Performance evaluation of the UVAPS: influence of physiological age of airborne bacteria and bacterial stress

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ABSTRACT

This study evaluated the effect of bacterial physiology, such as physiological age and stress, on the performance of the Ultravioloet Aerodynamic Particle Sizer (UV-APS, model 3312, TSI Inc., St. Paul, MN). Intensity of the fluorescent signals was measured for three bacteria having various sensitivities to environmental stresses, *Bacillus subtilus* (spores and vegetative cells), *Pseudomonas fluorescens*, and *Micrococcus luteus.* The performance of the UVAPS was found to depend on the type of airborne bacteria. In addition, the fluorescence signals for stationary-phase bacteria were generally stronger than for their log-phase counterparts. These results indicated that bacterial injury due to environmental stresses has a strong influence on the measured fluorescence signals. This hypothesis was confirmed by obtaining a linear relationship between the percentage of fluorescent particles and the proportion of injured bacteria in the total population of cultivable bacteria in samples simultaneously collected with the AGI-30 impingers. This indicates that the amount of fluorophors (specifically NADH) within injured bacteria is below the UVAPS sensitivity level. The practical implications of these findings are discussed in the paper. The reported results contribute to broadening our understanding of the method and may assist in developing sampling strategies for the application of the UVAPS to various bioaerosol studies.

Keywords: Bacterial aerosols; Real-time monitoring; Intrinsic fluorescence; Performance evaluation

1. Introduction

Heterogeneity is one of the major properties of natural bacterial populations. The individual bacterial cells may be in a condition ranging from physiological dormancy to active metabolism and cell division. Examples of metabolically active microorganisms include exponentially growing (log-phase) cells, cultures that have reached a stationary phase and organisms that have shifted from exogenous to endogenous metabolism to survive starvation conditions. Dormant microorganisms are metabolically inactive but are capable of returning to an active state. Examples include spores that are metabolically "frozen," freeze-dried cells, and cells that remain viable at suboptimal or freezing temperatures. Bacterial dormancy is the fate of non-sporulating microbes in most natural environments and is the result of nutrient deficiency and other growth limiting factors (Reinhardt et al., 1990).

Many unique characteristics, which jointly define the microbial physiological state, distinguish bacteria of different ages. Specifically, the physiological state of a microorganism refers to complex attributes including nutritional patterns, physiological age, metabolism, and biochemical composition. The physiological state of microorganisms influences their ability to survive extreme environmental conditions (Caldwell et al., 1989; Olsen and Bakken, 1987). As a result, bacterial populations tend to become heterogenous, due to changes in microenvironments as well as genetic differences among individuals.

To accurately assess exposure to airborne viable bacteria, the performance of the bioaerosol monitors ideally should not be affected by the physiological state of the

microorganisms. However, this is not the case with the conventional bioaerosol samplers. For example, bacterial cells in log-phase of growth, which are generally more sensitive to stresses than the stationary-phase cells (Abbe and Wouters, 1999; Neidhardt et al., 1990; Cox, 1987), are also more susceptible to the sampling stresses caused by conventional biological samplers (Griffiths et al., 1996). The biological efficiency of such sampling practices, which is defined as a capability of the method to maintain the viability of airborne microorganisms, may therefore be diminished. This sampling efficiency drawback is further amplified during analysis of the collected samples. Culture onto agar or into broth remains the most popular method for both identification and enumeration of viable bioaerosols. As only a small proportion of microorganisms will grow on culture agars (Amann et al, 1995), enumeration by culture of bioaerosol samples significantly underestimates the actual exposure levels. In addition, the conventional culture methods are very laborious and time-consuming. Real-time *in-situ* bioaerosol monitors not only enhance efficiency of the bioaerosol studies but may also improve accuracy of data, compared to the conventional methods.

The Ultraviolet Aerodynamic Particle Sizer (UVAPS, Model 3312, TSI Inc., St. Paul, MN) spectrometer is a novel, commercially available aerosol counter for real-time, continuous monitoring of viable bioaerosols. The monitor measures aerodynamic diameter, scattered light intensity, and fluorescence of the aerosol particles within a size range of 0.5-15 μm. Fluorescence is produced by exciting particles with an ultraviolet laser beam at an excitation wavelength of 355 nm and detected at an emission region between 420 and 575 nm. At such conditions, the measured fluorescence is considered to be mainly produced by reduced pyridine nucleotides (e.g., NAD(P)H) which are specific

to all living cells and can be therefore used to distinguish viable aerosol particles (i.e. those that contain viable microorganisms) from the non-viable counterparts (Hairston et al., 1997). The fluorescence detected for each particle is registered in one out of 64 channels, depending on the intensity of the signal. The increasing channel numbers represent gradually increased fluorescence intensity of the particles. The proportion of measured fluorescent particles in the total particle population gives an indication of the concentration of viable bioaerosols in the environment of concern. A detailed description of the UVAPS can be found elsewhere (Agranovski et al., 2003, Brosseau et al, 2000; Hairston et al, 1997).

Although the UVAPS cannot be used to identify airborne microorganisms (i.e., to differentiate them at the genus and species level), it can be very beneficial in applications such as studying dispersion of bioaerosols, controlling the spread of bacterial diseases, and source identification. This is particularly so in situations when a risk from an exposure to bioaerosols has to be acknowledged urgently to allow the application of appropriate control measures (Ho, et al., 2002).

While the potential of this monitor for detection of airborne bacteria has been demonstrated in a number of studies by its designers (Hairston et al., 1997; Ho et al., 1999; Brosseau et al., 2000), the efficient application of the UVAPS for routine aerosol sampling is still not possible without a thorough understanding of its capabilities and limitations.

The study presented in this paper is a continuation of an investigation aimed towards systematic evaluations of the performance of the UVAPS. The previous tests, concerned with the instrument counting efficiency (Agranovski et al., 2003), have

demonstrated influence on the performance of the UVAPS by the type of bacteria in the aerosol and indicated a correlation of the fluorescent signals with the physiological state of the bacteria. Thus, it was an objective of this study to evaluate the effect of bacterial physiology, such as physiological age and sensitivity to stress, on the UVAPS performance. The tests were performed with three bacteria having various sensitivities to the environmental stresses: *Bacillus subtilis* (spores or vegetative cells), *Micrococcus luteus,* and *Pseudomonas fluorescens*. The test aerosols consisted of homogenous organisms of a defined physiological age including dormant or germinated endospores, exponential (i.e. log), or stationary growth phase cells. The aerosols were produced with carefully washed organisms, as growth media can contribute to producing fluorescence signals detected by the UVAPS and thus subsequently complicating data interpretation (Agranovski et al., 2003).

Results presented in this paper enhance the understanding of the UVAPS, particularly the capabilities and limitations of the instrument, and thus assist in developing methodologies for routine application of the UVAPS in various environments. The findings may also be useful for the designing new detection methods for the investigation of bacterial aerosols in real-time.

2. Materials and methods

2.1 Experimental design and setup

The overall design of this study was intended to compare the UVAPS response to the bacterial aerosols made up of homogenous organisms of a defined physiological age,

specifically dormant endospores, germinated endospores, and either exponential or stationary growth phase cells. In addition to physiological age, the study was concerned with the effect of bacterial viability on the UVAPS signals, particularly on establishing a correlation between the fluorescence signals and the proportion of injured/healthy cells in the viable bacterial populations. In an attempt to make the effect of the bacterial viability on the fluorescence signals more profound, bacterial cells were stressed by a heat shock. To monitor the viability of the aerosolised bacteria, the aerosols were collected with a reference sampler and then analysed for the concentration of both the total and the injured culturable population. The details of the experiments are given below.

The experimental setup used in this study is schematically shown in Fig. 1. The aerosols were generated by a 6-jet Collison nebulizer (BGI Inc., Waltham, MA) at a flow rate of 6 L/min, passed through a neutraliser (10-mCi ^{85}Kr, model 3012, TSI Inc., St. Paul, MN) and then mixed with filtered compressed air (30-40 L/min), to evaporate water and to control particle concentration. To restrain possible fluctuations in aerosol concentration, the aerosols were afterwards introduced into a mixing chamber, which was also used as a manifold for simultaneous sampling by the UVAPS and the AGI-30 impingers. The AGI-30 was selected as a reference sampler for monitoring viable microorganisms for the following reasons: (1) it is one of the most frequently used biosamplers, (2) it was readily available for the use, (3) it was most convenient sampler to us, (4) its use allowed comparison with the previously generated data (Agranovski et al., 2003).

The experiments were performed with the air temperature in the chamber between 20 and 23 $^{\circ}$C and the relative humidity (RH) within 45-55 %. The entire setup was placed

inside a Class II, Type A, Biological Safety Cabinet (SG-400 SterilGARD, Email Westinghouse Pty Ltd, Australia).

2.2. Microorganisms

Bacterial aerosols were generated using following microorganisms: *Bacillus subtilis* spores (ATCC 6633), vegetative cells of *Bacillus subtilis* (grown from spores of ATCC 6633), *Pseudomonas fluorescens* (QUT 0980; obtained from the QUT culture collection), and *Micrococcus luteus* (QUT 0982; obtained from the QUT culture collection). These bacteria are commonly found in various environments and therefore are typical airborne microorganisms.

B. subtilis is a gram-positive, facultatively anaerobic, endospore forming, rod-shaped bacterium. The vegetative cells are 0.7-0.8 μm in width and 2.0-3.0 μm in length (Bergy's 1994). The endospores are rod-shaped and are approximately 0.5 x 1.0-1.8 μm.

P. fluorescens is a gram-negative, strictly aerobic, non-sporulating bacillus (straight or slightly curved rods) with the size of about 0.7 – 0.8 μm in width by 2.0 – 2.8 μm in length (Bergy's 1994).

M. luteus is a gram-positive, strictly aerobic, non-sporulating coccus with cells 0.9 - 1.8 μm in diameter occurring singly, in pairs and dividing in more than one plane to form tetrads, irregular clusters or regular packets of 2-8 cells (Bergy's 1994).

All bacteria were grown aerobically in Trypticase Soy Broth (TSB; Difco®, Bacto Laboratories Pty Ltd, Liverpool, NSW, Australia) on a rotary shaker at the relevant optimal temperature: *B. subtilis* at 37 ^{o}C, *P. fluorescens* at 30 ^{o}C, and *M. luteus* at 35 ^{o}C. The physiological age of the bacterial cells was controlled by using growth curves

obtained by both viable counts and turbidity (optical densities) measurements, which were determined hourly. Viable counts were performed on Trypticase Soy Agar (TSA; Difco®) plates and the turbidity was monitored at 540 nm. In addition, to monitor the *B. subtilis* sporulation process, the post-exponential cells were periodically examined under phase contrast microscope.

2.3. Preparation of bacterial suspensions

Suspensions of the dormant *B. subtilis* spores were prepared by suspending one vial of the supplied material in 50 ml of sterile distilled water (SDW) to yield a concentration of culturable microorganisms in the order of 10^9 CFU/ml. The suspension was subsequently heated at 75 ^oC for 10-15 min in a temperature-control water bath to kill any vegetative cells that may have possibly been present.

For preparation of the suspensions of the germinated *B. subtilis* spores, one vial of the supplied spore material was suspended in 30 ml of TSB and heated at 75 ^oC for 15 min to activate the spores and to kill any vegetative cells present. After that, the suspensions were quickly cooled down and then incubated at 30 ^oC on a rotary shaker for 15-20 min. The germination process was monitored by measuring the drop in optical density (at 540 nm) coupled with the examination of spores with phase contrast microscope as previously described by Coote (1982). The germinated spores were harvested by centrifugation (2,000 x g, 15 min, room temperature), the pellet was resuspended in SDW, and then centrifuged again. The procedure was repeated twice before a final resuspension in 50 ml of SDW.

The suspensions for aerosolisation of bacterial cells were prepared by harvesting bacteria from broth as follows. One millilitre of the overnight starter culture (50 ml TSB) was subcultured in 100 ml of TSB and incubated at the relevant optimal temperature until the desired growth phase was reached. Cells were harvested by centrifugation (2,000 x g, 15 min, room temperature), washed three times and then resuspended in 50 ml of SDW. Using SDW as the suspension medium was intended to avoid the interference with the UVAPS fluorescence signals of bacterial cells (Agranovski et al., 2003). Although SDW is not considered an optimal washing and resuspension medium for preserving the viability of bacteria, preliminary experiments had demonstrated that it was an appropriate medium for all microorganisms used in this study. The recovery of viable cells by using SDW was comparable with that of using peptone water.

Harvesting the bacteria at a specific stage of growth was critical for this study since the cellular chemical composition, in particular the NADH content, varies during the growth phases (Setlow and Setlow, 1977; Neidhardt et al., 1990). For *B. subtilis* cultures, harvesting at the early stationary phase is important in order to avoid the sporulation process, which is known to begin at the end of exponential growth and requires approximately 8 to 10 hours for completion, under standard laboratory conditions (Sonenshein et al., 1993; Errington, 1993). Avoiding sporulation was desirable, as it could affect the concentration of fluorophores within the *B. subtilis* cells and subsequently the UVAPS signals. Harvesting the other test microorganisms at the early stationary phase was important to insure the consistency of the results and to assist data interpretation. This was necessary as the bacterial cells in the prolonged stationary-phase of growth are known to undergo compositional changes, which also makes them more

resistant to the environmental stresses (Neidhardt et al., 1990; Watson, 1990; Kolter et al., 1993; Hengge-Aronis, 1993).

The suspensions with the heat-stressed *M. luteus* cells were prepared as follows. The bacterial cultures were grown, harvested and washed as described above. To assure an even distribution of the bacteria throughout the liquid, the final suspensions (50 ml) were thoroughly mixed by vortexing at maximum speed for 10 sec and then equally divided between two centrifuge tubes containing 25 ml of SDW. The first tube was designated for the tests with "healthy" bacteria and maintained at room temperature. The second tube, designated for "heat-stressed" bacteria, had been preheated and maintained at 60^{o}C. After addition of the bacterial cells, the tube was subsequently heated at 60^{o}C for 5 min on a rotary shaker and then quickly cooled in ice. The total viable and injured bacterial populations of both suspensions were subsequently counted as discussed in the next sections.

Since prolonged nebulisation over 2 hours may affect viability of bacterial cells (Jensen et al, 1992; Agranovski et al., 2002), only fresh suspensions were used for the trials conducted on different days.

2.4. Enumeration of viable bacteria

The Spread-Plate Count technique (Greenberg et al., 1992) was used to enumerate viable bacteria in the suspensions used to generate the test bioaerosols (both before and after aerosolisation) and in the collection medium of the AGI-30 impingers. Aliquots (0.2 mL) of serially diluted samples were inoculated onto the surface of the respective agar plates (see next section for the details) in triplicates and incubated at the relevant

temperatures. The plates with *B. subtilis* were incubated at 37˚C for 1 day, the plates with *P. fluorescens* at 30˚C for 1-2 days, and with *M. luteus* at 35˚C for 1-2 days. All plates were inspected daily.

The concentration of airborne viable bacteria were determined by converting number of colony forming units (CFU) into an aerosol concentration, taking into account the dilution factor, the volume of sample plated, the elute volume, and the volume of sampled air. The proportion of viable bacteria (further referred to as culturable fraction) in the total population of aerosol particles was determined by dividing the total aerosol count measured by the UVAPS to viable aerosol count measured by plate technique. The culturable fractions were subsequently compared with the fluorescent aerosol fractions, which were determined by dividing the total aerosol count to the fluorescent count measured by the UVAPS.

2.5. Discrimination of injured bacteria

Microorganisms subjected to environmental stresses can undergo sublethal physiological and structural changes of varying degrees, termed injury (McFeters, 1990). The most prominent characteristic of injured cells is the inability to form colonies on selective agars on which uninjured cells can recover and grow (Hurst, 1977; Jay, 1986; McFeters, 1990). Thus, the criterion often used to discriminate injured airborne bacteria is their ability to grow on non-selective but not on selective agar media (McFeters, 1990). The difference in counts between selective and non-selective reference media is used as a means to quantify the fraction of sublethally injured population, in accordance with the following equation:

$$\%\ Injured\ bacteria = \left(1 - \frac{CFU_{selective}}{CFU_{non-selective}}\right) \times 100\,\% \quad (1)$$

Cetrimide Agar (CA; Difco®), which is often used as the selective media for *Pseudomonas* species (Bergy's, 1994), was used in this study as a selective agar for *P. fluorescens*. Mannitol Salt Agar (MSA; Difco®) was used as the specific selective media for *M. luteus*. TSA was used as the non-selective reference medium for both bacteria. It has to be noted, that the agar selection was to a certain extent arbitrary, as different media recover bacteria with varying efficiencies. Thus, the obtained values should be considered as the indicators of the relative levels of injured populations, rather than the absolute.

2.6. Experimental procedure

Before starting the experiments, the UVAPS sensitivity was adjusted using the procedure described by Agranovski et al (2003) with Polystyrene Latex (PSL) Particles (Duke Scientific Corporation, Palo Alto, CA). Afterwards, every measurement of the bacterial aerosols was started and finished with measuring the PSL (0.993 μm) aerosol. The intensity of the fluorescence signals for the PSL aerosols was converted into the percentage of fluorescent particles (referred to as "percent fluorescence") and used as a reference. The "percent fluorescence" of the PSL reference aerosols was subsequently compared with the "percent fluorescence" of the bacterial aerosols. Since the counting efficiency of the UVAPS was found to depend on the particle concentration (Agranovski et al., 2003), the total concentration of the test aerosols was maintained at arbitrary level

of about 2 x 10^7 particles/ m^3. The aerosol concentrations were adjusted by altering the flow rate of the diluting air.

After cleaning the chamber with filtered air, continuous bioaerosol measurements were taken with a sampling time of 3 seconds. To obtain statistically significant results, all measurements were repeated at least three times and the results have been presented as the averages. For comparative tests, the bacterial aerosols were simultaneously measured with the UVAPS and the AGI-30 impingers over 10-15 min sampling periods. Samples collected with the AGI-30 impingers were analysed by the culture techniques described above.

2.7. Statistical analysis

Statistical analysis was conducted on the complete data set using the Student's *t*-test. Statistical significance was accepted at the $P<0.05$ level of probability.

3. Results

3.1. Effect of physiological age

The first series of experiments concerned the effect of bacterial physiology on the UVAPS performance and was conducted by aerosolising test microorganisms of defined age. The organisms were prepared under specific conditions to represent the complete range of physiological ages. The results presented in this paper correspond to the measurements undertaken for the aerosols of matching particle concentrations, which were supplied at arbitrary level of approximately 20 particles/ cm^3. The UVAPS data

were expressed in a percent fluorescence which was calculated as a percentage of the fluorescent particles in the total particle population.

Very weak or no fluorescent signals were detected for both dormant and germinated *B. subtilis* spores. The corresponding spectra were not statistically different from the UVAPS signals measured for the background PSL aerosols ($P>0.05$). The mean percent fluorescence for these three aerosols (dormant spores, germinated spores and PSL particles) was in order of 2.0 ± 0.1 % ($n = 9$), whereas a simultaneous control sampling of *B. subtilis* (spores) aerosols with the AGI-30 liquid impingers showed that at least 30–45% of the particles were viable.

Similar results were obtained for the *P. fluorescens* aerosols (Fig. 2). The difference between the mean percent fluorescence of the PSL aerosols and both stationary and log-phase *P. fluorescens* aerosols was not significant ($P>0.05$). The control samples collected with the AGI-30 revealed, however, that the fraction of culturable particles was slightly higher for the aerosols generated from the suspensions of stationary-phase cells than those with log-phase cells. The mean culturable fractions of aerosolised *P. fluorescens* cells were found to be 32.2 ± 1.1 % ($n = 3$) and 27.9 ± 1.2 % ($n = 3$) for the stationary and log-phase cells, respectively (Fig. 3).

The fluorescent spectra of aerosolised cells of *B. subtilis* and *M. luteus* were stronger for stationary-phase microorganisms (Fig. 2; $P<0.05$). These results were consistent with the results of culture analysis of the AGI-30 samples grown on the TSA plates, which showed higher proportion of culturable particles in the aerosols generated with stationary-phase cells than in those with the log-phase cells (Fig. 3). The difference between the fractions of fluorescent particles was however bigger than the difference

between the corresponding culturable fractions for the aerosolised cells of different physiological age, particularly for *B. subtilis* aerosols. The larger than 100 % culturable fractions of bacteria in the *M. luteus* samples (Fig. 3) indicate that the majority of the cells were aerosolised as clumps, which were subsequently broken down into individual cells during aerosol collection by the AGI-30 impingers. This was confirmed by observing the samples under an optical microscope.

The mean particle diameters of the aerosols generated from stationary-phase cells of *B. subtilis* and *M. luteus* bacteria were bigger than those for log-phase cells ($P < 0.05$; Table 1). The size difference for *P. fluorescens* particles was not statistically significant ($P > 0.05$; Table 1).

3.2. Effect of stress

The second series of experiments was conducted to establish an association of the UVAPS fluorescence signals with bacterial stress. The percent fluorescence of the bacterial aerosols was compared with the proportion of injured cells in the total population of culturable bacteria (Table 2). To estimate the extent of the injured population, the aerosol particles collected by the AGI-30 impingers were analysed by cultivation on both selective and non-selective agar media (see Materials and Methods).

While only about 4.5 % ($n = 3$) of the *P. fluorescens* stationary cells were found injured in the original suspensions, which were then used to aerosolise the bacterium, all cells were injured as a result of the aerosolisation (Table 2). The corresponding measurements with the UVAPS resulted in no fluorescence detection, which indicated that amount of fluorophors in the airborne (injured) cells were below the sensitivity level

of the instrument. Since the log-cells were expected to be even more sensitive to the aerosolisation stresses than the stationary one, they were not further used for investigation.

The results of the UVAPS measurements for the *M. luteus* aerosols were consistent with the results of culture analysis of the samples collected with the AGI-30 (Table 2; see results for the "healthy" bacteria). Aerosolisation stress was more damaging for the log-phase cells than those harvested at stationary phase of growth. Almost half of the log-cells from the original suspension were injured during aerosolisation, whereas only about 16 % of stationary-cells were affected while airborne (Table 2). The fluorescence signals were also stronger for the stationary-cells. The proportion of both fluorescent and culturable particles was however smaller than in the corresponding tests of the first series (Figs 2 and 3). The total culturable fractions of cells in the samples collected by the AGI-30 and then cultivated on the TSA media were also smaller than in the earlier tests: about 25.2 ± 0.3% and 80.5 ± 0.2 % for log and stationary cells, respectively. These results were a consequence of using half concentrated solutions of the *M. luteus* suspensions in the second series of tests, which restricted formation of the bacterial clumps in the aerosols. Such an effect was reflected also on the mean diameters of both total and fluorescent aerosol particles (Fig. 5).

To better understand the link between bacterial viability and the fluorescent signals of the UVAPS, a third series of experiments was performed with *M. luteus,* using cells stressed by a heat-shock before aerosolisation. The stressed bacterial samples were tested against the control "healthy" populations. The results for the "healthy" cells were not statistically different from those in the second series of tests. The mean particle diameters

(Fig. 5), the fluorescent signals, and results of the culture analysis compared favourably with each other.

There was a significant difference between the injured fraction of the heat-stressed and "healthy" bacterial samples, particularly for the log-phase cells (Table 2). The UVAPS signals for the *M. luteus* aerosols were consistent with the proportion of the injured airborne bacteria (Fig. 4). The mean particle size of aerosolised "healthy" bacteria was consistently bigger than those for the heat-stressed cells (Fig. 5).

4. Discussion

The main focus of the study presented in this paper was on examining whether the performance of the UVAPS was dependent on the physiological age of bacteria and on establishing a correlation of the fluorescence signals with bacterial viability.

The primary fluorophor in bacteria illuminated with 355 nm light and detected at 420-575 nm is NADH (nicotinamide adenine dinucleotide) (Li et al., 1991), which is involved in cell growth and metabolism. While part of the fluorescence measured under the UVAPS operational conditions may also originate from NADPH and riboflavin, the contribution of these fluorophors is not significant (Hairston et al., 1997; Ho et al., 1999) and is not a major concern. Thus, the UVAPS fluorescence signals were assumed to be proportional to the NADH level in aerosol particles.

The amount of intracellular NADH in bacteria is known to depend on their metabolic activity, thus on their physiological age, and growth conditions (Karl, 1980). The concentration of NADH in different bacteria grown under similar conditions, such as growth media and oxygen supply, is however almost identical (Wimpenny and Firth,

1972; Neidhardt et al., 1990). The UVAPS signals were therefore expected to be comparable for the bacteria of similar sizes and physiological ages. However, these assumptions were not supported experimentally.

Despite the fact that *B. subtilis* and *P. fluorescens* vegetative cells have similar sizes and hence were expected to produce similar UVAPS responses, their fluorescence signals were found to be radically different. While relatively strong fluorescence has consistently been measured for the *B. subtilis* cells, the signals for the *P. fluorescens* aerosols (Fig. 2) were comparable with those for the background PSL aerosols (results are not shown). Simultaneous sampling with the AGI-30 impingers, however, showed that at least 35 % of the *P. fluorescens* cells were viable (Table 2). These results indicate that amount of NADH in the airborne *P. fluorescens* was considerably smaller than that within the *B. subtilis* cells. The most obvious explanation for this effect is that aerosolisation caused some intracellular compositional changes in the bacterial cells, which were more profound in the *P. fluorescens* cells than in the *B. subtilis* cells.

As NADH is a metabolic product, dormant bacterial spores lack this compound. NADH starts being actively produced during spore germination process at levels comparable with those in the log-phase cells (Setlow and Setlow, 1974). The germinated *B. subtilis* spores were therefore expected to produce some fluorescence, whereas metabolically dormant spores were used only as a reference aerosol. However, no measurable fluorescence was recorded by the UVAPS for both physiological states of the *B. subtilis* spores. There are two possible explanations for these results. First, this phenomenon may be attributable to the size of spores, with the amount of NADH molecules per spore being lower than the sensitivity level of the UVAPS. The second

reason is a sensitivity of the germinated spores to the aerosolisation stresses, as germination makes the spores vulnerable to stress similar to the log-phase cells (Neidhardt et al., 1990). Our numerous attempts to generate spore clumps with the Collison nebuliser by using more concentrated spore suspensions failed. To produce the clumps, other generation methods would need to be employed. However, these methods would affect not only the size of the aerosolised particles but also the viability of spores. As the viability of airborne microorganisms is known to depend on generation procedure, the use of different generation methods would greatly complicate an interpretation of the results and make the comparison of data almost impossible.

In regards to the vegetative cells of different age, the stationary phase cells produced considerably stronger signals than the log-phase cells (Fig. 2). The only exception was the *P. fluorescens* aerosols, for which no fluorescence was measured for both cases. These results indicate that the amount of NADH in the airborne log-phase cells was smaller than in the stationary-phase cells. It is difficult to conclude whether the observed difference in the NADH level of the airborne cells is consistent with those in the original suspensions or whether this is predominantly the result of aerosolisation, as log-phase cells are more susceptible to stresses (Neidhardt et al., 1990; Cox, 1987). Although intracellular levels of pyridine nucleotides play an important role in the cellular metabolic network of microorganisms, limited data are reported in the literature in regards to their level during growth phases. Moreover, the majority of the information available concerns either anaerobic bacteria, or eukaryotes cells, which may have different metabolism framework than aerobic bacteria. Unfortunately, methods (such as enzymatic ones) for direct measurements of NADH in the cells in question were not available for this study.

Nevertheless, the predictions on the relative amount of NADH within bacteria can be made by considering general principals of bacterial metabolism, cellular biochemistry, and the related experimental research.

The amount of NADH, which is mostly involved in producing energy by respiration in actively growing cells and in biosynthesis of proteins, should generally decline when nutrients essential for active growth of cells are depleted and cells enter stationary phase (Andersen and Meyenburg, 1977). Similarly, starvation will reduce the amount of NADH as the compound will be consumed for needed biosynthetic and catabolic enzymes (Neidhardt et al., 1990). Thus, the log-phase cells may be expected to have a higher content of NADH than the stationary-phase cells. This assumption is supported quantitatively by the relative amount of NADH in *Bacillus megaterium* at different phases of growth (Setlow and Setlow, 1974). However, on-line monitoring of NADH-related fluorescence for controlling fermentation processes indicates the possibility of rather contradictory trends. For instance, in case of *Pseudomonas putida* (Li and Humphrey, 1991) the NADH fluorescence signals are slightly stronger during stationary phase than during log-phase of growth. An opposite tendency was however observed for *Corynebacterium glutamicum* (Kwong and Rao, 1994) and for some yeast cultures (Zabriskie and Humphrey, 1978; Li et al., 1991). Such controversy is another confirmation of the complexity of microbial metabolism, making the prediction of the NADH levels in bacteria a rather difficult task.

Regardless of the absolute amount of NADH in the log and the stationary cells, the difference between the two groups of intact cells seems unlikely to be as dramatic as follows from the fluorescence signals (Fig. 2). Considering the relative sensitivity of the

tested bacteria to stress, the results of the first series of experiments are indicative of cell injury caused by aerosolisation. The results of the succeeding experiments, performed with the *P. fluorescens* and *M. luteus* aerosols, have confirmed this hypothesis. The values presented for these two bacteria in Table 2 demonstrate that the measured fluorescence was proportional to the amount of injured aerosolised bacteria, as determined by cultivation on selective media. These results indicate that sublethally injured cells responded to stress (both heat shock and aerosolisation) by entering a physiological state which: (1) was accompanied by a decrease in the NADH level and (2) required specific reparative processes for restoring the ability of the cells to multiply. The latter was reflected in the ability of the cells to grow only on a non-selective medium, which supported cell recovery before growth. It has to be pointed out, however, that the presented values for the percentage of injured cells must be treated only as indicative, since the choice of the selective media used for calculations was arbitrary. Different selective media could have been used for evaluating the extent of the injured population. Those different selective media may have recovered the injured bacteria with varying efficiencies. Thus, the respective values in Table 2 are rather indicative then absolute. In addition, the injury of bacteria collected by the AGI-30 impingers, which were used as the reference sampler in this study, is a reflection of not only the stresses imposed on the cells during aerosol generation and while airborne, but also during aerosol collection by impingment. Thus, assuming that cell damage during collection by the UVAPS is negligible, the percentage of injured bacterial cells shown in Table 2 is probably an overestimate of the concentration of injured cells in the aerosols measured by the spectrometer. Nevertheless, the results can be used to draw the trends for the correlation

of the UVAPS signals with the physiological state of measured bacteria, particularly injured bacteria. The correlation between the fluorescence signals and the amount of injured bacteria is particularly convincing for the *M. luteus* aerosols (Fig. 4). This correlation can be explained by considering the structural and physiological consequences of stress on bacterial cells. The inability of cells to grow on selective media is indicative of membrane damage (Ray, 1979). The cell membrane is known to be the site of the respiration processes, with all the essential respiration proteins and components of the respiratory chain, including NADH, being embedded in the cytoplasmic membrane (Neidhardt et al., 1990). Thus, membrane damage was accompanied by an inactivation of the membrane-bound transport system that was consequently reflected in a decreased level of NADH. These findings are consistent with the previously reported observations of reduced intracellular ATP concentrations (Camper and McFeters, 1979) and major metabolic alterations, including reduced (>75%) aerobic respiration (Domek et al. 1987).

Information on the damaged cellular components and altered physiological activities of stressed bacteria is limited. The results of the present study provide indirect evidence that the compromised physiological functions relate to membrane damage as discussed above. These findings might have a more universal significance for those concerned with applying methods for the determination of bacterial metabolic activity, such as the use of monotetrazolium redox dye (CTC) as a measure of actively respiring bacteria, to enumerate viable airborne bacteria. The alteration of bacterial metabolism caused by cellular damage during aerosol sampling may diminish the usefulness of these methods for the enumeration of viable bacteria in ambient air. It can be assumed that the portion of metabolically active organisms will be underestimated by these methods.

5. Conclusions and implications

Perhaps the most interesting conclusion, which can be drawn from this study, has been the strong influence on the performance of the UVAPS by the physiological state of bacteria, specifically injured cells. The amount of NADH in the injured cells seems to be below the sensitivity level of the instrument. The same may apply to the smaller particles, specifically those of the size of *B. subtilis* spores.

In light of the findings of the present study, it can be concluded that the application of NADH-related fluorescence for enumerating viable airborne microorganisms has a similar drawback as classical cultivation technique. Specifically, both methods have limitations in detecting viable microorganisms with poorly defined physiological attributes such as apparent cell integrity and measurable cellular activity. However, the results presented in this paper are limited to pure bacterial cultures. In real-life situations, microbial populations are highly heterogeneous which may diminish the accuracy of culture-based techniques dramatically. Bacterial competitiveness is known to cause some species to overgrow others. Hence, stressed organisms, which grow slower than their healthy counterparts, may be swamped during cultivation. Under these circumstances, culture-based methods may significantly underestimate exposure to the total population of viable microorganisms. Thus, the efficiency of the UVAPS to measure heterogeneous bioaerosols under field conditions may be higher than the efficiency of the traditional culture-based methods. As with any other method, the suitability of the UVAPS to study bioaerosols in a particular case has to be evaluated under the specific field conditions by its direct comparison with the alternative sampling techniques. The results of this study

demonstrate the sensitivity of the UVAPS to the physiological state of bacteria and the need for the potential users to be cautious when interpreting results. In addition, the results obtained in this study may be of interest for those concerned with applying the NADH-dependent fluorescence, or other markers of microbial metabolism, for monitoring airborne viable bacteria.

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Figure Legends

Table 1. Effect of physiological age on characteristics of bacterial aerosols.

Table 2. Correlation of bacterial viability with the fluorescent signals of the UVAPS.

Fig. 1. Schematic diagram of the experimental setup.

Fig. 2. Comparison of the UVAPS data obtained for three bacterial aerosols generated from the aquatic suspensions of washed bacterial cells of defined physiological age. The results expressed as the concentration ratio of the fluorescent particles to total aerosol particles, which is then converted to a percentage. Separate datum values indicate the average determined for $n \geq 3$ replicate samples.

Fig. 3. Fraction of viable particles in aerosols generated from the suspensions of bacterial cells of defined physiological age. The results expressed as the concentration ratio of viable particles in the samples collected with the AGI-30 impingers and then cultivated on TSA plates to the total aerosol particles measured by the UVAPS, which was afterwards converted to a percentage. The results for *M. luteus* aerosols indicate that the majority of cells were aerosolised as the clumps, which were broken down into individual cells during aerosol collection.

Fig. 4. Correlation of the fluorescent signals with the bacterial viability. These results based on the tests with the *M. luteus* and *P. fluorescence* aerosols. The linear trendline corresponds to the *M. luteus* results.

Fig. 5. Size of aerosol particles in *M. luteus* aerosols.

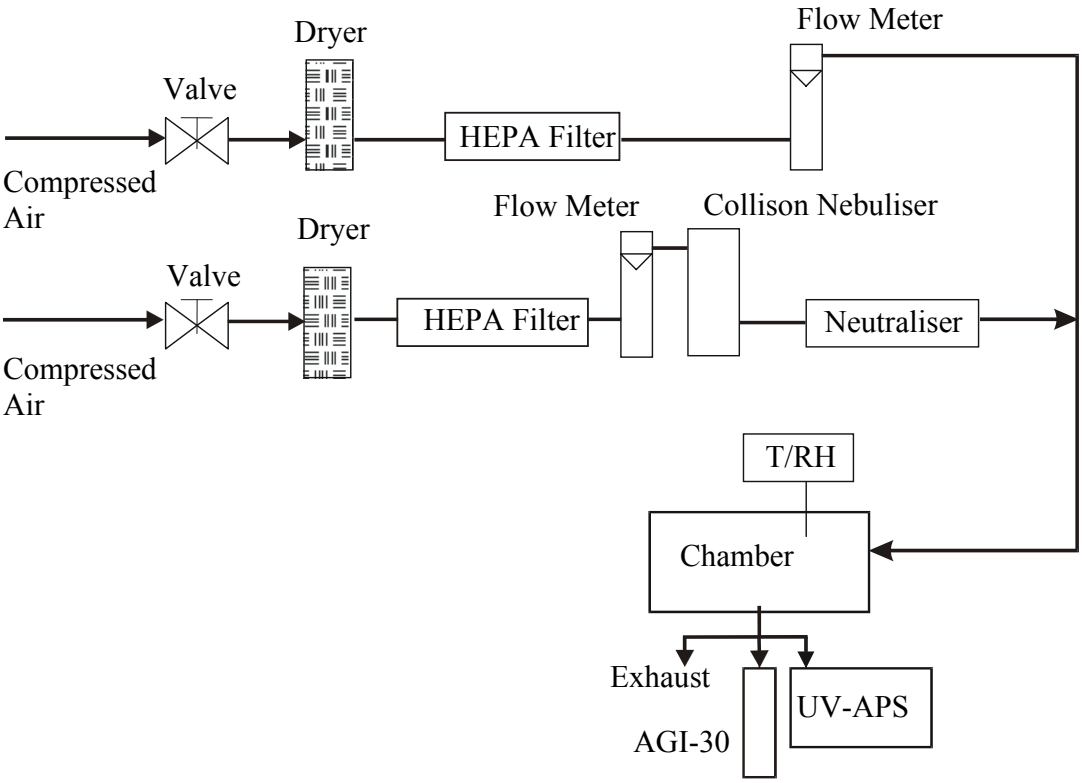

Fig 1 Experimental set-up.

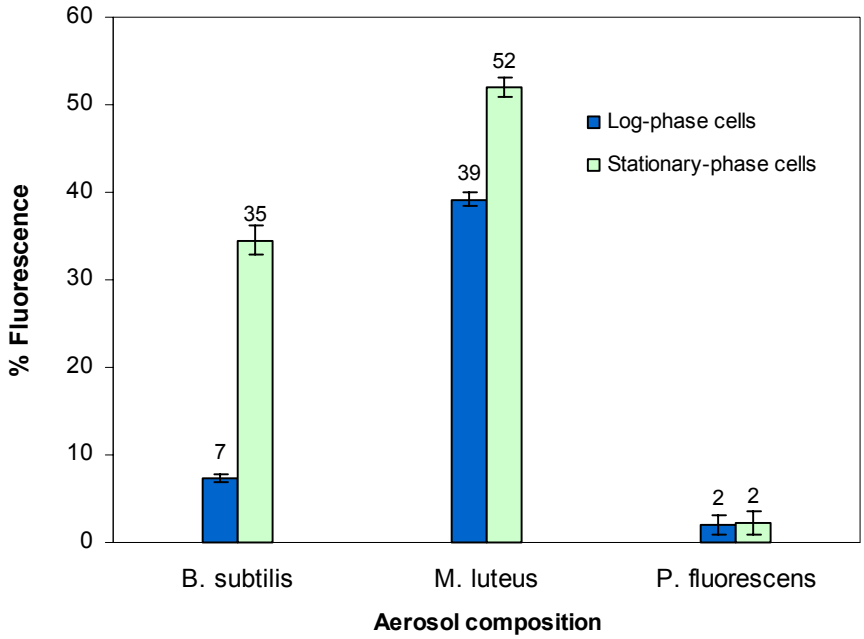

Fig 2. Comparison of the UVAPS data obtained for three bacterial aerosols generated from the aquatic suspensions of washed bacterial cells of defined physiological age.

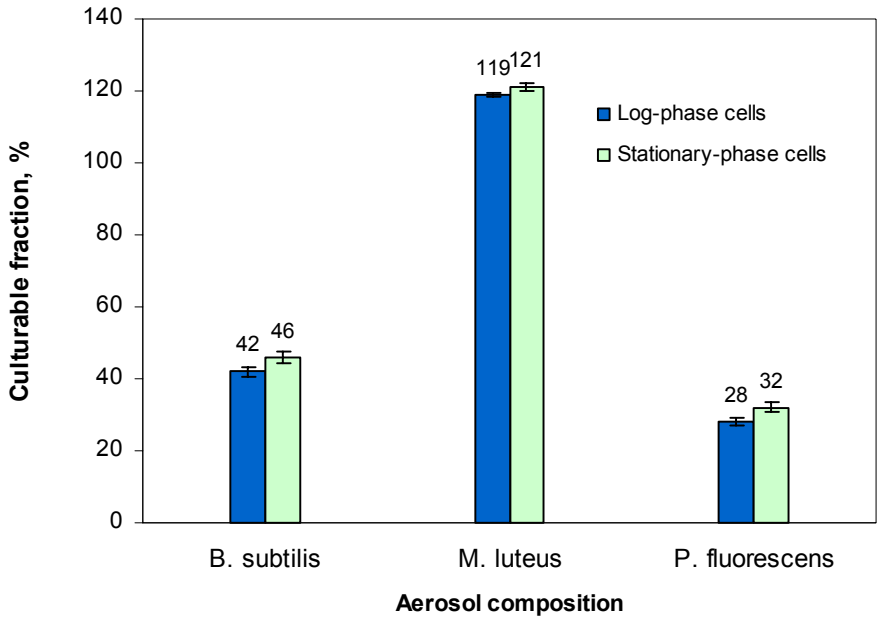

Fig 3. Fraction of viable particles in aerosols generated from the suspensions of bacterial cells of defined physiological age.

Table 1. Effect of physiological age on characteristics of bacterial aerosols

Organism/Age[a]	Particles	Aerosol statistics[d] Conc., #/cm^3	Size, μm	GSD
B. subtilis				
Log-phase	total[b]	19.80 (0.19)	0.92 (0.01)	1.28 (0.01)
	fluorescent[c]	1.44 (0.09)	1.08 (0.01)	1.57 (0.12)
Stationary-phase	total	20.10 (0.53)	0.97 (0.02)	1.33 (0.01)
	fluorescent	6.94 (0.44)	1.09 (0.05)	1.22 (0.01)
M.luteus				
Log-phase	total	21.05 (0.18)	1.51 (0.06)	1.34 (0.02)
	fluorescent	8.25 (0.22)	1.72 (0.02)	1.18 (0.00)
Stationary-phase	total	20.81 (0.16)	1.63 (0.02)	1.31 (0.01)
	fluorescent	10.82 (0.08)	1.88 (0.01)	1.17 (0.00)
P.fluorescens				
Log-phase	total	21.03 (0.02)	0.78 (0.02)	1.25 (0.01)
	fluorescent	0.43 (0.08)	1.07(0.08)	0.90 (0.33)
Stationary-phase	total	20.81 (0.01)	0.77 (0.01)	1.31(0.01)
	fluorescent	0.44 (0.27)	1.09 (0.07)	0.81 (0.01)

[a] As determined by optical density of the corresponding bacterial cell suspension.

[b] As determined by counting aerosol particles through the channels 1 to 64.

[c] As determined by counting aerosol particles through the channels 2 to 64.

[d] Each set of values is the average with the standard deviations in parentheses

Table 2 . Correlation of bacterial viability with the fluorescent signals of the UVAPS.

Organism/Age[a]	Stress applied	Fluorescent particles[c], %	Injured bacteria, %	
			Suspension	Aerosol[d]
M. luteus				
Log-phase	Healthy[b]	34.5 (0.1)	0.7 (0.0)	46.3 (0.2)
	Heat-stressed	23.6 (0.1)	0.9 (0.0)	57.1 (0.3)
Stationary-phase	Healthy	44.0 (0.2)	0.2 (0.0)	16.1 (0.2)
	Heat-stressed	43.4 (0.1)	0.5 (0.0)	20.4 (0.3)
P. fluorescens				
Stationary-phase	Healthy	0.0 (0.0)	4.5 (0.0)	100.0 (0.0)

[a] As determined by optical density.

[b] Washed bacterial cells suspended in SDW.

[c] Average values expressed as the concentration ratio of the fluorescent particles to the total aerosol particles measured by the UVAPS, which is then converted to a percentage; the standard deviations given in parentheses.

[d] As determined from the samples collected with the AGI-30 impingers by cultivation (for details see Materials and Methods).

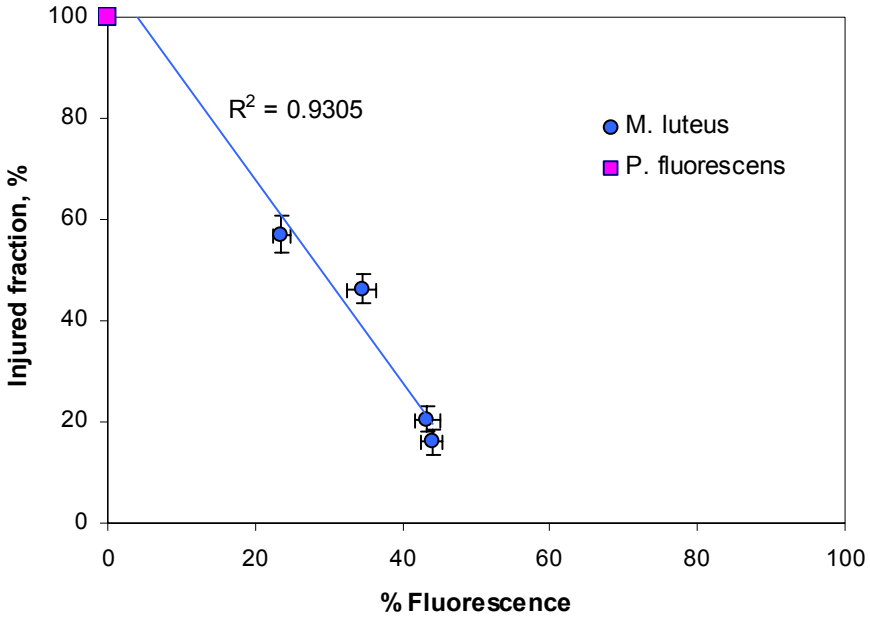

Fig. 4. Correlation of the fluorescent signals with the bacterial viability. These results based on the tests with the *M. luteus* and *P. fluorescence* aerosols. The linear trendline corresponds to the *M. luteus* results.

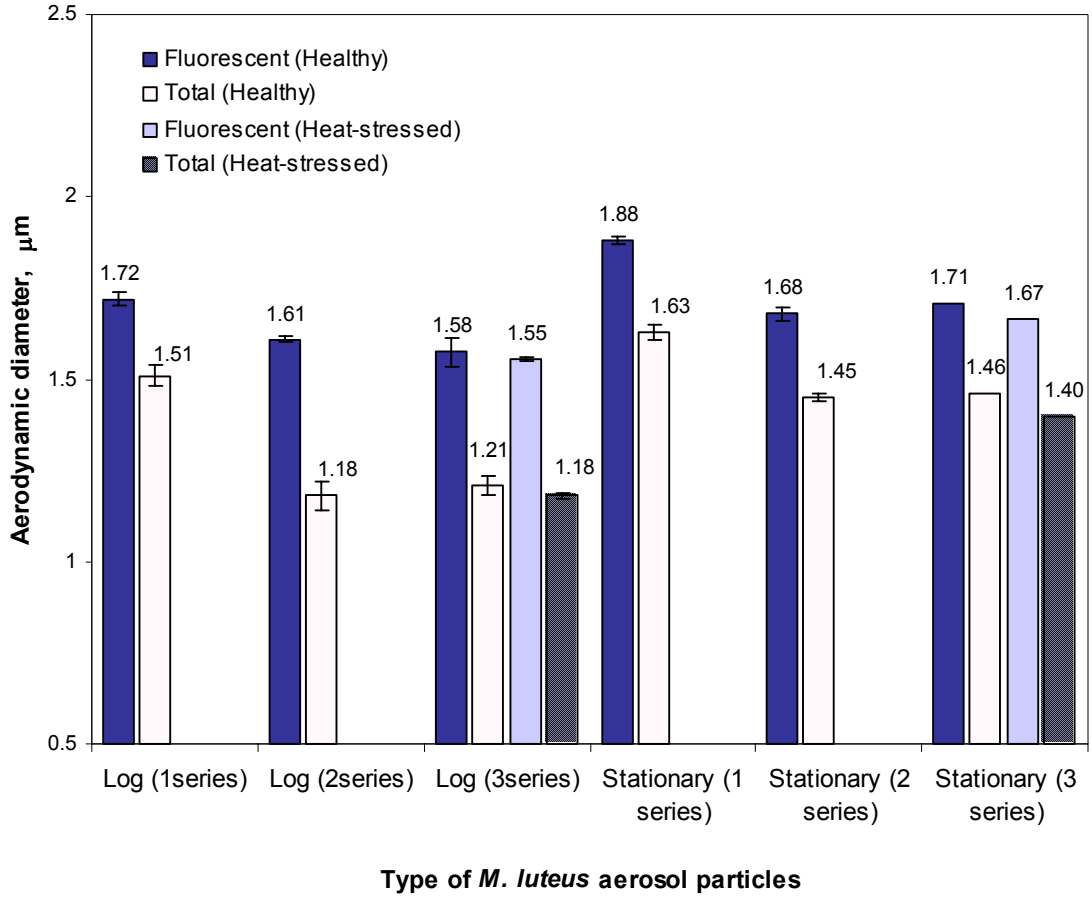

Fig 5. Size of aerosol particles in *M. luteus* aerosols.