 20 ml of M7H9 supplemented with 10% OADC, 0.1% Tween 80, and 0.004% Antifoam B silicone emulsion (JT Baker, B531-05) that prevents froth formation particularly during collection of hydrophobic bacteria.
b. Aerosols should be collected in the biosampler for 5 minutes.
c. Impinged solution should then be transferred into 50 ml conical vials.
d. Impinged solutions should then be vortexed for 30 s at 1,500 RPM (VWR) and 10-fold serial dilutions should be prepared by diluting 1 ml of solution into 9 ml of phosphate buffered saline (Fisher). Samples should either be collected by vacuum filtration onto 0.45µm filters (Millipore) or by spread plating 100 µl onto M7H10 agar plates.
e. Samples may be plated as a single replicate at three different dilutions.

P5. Additional precautions and considerations

The major organism of concern is Mycobacterium chimaera, and aerosolization of this bacterium poses a high risk for the user especially in cases of leaks from the test setup. Therefore, additional precautions are required.

a. After setting up the protocol, user should use a particle counter (for example: TSI Inc. Condensation Particle Counter Model 3775) at various points near the test setup to ensure that no additional particle counts are detected across various set-ups (without water, with water, or with water contaminated with *M. chimaera*).
b. For the safety of the user, this leak testing can also be performed using *M. smegmatis*, which is a lower risk microorganism compared to *M. chimaera*.
c. If the counts across various set-ups are similar that provides assurance to the user that there are no leaks. However, if the particle counter determines relatively higher particle counts when the water is contaminated with *M. smegmatis*, then the bubbler setup connections would need to be tightened or modified.
d. If the counts are found to be similar after tightening the test setup, then the above tests would need to be repeated with *M. chimaera* to ensure leak proof setup.

Other considerations include taking different precautions for preventing aggregation for different subspecies of NTM. For example, 0.1 % Tween 80 should be added to M7H9 buffer specifically when culturing *M. smegmatis* to ensure uniform growth and to reduce aggregate formation. NTM concentrations above 107 CFU/mL should be avoided as large concentrations tend to result in aggregate formation, settling of these aggregates from the water, and hence depletion of single units of NTM in solution.

References

1. FDA's Ongoing Evaluation and Continued Monitoring of Reports of Nontuberculous Mycobacteria Infections Associated with Water-Based Heater-Cooler Devices
2. Guha, S., Wolloscheck, D., Abdali, N., Wentz, C., Gillette, N., Bauer, K. A., & Weeks, J. W. (2022). *A methodology for investigating aerosolization of nontuberculous mycobacteria from contaminated heater cooler devices*. Frontiers. https://doi.org/10.3389/frwa.2022.902872External Link Disclaimer
3. Weeks, J.W., Segars K., & Guha S. (2020) *The Research Gap in Non-tuberculous Mycobacterium (NTM) and Reusable Medical Devices. Frontiers in Public Health*. https://doi.org/10.3389/fpubh.2020.00399