Technical Report

Evaluation of the virus removal effectiveness of Air Water Global's Airofresh Intl technology using the model virus *Escherichia coli* bacteriophage MS2 virus

Executive summary

The capability of the Air Water Global technology, to reduce virus numbers was tested in a controlled sealed room using the model virus *Escherichia coli* bacteriophage MS2 (American Type Culture Collection (ATCC) 15597-B1) delivered as an aerosol. The MS2 viral particles were directed through the device using a fan and captured at the terminal end of the device using an air sampler that captures the MS2 aerosol and separates the MS2-containing aerosol droplets into different sizes.

Loss of MS2 viral particles through factors other than the effect of the device (e.g. adhesion and loss through physical damage by being aerosolised) was quantified (negative control) and this number (10^2) subtracted from the total viral particle loss observed when the Airofresh Intl device was turned on. This allows us to calculate the loss caused by the device itself.

The Airofresh Intl device significantly reduced MS2 viral particle concentrations. Estimated removal of 1 $\log_{10}$, 4 $\log_{10}$, and 13 $\log_{10}$ reductions were achieved, with a maximum removal of 13 $\log_{10}$. This demonstrates that the Airofresh Intl can achieve a 13 $\log_{10}$ reduction in MS2 viral particles, a reduction equivalent to >99.99999%.

Key findings

This study demonstrates that the Airofresh Intl technology was effective in reducing the concentrations of viable MS2 (a model virus) and at the highest concentration of MS2 that was tested, the device reduced the viral numbers by 13 $\log_{10}$, a reduction equivalent to >99.99999%.

BACKGROUND

Bacteriophage MS2 is a single stranded RNA virus that infects *Escherichia coli* and other members of the Enterobacteriaceae. It is a recognised indicator organism for enteric viruses and faecal contamination (McMinn et al., 2017). It is a suitable surrogate for viral pathogens and has been used as a model organism in virus inactivation studies (Hosseini et al., 2017, Kohn et al., 2016, Silverman et al., 2019) and to investigate virus survival during aerosol dispersion (Turgeon et al., 2014, Zuo et al., 2014).

METHODS

The capability of the Air Water Global device Airofresh Intl technology to remove viruses was tested under laboratory conditions using the experimental set up shown in the diagram and photo below (Figures 1 and 2). The protocol used was developed and validated in our laboratory for assessing the efficacy of air purification equipment in reducing bacteria, viruses and fungi.

All necessary biosafety training has been undertaken by all personnel involved in the project.

The Airofresh Intl unit was provided by Air Water Global and installed according to the manufacturer's instructions in the Flinders University PC2 (Physical Containment level 2) room in the Health Sciences Building, on level 5 at the Bedford Park campus, Flinders University. Microorganisms were aerosolised using the nebuliser (1-jet collusion nebulizer, CH Technologies, USA). Fore and aft delivery tunnels (removable piping) were constructed with stainless steel to facilitate aerosol introduction into the device (fore tunnel) and aerosol collection into the air sampler (Staplex® MBS-6 Six Stage Microbial Air Sampler, USA) (aft tunnel) (Figures 1 and 2). Each stage of the air sampler contains a different filter size. Stage 6 contains the finest filter and collects particles of 0.65 to 1.1 µm. Stages 5, 4, 3, 2 and 1 collects 1.1 µm to 2.1 µm, 2.1 µm to 3.3 µm, 3.3 to 4.7 µm, 4.7 to 7.0 µm and 7.0 µm and above, respectively. The air flow rate was controlled at 1 m/s which was confirmed using an anemometer (Pocket Pro™ + Multi 2, HACH Company). The air pressure released from the air cylinder was set to 10 psi. All the experiments were conducted in triplicate

and all steps were taken to ensure the environment was sanitised prior to the addition of the test organism.

Surviving microorganisms were enumerated with the unit turned on and turned off (negative control/baseline). All tests were conducted for the Airofresh Intl technology device in triplicate under the following conditions:

- Test with fan on and AF device off with MS2 (negative control). This allows us to quantify the viral particle load lost due to factors other than the action of the device itself, for example, loss through particles adhering to the device, or being lost by damage through the aerosolising process.
- Test with fan on and AF device on with microorganisms. This was undertaken with increasing concentrations to determine the highest reduction in viral particle numbers.

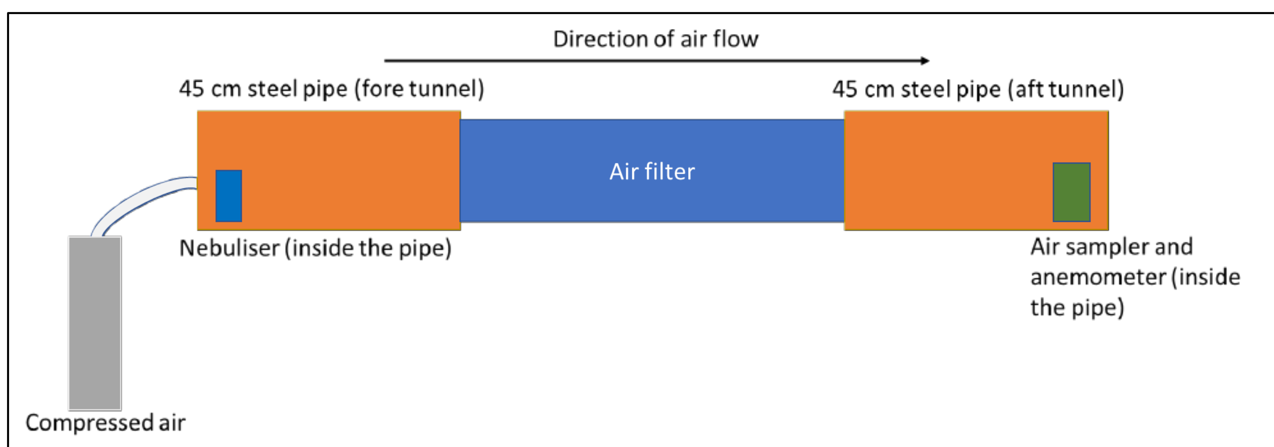

Figure 1: Schematic diagram showing experimental setup

Figure 2: Photo showing experimental setup

MS2 (ATCC 15597-B1) stock was diluted in Tryptone Soya Broth (TSB) to obtain cultures of varying concentrations. Each concentration was confirmed using the double agar layer method. The bottom layer of the agar plates consisted of 1.5% Tryptone Soya Agar (TSA), prepared by mixing 15 g technical agar (Oxoid) and 30 g TSB (Oxoid) in 1 L of Milli-Q water which was sterilized by autoclaving for 15 minutes at 121^{0}C. The top agar layer consisted of 5 mL of a 10:1 ratio of TSA and overnight culture of *E. coli* in TSB (ATCC 700891, used as the host for the virus MS2 (which was kept in a water bath with water heated up to 50^{0}C to prevent solidifying) and 5 mL of MS2 in TSB. This was mixed, poured on top of the bottom layer and allowed to set (referred to as TSA- *E. coli* plates). The plates were incubated overnight at 37 ^{0}C and the number of plaques counted.

A highly concentrated stock of MS2 was prepared by adding 10 mL of ½ strength tryptone water to a TSA - *E.coli* plate with MS2 growth (indicated by the presence of plaques). The plate was swirled gently and placed in the incubator for 45 min at 37^{0}C with swirling every 10 mins. Following 45 min of incubation, the plate was removed and tryptone water containing MS2 and *E. coli* was extracted using a 10 mL syringe**.** The tryptone water in the syringe was added to another TSA - *E.coli* plate with MS2 growth and the process repeated three more times. The final time the tryptone water containing MS2 and *E. coli* was syringed off and was centrifuged at 3000 rpm for 15 minutes to separate the *E. coli* cells which have been extracted with the MS2.

The supernatant was transferred into a sterile 50 mL tube and filtered using 0.22 μm filter to obtain a pure MS2 stock. The concentration was determined by serially diluting and plating onto TSA- *E. coli* plates*.* The Airofresh Intl AF4000 device was tested against a number of phage concentrations up to 10^{17} PFU/mL using the previously described methods.

Aerosols were generated for 30 minutes (1 mL delivered) with the Airofresh Intl device turned off (negative control) and the system turned on (trials), with the system cleaned in between each experiment. The surviving MS2 were enumerated using an air sampler (Staplex® MBS-6 Six Stage Microbial Air Sampler, USA) with 6 plates of TSA- *E.coli* (see above for method), which were incubated (Memmert *In vitro* incubator) at 37°C overnight following the experiment. The plaques were counted using a colony counter (Bacto Laboratories Pty Ltd, NSW, Australia) and recorded. The average plaque counts were calculated.

RESULTS AND DISCUSSION

REDUCTION IN MS2 BY FACTORS OTHER THAN THE AIROFRESH INTL DEVICE

To quantify the viral particle load lost due to factors other than the action of the device itself, initial tests were conducted with MS2 concentations of 10^8 and 10^5 MS2 plaque forming units (PFU)/mL, with 1 mL delivered into the device. These concentrations were found to be too high to calculate a loss, as the numbers of particles exiting the aft tunnel were too high to count. A third and fourth test were therefore run with starting concentrations of 10^3 and 10^2 MS2 PFU/mL, with 1 mL delivered. From this test, it was found that loss attributable to non-device factors could be calculated to be 10^2 MS2 PFU/trial (Table 4 and confirmed in Table 5). This number was used to correct for the removal of viral particles that could not be attributed to the unit itself.

REMOVAL OF MS2 BY THE AIROFRESH INTL DEVICE

The Airofresh Intl device was tested in triplicate against three concentrations of MS2, namely 6 x10^5 (Table 1), 6 x 10^8 (Table 2) and 6 x 10^{17} plaque forming units (PFU)/ mL (Table 3) and there was a significant reduction of the MS2 virus in all three concentrations tested.

When the filter was turned off, all plates in all concentrations had an uncountable number of colonies. This meant that the contribution to the reduction of MS2 as a result of the viral particles being physically removed (by landing on a surface inside the device, for example) had to be separated from the removal of the viral particles by destruction by the device itself. To do this, two low concentrations of MS2 were tested with the unit off (as described above). Removal with the unit off was estimated to be 10^2. As noted above, this number was used to correct for the removal of viral particles that could not be attributed to the filter itself. It is therefore reported that when the filter was turned on there was a significant reduction in the number of surviving viruses, with an estimated total removal efficiency increasing with each trial, and final results of 1 log_{10}1 (Table 1) 4 log_{10} (Table 2) and 13 log_{10} (Table 3), a maximum reduction equivalent to >99.99999%.

The virus concentration did not appear to be dependent on aerosol size with no significant difference observed in aerosol size.

REFERENCES

Hosseini, S.R.S., Dovom, M.R.E., Yavarmanesh, M. and Abbaszadegan, M., 2017. Thermal inactivation of MS2 bacteriophage as a surrogate of enteric viruses in cow milk. Journal of Consumer Protection and Food Safety, 12(4), pp.341-347.

Kohn, T., Mattle, M.J., Minella, M. and Vione, D., 2016. A modelling approach to estimate the solar disinfection of viral indicator organisms in waste stabilization ponds and surface waters. Water Research, 88, pp.912-922.

McMinn, B.R., Ashbolt, N.J. and Korajkic, A., 2017. Bacteriophages as indicators of faecal pollution and enteric virus removal. Letters in Applied Microbiology, 65(1), pp.11-26.

Silverman, A.I., Tay, N. and Machairas, N., 2019. Comparison of biological weighting functions used to model endogenous sunlight inactivation rates of MS2 coliphage. Water Research, 151, pp.439-446.

Turgeon, N., Toulouse, M.J., Martel, B., Moineau, S. and Duchaine, C., 2014. Comparison of five bacteriophages as models for viral aerosol studies. Journal of Applied Environmental Microbiology, 80(14), pp.4242-4250.

Zuo, Z., Kuehn, T.H., Bekele, A.Z., Mor, S.K., Verma, H., Goyal, S.M., Raynor, P.C. and Pui, D.Y., 2014. Survival of airborne MS2 bacteriophage generated from human saliva, artificial saliva, and cell culture medium. Journal of Applied Environmental Microbiology, 80(9), pp.2796-2803.

Disclaimer:

Any interpretations, research, analysis, recommendations, opinions, conclusions or advice provided by Flinders University are opinions based upon inferences from measurements, assumptions and industry practice. Data and conclusions provided in this report are the findings and opinions of Flinders University and are not an expressed or implied representation, warranty or guarantee. This report prepared for Air Water Global is for their exclusive use, and Flinders does not accept liability for any loss incurred by either the Air Water Global or a third party as a result of reliance on this report.

Appendix: Raw data

Table 1: Survival of aerosolised *E. coli* bacteriophage MS2 (starting concentration of $6x10^5$ PFU/mL) after passage through the Airofresh Intl unit experimental set up (shown in Figures 1 and 2). Tests were conducted in triplicate with the filter turned off (negative control) and filter turned on (trial)

Stage	Aerosol size (µm)	Filter off (PFU per plate)				Filter on (PFU per plate)			
		Trial 1	Trial 2	Trial 3	Avg	Trial 1	Trial 2	Trial 3	Avg
1	0.65-1.1	+++	+++	+++	+++	16	18	16	17
2	1.1-2.1	+++	+++	+++	+++	9	89	36	45
3	2.1-3.3	+++	+++	+++	+++	8	10	26	15
4	3.3-4.7	+++	+++	+++	+++	14	40	13	22
5	4.7-7	+++	+++	+++	+++	301	344	215	287
6	>7	+++	+++	+++	+++	429	449	315	398
		Total			+++	Total			784

The loss of MS2 not attributed to the filter (negative control) could not be calculated as there was too many plaques to count
The total loss of MS2 (trial) attributed to the filter and other external factors was a 3 log_{10} reduction
Using the loss attributed to external factors (shown in Tables 4 and 5) the MS2 removal due to the filter is calculated to be a 1 log_{10} reduction

+++ = too many to count

Table 2: Survival of aerosolised *E. coli* bacteriophage MS2 (starting concentration of $6x10^8$ PFU/mL) after passage through the Airofresh Intl experimental set up (shown in Figures 1 and 2). Tests were conducted in triplicate with the filter turned off (negative control) and filter turned on (trial)

Stage	Aerosol size (µm)	Filter off (PFU per plate)				Filter on (PFU per plate)			
		Trial 1	Trial 2	Trial 3	Avg	Trial 1	Trial 2	Trial 3	Avg
1	0.65-1.1	+++	+++	+++	+++	88	696	518	434
2	1.1-2.1	+++	+++	+++	+++	145	0	601	249
3	2.1-3.3	+++	+++	+++	+++	133	0	798	310
4	3.3-4.7	+++	+++	+++	+++	356	0	636	331
5	4.7-7	+++	+++	+++	+++	+++	0	+++	+++
6	>7	+++	+++	+++	+++	232	0	444	225
		Total			+++	Total			310
The loss of MS2 not attributed to the filter (negative control) could not be calculated as there was too many plaques to count The total loss of MS2 (trial) attributed to the filter and other external factors was a 6 log_{10} reduction Using the loss attributed to external factors (shown in Tables 4 and 5) the MS2 removal due to the filter is calculated to be a 4 log_{10} reduction									

+++ = too many to count

Table 3: Survival of aerosolised *E. coli* bacteriophage MS2 (starting concentration of $6x10^{17}$ PFU/mL) after passage through the Airofresh Intl unit experimental set up (shown in Figures 1 and 2). Tests were conducted in triplicate with the filter turned off (negative control) and filter turned on (trial)

Stage	Aerosol size (µm)	Filter off (PFU per plate)				Filter on (PFU per plate)			
		Trial 1	Trial 2	Trial 3	Avg	Trial 1	Trial 2	Trial 3	Avg
1	0.65-1.1	+++	+++	+++	+++	+++	+++	45	+++
2	1.1-2.1	+++	+++	+++	+++	232	256	195	228
3	2.1-3.3	+++	+++	+++	+++	173	251	37	154
4	3.3-4.7	+++	+++	+++	+++	25	37	17	26
5	4.7-7	+++	+++	+++	+++	31	28	0	40
6	>7	+++	+++	+++	+++	22	28	9	20
		Total			+++	Total			468
The loss of MS2 not attributed to the filter (negative control) could not be calculated as there was too many plaques to count The total loss of MS2 (trial) attributed to the filter and other external factors was a 15 log_{10} reduction Using the loss attributed to external factors (shown in Tables 4 and 5) the MS2 removal due to the filter is calculated to be a 13 log_{10} reduction									

+++ = too many to count

Table 4: Survival of aerosolised E. coli bacteriophage MS2 (starting concentration of 1x10^3 PFU/mL) after passage through the Airofresh Intl unit experimental set up (shown in Figures 1 and 2). Tests were conducted in triplicate with the filter turned off (negative control) to determine the baseline viral particle removal due to factors other than the Airofresh Intl AF4000 device

Stage	Aerosol size (µm)	Filter off (PFU per plate)			
		Trial 1	Trial 2	Trial 3	Avg
1	0.65-1.1	0	0	0	0
2	1.1-2.1	0	0	0	0
3	2.1-3.3	0	0	0	0
4	3.3-4.7	0	0	0	0
5	4.7-7	0	1	1	1
6	>7	3	3	3	3
		Total			4
There is a loss of 10^2 PFU not attributed to the activity of the air filter					

Table 5: Survival of aerosolised E. coli bacteriophage MS2 (starting concentration of 1x10^2 PFU/mL) after passage through the Airofresh Intl unit experimental set up (shown in Figures 1 and 2). Tests were conducted in triplicate with the filter turned off (negative control) to determine the baseline viral particle removal due to factors other than the Airofresh Intl device

Stage	Aerosol size (μm)	Filter off (PFU per plate)			
		Trial 1	Trial 2	Trial 3	Avg
1	0.65-1.1	0	0	1	0
2	1.1-2.1	0	0	0	0
3	2.1-3.3	0	0	0	0
4	3.3-4.7	0	0	0	0
5	4.7-7	0	0	0	0
6	>7	0	0	0	0
		Total			0
There is a loss of 10^2 PFU not attributed to the activity of the air filter					

Appendix 2: Sample photos showing experimental results

Figure 3: [MS2] = 10^{17}, Air sampler Stage 1, with the filter on

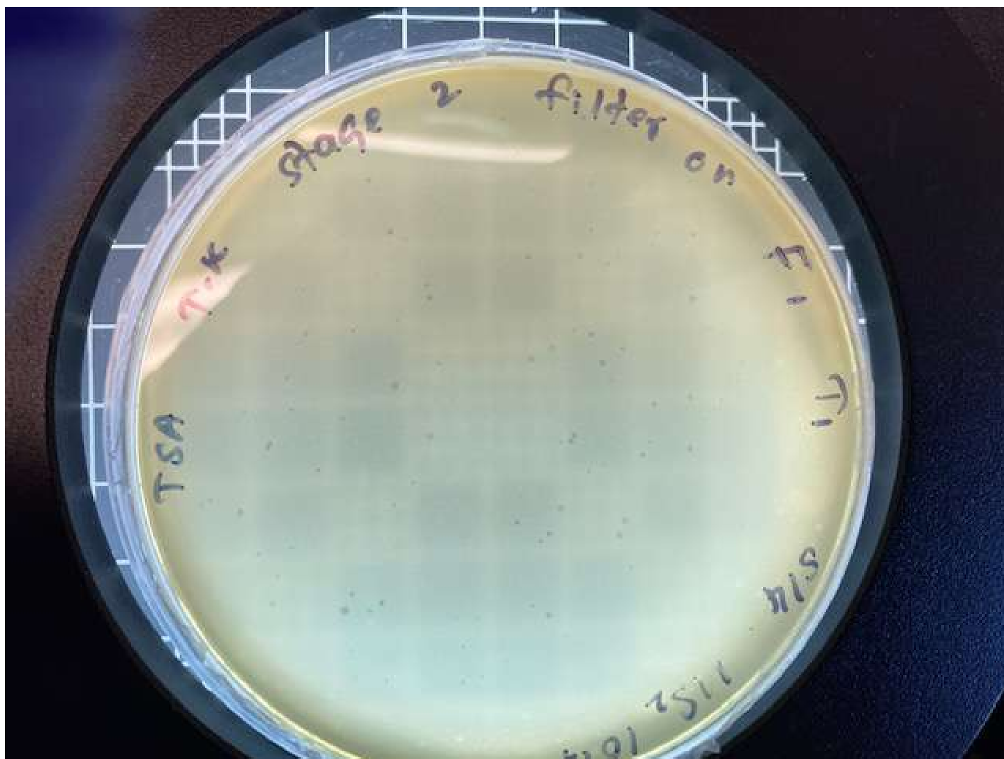

Figure 4: [MS2] = 10^{17}, Air sampler Stage 2, with the filter on

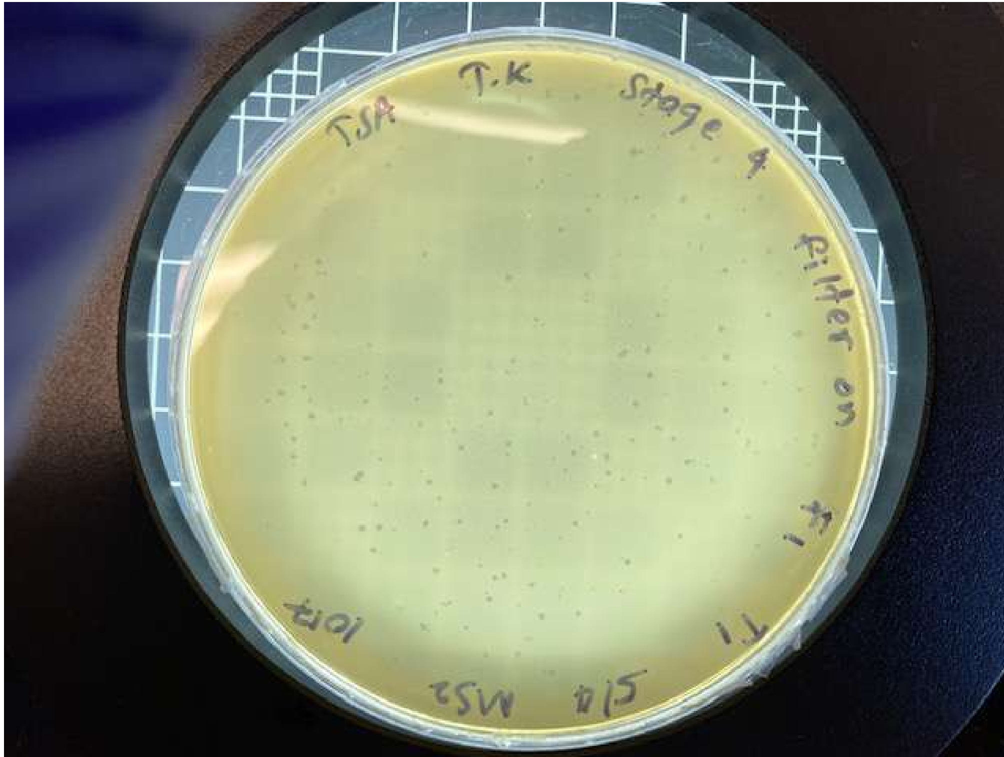

Figure 5: [MS2] = 10^{17}, Air sampler Stage 4, with the filter on

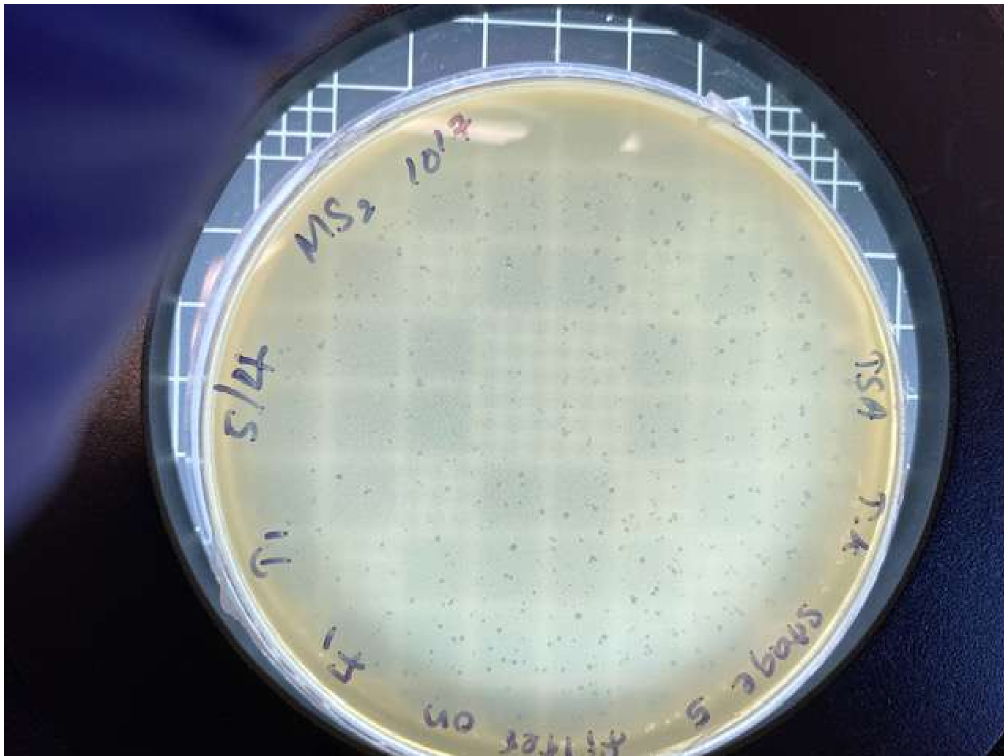

Figure 6: [MS2] = 10^{17}, Air sampler Stage 5, with the filter on