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螢光顯微鏡、流式細胞儀與定量 PCR 用於生物氣膠之分析 (2/3)

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SAMPLING PERFORMANCE FOR BIOAEROSOLS

BY FLOW CYTOMETRY WITH FLUOROCHROME

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一、中文摘要

雖然培養分析仍是環境生物氣膠的主要分析方法，但為了要更了解及定量生物氣膠，應該一起使用培養及非培養的方法並加以比較。在此流式細胞儀及螢光染色法用不同的螢光染劑染色（細胞膜的完整性及代謝）用於去評估衝擊（**AGI-30 all-glass impinger**）及過濾**(track-etched polycarbonate filter)**的採樣成效，然後和傳統的培養方法做比較。兩種細菌性氣膠和兩種真菌性氣膠被作來研究。生物氣膠的特性（堅硬的或脆弱的）在採樣過程中高度影響著生物氣膠的活性，就如螢光染劑會有不同的物理機制一樣。對於被採樣的生物氣膠活性方面，衝擊器比過濾器更好。此外，發現在過濾時採樣的壓力對生物氣膠代謝機制的影響較細胞膜的完整性大。再者，也發現由於採樣壓力所造成不同的細胞膜完整性及代謝性和生物氣膠的種類有關。

關鍵詞：生物氣膠、採樣效率、流式細胞儀

Abstract

Although culture-based analysis remains the primary method for environmental bioaerosol analysis, for better understanding and quantifying of bioaerosols, both culture and nonculture-based methods should be used and compared. Here, flow cytometry with fluorochrome (FCM/FL) was applied to evaluate the sampling performance of impingement (AGI-30 all-glass impinger) and filtration (track-etched polycarbonate filter) with different types of fluorescent dye staining (cell membrane integrity and metabolism) and then compared with a traditional culture method (culturability). Two bacterial aerosols (Escherichia coli and endospores of Bacillus subtilis) and two fungal aerosols (Candida famata and Penicillium citrinum spores) were studied. The bioaerosol viability during the sampling processes was highly influenced by bioaerosol characteristics (hardy or fragile), as well as by the fluorescent dyes with different physiological mechanisms. For better viability of the sampled bioaerosol, the impinger was superior to the filter. Moreover, it was found that sampling stress from filtration had more influence on the bioaerosol metabolism mechanism than cell membrane integrity. Furthermore, the differences between cell membrane integrity and the metabolism by sampling stress were found related to the bioaerosol species.

Keywords: bioaerosol, sampling efficiency, flowcytometry

二、前言

Bioaerosols are associated with respiratory and other related health disorders. Currently, infectious indoor agents (e.g., *Legionella* spp., *Mycobacterium tuberculosis,* and *Staphylococcus* spp.), indoor allergens (e.g., *Penicillium* spp., *Alternaria* spp., *Bacillus subtilis*, *Bacillus cereus*, and *Actinomyces* spp.), and invasive fungal agents (e.g., *Aspergillus fumigatus*) can be sampled and quantified by numerous combinations of sampling and analytical techniques. Although samplers for bioaerosols are similar in design to general aerosol samplers, the ability of bioaerosol samplers to preserve the viability (including culturability) of airborne microorganisms is an additional vital factor that must be evaluated (Henningson and Ahlberg, 1994).

三、研究目的

In our current investigation, FCM/FL was applied to evaluate the sampling performance of impingement (AGI-30 all-glass impinger) and filtration (track-etched polycarbonate filter) with different types of fluorescent dye staining (cell membrane integrity and metabolism) and then compared with a traditional culture method (culturability). Two bacterial aerosols (*Escherichia coli* and endospores of *Bacillus subtilis*) and two fungal aerosols (*Candida famata* and *Penicillium citrinum* spores) were studied. In addition, the mechanisms of sampling stress due to impingement and filtration on bioaerosols were evaluated with different types of dye staining (cell membrane integrity and metabolism) and then compared with the traditional culture method (culturability).

四、文獻探討

Bioaerosols consist of both viable and nonviable microbes. Viability can be assessed by various methods, although the culture method is typically used. Many microorganisms, however, do not grow under the standard culture conditions used in laboratories (Wong et al., 2004). Resuscitation of microorganisms, such as *Virio vulnificus*, from a viable but nonculturable (VBNC) state to an infectious state has been demonstrated (Oliver and Bockian, 1995; Oliver et al., 1995). Furthermore, the plate count method generally yields poor precision and requires long incubation times (Henningson et al., 1998). Methods that do not require culturing could help detect and quantify VBNC bacteria in air samples and improve the accuracy of bioaerosol exposure assessment.

The most commonly used methods for quantifying airborne bacteria involve the capture of microorganisms directly on solid media, as with an Andersen sampler, in liquid buffer, as with all-glass impingers (AGI), or through a filter. Because the culture method remains the dominant analytical method for bioaerosol measurements, many studies have investigated sampling efficiency using culturability and the preservation of culturability measurements as a function of the composition of the collection medium. Few studies, however, have evaluated the sampling efficiency in terms of viability. Nonculture-based methods, such as epifluorescence microscopy (EFM), have recently been compared with culture-based methods for bioaerosol concentration assessment. Culture techniques tend to underestimate bacterial concentrations by two orders of magnitude (Heidelberg et al., 1997). Procedures that do not require plate counting are therefore needed to characterize bioaerosol samplers.

Microscopic analysis using stains can be used to evaluate the viability of bacterial cells, even those cells that have lost the ability to produce colonies on standard microbiological culture media (Henningson et al., 1998).

Flow cytometry (FCM) is useful for the rapid identification and quantification of bacteria in both aquatic and air environments (Day et al., 2002; Lange et al., 1997; Monfort and Baleux, 1992; Sincock et al., 1999). When used with a variety of dye stains, FCM could also provide a much more rapid and accurate viability assay than EFM (Button et al., 2001; Deleo et al., 1996; Gérald Grégori et al., 2001; Henningsen, et al., 1997; Lopez-Amoros et al., 1995; Sieracki et al., 1999).

Our group has successfully established FCM with fluorochrome (FCM/FL) for quick and accurate determination and quantification of total concentrations and viability of bioaerosols. We have applied the optimal conditions of FCM/FL for bacterial and fungal aerosols of laboratory samples (pure and mixture suspension) and environmental field samples (both the air and water samples from the aeration tank of hospital wastewater treatment plant). For an indicator of total cell concentration, AO (acrodine orange) was believed reliable due to its high staining efficiency. For a viability indicator, PI (propidium iodide), a membrane integrity dye, failed to distinguish the endospores of *B. subtilis* due to overlapping of viable and nonviable regions, whereas YOPRO-1 (quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene) methyl]-1- [3 (trimethylammonio) propyl]-, diiodide), another membrane integrity dye, clearly distinguished the regions of viable and nonviable cells. For a metabolic activity indicator, CTC (5-Cyano-2, 3-ditolytetrazolium chloride) was evaluated. Our findings demonstrated that the FCM/FL method can provide reliable information for bioaerosol evaluation by determining viability based on membrane integrity and metabolism. Possible applications of this powerful method include determining bioaerosol sampler performance, determining bioaerosol characteristics in different environmental samples, and controlling effectiveness of air cleaning techniques.

五、研究方法

Test bioaerosols

The two bacterial aerosols used here (*Escherichia coli* and endospores of *B. subtilis*) represent sensitive and hardy bacteria, respectively, and the two fungal aerosols (*C. famata* and *P. citrinum* spores) represent mold and yeast, respectively, that are frequently found in Taiwan. The two bacterial cultures were *E. coli* (Culture Collection & Research Center in Taiwan, CCRC 10675) and *B. subtilis* (CCRC 12145). *E. coli* is a sensitive bacterial strain that is gram-negative and rod-shaped (0.3 to 1 μm by 1 to 6 μm) with an aerodynamic diameter of 0.87 μm (Li et al., 1999), whereas *B. subtilis* endospores are resistant to many adverse conditions (Lin and Li, 2002), are gram-positive, and are rod-shaped, (0.7 to 0.8 μm by 1.5 to 1.8 μm) with an aerodynamic diameter of 1.35 μm (Li et al., 1999). The two fungal strains were spores of *P. citrinum* Thom (CCRC 33168) and vegetative cells of *C. famata* (CCRC 22304). Spores (conidia) of *P. citrinum* are spheroidal and 2.0 to 3.6 μm in diameter (Tzean et al., 1994) with an aerodynamic diameter of 2.32 μm (Li et al., 1999). *C. famata* are spheroidal and 2 to 10 μm in diameter (Tzean et al., 1994) with an aerodynamic diameter of 2.44 μm (Lin and Li, 2002).

Active cultures of *E. coli* and *C. famata* were respectively inoculated into a nutrient broth (Difco) and a YM broth (Difco Laboratories, Detroit) and respectively incubated for 24 hr at 37 ℃ and at 25 ℃. For *B. subtilis*, the cells were initially inoculated on TSA (trypticase soy agar, Difco Laboratories, Detroit, MI) for sporulation for 7 days at 37 ℃. *B. subtilis* growth was then harvested into sterile distilled water, agitated at 45 rpm for more than 24 hr at room temperature, and then heated for 10 min at 80 ℃ to kill vegetative cells. For *P. citrinum,* the sample strains were cultured on MEA (malt extract agar, pH 4.7, Difco Laboratories, Detroit, MI) and

incubated for 7 days at 25 ℃, and then washed out their spores by using Tween 80 prior to their generation. The resulting suspensions of *P. citrinum* were then aseptically washed with sterile phosphate-buffered saline (PBS) in a 15-ml sterile conical centrifuge tube, capped, and centrifuged twice at 4,000 rpm (Model 2010, Kubota, Japan) for 5 min, and then again for an additional 5 min. After the supernatant was removed, the pellets were resuspended in a PBS solution. Samples were washed twice with PBS and diluted to the proper concentration (~ 10^8 cells/mL) to optimize their generation. Optical microscopy confirmed that the centrifuged *P. citrinum* suspensions contained only pure spores (data not shown).

Aerosol generation system

The aerosol generation system to evaluate the bioaerosol samplers were described in detail elsewhere (Lin and Li, 1998). In brief, the sampling chamber is 12.5 cm in diameter and has a height of 27 cm. A collision three-jet nebulizer (BGI inc., Waltham, MA) is used to nebulize the microbe suspension at 3 L/min of dry, filtered and compressed laboratory air. The aerosol is then passed through a Kr-85 particle charge neutralizer (model 3077, TSI) and humidified and diluted with filtered air at 47 L/min to produce sufficient air flow for sampling in triplicate. The humidified gas stream is generated by passing compressed air through a humidity saturator. The relative humidity is maintained at 65% by adjusting the ratio of humidified gas stream to dry gas stream flow rate, and monitored with a hydrometer (Testo, Sekunden-Hydrometer 601) located in the sampling chamber.

Bioaerosol samplers and sample processing

In the present experiments, 20 mL of sterile deionized water with 1% peptone and 0.01% Tween 80 was placed into an autoclaved AGI-30 impinger by the method of Thorne et al. (1992). Then, 0.005% antifoam A (Sigma Chemical Co., St. Louis, MO) was added to reduce foaming and prevent excessive fluid loss. The AGI-30 sampler was operated at 12.5 L/min with a sampling time of 45 min to obtain sufficient cells for FCM analysis with a detection limit of 10^5 cells/mL. The suspension from this AGI-30 impinger was then vortexed for analysis by both FCM/FL and culture methods. Triplicate tests were performed for each experimental set.

A Nuclepore filter (Costar, Cambridge, MA) is a track-etched polycarbonate filter consisting of a polycarbonate membrane with straight-through pores of uniform size. In this study, 37-mm-diameter filters with 0.4-μm pores were loaded into open-face three-piece plastic cassettes on cellulose pad supports. Before sampling, filters and support pads were autoclaved and plastic cassettes were sterilized with ethylene oxide. The filter sampler was operated at 4 L/min for 45 min. The collected bioaerosols were removed from the Nuclepore filter by first placing the filter in a test tube containing 4 mL of sterile deionized water and then vortexing the tube for 60 sec. This vortexed suspension samples was then used for analysis by both FCM/FL and culture method.

CFU counting

The numbers of CFUs in vortexed suspension samples were determined using TSA and MEA plates, plated with 10 serial dilutions and incubated for 24 hr at 37 ^{o}C for bacteria or 48 hr at 25 ^{o}C for fungi (Jensen et al., 1992).

Dye and staining protocols

For bioaerosol viability measurements, four stains were evaluated: AO, PI, YOPRO-1, and CTC. AO was used to stain all of the microorganisms by penetrating all cell membranes and staining the nucleic acid; PI and YOPRO-1 were used as cell

membrane integrity indicators. From our previous findings, AO and YOPRO-1 are suitable to stain the four evaluated microorganisms. However, PI can not be used for *B. subtilis* endospores due to the poor separation of viable and unviable regions of this species. Moreover, CTC can be used only for cell type bioaerosols (*E. coli* and *C. famata*), not for *B. subtilis* endospores and spores of *P. citrinum* (the low staining efficiency). Therefore, no experiments were conducted for *B. subtili* with PI and *B. subtilis* endospores and spores of *P. citrinum* with CTC. Regarding the optimal staining conditions, each sample was individually stained with all four stains at optimal dye concentrations and optimal staining times of 5 µg/mL and 5 min for AO, 20 µM and 15 min for YOPRO-1, 25 µM, and 50 µM for PI, and 5 min and 8 hr for CTC as previously determined.

FCM

FCM was used to analyze the cell concentration in suspensions labeled as AO, PI, YOPRO-1, and CTC. The vortexed suspension samples were diluted in PBS that had been filtered by using a 0.22-µm-pore-size filter. FCM samples were prepared by mixing 0.5 mL of a stained cell suspension and 20 µl of a fluorescent bead suspension (7.37×10^7 beads/mL). The beads were monodispersed fluorescein-tagged 1.0-µm-diameter spherical polystyrene beads (Fluoresbrite; Polyscience, Inc., Warrington, PA) and were used to enable quantifications of cells in the FCM samples.

Analysis by FCM immediately after staining was done using a FacsCalibur flow cytometer (Becton Diskinson, San Jose, CA) equipped with an air-cooled argon laser (488 nm, 15 mW). The samples were vortexed prior to their analysis. The sample was delivered at a low flow rate to yield 300 to 600 counts per second. In this study, at least 10,000 counts of target cells were used for data acquisition. Five readings were recorded for each cell: forward scatter, side scatter, green fluorescence (515 to 545 nm), yellow-orange fluorescence (564 to 606 nm), and red fluorescence (< 670 nm). The fluorescence of AO, PI, YOPRO-1, and CTC fell into green, red, green, and red fluorescence range, respectively.

Indicators for sampling efficiency evaluation

(1) Culturability and viability with fluorescent dyes

The total cell concentration was determined using the AO stain, and viability was determined using the PI, YOPRO-1, and CTC stains. Because both PI and YOPRO-1 stained nonviable cells, viability with PI and YOPRO-1 staining was defined as

$$V_{PI} = \frac{(\text{total cell/ml})_{AO} - (\text{nonviable cell/ml})_{PI}}{(\text{total cell/ml})_{AO}}$$

$$V_{YOPRO\text{-}1} = \frac{(\text{total cell/ml})_{AO} - (\text{nonviable cell/ml})_{YOPRO\text{-}1}}{(\text{total cell/ml})_{AO}}$$

In contrast, because CTC stained metabolically active cells, viability with CTC was defined as

$$V_{CTC} = \frac{(\text{viable cell/ml})_{CTC}}{(\text{total cell/ml})_{AO}}$$

(2) Culturability and viability in bioaerosol samplers and the nebulizer (before bioaerosol generation)

The viabilities (V_{test} and V_o) and culturabilities (C_{test} and C_o) of the microorganisms in the samplers and in the nebulizer, respectively, were defined as

$$C_{test} = \frac{(\text{culturable cells/ml})_{test}}{(\text{total cells/ml})_{test}}$$

$$C_o = \frac{(\text{culturable cells/ml})_{nebulizer}}{(\text{total cells/ml})_{nebulizer}}$$

$$V_{test} = \frac{(\text{viable cells/ml})_{test}}{(\text{total cells/ml})_{test}}$$

$$V_o = \frac{(\text{viable cells/ml})_{nebulizer}}{(\text{total cells/ml})_{nebulizer}}$$

(3) Culturability ratio and viability ratio

For assessing culturability and viability differences in bioaerosols between the samplers and the nebulizer, the culturability ratio (CR) and viability ratio (VR) were used to adjust the initial culturability and viability as the biological efficiency indicator and defined as

$$CR = \frac{C_{test}}{C_o} \qquad VR = \frac{V_{test}}{V_o}$$

六、結果與討論

The FCM/FL method was applied here to evaluate the sampling performance of impingement and filtration with different types of dye staining (cell membrane integrity and metabolism) and then compared with a traditional culture method (culturability).

Culturability and viability in the nebulizer

Table 1 lists the measured culturability and viability with PI, YOPRO-1 and CTC before generation of *E. coli*, *B. subtilis* endospores, *C. famata* and spores of *P. citrinum*, respectively. For *E. coli*, the viabilities before generation with the cell membrane integrity stains, PI and YOPRO-1, were higher than the culturability. The viability of *B. subtilis* before generation with the cell membrane integrity stains, YOPRO-1, was also higher than the culturability. In addition, the viability of *B. subtilis* endospores with YOPRO-1 before generation was much lower than the viabilities of the other bioaerosols with YOPRO-1 before generation. This low culturability and viability of *B. subtilis* might be due to the endospore preparation process, such as heating at 80 ℃ for 10 min and/or agitating at 45 rpm for more than 24 hr, both of which might cause viability loss. However, for both *E. coli* and *C. famata,* the viability with the metabolic indicator (CTC) was lower than the culturability. For all four bioaerosols, the culturability was higher than the viability with the metabolic indicator (CTC), but lower than the viability with the membrane integrity indicators (PI and YOPRO-1). The different stains and culture methods apparently reflect different physiologic state of bioaerosols (e.g. metabolic active, membrane integrity damaged or culturable bioaerosols). In a previous FCM study (Henningson et. al., 1998), the viability before generation of *F. tularensis* (cell-type bacteria) in the same nebulizer was 0.93. In comparison to our study, the viability before generation of the cell-type bacteria, *E. coli*, was 0.96, 1.00, and 0.82 with PI, YOPRO-1, and CTC, respectively. Therefore, the initial viability for a sampled bioaerosol might depend on its preparation, its species, and the physiologic indicators used. In our study, different measured viabilities based on different physiologic indicators coupled with culturabilities should provide more detailed information about the bioaerosols.

Culturability and viability in bioaerosol samplers

For both vegetative cell-type bioaerosols (*E. coli* and *C. famata*), the culturability of the filter sample was much lower than that of the impinger sample. For *B. subtilis* endospores and spores of *P. citrinum*, the culturability of the impinger sample was similar to that of the filter sample (Table 1). The culturability of *E. coli* in theAGI-30 impinger in our study was similar to those previously reported for *S. marcescens* (0.07)*, K. planticola* (0.019*),* and *C. allerginae* (0.006) in AGI-30 impinger

stained with AO and analyzed by EFM (Heidelberg et al., 1997). As for the cell type *F. tularensis* (Henningson et. al., 1998), the culturability was also found to be smaller (0.0057) than the culturability of *E. coli* in our present study. From a previous report (Hernandez et al., 1999), the culturabilities of cell type bacteria, *E. coli* and *M. luteus,* in both filter samples (0.2 - 0.7) and AGI-30 impinger samples (0.1 - 0.4) were all higher than the culturabilities of *E. coli* in our studies. These observed differences in culturabilities might be related to the species of evaluated bioaerosols and the different analytical methods. All of these previous studies mentioned above, however, only assessed cell-type bacteria, whereas in our study here, we assessed the difference between bacterial and fungal bioaerosols, as well as between spore- and cell-type bioaerosols.

From staining with AO and YOPRO-1, it was found that the viabilities in both the AGI-30 impinger and filter samples were much higher than the culturabilities of *E. coli*, *B. subtilis* endospores, *C. famata* and spores of *P. citrinum* bioaerosols (Table 1). For PI stained *E. coli*, *C. famata* and spores of *P. citrinum* bioaerosols, the viabilities were also much higher than the culturabilities in both impinger and filter samples. In both inpinger and filter samples, the viabilities of CTC stained *E. coli* were higher than the culturabilities of *E. coli* but the viabilities of CTC stained *C. famata* were lower than the culturabilities of *C. famata*. This lower culturability for *C. famata* might be explained by the low staining efficiency of CTC for *C. famata*.

From our results, the viabilities in the filter samples for all four bioaerosols were lower than those in the impinger samples. Previously reported viabilities in impingers for *S. marcescens, K. planticola,* and *C. allerginae* were 0.64, 0.81, and 0.84 respectively, by the AO/AODVC method (Heidelberg et al., 1997), and 0.2 for *P. fluorescens* by the SYTO9/PI-EFM method (Terzieva et al., 1996). In addition, the viability of *E. coli* with CTC in the impinger samples in our study agrees well with that reported by Hernandez et al. (1999), but is about half that reported for *F. tularensis* by Henningson et al. (1998). In those previous studies (Heidelberg et al., 1997; Hernandez et al. 1999; Terzieva et al., 1996), only cell type bacteria were under evaluation by a single dye staining with the determinations of viability in bioaerosol samplers without considering those in the nebulizer. There is only one previous investigation which considered viabilities in both samplers and nebulizer for cell-type bacteria (Henningson et al., 1998). Based on our study, however, the culturability or viability in a sampler is not a representative indicator of the sampling efficiency of the sampler because the initial culturabilities and viabilities for different bioaerosol species vary widely. In our study, VR and CR were therefore proposed as indicators to characterize the effect of the sampling process on the culturability and viability. Based on our current study, viability during sampling is strongly influenced by the hardiness of the bioaerosol (i.e., cell type or spore type), as well as by the dye type used in the viability measurement. Therefore, the detailed evaluations of bioaerosol sampler performance for both bacterial and fungal bioaerosols (including cell types and spore types) with three different viability indicators and culturability indicator might be useful for sampler selection.

CR and VR

Table 2 lists the measured CR and VR with PI, YOPRO-1 and CTC of cell type *E. coli* and *C. famata*. In addition, it was also showed the measured CR and VR with YOPRO-1 of *B. subtilis* endospores and spores of *P. citrinum*. The *P. citrinum* spore had the highest CR and VR of impingement and filtration. For the two vegetative cell-types (*E. coli* and *C. famata*), CR of filtration was much smaller than that during impingement. However, CR of

impingement in our findings was much smaller than that (CR=0.88) reported by Henningson et al. (1998). This might be explained by different fluorescence labeling techniques**.** For the two spore types (*B. subtilis* endospores and spores of *P. citrinum*), CR of impingement was similar to that of filtration (Table 1). Our results strongly suggest that sampling stress differed significantly between that induced by impingement and by filtration for vegetative cell types, but not for spore types. In addition, impingement is reportedly the superior method for preserving the culturability of vegetative cell-type bioaerosols (Lin and Li, 1999a).

Table 2 shows that VRs of *C. famata* with PI, YOPRO-1, and CTC during impingement were similar to that of *E. coli*. However, VRs of *P. citrinum* with PI and YOPRO-1 during impingement were similar to that of *B. subtilis* with YOPRO-1. Of filtration, VRs of *E. coli* with PI and YOPRO-1 was similar to that of *C. famata*, whereas VR of *E. coli* with CTC was much smaller than that of *C. famata*. In addition, VR values of *P. citrinum* were slightly higher than that of *B. subtilis*. Therefore, VRs obtained with PI and YOPRO-1 indicated that the effect of sampling stress on the integrity of the cell membrane strongly depended on whether the bioaerosol strains were hardy or fragile. In addition, the metabolic activity of *E. coli* was more sensitive to the filtration process compared with that of *C. famata*, possibly due to the effect of dehydration on the metabolic activity of *E. coli* during filtration. The VR values of the four bioaerosols of impingement were all higher than those of filtration, possibly due to higher stress on the bioaerosols from filtration than that from impingement when the viability mechanism was assessed. Therefore, for better preservation of viability in bioaerosol sampling, an impinger is superior to a filter, especially when studying metabolic mechanisms. Regarding VR of the vegetative cell-type bioaerosols in the AGI-30 impinger, the VR (0.79) of *F. tularensis* was reported to be higher than those of *E. coli* observed in our study (Henningson et. al., 1998). To summarize, the differences in effect on cell membrane integrity and on cell metabolism due to sampling stress might be related to the characteristics of the bioaerosol (e.g., hardy or fragile) and to the methods used to analyze the viability of the bioaerosol.

The differences between culturability and viability during impingement are revealed by the ratio VR/CR. For *E. coli*, VR/CR was 11 with PI, 9.6 with YOPRO-1, and 5.7 with CTC, and for *C. famata*, it was 7.8, 7.4, and 3.9, respectively. For *P. citrinum*, VR/CR was 2.5 with either PI or YOPRO-1, and 6.2 for *B. subtilis* with YOPRO-1. These results clearly show that the traditional culture method to determine culturability underestimated the viability of the bioaerosols. The difference in effect of sampling stress induced by impingement and by filtration on culturability is revealed by the ratio $CR_{Filtration}$ / $CR_{Impingement}$. This ratio was 0.07 for *E. coli* and 0.38 for *C. famata*, indicating that the culturability preservation by filtration was much smaller than that by impingement for vegetative cell-type bioaerosols. In contrast, this ratio was 1.07 for *B. subtilis* and 1.34 for *P. citrinum*, indicating that the culturability preservation by filtration was similar to that by impingement for spore-type bioaerosols.

七、計畫成果自評

本計畫已在實驗室中建立以流式細胞儀輔以螢光染色對生物氣膠採樣器進行評估，並與傳統培養方法作比較，此成果可運用於環境中生物氣膠採樣方法的選擇，並提供更完整的生物氣膠暴露評估結果。

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Table 1. Culturabilities and viabilities of impinger and filter.

		culturability	Viability		
			PI	YOPRO	CTC
Before generation	*E. coli*	0.91	0.96	1.00	0.82
	B. s.	0.44	-	0.60	-
	C. famata	0.95	0.96	0.96	0.07
	P. citrinum	0.93	0.94	0.93	-
Impinger samples	*E. coli*	0.07	0.75	0.67	0.33
	B. s.	0.06	-	0.56	-
	C. famata	0.10	0.75	0.71	0.03
	P. citrinum	0.35	0.89	0.88	-
Filter samples	*E. coli*	0.004	0.60	0.55	0.01
	B. s.	0.07	-	0.44	-
	C. famata	0.04	0.61	0.56	0.01
	P. citrinum	0.47	0.78	0.76	-

The missing values with CTC were due to the low staining efficiency of spore type microorganisms. For missing values with PI, it was because of the poor separation of viable and unviable regions of *B. subtilus*.

Table 2. CR and VR of impinger and filter.

sampler	bioaerosols	CR[a]	VR[b]		
			PI	YOPRO	CTC
Impinger	*E. coli*	0.07	0.77	0.67	0.40
	B. subtilis	0.15	-	0.93	-
	C. famata	0.1	0.78	0.74	0.39
	P. citrinum	0.38	0.95	0.95	-
Filter	*E. coli*	0.005	0.62	0.55	0.008
	B. subtilis	0.16	-	0.72	-
	C. famata	0.038	0.64	0.58	0.070
	P. citrinum	0.51	0.83	0.81	-

a. culturability ratio (CR) = Ctest / Co, where Ctest and Co are culturability in the tested sampler and in nebulizer
b. viability ratio (VR) = Vtest / Vo, where Vtest and Vo are viability in the tested sampler and in nebulizer
* The missing values of VR with CTC were due to the low staining efficiency of spore type microorganisms. For missing values of VR with PI, it was because of the poor separation of viable and unviable regions of *B. subtilus*.