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Association between increased DNA mutational frequency and thermal inactivation of aerosolized *Bacillus* spores exposed to dry heat

Elisabet Johansson, **Atin Adhikari**, **Tiina Reponen**, **Michael Yermakov**, **Sergey A Grinshpun***

Center for Health-Related Aerosol Studies, Department of Environmental Health, University of Cincinnati, Cincinnati, OH 45267-0056, USA

Abstract

Inactivation of viable bioaerosol particles, especially stress-resistant microorganisms, has important implications for biodefense and air quality control. It has earlier been shown that the loss of viability of bacterial endospores due to exposure to dry heat is associated with mutational damage. Previous studies, however, used non-aerosolized spores, long exposure times, and moderately elevated temperatures. This study was designed to investigate the mechanism of inactivation of aerosolized *Bacillus* endospores exposed to high temperatures for sub-second time periods. Bioaerosol was tested in a continuous air flow chamber under two flow rates, 18 L/min and 36 L/min. The chamber had a cylindrical electric heating element installed along its axis. The estimated characteristic exposure temperature ($T_{exposure}$) ranged from 164°C to 277°C (with an uncertainty of 21–26°C). To quantify mutational frequency, spores were cultivated after dry heat exposure on tryptic-soy agar and on antibiotic nalidixic acid media. Increases in the exposure temperature caused viability loss and increase in mutational frequency of the spore DNA. Significant association was found between the inactivation factor and the mutational frequency ratio (heat exposed versus non-exposed) with R^2 of 0.985 for both flow rates combined. The results suggest that mutational damage is involved in the causal chain of events leading to inactivation of aerosolized endospores exposed to heat for sub-second time periods.

Keywords

bioaerosol; thermal inactivation; DNA mutations; *Bacillus* spores

Introduction

Inactivation (neutralization) of viable bioaerosol particles has several potential applications. For instance, effective destruction of aerosolized biological agents (primarily bacterial spores) over a relatively short time is a prominent part of defense research programs in the US (Henderson, 2004; Hitchcock et al., 2006; Koch, 2006) and abroad (Tan et al., 2006; Nadasi et al., 2007). If a bio-agent aerosolized from a bio-weapon facility in an explosion or

*Corresponding author. Tel: 513-558-0504; fax: 513-558-2263; sergey.grinshpun@uc.edu.

fire remains pathogenic, it can contaminate large areas, thus posing a major threat (Nelson, 2004). Inactivation of viable airborne microorganisms has also been explored in applications related to indoor air quality control. Methods such as germicidal ultraviolet (UV) radiation, ion emission, and thermal treatment have been studied with respect to their biocidal effects against bacterial spores (Luna et al. 2008; Lin and Li (2002); Jung et al. 2009; Grinshpun et al. 2005, 2007, 2010a).

Bacterial spores are among the hardiest microorganisms on earth, and dormant spores can remain viable under unfavorable environmental conditions for extremely long periods of time. The factors causing this resistance include a very thick spore coat surrounding the outer spore membrane, low core water content, high core mineral content, and a group of DNA-stabilizing proteins called α/β-type small acid soluble spore proteins (SASPs) (Nicholson et al. 2000). Most of what is known about the factors that determine the remarkable stability of bacterial spores and the mechanisms involved in their inactivation come from studies utilizing *Bacillus* species as model organisms, with *Bacillus subtilis* being the most commonly used because it is readily manipulated genetically (Setlow 2006). Spores can be inactivated by various stress-inducing factors, including wet and dry heat, UV radiation, γ-radiation, alkylating agents, oxidizing agents, acids, and extreme desiccation. DNA damage of various kinds has been shown to be a major cause of the spore inactivation by most of these factors (Setlow 2006; Roth et al. 2009). Conversely, DNA-stabilizing α/β-type SASPs have a key role in spore resistance, and although wet heat appear to inactivate wild-type spores by a DNA-independent mechanism, $\alpha^{-}\beta^{-}$ spores, which lack α/β-type SASPs, are significantly more sensitive to wet heat treatment and appear to be killed in part by DNA damage (Fairhead et al. 1993). Previous studies have demonstrated that dry heat treatment of lyophilized (non-aerosolized) spores at temperatures <155°C for time periods from five minutes to several hours is associated with increases in mutational frequency (Zamenhof 1960; Northrop and Slepecky 1967; Setlow and Setlow 1995; del Carmen Huesca Espitia et al. 2002). A recent interesting study by Jung et al. (2009) addressed cellular processes that may potentially be involved in a short-term thermal inactivation of vegetative cells of *airborne* bacteria. To our knowledge, no previous studies have identified the mechanism(s) of inactivation of *aerosolized bacterial spores* exposed to dry heat.

Our group has studied the short-term (sub-second) thermal inactivation of aerosolized *Bacillus* endospores in a controlled heated air flow at characteristic exposure temperatures ranging approximately from 150°C and 400°C (Grinshpun et al. 2010). The conditions (in terms of air temperature and exposure time) causing effective heat-induced inactivation, were determined experimentally by quantifying the spore culturability loss. It was found that the thermal exposure produced no effect or only a moderate inactivation when the characteristic exposure temperature remained below ~200°C for a tested air flow rate of 18 L/min and ~250°C for 36 L/min; however, inactivation rapidly increased with the exposure temperature once the above values were exceeded. Although some speculations about causative mechanisms of the inactivation of airborne spores were shared in that paper as a part of data interpretation, the investigation of these mechanisms was beyond the scope of the quoted study.

We hypothesized that the thermal inactivation of aerosolized *Bacillus* endospores exposed to dry heat over sub-second time intervals is associated with DNA mutation damage. The present investigation was initiated to test this hypothesis under experimental conditions when significant inactivation emerges from increase of air temperature. The relative mutational frequency, which served as a quantitative measure of the mutational damage, was determined by culturing exposed spores on tryptic-soy agar (TSA), as well as on TSA containing the antibiotic nalidixic acid. The latter allowed detecting mutations producing nalidixic acid resistance. To our knowledge, this is the first study focused on the mechanism governing inactivation of *aerosolized* bacterial spores exposed to dry heat.

Materials and methods

Challenge bioaerosol

B. atrophaeus (also known as *B. subtilis* var. *niger* and *B. globigii* [BG]) was chosen as the challenge microorganism for this study because it is well-characterized and has been extensively used as a stimulant of biological warfare agents, including *B. anthracis* (Johnson et al. 1994; Franz et al. 1997; Hill et al. 1999; Helfinstine et al. 2005; Luna et al. 2008). As a genus-level representative, *B. atrophaeus* has been utilized to evaluate inactivation techniques such as UV radiation (Shafaat and Ponce 2006), plasma sterilization (Muranyi et al. 2007) and thermal sterilization (Kempf et al. 2008). *B. atropheus* is not identical with, but closely related to *B. subtilis*.

B. atrophaeus endospores were aerosolized from liquid suspension using a six-jet Collison nebulizer (BGI Inc., Waltham, MA). For the preparation of the aqueous spore suspension, freeze-dried spores were suspended in sterile deionized water, vortexed to remove clumps, and centrifuged at approximately 6,300×g for 7 minutes. The spores were washed twice with sterile water by vortexing and centrifugation, and resuspended in sterile water to a concentration of approximately $(1 – 2)\times10^9$ colony forming units (CFU) per mL. Spore concentrations were determined by making serial dilutions in sterile water and cultivating aliquots on TSA (BD, Franklin Lakes, NJ) at 37°C for 24 hours.

Exposure of spores to dry heat

The experimental method and setup for exposing bioaerosol particles to a continuous heated air flow have been described in detail in Grinshpun et al. (2010a,b). Briefly, a cylindrical electric heating element (Mighty Watt Heater, Gordo Sales, Inc., Layton, UT) installed along the axis of a cylindrical continuous-flow exposure chamber was operated at voltages ranging approximately from 50 to 200 V, producing different heating conditions in the chamber. The axial geometry was chosen because it is relevant to a simultaneously performed laboratory study on the bio-agent defeat during explosion and combustion. Also, the continuous flow design is considered feasible for the development of air purifiers utilizing thermal energy (Jung et al., 2009). The air temperature profiles inside the chamber were determined for each set temperature and air flow rate using thermocouple probes (Type J, Model 5J36-ICIN-116, Omega Engineering, Inc., Stamford, CT). The characteristic exposure temperature ($T_{exposure}$) was estimated based on the measured air temperature profiles and corrections made for heat radiation (Grinshpun et al. 2010a). The nebulizer-generated *B. atrophaeus*

aerosol flow (6 L/min) was diluted with HEPA-filtered air at two flow rates of 12 and 30 L/min producing the total flows of Q=18 L/min and Q=36 L/min, respectively, which represented two different thermal flow regimes and exposure times, as explained below. The aerosol was passed through a 10-mCi 85Kr charge equilibrator (model 3012, TSI Inc., St. Paul, MN) and then entered the exposure chamber. The setup was housed in a Class II biosafety cabinet (Model 6TX, Baker Co., Inc., Sanford, ME).

The aerosol concentration and particle size distribution were measured in real time at the inlet and outlet of the exposure chamber using an optical particle size spectrometer (Model 1.108, Grimm Technologies, Inc., Douglasville, GA, USA). After exposure, the aerosol was sampled into three identical probes at 5 L/min and collected on three sterile 25-mm filter cassettes (SKC Inc., Eighty Four, PA) equipped with 25-mm polycarbonate filters (Millipore Corp., Billerica, MA) (pore size = 0.4 μm). The sampling time was 10 min. In a control experiment, endospores were passed through the system with the heating element turned off, i.e. at room temperature ($T_{control}$). Immediately after collection, the filters were removed. Each filter was submerged in 1 mL sterile de-ionized water and vortexed for 2 min to extract the spores. Cultivation of surviving spores was carried out as described below.

Sample analysis for quantifying the spore inactivation

Fifty μL of the spore suspension obtained after exposure was serially diluted with sterile water and 100-μL aliquots were spread on TSA. Cultures were incubated 24 h at 37°C, after which colonies were enumerated to obtain CFU_{TSA}. An average CFU value was determined from three separate counts on three replicate agar plates.

The culturable counts were compared for each test-control combination to determine the loss in spore culturability as a result of a specific thermal exposure. The loss in culturability served as a surrogate for the viability loss. Although culturable count may be lower than the viable count, culturability is widely used as a measure of viability, which is relevant to pathogenic species given that their hazard level is usually associated with the ability to multiply. Thus, the inactivation factor, IF, was defined as the control-to-test ratio of culturable spores:

$$IF = \frac{CFU_{TSA}\left(T_{control}\right)}{CFU_{TSA}\left(T_{exposure}\right)} \quad (1)$$

Experimentally determined inactivation factors calculated by Eq. (1) were corrected by multiplying by 0.667 to account for temperature profiles in the axially heated air flow This correction, introduced in Grinshpun et al. (2010a), reflects the fraction of the annular flow cross-section (50% pre-wall by the axial flow dimension), for which the characteristic exposure temperature was designated. The corrected IF-values serve as the lower approximation of the actual inactivation (Grinshpun et al., 2010a).

Sample analysis for quantification of DNA mutational damage

The remaining spore suspension from each filter was centrifuged for 2 min at 5,200×g and resuspended in 100 μL of sterile water. Mutational damage in *Bacillus* endospores results in nalidixic acid-resistance (nal^r) in a portion of exposed cells through specific mutations in the *GyrA* gene (Munakata et al. 1994; del Carmen Huesca Espitia et al. 2002). To quantify mutational damage we used an assay that involves the detection of resistant colonies formed on solid medium containing the antibiotic nalidixic acid (del Carmen Huesca Espitia et al. 2002). The frequency of nal^r mutations in the spore is assumed to be proportional to the total number of mutations in the genome. Although most mutations are unlikely to completely inactivate the spore, any mutation that causes the loss of expression or function of an essential gene product will lead to loss of viability.

To determine the frequency of mutations producing nal^r, the resuspended endospores were spread on TSA containing 20 μg nalidixic acid (Sigma-Aldrich, St. Louis, MO). Only spores that carry nal^r-inducing mutations are able to form colonies on this medium. Because the number of mutant spores in these experiments was always low, the entire aliquot from each filter was spread on one plate to increase the limit of detection. After incubation at 37°C for 48 h, nal^r colonies (CFU_{nal^r}) were enumerated. The mutational frequency (MF) for each filter was obtained by relating the colony counts on TSA without nalidixic acid and TSA containing nalidixic acid:

$$MF = \frac{CFU_{nal^r}}{CFU_{TSA}} \quad (2)$$

For each characteristic exposure temperature, we related the MF-values obtained for spores exposed and non-exposed (control) to heat. This was quantified by the mutational frequency ratio (*MFR*) defined as:

$$MFR = \frac{MF(T_{exposure})}{MF(T_{control})} \quad (3)$$

Experimental conditions

In accordance with the hypothesis of this study, the test conditions were chosen to examine the relationship between DNA mutational damage and heat-induced inactivation of endospores. The test temperatures were selected to cover the range in which *IF* rapidly changes from 1 (no effect) to ~10^2. We anticipated that the endospore inactivation of higher levels (several $\log_{10}$ reduction) may become too complex to be described by a single

mechanism or a simple kinetics relationship. Ababouch et al. (1995) suggested that multiple mechanisms may be involved in injury and repair of *B. subtilis* spores and that spores may become more resistant during heating at relatively high levels. Based on our previous findings, the chosen *IF*-range (1–100) corresponds to the following ranges of the estimated characteristic exposure temperature: $T_{exposure}$ ~ 160°C to 240°C for Q=18 L/min and $T_{exposure}$ ~ 190°C to 300°C for Q=36 L/min (Grinshpun et al., 2010a). These ranges translate to the following chamber wall temperatures, which served in the experiments as the set conditions: 150°C–225°C for 18 L/min and 200°C–325°C for 36 L/min. The above-referred estimate of the characteristic exposure temperature was made with an uncertainty of 21°C to 26°C, depending on Q and $T_{exposure}$.

Although the time of heat exposure was not clearly defined by this study design because the spores were exposed to different temperatures while moving in the flow, the characteristic exposure time was always below 1 s. For example, based on our earlier estimates (Grinshpun et al. 2010a,b), it is approximately 0.24 s at Q=18 L/min and $T_{exposure}$ =170°C and approximately 0.10 s at Q=36 L/min and $T_{exposure}$ = 250°C.

It should be noted that this study targeted temperatures in excess of those used in previous investigations addressing the spore inactivation mechanism (e.g., 155°C in Setlow and Setlow 1995). At the same time, we used much shorter duration for the thermal exposure than the quoted research.

Statistical methods

For each test temperature, the average values and the standard deviations of *IF* and *MFR* were calculated for 3–8 filter samples, each of which generated three replica plate counts. A One Way Analysis of Variance (ANOVA) was used to test the differences in *IF* and *MFR* at different $T_{exposure}$. The Pearson product moment correlation analysis was applied to test the relationship between the spore thermal inactivation and the DNA mutational frequency. The linear regression analysis was used to relate the natural logarithm of *IF* to *MFR*.

Results and discussion

Following a 10-min nebulization period, a stable concentration of the challenge aerosol was achieved upstream and downstream of the exposure chamber. The optical particle size distribution exhibited a peak at 0.7 – 0.8 μm, which corresponds to intact *B. atrophaeus* endospores (An et al. 2004). The distribution remained the same at both air flow rates tested in this study. Furthermore, axial heating did not affect the size distribution of the test aerosol (within the tested temperature range). These findings suggest that (i) the physical characteristics of the challenge bioaerosol used in this study were consistent in all the tests, (ii) the particle losses in the system were negligible, and (iii) the challenge aerosol is represented primarily by single spores. Testing with single spores (as opposite to spore agglomerates) is advantageous because it eliminates undesirable effects such as shielding and uneven heat transfer to the spore, which can potentially complicate the data interpretation.

Table 1 presents *IF* and *MFR* as functions of $T_{exposure}$ for each of the tested flow rates. As expected, increase in the characteristic exposure temperature resulted in increase in *IF*. For both flow rates, the *IF* data agreed with the results reported in Grinshpun et al. (2010a). The more important finding was that *MFR* also increased with increase of the temperature. For example, at Q=18 L/min, the average *MFR* increased from approximately 3.6 at $T_{exposure}$ ~ 164°C to 20.7 at $T_{exposure}$ ~ 212°C. At Q=36 L/min, it increased from 2.5 at $T_{exposure}$ ~ 191°C to 33 at $T_{exposure}$ ~ 277°C. Thus, differences in each of the two outcomes – the loss of spore viability (represented by *IF*) and the change in frequency of DNA mutation (represented by *MFR*) – were associated with the differences in exposure temperature (ANOVA, $p < 0.001$).

In a single mechanism model, the inactivation should follow the first-order kinetics, i.e.

$$IF \propto e^{kE_a} \tag{4}$$

where E_a is the activation energy. Previous investigators who tested UV and heavy ion radiation of *Bacillus* spores reported a linear relationship between mutational frequency and radiation dose (Tanooka et al. 1978; Baltschukat and Horneck 1991). It seems reasonable to assume the same type of linear relationship for mutations caused by other types of energy transfer. Consequently, if mutational damage is the main cause of spore inactivation, *IF* must be described by an exponential function of *MFR*:

$$IF \propto e^{k_1 MFR} \tag{5}$$

Here k and k_1 are proportionality constants, which are generally dependent of the exposure time and other parameters. Eq. (5) yields a linear relationship between $\ln(IF)$ and *MFR*.

To quantitatively characterize the relationship between the *IF* and *MFR* experimentally obtained for the same heat exposure, their measures were plotted against each other as

$$\ln(IF) = f(MFR) \tag{6}$$

and tested for linearity. When the data points obtained in the study were averaged [as ln(*IF*) and MFR for $T_{exposure}$=const, Q = 18 and 36 L/min] and subjected to a linear regression analysis, the calculated R^2 value was as high as 0.951 ($p<0.001$), see Fig. 1.

The Pearson product moment correlation analysis indicates that the spore thermal inactivation in these tests can be attributed almost entirely to increased mutational frequency, at least for *IF* below ~10^2. It is anticipated that a single (or dominant) mechanism may govern the spore inactivation process up to a certain level; at higher levels, more mechanisms or more complex kinetics may be involved. Higher inactivation levels corresponds to greater E_a values which may affect the kinetics of the process, e.g., by suppressing linearity of $MFR = f(E_a)$. More research is needed to quantify the boundaries of the first-order kinetics and potentially develop a predictive model for the process.

It is notable that the relationships obtained at two different flow rates (corresponding to different exposure times) were essentially identical. The strong correlation between inactivation and mutational frequency suggests that mutational damage is causally linked to viability loss for aerosolized endospores exposed to dry heat for sub-second time periods. It does not, however, prove direct causality. The precise mechanism by which dry heat causes mutations, or the nature of the lesions, will be explored in a follow-up study. Cell death may occur as a direct result of mutations in, and inactivation of, essential genes, or it may be caused by failure to repair DNA lesions during germination. Loss of, or disrupted binding by, SASPs would also be expected to increase the sensitivity of DNA to mutational damage Previous studies have indicated that dry heat generates abasic sites in DNA at least in part through depurination, which SASPs protects against, and $\alpha^-\beta^-$ spores are less resistant to dry heat than wildtype spores (Setlow and Setlow, 1995). A recent study by Barraza-Salas et al. (2010) showed that over-expression of the endogenous endonuclease Nfo enzyme, which repairs apurinic-apyridinic DNA damage, accumulated in dormant or germinating spores, increases the resistance of *Bacillus* spores to dry heat. Interestingly, the protective effect was much smaller in wildtype spores than in spores lacking SASPs suggesting that the efficiency with which SASPs protects DNA against apurinic damage makes repair enzymes such as Nfo in part dispensable. Although associations between dry heat inactivation of *Bacillus* spores and mutational damage have been reported earlier (Setlow and Setlow, 1995; del Carmen Huesca Espitia et al., 2002) these studies addressed a different type of exposure situation: much longer exposure periods (up to several hours) and lower temperatures (90°C–155°C). Furthermore, previous studies made use of lyophilized spores on solid support sealed under vacuum, whereas we investigated *aerosolized* spores. Given the differences in heat transfer for aerosolized and non-aerosolized spores exposed to heat, the similarity in mechanisms governing their inactivation is remarkable.

The present investigation has limitations. One comes from a less than straight-forward definition of the characteristic exposure temperature, which attempts to integrate the non-uniformities of air temperature produced by the continuous flow design, but causes uncertainties leading to fairly large coefficients of variation for *IF* and *MFR*. The same constraints of the continuous flow design limited our ability to quantify the time of exposure to a specific temperature in a more definitive way as compared to the approximation offered in this study.

Conclusion

A significant association was established between short-term thermal inactivation of aerosolized endospores of *B. atrophaeus* and the spore DNA mutation. Both the inactivation factor and the mutational frequency ratio increased as the exposure temperature increased. The strong correlation between *IF* and *MFR* appeared to be independent of the flow rate in the test system, which is linked to the exposure time (sub-second time periods were tested). The experimental findings are in agreement with the first-order kinetics of the inactivation process up to the inactivation factor of 100. Although similar associations have been reported for non-aerosol applications, long-term exposures and moderate heating temperature, this study is the first one providing strong evidence that short-term heat-induced inactivation of *aerosolized* endospores is causally associated with DNA mutational damage.

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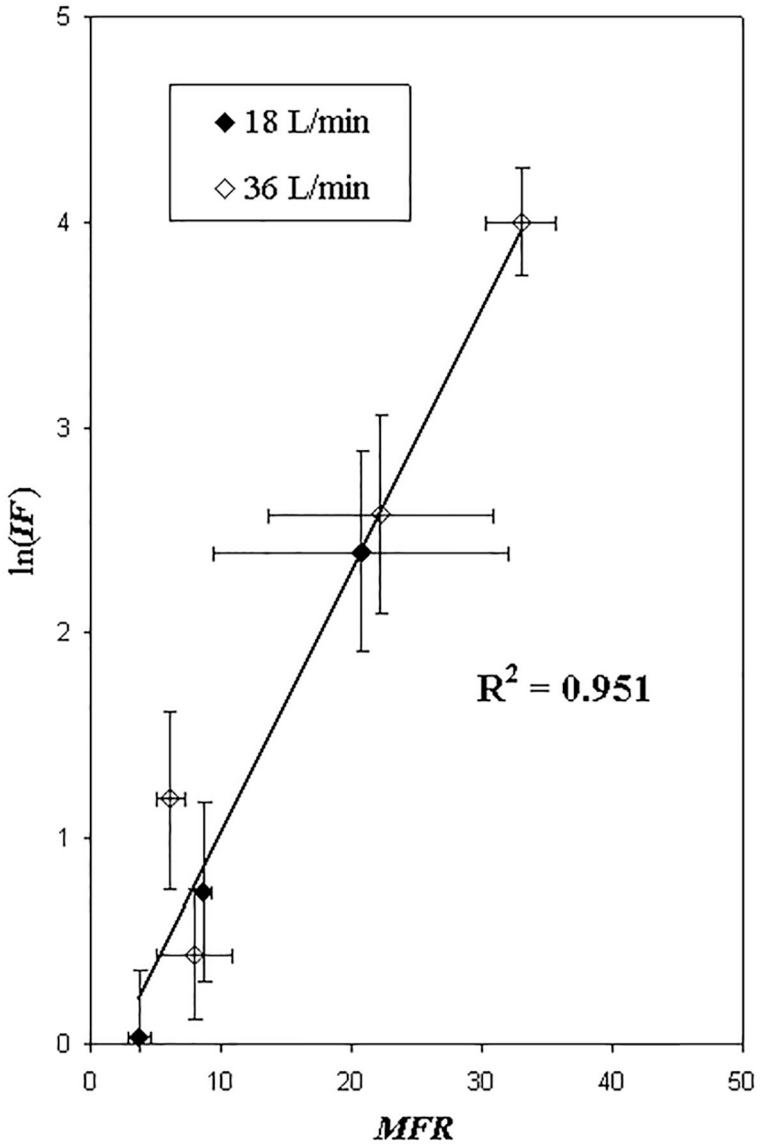

Figure 1.

Relationship between the inactivation factor (*IF*, corrected) and the mutational frequency ratio (*MFR*) for aerosolized *Bacillus subtilis* var. *niger* endospores exposed to dry heat. Each data point represents an average value for n = 3 – 7 filter samples, each of which generally generated three replica plate counts. Each bar represents the standard deviation. The line represents the linear regression.

Table 1.

The inactivation factor (*IF*, corrected) and the mutational frequency ratio (*MFR*) for aerosolized *Bacillus subtilis* var. *niger* endospores exposed to dry heat at different characteristic exposure temperatures and air flow rates. Each value represents an average or standard deviation (SD) of n = 3 – 7 filter samples, each of which generally generated three replica plate counts.

Flow rate, L/min	$T_{exposure}$, °C	Uncertainty in estimating $T_{exposure}$, °C	*IF*		*MFR*	
			Average	SD	Average	SD
18	164	25	1.07	0.20	3.60	0.89
	188	26	2.15	0.35	8.59	0.63
	212	26	10.8	6.53	20.7	11.3
36	191	18	1.69	0.60	2.50	0.72
	228	20	3.72	1.75	7.91	2.90
	255	21	13.0	6.57	22.2	8.65
	277	22	86.0	24.9	33.0	2.71