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Evaluation of sampling techniques for detection and quantification of airborne legionellae at biological aeration basins and shower rooms

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ABSTRACT

Inhalation of legionellae aerosolized from biological aeration basins (AB) and shower rooms (SR) has been linked to severe pneumonia and Pontiac fever. However, comprehensive evaluations at these facilities using various bioaerosol sampling techniques are lacking. This study assessed two agar-based (Andersen one-stage sampler and MAS-100/A), three liquid-based (BioSampler, AGI-30 and MAS-100/L) and two filter-based sampling methods (cassette/polycarbonate filter and IOM/gelatin filter) at AB. The BioSampler obtained the highest positive rate of culturable legionellae (8–50%) ($P<0.05$). As for collecting total and viable legionellae, the IOM consistently showed the highest efficiencies regardless of sampling time (30–270 min). When assessing the cassette, BioSampler and AGI-30 at SR, the cassette was more efficient in collecting total and viable legionellae ($P<0.05$), whereas only the BioSampler and AGI-30 recovered culturable legionellae. The viability percentages (calculated as viable cell counts to total cell counts, 12–77%) were not significantly different between the samplers regardless of sampling location and sampling time ($P>0.05$). Overall, the IOM and cassette performed better for collecting total and viable legionellae, and culturable legionellae may be detected more frequently by the BioSampler at AB and SR.

1. Introduction

Legionellae have been detected in the air of shower rooms (SRs) (Deloge-Abarkan et al., 2007) and biological aeration basins (ABs) (Blatny et al., 2008). Inhalation of airborne legionellae may cause lethally pneumonic Legionnaires' disease (LD) and nonpneumonic Pontiac fever (PF) in humans, collectively named legionellosis. Recent studies indicate that legionellae in shower aerosols may increase the risk of PF among older residents in nursing homes (Bauer et al., 2008), and the ABs of a biological treatment plant are possibly the main amplifiers and primary disseminators of legionellae linked to LD outbreaks (Olsen et al., 2010). Thus, an efficient monitoring of airborne legionellae at the aerosol-generating AB and SR is essential in order to characterize the microbial exposure and prevent the risk posed to human health, which demands appropriate sampling and analytical techniques.

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Culture assays are the commonly used methods for detecting culturable legionellae in the air of the AB (Blatny et al., 2008; Nguyen et al., 2006) and SR (Bollin et al., 1985; Deloge-Abarkan et al., 2007). However, in addition to culturable cells, the human infection may be caused by viable but non-culturable (VBNC) legionellae (Dusserre et al., 2008). Viable legionellae (including culturable and VBNC cells) can be assayed by real-time quantitative PCR with ethidium monoazide (EMA-qPCR) (Chen & Chang, 2010) which selectively amplifies the DNA of target cells with intact cytoplasmic membrane, defined as viable cells (Stocks, 2004). Previous studies show that EMA could penetrate into viable *Listeria monocytogenes* (Nocker et al., 2006; Pan & Breidt, 2007) and *Enterobacter sakazakii* (Cawthorn & Witthuhn, 2008), inhibit DNA amplification during qPCR and result in an underestimation of viable counts. However, this does not occur in *Legionella pneumophila* (a species responsible for 90% of *Legionella*-related infections (Murray et al., 2009)) when treating the cells with EMA of 2.3 μg/mL (Chen & Chang, 2010). Moreover, a great linearity ($R^2=0.98$) over a range of 1–7 log CFU/mL of *L. pneumophila* is observed with EMA-qPCR (Chen & Chang, 2010). Indeed, the EMA-qPCR assay has been successfully applied to quantify viable *L. pneumophila* and *Escherichia coli* in air (Chang & Chou, 2011a). On the other hand, the qPCR alone can be used to assess the PF risk as the PF may be induced by viable and non-viable legionellae (Miller et al., 1993) and qPCR detects total legionellae regardless of cell viability.

With regard to sampling methods, various samplers have been adopted in field studies to recover airborne legionellae, including Microbiological Air Sampler (MAS-100, Merck, Darmstadt, Germany) (Bauer et al., 2008; Blatny et al., 2008; Deloge-Abarkan et al., 2007; Pascual et al., 2001), Andersen sampler (Andersen Samplers, Inc., Atlanta, GA, USA) (Bollin et al., 1985), slit-to-agar sampler (STA-204, New Brunswick Scientific, Enfield, CT, USA) (Blatny et al., 2008), BioSampler (SKC, Eighty Four, PA, USA) (Deloge-Abarkan et al., 2007), wetted-wall cyclone (SASS 2000, Research International, Monroe, WA, USA) (Blatny et al., 2008), cassette (Millipore, Bedford, MA, USA) (Deloge-Abarkan et al., 2007) and IOM personal inhalable air sampler (IOM, SKC) (Dutil et al., 2007). However, comparative assessment of the performance of bioaerosol samplers for capturing legionellae at the SR and AB is limited. By sampling at the AB with a wetted-wall cyclone (SASS 2000) and two agar-based impactors, Blatny et al. (2008) reported that the SASS 2000 was suitable for collecting culturable *Legionella*. While sampling in the SR with three types of samplers, Deloge-Abarkan et al. (2007) revealed a significantly higher detection of *Legionella* by the BioSampler. Although both studies provide useful information, only three kinds of samplers were assessed in their investigations (Blatny et al., 2008; Deloge-Abarkan et al., 2007).

Considering limited information in the literature and noticeable exposure risk at AB and SR, two agar-based (Andersen 1-STG and MAS-100/A), three liquid-based (BioSampler, AGI-30 and MAS-100/L) and two filter-based samplers (IOM and cassette) were first evaluated at the AB of an industrial wastewater treatment plant (WWTP). The MAS-100 was assessed with agar and liquid collection media (denoted as MAS-100/A (MAS-100 with agar) and MAS-100/L (MAS-100 with liquid), respectively), as has been successfully demonstrated in field studies (Pascual et al., 2001). The BioSampler, AGI-30 and cassette were further assessed at two SRs of nursing homes. The aim of this study was to explore the performance of various sampling techniques for collecting culturable, viable and total legionellae at the AB and SRs.

2. Materials and methods

2.1. Air sampling

Air sampling was performed at an operating AB of an outdoor WWTP and two SRs of two nursing homes. According to our preliminary surveys with qPCR analysis, *L. pneumophila* at mean concentrations of 5.9×10^4 cells/L (range: 5.3×10^4–6.7×10^4 cells/L) and 1.6×10^4 cells/L (range: 1.5×10^3–7.3×10^4 cells/L) were found in the water of AB and SRs, respectively, while the respective mean concentrations of *Legionella* spp. were 7.3×10^5 cells/L (range: 4.1×10^5–1.0×10^6 cells/L) and 2.6×10^5 cells/L (range: 7.4×10^4–8.1×10^5 cells/L) (data not shown). Thus, legionellae-containing aerosols were generated from these facilities and could be used to assess the performance of the samplers.

For sampling at the AB, the inlets of test samplers were positioned at a height of 1.2 m and 1 m downwind from the AB. The characteristics of the operation for test samplers are summarized in Table 1. The Andersen 1-STG (Andersen Samplers Inc., Atlanta, GA, USA) and MAS-100 (Merck, Darmstadt, Germany) were operated for 5–30 min with buffered charcoal yeast extract agar supplemented with α-ketoglutarate (BCYEα agar) (Sigma Chemical Co., St. Louis, MO, USA) and DGVP agar (i.e. BCYEα agar supplemented with dyes (bromothymol blue and bromocresol purple), glycine, vancomycin and polymyxin B (Sigma)). The MAS-100 with agar plate (MAS-100/A) was turned 90° from vertical to horizontal to face the direction of the wind. The BioSampler (SKC Inc., Eighty Four, PA, USA), AGI-30 (Ace Glass Inc., Vineland, NJ, USA) and MAS-100/L were filled with deionized water (DW, 20 mL) (Chang et al., 2010) and run for 15–60 min. The DW was replenished to 20 mL every 15 min during sampling to increase *Legionella* recovery (Chang & Chou, 2011a). As for filter-based samplers, the IOM (SKC) and the closed-face three-piece cassette (SKC) were respectively loaded with 25-mm gelatin filter (3 μm porosity, Sartorius, Goettingen, Germany) and 37-mm Isopore™ polycarbonate filter (PC, 0.2 μm porosity)/cellulose support pad (Millipore, Bedford, MA, USA), and were operated for 30–270 min. Before use, the Andersen 1-STG, AGI-30, BioSampler, the sieve portion of MAS-100, IOM, PC filters and cellulose support pads were autoclaved, the cassettes were sterilized with ethylene oxide, and the flow rate of sampling pumps was calibrated.

The BioSampler, AGI-30 and cassette were further assessed at two SRs. Prior to sampling, the mechanical ventilation of SR was shut down and the doors and windows were closed. The hot water from showerheads was then run for 7 min (∼duration of a shower, Deloge-Abarkan et al., 2007) at a maximum flow of approx. 8.2 L/min. After turning off hot water,

Table 1
Air sampling techniques tested at aeration basin (AB) and shower room (SR).

Sampler	Collection medium	Flow rate (L/min)	Sampling time (min)	d_{50} (μm)[a]	Sampling site
Agar-based sampler					
Andersen 1-STG	BCYEα, DGVP	28.3	5, 10, 15, 30	0.65	AB
MAS-100/A	BCYEα, DGVP	100	5, 10, 15, 30	1.7–2.5	AB
Liquid-based sampler					
BioSampler	DW, refilling[b]	12.5	15, 30, 60	0.3	AB, SR
AGI-30	DW, refilling	12.5	15, 30, 60	0.31	AB, SR
MAS-100/L	DW, refilling	100	15, 30, 60	1.7–2.5	AB
Filter-based sampler					
IOM	Gelatin filter	2	30, 60, 270	–	AB
Cassette	Polycarbonate filter	4	30, 60, 270	–	AB
			30, 60		SR

[a] References of d_{50}: Andersen 1-STG and AGI-30 (Macher & Burge, 2001), MAS-100 (Yao & Mainelis, 2006), BioSampler (Deloge-Abarkan et al., 2007).
[b] Replenishment of sterile distilled water (DW) to 20 mL every 15 min during 30- and 60-min sampling.

the air sampling began at a height of 1.2 m and 1 m from the showerheads. The hot water was flushed again for 7 min to create bioaerosols before conducting the next sampling. The operation of three samplers tested at the SRs was similar to that at the AB except for no 270-min sampling by the cassette (Table 1) due to the consideration that most airborne droplets would have settled out of the atmosphere and the residents of nursing homes unlikely stay in the SRs for such long time.

The samplers were tested on three different days at each AB and SR. On every sampling day, test samplers were operated for two to four different durations (Table 1). The samplers set at the same sampling time were tested simultaneously with a 0.3-m distance between each other. In total, three and six air samples were respectively obtained from the AB and SRs for each sampler operated at a specified sampling time. One field blank sample was also taken for each test sampling method on every sampling day at the AB and SRs, respectively. A total of 141 air samples and 45 field blanks were collected from the AB and SRs.

After sampling, agar plates and cassettes were sealed with parafilm and gelatin filters were transferred to sterile centrifuge tubes containing 10 mL of extraction fluid (0.1% peptone and 0.01% Tween 80) (Chang & Chou, 2011b). Liquid samples from the AGI-30, BioSampler and MAS-100/L were also transferred to sterile tubes and stored at 4 °C along with gelatin samples. All samples were transported to the laboratory at 4 °C and processed immediately. Culture assays were then applied to all samples, while the qPCR and EMA-qPCR were adopted to analyze the samples from the liquid and filtration sampling to determine the recovery of total and viable legionellae, respectively.

2.2. Sample processing, EMA treatment and DNA extraction

The remaining volume of DW from the AGI-30, BioSampler and MAS-100 was measured. The tubes containing gelatin filters were heated at 37 °C for 5 min in a water bath (Firstek, B206-T1, Taipei, Taiwan) to completely dissolve the filters. Following a 30-s vortexing, an aliquot (0.5 mL) was taken from each of liquid samples, tenfold diluted and spread onto duplicate plates of BCYEα and DGVP agar along with undiluted ones (0.1 mL/plate). The remaining liquid was filtered using a 0.4-μm HTTP Isopore membrane (Millipore). Membrane filters were treated with 20 mL of acid buffer (0.2 M HCl–KCl [pH 2.2], Ishimatsu et al., 2001) for 3 min to reduce the growth of non-legionellae microflora. The filters were then gently washed by filtering 20 mL of DW repeatedly until the pH of the filtrate was measured to be between 6 and 8. Acid-treated filters were cut into pieces, suspended in 3 mL of extraction fluid and vortexed for 30 s. An aliquot (1 mL) of samples was tenfold diluted and spread on BCYEα and DGVP agar. The remaining aliquot (2 mL) was vortexed for 5 min, followed by filter removal and centrifugation ($8200 \times g$, 10 min, 4 °C), resulting in cells suspended in 1 mL. The PC filters of the cassettes were also acid-treated and processed, as described above.

Concentrated cell suspension (1 mL) was divided in two equal parts. One part was added with 50 μL of EMA (ethidium monoazide, Sigma, 25 μg/mL) in an Eppenddorf tube (1 mL) and kept in the dark for 5 min. The sample was then placed on chipped ice, followed by an exposure to a 500-W halogen light for 20 min at a 15-cm distance (Chen & Chang, 2010). The other part was not treated with EMA and was used for quantification of total *L. pneumophila* and *Legionella* spp. Both parts were concentrated at $20{,}000 \times g$ for 5 min (twice, 4 °C), and the DNA of cell pellets was extracted using the QIAamp DNA mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions but with the volume of DNA elution buffer modified to 100 μL. Extracted DNA was diluted with TE buffer and analyzed by qPCR along with undiluted DNA for quantification of viable and total legionellae, as described below.

2.3. Legionella identification from culture samples

BCYEα and DGVP plates from the Andersen 1-STG and MAS-100/A and from the processing of liquid- and filter-based samples were incubated at 37 °C with 5% CO_2 for 7 d. Bacteria-like colonies were gram-stained (Rapid Gram Stain kit, Baso diagnostic Inc., Taiwan) and tested for L-cysteine requirement using BCYEα and nutrient agars. DNA from 51 colonies that grew exclusively on BCYEα agar and were determined to be gram-negative was extracted using the QIAamp DNA mini Kit (Qiagen GmbH). Extracted DNA (5 μL) were analyzed on the LightCycler 480 System (Roche Diagnostics GmbH, Basel, Switzerland) using the primers and TaqMan MGB probes targeting on *mip* and 16S rRNA genes for identification of *L. pneumophila* and *Legionella* spp., respectively (Chen & Chang, 2010). The number of colonies identified as *Legionella* spp. and *L. pneumophila* was determined for each plate, and the positive rate of legionellae was calculated for each sampling method.

2.4. Quantification of viable and total legionellae

The primers, probes and thermal settings described by Chen & Chang (2010) were applied to diluted and undiluted DNA of air samples and field blanks to quantify viable and total *L. pneumophila* and *Legionella* spp. An internal inhibition control (IIC, 1 μL) and IIC-specific probe (300 nM) was added in the qPCR reaction mixture to monitor the qPCR inhibition (Chen & Chang, 2010). Standard DNA of *Legionella* spp. was prepared by extracting DNA from *L. pneumophila* (ATCC 33152) and quantified by the absorbance at 260 nm. Standard DNA of *L. pneumophila* was synthesized by the Mission Biotech Co. (Taipei, Taiwan), and the copy number/μL of the DNA was determined. Each qPCR run consisted of sample DNA, a serially diluted DNA standard (10 fg/μL–1 ng/μL for *Legionella* spp. and 0.31–3.1 × 10^4 copies/μL for *L. pneumophila*), a non-template control and an IIC control (i.e. IIC in legionellae-free and PCR-grade water). For reactions where IIC amplification was inhibited, DNA extracts were further diluted and the qPCR was re-run until the cycle threshold (Ct) values of the IIC were within one standard deviation of the mean IIC Ct values that were obtained using the IIC control.

Standard curves (i.e. Ct vs. log concentration of standard DNA (log fg/μL or log copies/μL)) were constructed for *L. pneumophila* and *Legionella* spp. and used to quantify the number of legionellae in sample DNA based on Ct value, DNA elution volume (100 μL), dilution fold of the DNA, and the copy number of target gene or the genomic DNA quantity in a single cell (i.e. a single copy of *mip* gene in *L. pneumophila* and approx. 4.3 fg in a legionellae cell) (Morio et al., 2008). In detail, the number of *L. pneumophila* in sample DNA was calculated as copies/μL × 100 μL × dilution fold × 1 cell/copy, and the number of legionellae was calculated as fg/μL × 100 μL × dilution fold × 1 cell/4.3 fg. Because only a portion of environmental sample was processed with DNA extraction and qPCR, the sample volume analyzed by qPCR was adjusted to obtain the number of viable and total legionellae collected in the air sample. Airborne concentrations of total and viable legionellae (cells/m^3) were further determined according to the number of legionellae cells, sampling flow rate and sampling time.

2.5. Data analysis

The concentration of viable legionellae was divided by that of total cells for each sample to determine the viability percentage (VP). Moreover, by taking the concentration of viable and total legionellae determined from the BioSampler (for 15-, 30- and 60-min sampling) and IOM (for 270-min sampling) as a reference, the relative efficiency (RE, %) of legionellae collection was calculated for simultaneously tested sampling methods according to the following equation:

$$\mathrm{RE}\ (\%) = [C_{\mathrm{SAMPLER}}/C_{\mathrm{REF}}] \times 100\%$$

C_{SAMPLER} is the concentration of legionellae determined by a test sampler operated simultaneously with the reference sampler and C_{REF} the concentration of legionellae determined by the reference sampler.

The one-way analysis of variance (ANOVA) was applied to compare the log-transformed RE values of various sampling methods because the log-transformed RE values were distributed normally according to the result of Kolmogorov–Smirnov normality test ($P > 0.05$). Due to the non-normality and/or limited sample size (≤ 18), the Kruskal–Wallis test was conducted to examine the positive rate of culturable legionellae and the VP value among sampling methods as well as the effect of sampling time on RE and VP values of a given sampler. The post hoc analysis using the Least Significance Difference (LSD) test was further undertaken when observing a statistical significance by the ANOVA or Kruskal–Wallis test. All statistical analyses were conducted with SAS software version 9.1 (SAS Institute Inc., NC, USA). Statistical significance was considered as $P < 0.05$.

3. Results

3.1. Airborne concentrations of total and viable legionellae at aeration basins

Legionellae were detected in every air sample analyzed by qPCR or EMA-qPCR but were absent in field blanks. According to the results of tested samplers (Table 2), the mean concentrations of total *L. pneumophila* and *Legionella* spp. at AB were ranged from 4.2 × 10^3 to 2.3 × 10^5 cells/m^3 and from 7.0 × 10^4 to 3.5 × 10^6 cells/m^3, respectively. As for viable

Table 2
Airborne concentrations of viable and total *Legionella pneumophila* (Lp) and *Legionella* spp. (Leg) collected by various samplers around aeration basin.

Sampler (sampling time (min))	N[a]	Mean cell concentration (range) (cells/m^3)			
		Total Lp	Total Leg	Viable Lp	Viable Leg
AGI-30 (15, 30, 60)	9	8.4×10^4 (4.7×10^3–3.5×10^5)	1.3×10^6 (3.8×10^4–8.8×10^6)	2.9×10^4 (8.1×10^2–1.4×10^5)	1.7×10^5 (3.2×10^4–2.8×10^5)
BioSampler (15, 30, 60)	9	4.7×10^4 (7.6×10^3–1.3×10^5)	8.7×10^5 (1.9×10^4–5.6×10^6)	1.4×10^4 (2.8×10^3–3.8×10^4)	1.1×10^5 (7.9×10^3–4.5×10^5)
MAS-100/L (15, 30, 60)	9	4.2×10^3 (1.4×10^2–1.4×10^4)	7.0×10^4 (4.8×10^3–2.2×10^5)	1.9×10^3 (9.8×10^1–5.9×10^3)	1.2×10^4 (1.6×10^3–5.2×10^4)
Cassette (30, 60, 270)	9	9.9×10^4 (6.2×10^3–2.9×10^5)	9.3×10^5 (2.4×10^4–3.9×10^6)	2.8×10^4 (4.9×10^3–9.0×10^4)	1.8×10^5 (9.4×10^3–4.7×10^5)
IOM (30, 60, 270)	9	2.3×10^5 (8.8×10^3–1.1×10^6)	3.5×10^6 (6.4×10^5–7.1×10^6)	9.6×10^4 (3.2×10^3–3.1×10^5)	1.1×10^6 (5.5×10^4–3.1×10^6)

[a] Number of samples.

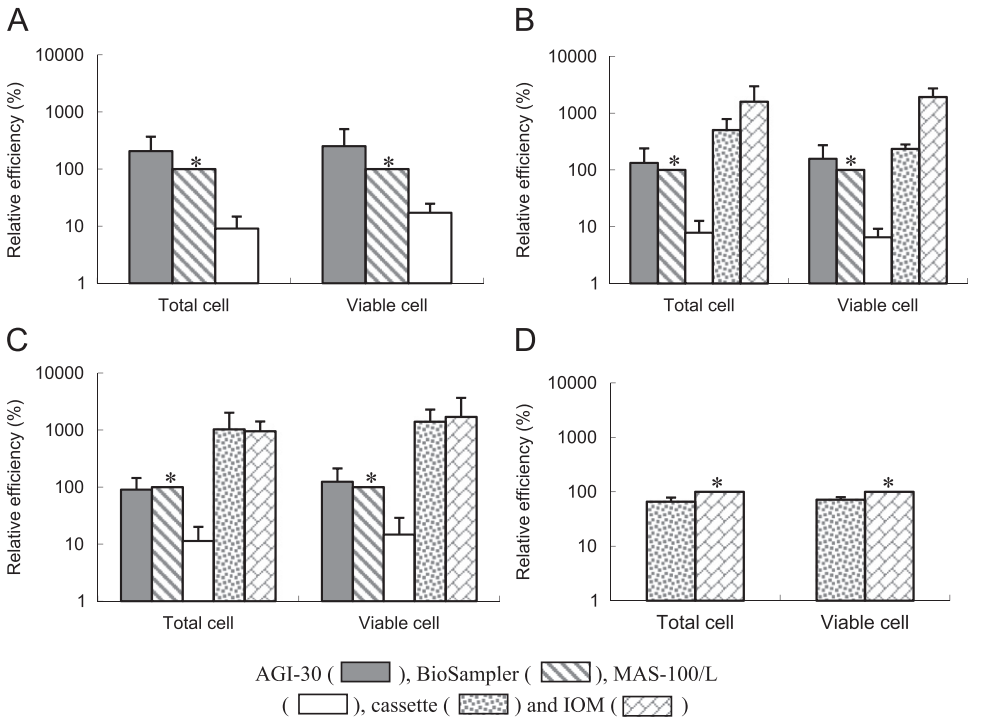

Fig. 1. Relative efficiencies of AGI-30 ( ), BioSampler ( ), MAS-100/L ( ), cassette ( ) and IOM ( ) for capturing total and viable *Legionella pneumophila* at (A) 15 (B) 30 (C) 60 and (D) 270 min of sampling around aeration basin ($n=3$). Each error bar represents one standard deviation from a mean of repeated samples. ∗ Is the reference sampler.

cells, the averaged concentrations were between 1.9×10^3 and 9.6×10^4 cells/m^3 for *L. pneumophila* and between 1.2×10^4 and 1.1×10^6 cells/m^3 for *Legionella* spp.

3.2. Relative efficiencies for total and viable legionellae at aeration basins

Air concentration data were transformed to RE values and categorized by sampling time in order to compare the collection efficiency of samplers operated simultaneously. For total and viable *L. pneumophila* sampled at the AB using three liquid-based samplers for 15–60 min (Fig. 1A–C), the RE values of the AGI-30 (90–251%) and BioSampler (100%) were significantly greater than those using the MAS-100/L (7–17%) ($P<0.05$, Table 3). An increase in sampling time did not significantly affect the RE of AGI-30 and MAS-100/L ($P>0.05$). Statistical testing on sampling time was not applied to

Table 3
Statistical results on relative efficiency (RE) of samplers for recovering viable and total *Legionella pneumophila* (Lp) and *Legionella* spp. (Leg).

Sampling site	Sampler (sampling time (min))	Least Significance Difference (LSD) test[a]			
		Total Lp	Viable Lp	Total Leg	Viable Leg
Aeration basin	Liquid-based samplers (15, 30 and 60)				
	AGI-30	A	A	A	A
	BioSampler	A	A	A	A
	MAS-100/L	B	B	B	B
	Filter-based samplers (30, 60 and 270)				
	IOM	A	A	A	A
	Cassette	A	A	A	A
	Liquid- and filter-based samplers (30 and 60)				
	IOM	A	A	A	A
	Cassette	A	A	B	B
	AGI-30	B	B	C	C
	BioSampler	B	B	C	C
	MAS-100/L	C	C	D	D
Shower room	Liquid-based samplers (15, 30 and 60)				
	AGI-30	A	A	A	A
	BioSampler	A	A	A	A
	Liquid- and filter-based samplers (30 and 60)				
	Cassette	A	A	A	A
	AGI-30	B	B	B	B
	BioSampler	B	B	B	B

[a] LSD test was applied to collectively analyze all individual RE data obtained by the specified samplers operated at various sampling durations. Samplers with the same letter have relative efficiencies that are not statistically different ($P > 0.05$, LSD test).

BioSampler because it was served as a reference sampler. As for two filter sampling methods, lower mean RE values were observed for the cassette (234–504%) compared to the IOM (1592–1929%) when sampling total and viable *L. pneumophila* for 30 min (Fig. 1B); however, comparable efficiencies were found when sampling for 60 and 270 min (Fig. 1C and D). Statistical insignificant results were revealed when collectively analyzing the RE data of two filter samplers shown in Fig. 1B–D ($P > 0.05$, Table 3). The RE values of the IOM were re-calculated by taking the cassette as a reference for 30–270 min sampling in order to assess the effect of sampling time, and statistical comparable results among three sampling durations were observed in the IOM for collecting total and viable *L. pneumophila* ($P > 0.05$).

Three liquid- and two filter-based samplers were simultaneously tested for 30 and 60 min (Fig. 1B and C). Analyses on these RE data indicated that the efficiencies of the IOM and cassette were statistically comparable for recovering total and viable *L. pneumophila*, but were significantly greater than those of three liquid sampling methods ($P < 0.05$, Table 3).

The RE for total and viable *Legionella* spp. is presented in Fig. 2. Similar to the findings of *L. pneumophila* (Fig. 1), the mean RE values of the AGI-30 (110–223%) and BioSampler (100%) were consistently higher than those of the MAS-100/L (6–29%) when sampling for 15–60 min (Fig. 2A–C) ($P < 0.05$, Table 3). Sampling time showed no significant influence on the RE of AGI-30 and MAS/100/L ($P > 0.05$). With regard to two filter-based samplers operated for 30–270 min, the cassette had the mean RE values less than the IOM regardless of sampling time (Fig. 2B–D). Taking the cassette as a reference, no statistical difference was observed among sampling times in the IOM for collecting viable *Legionella* spp. ($P > 0.05$), whereas a significant positive effect of sampling time was revealed for recovering total *Legionella* spp. ($P < 0.05$). Comparison of five samplers simultaneously operated for 30 and 60 min (Fig. 2B and C) indicated that the IOM had the highest efficiencies for collecting total and viable *Legionella* spp. (1480–3873%), significantly greater than the other four samplers ($P < 0.05$, Table 3). The second highest RE values were determined using the cassette (311–953%), followed by the AGI-30 (110–223%), BioSampler (100%) and MAS-100/L (12–29%); the MAS-100/L had the RE values significantly lower than the other four sampling methods ($P < 0.05$, Table 3).

3.3. Airborne concentrations of total and viable legionellae at shower rooms

Since the MAS-100/L was significantly less efficient than the other four samplers in legionellae collection (Figs. 1 and 2 and Table 3), it was not used at the SR sampling. Moreover, preliminary sampling tests at the SR revealed a failure of the IOM in cell recovery due to the dissolution of gelatin filters at high relative humidity (86–92% RH). Thus, only the AGI-30,

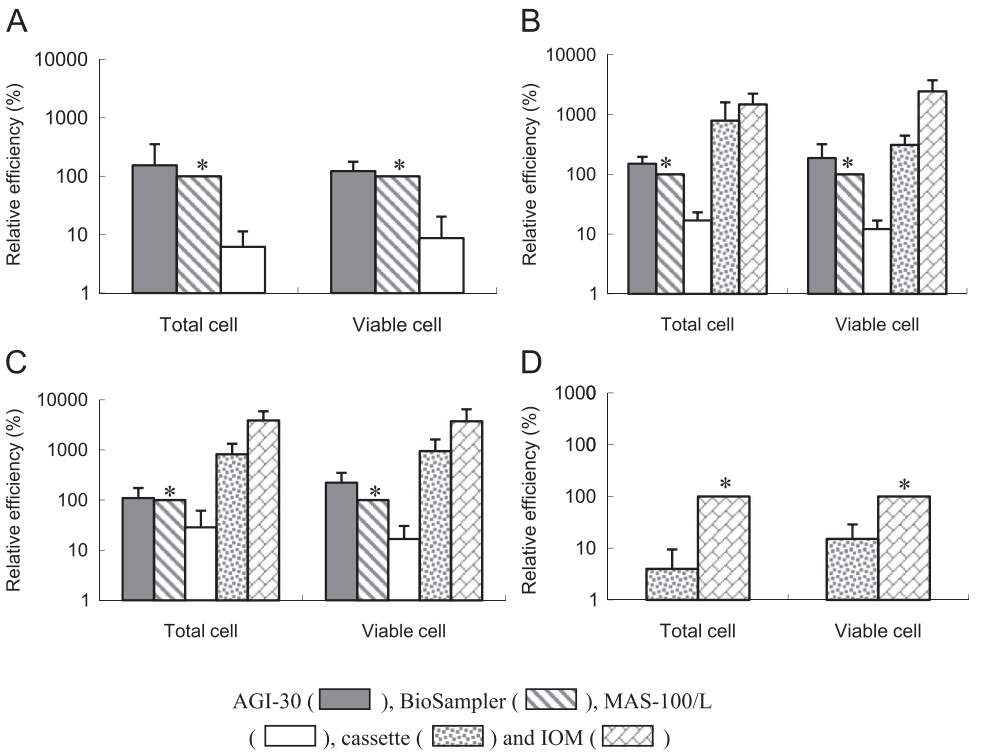

Fig. 2. Relative efficiencies of AGI-30 ( ), BioSampler ( ), MAS-100/L ( ), cassette ( ) and IOM ( ) for capturing total and viable *Legionella* spp. at (A) 15 (B) 30 (C) 60 and (D) 270 min of sampling around aeration basin ($n=3$). Each error bar represents one standard deviation from a mean of repeated samples. ∗ Is the reference sampler.

Table 4
Airborne concentrations of viable and total *Legionella pneumophila* (Lp) and *Legionella* spp. (Leg) collected by various samplers at shower room.

Sampler (sampling time (min))	N[a]	Mean cell concentration (range) (cells/m^3)			
		Total Lp	Total Leg	Viable Lp	Viable Leg
AGI-30 (15, 30, 60)	18	7.9×10^4 (4.3×10^3–4.0×10^5)	4.8×10^5 (4.2×10^4–2.2×10^6)	4.9×10^4 (1.0×10^3–2.4×10^5)	1.1×10^5 (7.8×10^1–6.2×10^5)
BioSampler (15, 30, 60)	18	6.0×10^4 (4.1×10^3–3.4×10^5)	3.2×10^5 (2.3×10^4–1.2×10^6)	3.2×10^4 (2.9×10^3–1.3×10^5)	8.3×10^4 (3.8×10^3–3.2×10^5)
Cassette (30, 60)	12	1.7×10^5 (1.6×10^4–5.6×10^5)	2.9×10^6 (2.8×10^5–1.8×10^7)	8.9×10^4 (1.5×10^4–3.7×10^5)	5.0×10^5 (9.0×10^4–1.8×10^6)

[a] Number of samples.

BioSampler and cassette were evaluated at two SRs on six days for legionellae collection. Table 4 shows the mean concentrations of legionellae determined by tested samplers were ranged from 6.0×10^4 cells/m^3 to 1.7×10^5 cells/m^3 for total *L. pneumophila*, 3.2×10^5 cells/m^3 to 2.9×10^6 cells/m^3 for total *Legionella* spp., 3.2×10^4 cells/m^3 to 8.9×10^4 cells/m^3 for viable *L. pneumophila* and 8.3×10^4 cells/m^3 to 5.0×10^5 cells/m^3 for viable *Legionella* spp.

3.4. Relative efficiencies for total and viable legionellae at shower rooms

The RE values of SR samplings are presented in Fig. 3 for *L. pneumophila* and Fig. 4 for *Legionella* spp. When sampling total and viable *L. pneumophila* and *Legionella* spp. for 30 and 60 min (Figs. 3B and C and 4B and C), the RE values of the cassette (289–493%, Fig. 3; 527–2236%, Fig. 4) were significantly greater than those of the AGI-30 (137–174%, Fig. 3; 102–169%, Fig. 4) and BioSampler (100%) ($P<0.05$, Table 3). On the other hand, comparable mean RE values were observed

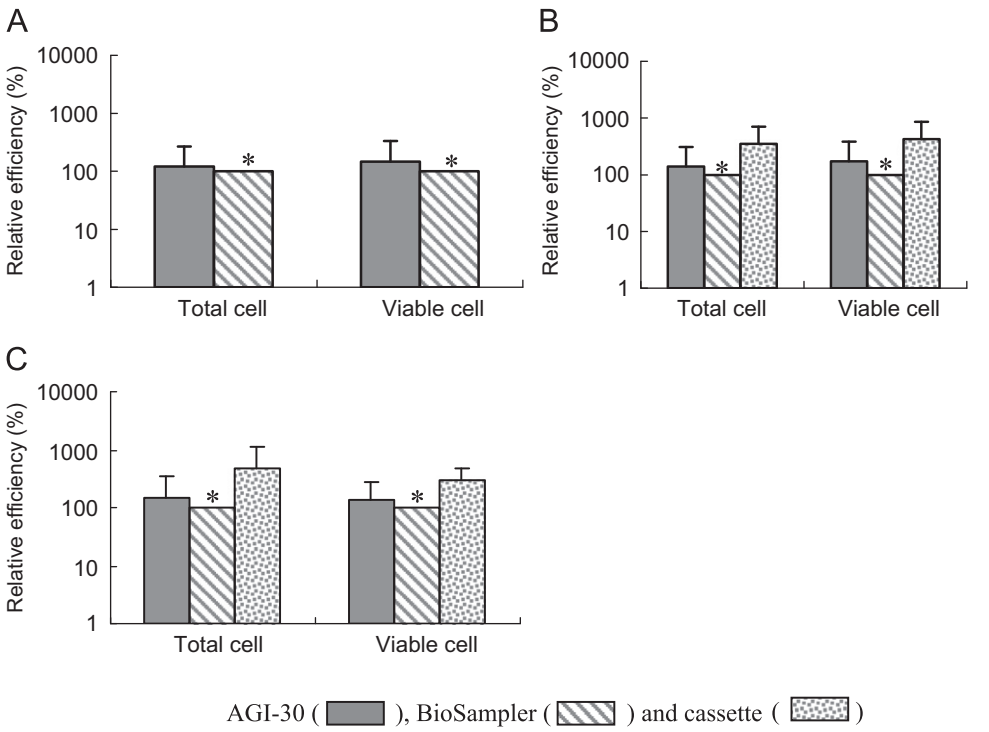

Fig. 3. Relative efficiencies of AGI-30 ( ), BioSampler ( ) and cassette ( ) for capturing total and viable *Legionella pneumophila* at (A) 15 (B) 30 and (C) 60 min of sampling in shower room ($n=6$). Each error bar represents one standard deviation from a mean of repeated samples. ∗ Is the reference sampler.

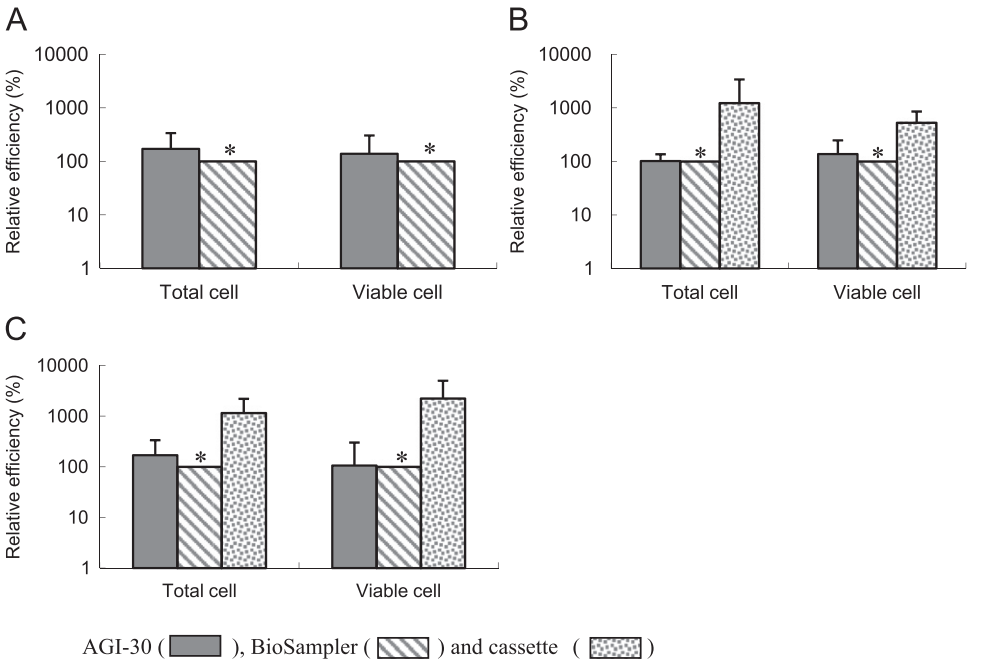

Fig. 4. Relative efficiencies of AGI-30 ( ), BioSampler ( ) and cassette ( ) for capturing total and viable *Legionella* spp. at (A) 15 (B) 30 and (C) 60 min of sampling in shower room ($n=6$). Each error bar represents one standard deviation from a mean of repeated samples. ∗ Is the reference sampler.

between AGI-30 and BioSampler when sampling total and viable legionellae for 15–60 min (Figs. 3 and 4) ($P > 0.05$, Table 3). Increasing the sampling time from 15 to 60 min did not significantly affect the RE of AGI-30 for recovering total and viable *L. pneumophila* and *Legionella* spp. ($P > 0.05$).

3.5. Viability percentages

The mean VP values for *L. pneumophila* and *Legionella* spp. varied from 17% to 55%, 19% to 59%, 24% to 60% and 19% to 77% when sampling for 15, 30, 60 and 270 min, respectively, at the AB (Fig. 5). As for SR testing, the mean VP values determined at 15, 30 and 60-min sampling were 31–60%, 35–71% and 12–68%, respectively (data not shown). No

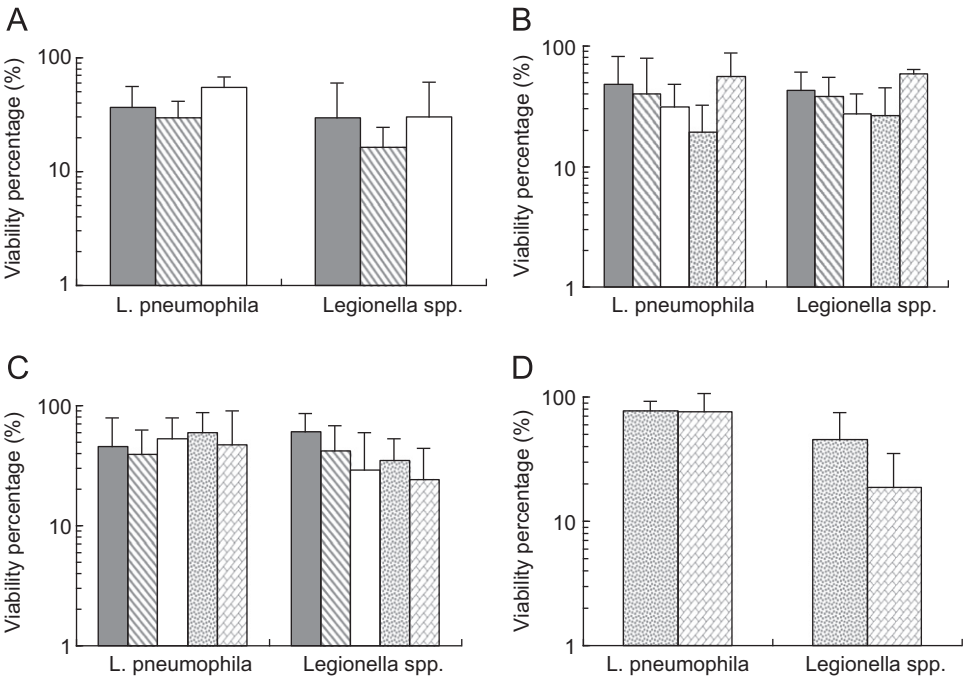

Fig. 5. Viability percentages of AGI-30 ( ), BioSampler ( ), MAS-100/L ( ), cassette ( ) and IOM ( ) for collecting *Legionella pneumophila* and *Legionella* spp. at (A) 15 (B) 30 (C) 60 and (D) 270 min of sampling around aeration basin ($n = 3$). Each error bar represents one standard deviation from a mean of repeated samples.

Table 5
Culturable legionellae detected from air samples by various sampling and analytical methods[a].

Sampling site (n[b])	Sampler	Sampling time (min)	% Positive for *Legionella pneumophila*					% Positive for *Legionella* spp.				
			BCYEα agar		DGVP agar		Overall	BCYEα agar		DGVP agar		Overall
			N[c]	F/A[d]	N	F/A		N	F/A	N	F/A	
Aeration basin (3)	MAS-100/A	15	0	0	0	0	0 (0/12)	33	0	0	0	8 (1/12)
		30	0	0	0	0	0 (0/12)	0	0	33	0	8 (1/12)
	AGI-30	30	33	0	67	0	25 (3/12)	33	0	67	0	25 (3/12)
		60	0	33	33	33	25 (3/12)	0	33	33	33	25 (3/12)
	BioSampler	15	0	33	0	0	8 (1/12)	0	33	0	0	8 (1/12)
		30	33	67	33	67	50 (6/12)	33	67	33	67	50 (6/12)
		60	67	33	67	33	50 (6/12)	67	33	67	33	50 (6/12)
	MAS-100/L	60	33	0	0	0	8 (1/12)	33	0	0	0	8 (1/12)
	Cassette	270	0	0	0	0	0 (0/12)	0	0	0	33	8 (1/12)
Shower room (6)	AGI-30	60	0	0	0	0	0 (0/24)	17	0	0	0	4 (1/24)
	BioSampler	30	0	0	17	0	4 (1/24)	17	0	17	0	8 (2/24)
		60	17	0	0	0	4 (1/24)	17	0	0	0	4 (1/24)

[a] Only the sampling methods with at least one legionellae-positive sample are included.
[b] n: number of samples on BCYEα or DGVP agar.
[c] N: direct plating on agar without filtration and acid treatment.
[d] F/A: filtration and acid treatment prior to plating on agar.

significant differences were found between the VP values of any two samplers tested at the AB or SR regardless of sampling time ($P > 0.05$). Moreover, the sampling time did not significantly affect the VP value of any test sampler for collecting legionellae ($P > 0.05$).

3.6. Detection of culturable legionellae

Culturable legionellae in the air of the AB were not detected by the Andersen 1-STG and IOM regardless of sampling time. Instead, legionellae were recovered by the MAS-100/A (sampling for 15 and 30 min), AGI-30 (30 and 60 min), BioSampler (15–60 min), MAS-100/L (60 min) and cassette (270 min) (Table 5). The highest positive rate was obtained by the BioSampler (8–50%), which was significantly greater than the AGI-30 (25%), MAS-100/L (8%), cassette (0–8%), MAS-100/A (0–8%), Andersen 1-STG (0%) and IOM (0%) ($P < 0.05$). As for the three samplers tested at the SR, the BioSampler and AGI-30 also recovered culturable legionellae after sampling for 30 min or longer with a positive rate $\leq 8\%$ (Table 5), whereas the cassette did not. Statistical tests were not applied to the SR data due to the low positive rate.

Despite that the positive rate of culturable legionellae varied among samplers, the mean colony count was similar, i.e. 1–2 CFU/plate for AB and SR samples (data not shown). The CFU number was too low to be used to assess sampler performance. However, $9.6 (\pm 2.1) \times 10^3$ and $1.2 (\pm 0.5) \times 10^2$ CFU/L of culturable legionellae were found in the AB and SR water, respectively (filtration and acid treatment, plating on DGVP agar, qPCR identification, data not shown).

4. Discussion

4.1. Culturable legionellae

Although the water of the studied SRs and AB was contaminated with culturable legionellae, only a few colonies were detected on the plates of air samples regardless of sampler type, sampling volume, agar type and sample treatment. A similar finding was also reported by Deloge-Abarkan et al. (2007) who revealed 4×10^4 CFU/L of *Legionella* spp. in hot water of SRs but detected only one colony of *Legionella* spp. in aerosol samples using a MAS-100/A. Low CFU counts limit the potential to quantitatively assess the efficiency of test samplers to recover culturable legionellae. Future studies with spiking a high number of legionellae onto different samplers and running the samplers for various time periods using HEPA filtered air are warranted in order to investigate the recovery efficiency of samplers.

Our data indicate the BioSampler obtained the greatest positive rate of legionellae compared to the other six samplers tested at AB ($P < 0.05$). This sampler also recovered culturable legionellae from the air of SRs (Table 5). Both results demonstrate the superior ability of BioSampler to detect culturable legionellae. This finding accords with the observation of previous laboratory chamber studies, i.e. a greater efficiency of the BioSampler to collect culturable *L. pneumophila* compared to other agar-, liquid- and filter-based samplers (Chang et al., 2010; Chang & Chou, 2011b; Deloge-Abarkan et al., 2007). With regard to the negative result by Andersen 1-STG, it may be partly attributed to fungal growth, which was observed in all BCYEα agar plates and 58% of DGVP plates (data not shown). Although polymyxin B, a component of DGVP agar, may inhibit the growth of *Aspergillus sydowii* (Galloway & Krauss, 1959), *Saccharomyces cerevisiae* and *Candida albicans* (Schwartz et al., 1972) to some extent, fungal contamination was observed and could have interfered with the growth and/or enumeration of culturable legionellae. As for filter-based samplers, the stresses of filtration process and desiccation may affect the metabolic activities and/or multiplication abilities of legionellae, resulting in low or no recovery of culturable cells by the IOM and cassette.

4.2. Total and viable legionellae

This study shows the MAS-100/L was significantly less efficient to recover total and viable legionellae at AB compared to other test samplers by 1–2 orders of magnitude (Figs. 1 and 2 and Table 3). Previous chamber tests also revealed a significantly lower recovery of total *L. pneumophila* by the MAS-100/L (Chang et al., 2010; Chang & Chou, 2011b), which is probably due to the fact that the d_{ae} of monodispersed *L. pneumophila* (0.7 μm) is much smaller than the 50% cut-off diameter (d_{50}) of the MAS-100 (1.7–2.5 μm (Yao & Mainelis, 2006)). While the d_{ae} of airborne legionellae at AB could be increased due to cell aggregation or attachment to larger particles or droplets, the present result indicates that such increase, if occurred, was not large enough to improve legionellae recovery by the MAS-100/L.

In contrast to MAS-100/L, the IOM consistently obtained the highest efficiency for capturing total and viable legionellae regardless of sampling time (Figs. 1, 2 and Table 3). Previous chamber tests also indicate the IOM as one of the most appropriate sampling methods to collect total and viable *L. pneumophila* (Chang & Chou, 2011b). Consistent findings strongly support that the IOM is the best sampling technique to quantify viable and total legionellae in air. However, the dissolution of gelatin filters has occurred in every IOM sample when performing the sampling in the SRs for 30 min or longer, which was likely caused by the water mist generated from showerheads and continuously present in the air during the sampling, evident by high RH levels at 86–92%. This observation suggests that the IOM/gelatin filter may not be suitable for collecting legionellae from the air with high RH. The cassette/PC filter may be applied instead.

The efficiency of the cassette for recovering total and viable legionellae was statistically comparable to that of the IOM in most cases, and was consistently greater than that of the BioSampler and AGI-30 regardless of sampling location

(Table 3). Such high efficiency of the cassette was also revealed in previous chamber tests for collection of total *L. pneumophila*; however, the cassette was significantly less efficient in recovering viable *L. pneumophila* compared to the BioSampler and AGI-30 by a factor of 13 and 17, respectively, after sampling for 60 min (Chang & Chou, 2011b). This inconsistency between field sampling and chamber testing may be attributed to the difference in the magnitude of dehydration of legionellae cells. Legionellae-containing droplets generated from the AB and showerheads of SRs tended to be less dehydrated than those from a laboratory generation system in which airborne droplets were desiccated by passing through a Kr-85 particle charge neutralizer and diluted with a high volume of air (Chang & Chou, 2011b). Considering that dehydration adversely affects the recovery of viable *L. pneumophila* from the PC filter of the cassette (Chang & Chou, 2011b), less dehydrated legionellae from the AB and showerheads may have a better chance to preserve their viability, resulting in an increase in the recovery of viable cells.

As for the lower efficiency found in the AGI-30 and BioSampler relative to filter-based samplers (Figs. 1–4), it could be caused by the loss of bacteria in the sampler inlet (Seshadri et al., 2009) and inside the sampler (Han & Mainelis, 2012), and/or the reaerosolization of collected cells from the DW (Lin et al., 2000).

The VP values were statistically comparable between the samplers tested at AB and SRs, suggesting that the stresses arising from different sampling methods (e.g., desiccation on filters and impingement/shear forces in liquid) do not cause significant differences in terms of the magnitude of damage to legionellae membrane. Moreover, no statistical differences between the VP values of various sampling times support that the membrane integrity of legionellae aerosolized from the AB and showerheads is not compromised by extending the sampling duration. This was also supported by the statistical comparable RE values among various sampling durations in collection of viable legionellae. These VP and RE findings, however, differ from those reported with culture assays. When collecting culturable *L. pneumophila*, previous studies showed a lower collection efficiency in filter-based samplers than in liquid-based samplers (Chang & Chou, 2011b; Deloge-Abarkan et al., 2007; Li et al., 2003) and a decreased collection efficiency with increasing sampling time in filter- and liquid-based samplers (Chang et al., 2010; Chang & Chou, 2011b). The difference between the present and previous studies suggests that airborne legionellae that are faced with sampling stresses undergo physiological modulations to become less culturable but no significant alternation in cell membrane integrity. As for the positive effect of sampling time on the IOM for collecting total *Legionella* spp., it could be attributed to the characteristic that initially deposited cells plug microsized holes of the filter (Lundgren & Gunderson, 1975) and decrease the penetration of the cells coming later through the filter, resulting in an increase in collection efficiency.

5. Conclusions

This study shows the BioSampler performed better than the other test samplers for recovering culturable legionellae. The IOM/gelatin filter was the most appropriate method to quantify total and viable legionellae at AB, but was unsuitable for sampling at SR due to the dissolution of geltain filters. The cassette/PC filter may be used in such situation. These findings should be considered when assessing airborne legionellae at AB and SR during legionellosis investigation and/or at regular practice.

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