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Methods for Detection and Quantification of Airborne Legionellae Around Cooling Towers

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Inhalation of legionellae may cause severe pneumonia and Pontiac fever in humans; however, comprehensive field evaluations using various bioaerosol monitoring techniques are lacking. The performance of samplers for legionellae collection has been investigated in a chamber setting. This study further assessed the capabilities of two filter-based (cassette/polycarbonate filter and IOM/gelatin filter), two agar-based (Andersen one-stage sampler and MAS-100/A), and four liquid-based sampling methods (BioSampler, AGI-30, MAS-100/L, and SASS 2300) for monitoring culturable, viable, and total legionellae around cooling towers. The highest detection rate of culturable legionellae was obtained by the SASS 2300 (25%–31%), followed by the BioSampler (0%–23%), MAS-100/L (5%–9%), and IOM (5%), while no culturable legionellae were recovered by the other four sampling methods under the experimental conditions tested. As for the recovery of total and viable *L. pneumophila* and *Legionella* spp., the IOM consistently showed the highest efficiencies relative to the other samplers that were simultaneously tested regardless of sampling time (30–270 min), and these values were statistically comparable to those of the cassette but significantly greater than those of the other liquid- and filter-based sampling methods by 1–3 orders of magnitude ($p < .05$). In most cases, the biological efficiencies (calculated as a percentage of the viable cell counts to total cell counts, 19.6%–80.2%) were not significantly different between the samplers ($p > .05$). This study shows that viable and total legionellae can be efficiently sampled by the IOM equipped with a gelatin filter, and culturable cells may be detected more frequently by the SASS 2300.

INTRODUCTION

Legionella spp. may considerably proliferate and become aerosolized during the operation of cooling towers (CTs) (Nguyen et al. 2006). Inhalation of airborne legionellae could result in lethal Legionnaires' disease (LD) and nonpneumonic Pontiac fever (PF), collectively referred to as legionellosis (Fields 1997). Among the 52 *Legionella* species, *Legionella pneumophila* is responsible for 90% of *Legionella*-related infections (Murray et al. 2009). It is considered that, in addition to culturable cells, viable but nonculturable legionellae may also cause infection (Dusserre et al. 2008); on the other hand, PF can be induced by viable and nonviable legionellae (Miller et al. 1993). Significantly, severe legionellosis outbreaks have been associated with *Legionella*-contaminated CTs (Nguyen et al. 2006; Gilmour et al. 2007).

Efficient air sampling in combination with water surveillance is beneficial for preventing the occurrence of legionellosis. Monitoring the air around aerosol-producing devices may assist in tracking the greatest potential of legionellae aerosolization (Blatny et al. 2008), identifying the plausible infection source, and assessing the distance that legionellae have spread (Nguyen et al. 2006). Thus, characterization of various sampling techniques in terms of their capability of detection and quantification of airborne legionellae is essential in order to select an appropriate sampling method for monitoring legionellae.

The following bioaerosol samplers have been adopted in field studies for recovering legionellae: Andersen sampler (Andersen Samplers, Inc., Atlanta, GA, USA) (Bollin et al. 1985), slit-to-agar sampler (STA-204, New Brunswick Scientific, Edison, New Jersey, USA) (Blatny et al. 2008), Microbiological Air Sampler (MAS-100, Merck, Darmstadt, Germany) (Pascual et al. 2001; Deloge-Abarkan et al. 2007; Bauer et al. 2008; Blatny et al. 2008), BioSampler (SKC, Inc., Eighty Four, PA, USA) (Deloge-Abarkan et al. 2007), cassette (Millipore, Bedford, MA, USA) (Deloge-Abarkan et al. 2007), IOM personal

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inhalable air sampler (IOM, SKC) (Dutil et al. 2007), and wetted-wall cyclone (SASS 2000, Research International, Monroe, WA, USA) (Blatny et al. 2008). However, comparative evaluation of the performance of aerosol samplers for capturing environmental legionellae is limited. By sampling around hot water showers with three types of samplers, Deloge-Abarkan et al. (2007) reported a significantly high detection of *Legionella* by the BioSampler filled with deionized water (DW). While sampling airborne *Legionella* around biological aeration ponds with a wetted-wall cyclone (SASS 2000) and two agar-based impactors, Blatny et al. (2008) concluded that the SASS 2000 was suitable for collecting culturable *Legionella*. Although both studies provide useful information on the recovery of airborne *Legionella*, only three types of samplers were evaluated in those investigations (Deloge-Abarkan et al. 2007; Blatny et al. 2008).

In addition to field evaluations, sampler performance may be assessed in a laboratory chamber setting. We have recently tested a variety of bioaerosol sampling methods in a relative-humidity- and temperature-controlled chamber with monodispersed *L. pneumophila*. These tests were followed by sample analyses using culture assays, real-time quantitative PCR (qPCR), and qPCR with ethidium monoazide (EMA-qPCR) to determine the recovery of culturable, total, and viable *L. pneumophila*, respectively (Chang et al. 2010; Chang and Chou 2011a, 2011b). The EMA-qPCR is a qPCR-based technique that selectively amplifies the DNA of viable cells, which are defined as the cells with intact cytoplasmic membrane (Stocks 2004), and this technique has been successfully applied to bioaerosols (Chang and Chou 2011a), water (Inoue et al. 2008), and food samples (Rudi et al. 2005). The results of the chamber studies indicate that the IOM with a gelatin filter and the cassette with a polycarbonate (PC) filter were the most suitable methods for collecting total *L. pneumophila* (Chang and Chou 2011b). Meanwhile, culturable cells were sampled most efficiently using the BioSampler (Chang et al. 2010; Chang and Chou 2011b), and viable cells could be appropriately collected using the IOM/gelatin filter, AGI-30, and BioSampler (Chang and Chou 2011b). In contrast, *L. pneumophila* was recovered significantly less when using the MAS-100 sampler (Chang and Chou 2011b). One caveat of these results is that laboratory conditions are not exactly the same as those that occur in uncontrolled environments. For example, airborne legionellae that are found in fields could aggregate or attach to larger particles or droplets, which could increase their aerodynamic diameter (d_{ae}) and the cell recovery by inertial sampling devices. In addition, airborne droplets generated from field facilities such as CTs tend to be less dehydrated than those produced in the laboratory chamber, where bioaerosols are passed through a particle charge neutralizer and diluted with a high volume of air (Chang and Chou 2011a). It is possible that moisture may help in preserving the viability of bioaerosols, which are collected on filters, during sampling and transportation.

Considering the differences between the laboratory and the field and the limited information in the literature, this study

was initiated as the next step to our chamber studies to assess the performance of eight bioaerosol sampling methods around operating CTs. The sampling methods evaluated in this study included two agar-based [MAS-100 and Andersen one-stage sampler (Andersen 1-STG)], two filter-based (IOM and cassette), and four liquid-based sampling techniques [BioSampler, all-glass impinger (AGI-30, Ace Glass, Inc., Vineland, NJ, USA), MAS-100 and SASS 2300]. The MAS-100 was tested with agar and liquid collection media (denoted as MAS-100/A and MAS-100/L, respectively), as has been successfully demonstrated in field studies (Pascual et al. 2001). All sampling techniques were assessed for the ability to detect culturable *Legionella* spp. and *L. pneumophila*. Moreover, liquid- and filter-based samplers were evaluated for their capabilities to collect viable and total legionellae coupled with EMA-qPCR and qPCR assays, respectively.

MATERIALS AND METHODS

Air Sampling

The characteristics of the operation for each of the tested sampling techniques are summarized in Table 1. Buffered charcoal yeast extract agar supplemented with α -ketoglutarate (BCYE α agar) (Sigma Chemical Co., St. Louis, MO, USA) and DGVF agar (i.e., BCYE α agar supplemented with dyes [bromothymol blue and bromocresol purple], glycine, vancomycin, and polymyxin B [Sigma]) were used in the Andersen 1-STG (Andersen Samplers, Inc.) and MAS-100 (Merck). Both samplers were run for 5–30 min, and the MAS-100 with agar collection (MAS-100/A) was turned 90° from vertical to horizontal so that the samples were taken with the MAS-100/A positioned to face the direction of the wind.

The IOM (SKC, Inc.) and the closed-face three-piece cassette (SKC) were respectively loaded with 25-mm gelatin filter (3- μ m porosity, Sartorius, Goettingen, Germany) and 37-mm Isopore PC filter (0.2- μ m porosity)/cellulose support pad (Millipore), and both samplers were continuously operated for 30–270 min. For the four liquid-based sampling methods, the BioSampler (SKC), AGI-30 (Ace Glass, Inc.), and MAS-100/L were filled with 20 mL of DW (Chang et al. 2010) and operated for 15–60 min. The DW was refilled to 20 mL every 15 min during sampling to increase the recovery of *Legionella* (Chang and Chou 2011a). The SASS 2300 (Research International), a wetted-wall cyclone sampler that draws air into the device through a water curtain and traps bioaerosols in the collection medium, was filled with 5 mL of DW and operated for 30–270 min. There was no need to manually refill the DW in this apparatus because the evaporated DW was automatically replaced from water in an internal reservoir.

The MAS-100 and SASS 2300 were operated at higher flow rates (100–325 L/min) by built-in batteries and pumps, while the IOM, cassette, AGI-30, BioSampler, and Andersen 1-STG were run at lower flow rates (2–28.3 L/min) using external vacuum pumps (Andersen 1-STG and AGI-30: DOA Series,

TABLE 1
Air sampling techniques tested around cooling towers

Sampler	Collection medium	Flow rate (L/min)	Sampling time (min)	Sampled air volume (L)
Agar-based sampler				
Andersen 1-STG	BCYE α , DGVP	28.3	5, 10, 15, 30	141.5, 283, 424.5, 849
MAS-100/A	BCYE α , DGVP	100	5, 10, 15, 30	500, 1000, 1500, 3000
Filter-based sampler				
IOM	Gelatin filter	2	30, 60, 270	60, 120, 540
Cassette	Polycarbonate filter	4	30, 60, 270	120, 240, 1080
Liquid-based sampler				
BioSampler	DW, refilling ^a	12.5	15, 30, 60	187.5, 375, 750
AGI-30	DW, refilling	12.5	15, 30, 60	187.5, 375, 750
MAS-100/L	DW, refilling	100	15, 30, 60	1500, 3000, 6000
SASS 2300	DW, 5 mL	325	30, 60, 270	9750, 19,500, 87,750

^aSterile distilled water (DW) was replenished to 20 mL every 15 min during 30 and 60 min of sampling.

GAST Manufacturing, Inc., Benton Harbor, MI, USA; BioSampler: 0523 Series, GAST; IOM: Aircheck XR5000, SKC; Cassette: KS-N300, Reliance Electric, Inc., Gallipolis, OH, USA). The pumps were calibrated for the flow rate prior to sampling, and the AGI-30, BioSampler, Andersen 1-STG, the sieve portion of the MAS-100, IOM, PC filters, and cellulose support pads were autoclaved. The cassettes were sterilized with ethylene oxide, and the SASS 2300 was flushed with 1 L of sterile DW before use.

Air sampling was conducted at two operating CTs that were located on the roofs of two nursing homes. According to our preliminary surveys with qPCR analysis, *L. pneumophila* at concentrations of 4.2×10^4 to 3.6×10^6 cells/L and *Legionella* spp. at concentrations of 1.9×10^5 to 4.1×10^6 cells/L were revealed in the water of both CTs (data not shown). Thus, legionellae-containing aerosols were generated from these two operating CTs and could be used to assess the performance of the samplers. The sampling inlets of the tested samplers were positioned at a height of 1.2 and 1 m downwind from the waterfalls of the CTs. Each tested sampling method was run for at least three different sampling times (Table 1). The samplers set at the same sampling time were tested simultaneously. In addition to air samples, one blank sample from the field was taken for each sampling method on every sampling day. In total, six air samples were obtained for each sampler operated at a specified sampling time on six different days, except for the SASS 2300, which was unavailable on two occasions. The DGVP plates were not applied on one occasion. A total of 190 air samples and 46 field blanks were collected.

After sampling, agar plates and cassettes were sealed with parafilm. Gelatin filters were gently removed from the IOM and transferred to sterile centrifuge tubes containing 10 mL of extraction fluid (0.1% peptone and 0.01% Tween 80) (Chang and Chou 2011b). Liquid samples from the AGI-30, BioSampler, MAS-100/L, and SASS 2300 were also transferred to sterile

centrifuge tubes and stored at 4°C, along with the gelatin samples. All samples were transported at 4°C to the laboratory within 2 h and processed immediately.

Sample Processing

The remaining volume of the collection medium was measured for the AGI-30, BioSampler, and MAS-100/L. The tubes containing gelatin filters were placed in a water bath (B206-T1, Firstek, Taipei, Taiwan) at 37°C for 5 min to completely dissolve the filters. Following vortexing for 30 s (Delta Mixer-110, Taitec, Inc., Saitama-ken, Japan), an aliquot (0.5 mL) was taken from each of the liquid samples, diluted 10-fold, and spread on BCYE α and DGVP agars, along with undiluted samples (0.1 mL/plate, in duplicate). The residual liquid was filtered using a 0.4- μ m HTTP Isopore membrane (Millipore) and treated with 20 mL of acid buffer [0.2 M KCl-HCl (pH 2.2), Ishimatsu et al. 2001] for 3 min to reduce the growth of nonlegionellae microflora. The filters were gently washed with sterile DW until the pH of the filtrate was measured to be between 6 and 8. Acid-treated filters were cut into pieces using sterile scissors, suspended in 10 mL of extraction fluid, and vortexed for 30 s. An aliquot (1 mL) of samples was diluted 10-fold and spread on BCYE α and DGVP agars, as described earlier. The remaining aliquot (9 mL) was vortexed for 5 min. After removal of the filters, samples were centrifuged at $8200 \times g$ for 10 min at 4°C, resulting in cells suspended in 1 mL. The PC filters removed from the cassettes were also acid treated and processed, as described earlier.

Concentrated cell suspensions (1 mL) were divided in two equal parts. One part was added with 50 μ L of EMA (25 μ g/mL, Sigma) and kept in the dark for 5 min, followed by an exposure to a 500-W halogen light at a 15-cm distance for 20 min on chipped ice (Chen and 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

by centrifugation ($20,000 \times g$, 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 $100 \mu\text{L}$. Extracted DNA was diluted 5- and 10-fold with TE buffer and analyzed by qPCR along with undiluted DNA for quantification of viable and total legionellae, as described later.

Culture Assay and *Legionella* Identification

BCYE α 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 for 7 days with 5% CO_2 . Bacteria-like colonies were gram stained (Rapid Gram Stain kit, Baso Diagnostic, Inc., Taipei, Taiwan) and tested for the requirement of L-cysteine using BCYE α and nutrient agars. DNA from those colonies that grew exclusively on BCYE α agar and were determined to be gram-negative was extracted using the QIAamp DNA mini Kit (Qiagen GmbH). For identification of *L. pneumophila* and *Legionella* spp., the extracted DNA ($5 \mu\text{L}$) was analyzed on the LightCycler 480 System (Roche Diagnostics GmbH, Basel, Switzerland) using the primers, TaqMan MGB probes, and thermal programs described by Chen and 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.

Quantification of Viable and Total Legionellae

To quantify viable and total *L. pneumophila* and *Legionella* spp., the primers, probes, and thermal settings described by Chen and Chang (2010) were applied to diluted and undiluted DNA from air samples and field blanks. An internal inhibition control (IIC, $1 \mu\text{L}$) and IIC-specific probe (300 nM) were added in the qPCR reaction mixture to monitor inhibition of the qPCR (Chen and Chang 2010). Standard DNA of *Legionella* spp. was prepared by extracting DNA from *L. pneumophila* (ATCC 33152) and quantified by measuring the absorbance at 260 nm with a spectrophotometer (Shimadzu Co., Kyoto, Japan). 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 contained sample DNA, a serially diluted DNA standard ($10 \text{ fg}/\mu\text{L}$ – $1 \text{ ng}/\mu\text{L}$ for *Legionella* spp. and 0.31 – 3.1×10^4 copies/ μL for *L. pneumophila*), a nontemplate control, and an IIC control (i.e., IIC in legionellae-free and PCR-grade water). For reactions where IIC amplification was partially or completely inhibited, DNA extracts from the samples were further diluted and the qPCR was re-run until the Ct (cycle threshold) values of the IIC were within one standard deviation of the mean IIC Ct values that were obtained using the IIC control.

TABLE 2

Airborne concentrations of viable and total *L. pneumophila* (Lp) and *Legionella* spp. (Leg) around cooling towers determined by various samplers

Sampler (n^a)	Sampling time (min)	Mean cell concentration (\pm standard deviation) (cells/ m^3)			
		Total Lp	Total Leg	Viable Lp	Viable Leg
AGI-30 (6)	15	$4.3 (\pm 6.4) \times 10^5$	$2.1 (\pm 2.5) \times 10^6$	$2.1 (\pm 3.1) \times 10^5$	$1.0 (\pm 1.2) \times 10^6$
	30	$4.4 (\pm 6.3) \times 10^5$	$1.7 (\pm 0.9) \times 10^6$	$6.4 (\pm 5.4) \times 10^4$	$4.6 (\pm 4.2) \times 10^5$
	60	$4.4 (\pm 9.5) \times 10^5$	$1.8 (\pm 2.4) \times 10^6$	$1.0 (\pm 1.9) \times 10^5$	$2.5 (\pm 2.4) \times 10^5$
BioSampler (6)	15	$1.8 (\pm 1.4) \times 10^5$	$1.1 (\pm 1.0) \times 10^6$	$8.8 (\pm 13) \times 10^4$	$3.5 (\pm 2.5) \times 10^5$
	30	$9.5 (\pm 9.5) \times 10^4$	$6.5 (\pm 6.1) \times 10^5$	$3.5 (\pm 4.6) \times 10^4$	$3.1 (\pm 3.4) \times 10^5$
	60	$1.1 (\pm 1.2) \times 10^5$	$9.1 (\pm 11) \times 10^5$	$5.9 (\pm 7.0) \times 10^4$	$1.3 (\pm 0.8) \times 10^5$
MAS-100/L (6)	15	$3.4 (\pm 5.6) \times 10^4$	$1.6 (\pm 2.3) \times 10^5$	$1.9 (\pm 3.9) \times 10^4$	$9.2 (\pm 14) \times 10^4$
	30	$4.1 (\pm 5.9) \times 10^4$	$1.3 (\pm 1.0) \times 10^5$	$1.7 (\pm 2.8) \times 10^4$	$8.5 (\pm 8.5) \times 10^4$
	60	$6.4 (\pm 7.1) \times 10^3$	$4.5 (\pm 3.4) \times 10^4$	$2.8 (\pm 3.0) \times 10^3$	$1.6 (\pm 1.8) \times 10^4$
Cassette (6)	30	$1.1 (\pm 1.1) \times 10^6$	$8.0 (\pm 6.9) \times 10^6$	$8.2 (\pm 9.0) \times 10^5$	$3.2 (\pm 4.3) \times 10^6$
	60	$1.3 (\pm 1.7) \times 10^6$	$7.5 (\pm 5.9) \times 10^6$	$6.4 (\pm 11) \times 10^5$	$1.5 (\pm 1.0) \times 10^6$
	270	$4.6 (\pm 4.4) \times 10^5$	$1.5 (\pm 1.1) \times 10^6$	$1.6 (\pm 2.5) \times 10^5$	$8.1 (\pm 7.0) \times 10^5$
IOM (6)	30	$4.3 (\pm 3.2) \times 10^6$	$4.4 (\pm 8.4) \times 10^7$	$2.1 (\pm 2.3) \times 10^6$	$1.0 (\pm 1.4) \times 10^7$
	60	$2.4 (\pm 2.4) \times 10^6$	$8.7 (\pm 6.2) \times 10^6$	$1.2 (\pm 1.5) \times 10^6$	$4.1 (\pm 3.3) \times 10^6$
	270	$5.7 (\pm 5.1) \times 10^5$	$5.0 (\pm 7.2) \times 10^6$	$4.1 (\pm 4.6) \times 10^5$	$2.3 (\pm 3.1) \times 10^6$
SASS 2300 (4)	30	$3.2 (\pm 3.4) \times 10^4$	$1.7 (\pm 1.5) \times 10^5$	$4.1 (\pm 2.9) \times 10^3$	$3.9 (\pm 2.2) \times 10^4$
	60	$1.5 (\pm 1.8) \times 10^4$	$1.1 (\pm 1.2) \times 10^5$	$1.2 (\pm 1.9) \times 10^4$	$7.1 (\pm 11) \times 10^4$
	270	$1.5 (\pm 1.7) \times 10^3$	$1.1 (\pm 1.4) \times 10^4$	$2.3 (\pm 2.5) \times 10^2$	$2.1 (\pm 2.5) \times 10^3$

^aNumber of samples collected for each sampling time.

Standard curves (i.e., Ct vs. log concentration of standard DNA) were constructed for *L. pneumophila* and *Legionella* spp. and used to quantify the number of legionellae in the sample DNA based on the Ct value, DNA elution volume (100 μL), dilution fold of the DNA, and the copy number of the target gene or the quantity of the genomic DNA in a single cell (i.e., a single copy of the *mip* gene in *L. pneumophila* and approximately 4.3 fg in a legionellae cell) (Morio et al. 2008). The airborne concentrations of viable and total legionellae were further calculated for each sample by adjusting for the sample volume analyzed by qPCR, sampling flow rate, and sampling time.

Data Analysis

The biological efficiency (BE) was calculated for each sample by dividing the concentration of viable cells by that of total cells and was presented as a percentage. Moreover, by taking the concentrations 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:

$$RE, \% = [C_{\text{SAMPLER}}/C_{\text{REF}}] \times 100\%,$$

C_{SAMPLER} : the concentration of legionellae determined by a test sampler operated simultaneously with the reference sampler,

C_{REF} : the concentration of legionellae determined by the reference sampler.

As log-transformed RE values were distributed normally, one-way analysis of variance (ANOVA) was conducted to compare the log-transformed RE values of various sampling methods. The post hoc analysis by the Least Significance Difference

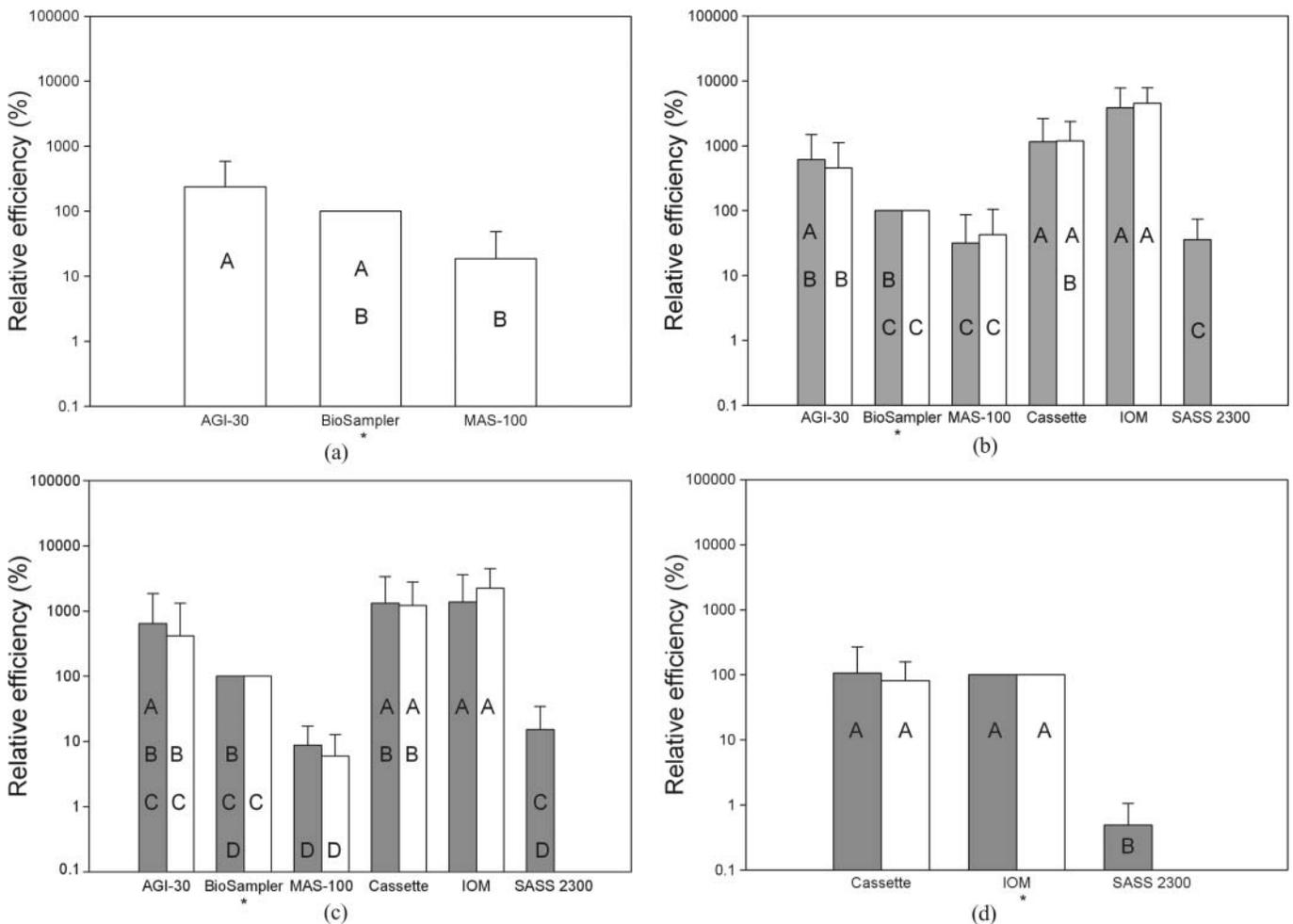


FIG. 1. Relative efficiencies of bioaerosol samplers for capturing total *Legionella pneumophila* around cooling towers at (a) 15, (b) 30, (c) 60, and (d) 270 min of sampling (■ $n = 4$; □ $n = 6$; n represents the number of repeated samples taken from the samplers that had been operated simultaneously). Samplers with the same letter in the histogram bar have relative efficiencies that are not statistically different ($p > .05$, LSD test). Each error bar represents one standard deviation from a mean of repeated samples. *: reference sampler.

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(LSD) test was further undertaken when observing a statistical significance as $p < .05$ by the ANOVA. The Kruskal–Wallis test with post hoc analysis using the LSD test was applied to examine the concentration of viable and total legionellae obtained using different sampling methods because neither the original nor the log-transformed concentration data were normally distributed. The Kruskal–Wallis and LSD tests were also used to examine the positive rate of culturable legionellae and BE value among sampling methods as well as the effect of sampling time on the BE value. All statistical analyses were conducted using the SAS software version 9.1 (SAS Institute, Inc., NC, USA), and statistical significance was considered as $p < .05$.

RESULTS

Airborne Concentrations of Total and Viable Legionellae

Legionellae were detected in every air sample analyzed by qPCR or EMA-qPCR but were absent in the field blanks. For

the five samplers that performed on six sampling days ($n = 6$), the IOM and cassette obtained the highest mean concentration of total *L. pneumophila* (4.6×10^5 to 4.3×10^6 cells/m³) and *Legionella* spp. (1.5×10^6 – 4.4×10^7 cells/m³) (see Table 2). The second highest concentration was determined using the AGI-30 and BioSampler (*L. pneumophila*: 9.5×10^4 to 4.4×10^5 cells/m³; *Legionella* spp.: 6.5×10^5 to 2.1×10^6 cells/m³), while the MAS-100/L had the lowest value (*L. pneumophila*: 6.4×10^3 to 4.1×10^4 cells/m³; *Legionella* spp.: 4.5×10^4 to 1.6×10^5 cells/m³). The LSD results showed that the IOM was comparable to the cassette but was significantly greater than the AGI-30, BioSampler, and MAS-100/L for recovering total legionellae ($p < .05$). As for viable legionellae, the IOM still obtained the highest mean concentration (4.1×10^5 to 2.1×10^6 cells/m³ for *L. pneumophila* and 2.3×10^6 to 1.0×10^7 cells/m³ for *Legionella* spp.), followed by the cassette (1.6×10^5 to 8.2×10^5 cells/m³ and 8.1×10^5 to 3.2×10^6 cells/m³), AGI-30 (6.4×10^4 to 2.1×10^5 cells/m³ and 2.5×10^5 to 1.0×10^6

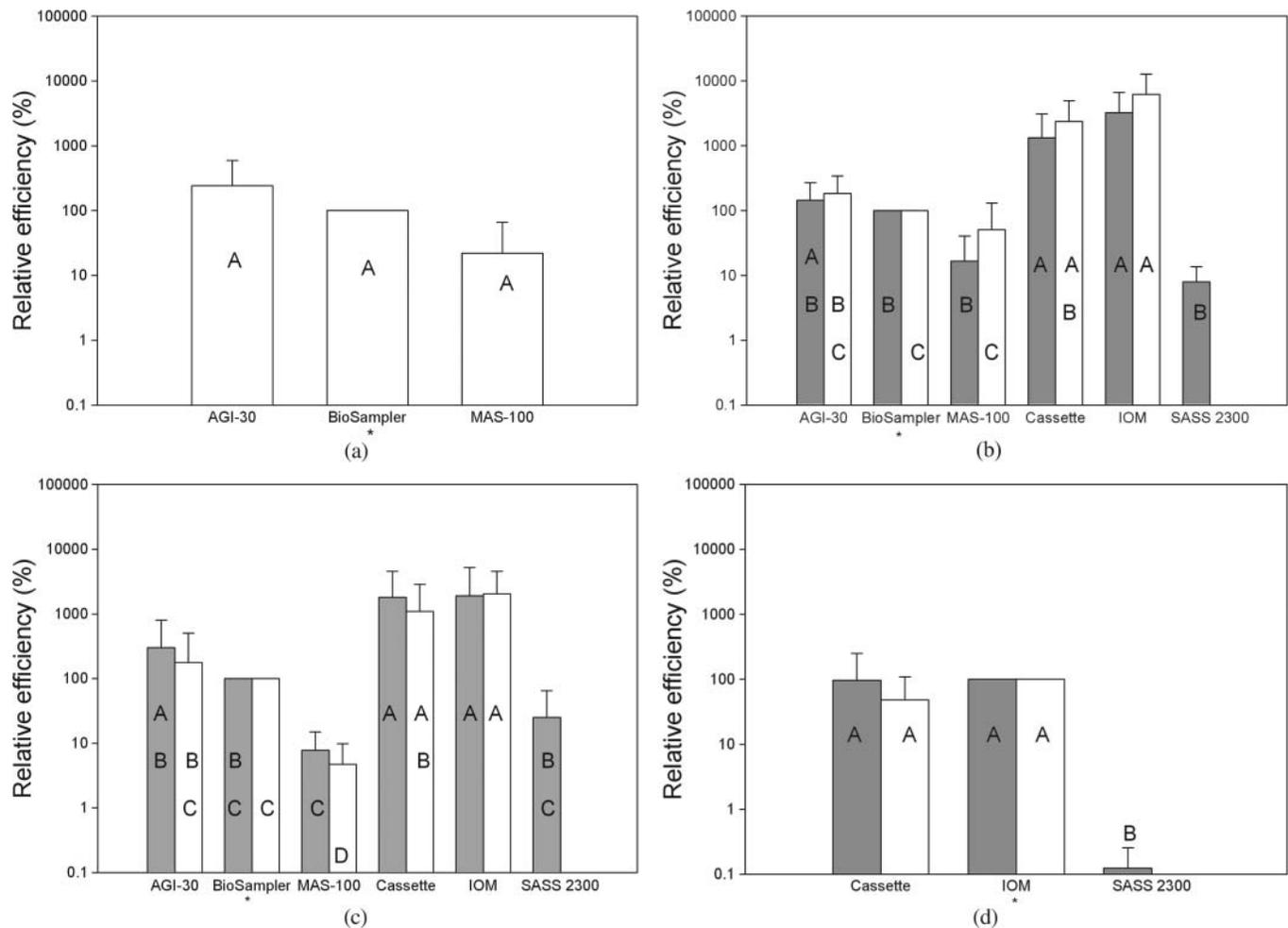


FIG. 2. Relative efficiencies of bioaerosol samplers for capturing viable *Legionella pneumophila* around cooling towers at (a) 15, (b) 30, (c) 60, and (d) 270 min of sampling (■ $n = 4$; □ $n = 6$; n represents the number of repeated samples taken from the samplers that had been operated simultaneously). Samplers with the same letter in the histogram bar have relative efficiencies that are not statistically different ($p > .05$, LSD test). Each error bar represents one standard deviation from a mean of repeated samples. *: reference sampler.

cells/m³), BioSampler (3.5×10^4 to 8.8×10^4 cells/m³ and 1.3×10^5 – 3.5×10^5 cells/m³), and MAS-100/L (2.8×10^3 – 1.9×10^4 cells/m³ and 1.6×10^4 to 9.2×10^4 cells/m³). The concentration of viable legionellae sampled by the IOM was significantly greater than that by the other four samplers ($p < .05$).

The SASS 2300 was tested on four sampling days and obtained total and viable legionellae of 1.5×10^3 to 1.7×10^5 cells/m³ and 2.3×10^2 to 7.1×10^4 cells/m³, respectively (see Table 2). Statistical analyses on legionellae concentration data collected simultaneously on four sampling days showed that the SASS 2300 recovered significantly less viable and total legionellae than the IOM ($p < .05$).

Relative Efficiency for Total *L. Pneumophila*

In order to compare the collection efficiencies of the samplers operated simultaneously, the concentration data were transformed to *RE* values and categorized by sampling time. The

RE for collection of total *L. pneumophila* is presented in Figure 1. After 15 min of sampling (Figure 1a), the *RE* of the AGI-30 was 2.4 times higher than that of the BioSampler, whereas the collection efficiency of the MAS-100/L was 19% relative to BioSampler and was significantly lower than that of the AGI-30 ($p < .05$).

For the 30-min samplings (Figure 1b), the IOM, cassette, and AGI-30 obtained the highest *RE* values, i.e., 38–45, 12, and 5–6 times that of the BioSampler, respectively. The LSD results showed that the *RE* of the AGI-30 was significantly greater than that of MAS-100/L ($p < .05$) and the collection efficiency by the IOM and cassette was significantly higher than that of the BioSampler, MAS-100/L, and SASS 2300 ($p < .05$).

The IOM, cassette, and AGI-30 remained the best three methods at the 60-min sampling time, with *RE* > 100%; in contrast, the MAS-100/L recovered only 6%–9% of *L. pneumophila* compared with the BioSampler and had the *RE* values that were

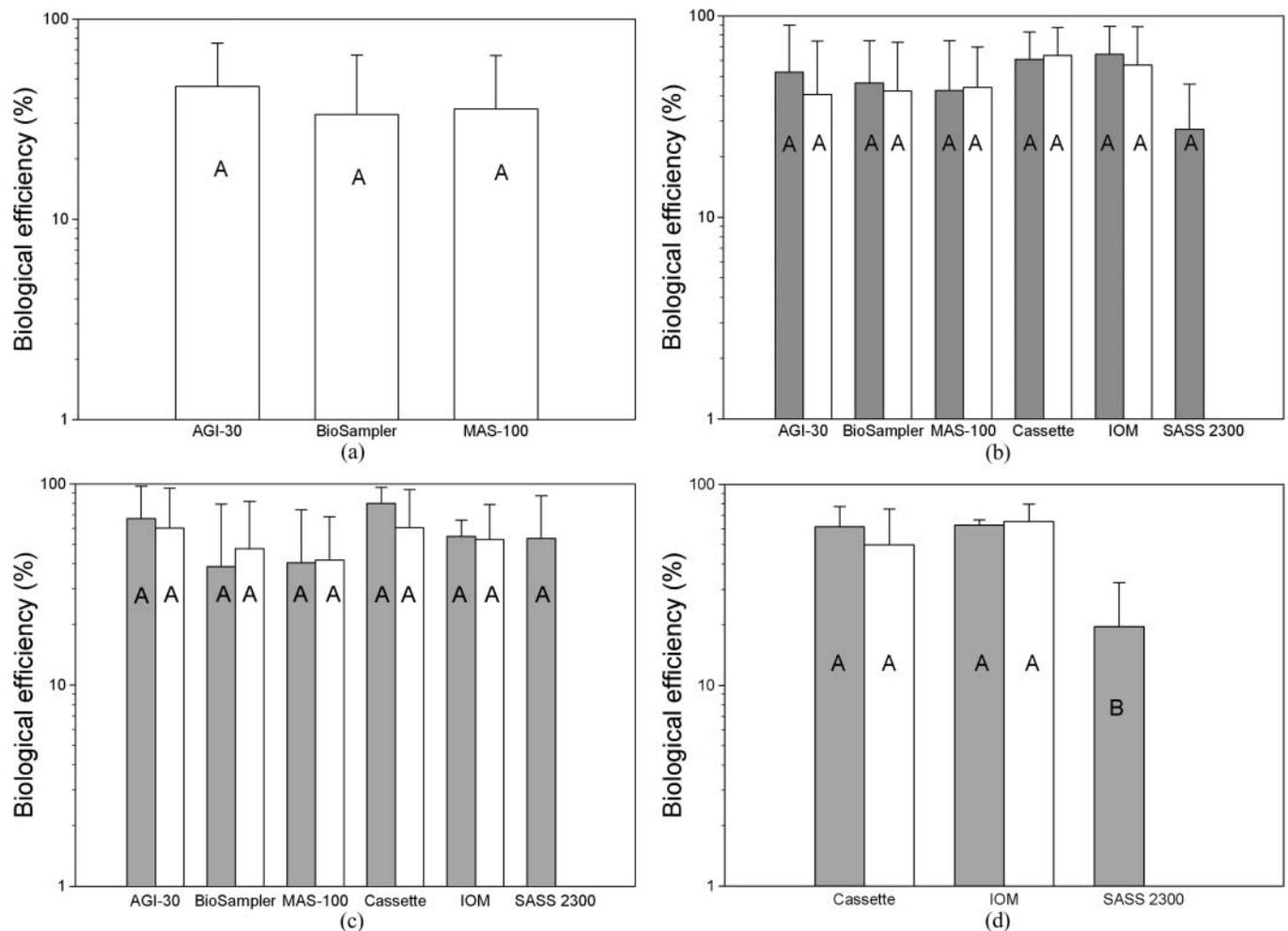


FIG. 3. Biological efficiencies of samplers for collecting *Legionella pneumophila* around cooling towers at (a) 15, (b) 30, (c) 60, and (d) 270 min of sampling (■ $n = 4$; □ $n = 6$; n represents the number of repeated samples taken from the samplers that had been operated simultaneously). Samplers with the same letter in the histogram bar have biological efficiencies that are not statistically different ($p > .05$, LSD test). Each error bar represents one standard deviation from a mean of repeated samples.

significantly lower than those of the IOM, cassette, and AGI-30 ($p < .05$) (Figure 1c). The SASS 2300 also obtained *RE* values that were lower than those of the IOM and cassette by two orders of magnitude after sampling for 60 and 270 min ($p < .05$), whereas a comparable efficiency was observed between the IOM and cassette (Figure 1c–d).

Relative Efficiency for Viable *L. Pneumophila*

When sampling viable *L. pneumophila* for 15 min (Figure 2a), the AGI-30 obtained the highest *RE* value (242%), while the MAS-100/L had the lowest value (22%). When extending the sampling time to 30 min (Figure 2b), the highest *RE* value was observed for the IOM (3252%–6170%) and cassette (1334%–2372%), followed by the AGI-30 (144%–183%) and BioSampler (100%). The *RE* values of the MAS-100/L (17%–50%) and SASS 2300 (8%) were relatively low and significantly different from those of the IOM and cassette ($p < .05$).

Such findings were also observed at the 60-min sampling time (Figure 2c). For the 270-min sampling (Figure 2d), comparable *RE* values were found between the IOM and cassette, which were significantly greater than that of the SASS 2300 by three orders of magnitude ($p < .05$).

Relative Efficiencies for Total and Viable *Legionella* spp.

The *RE* ranking of samplers for collecting total and viable *Legionella* spp. (data not shown) was similar to what is presented in Figures 1 and 2. The highest and lowest *RE* values were observed for the AGI-30 and MAS-100/L, respectively, after sampling for 15 min. When sampling for 30 and 60 min, the IOM obtained the highest *RE* value for viable and total *Legionella* spp. This value was followed by that of the cassette, AGI-30, and BioSampler, with the $RE \geq 100\%$, while the *RE* values of the MAS-100/L and SASS 2300 were less than 100%. The *RE* of the IOM was statistically comparable to that of the cassette and was

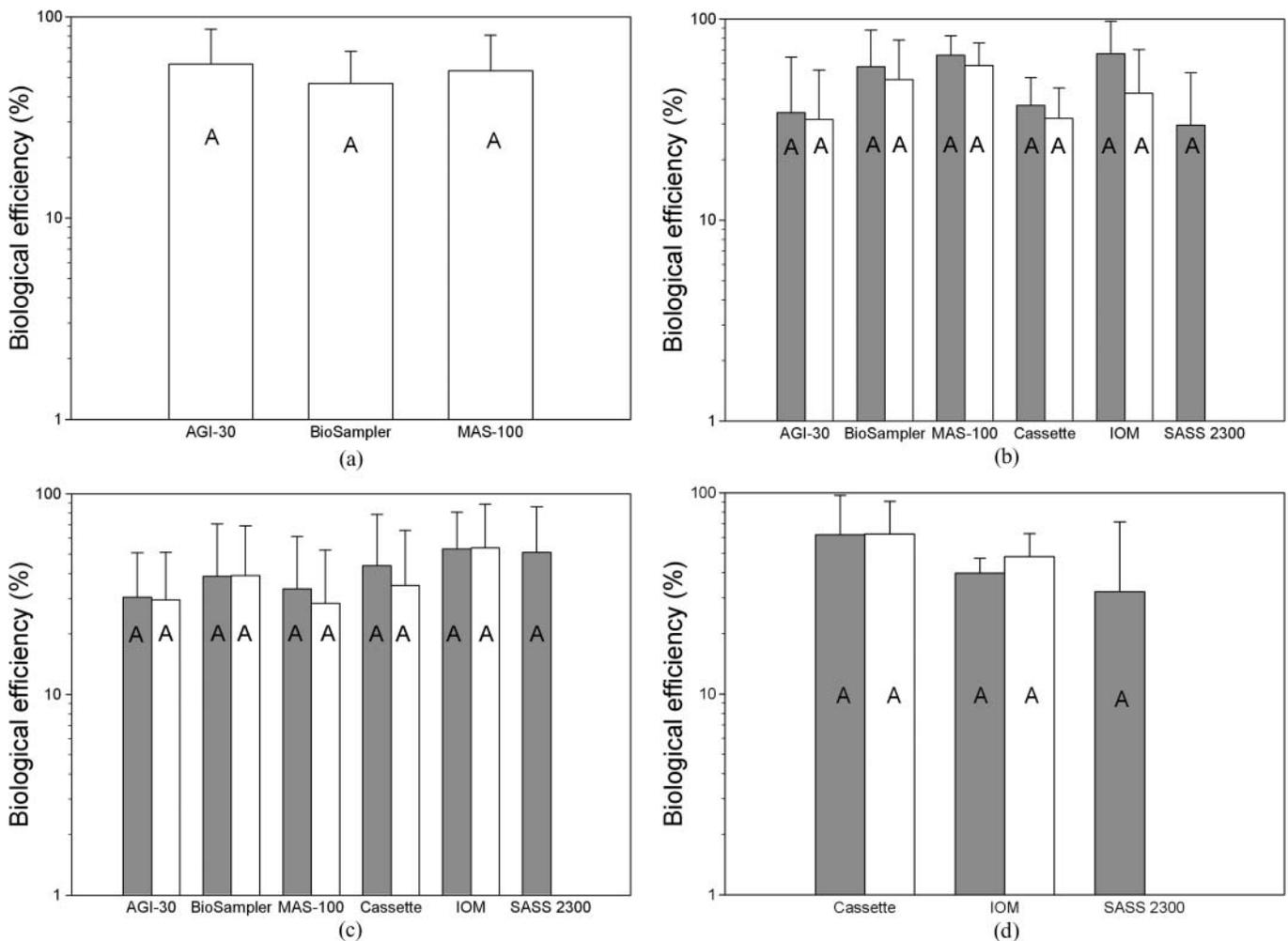


FIG. 4. Biological efficiencies of samplers for collecting *Legionella* spp. around cooling towers at (a) 15, (b) 30, (c) 60, and (d) 270 min of sampling (■ $n = 4$; □ $n = 6$; n represents the number of repeated samples taken from the samplers that had been operated simultaneously). Samplers with the same letter in the histogram bar have biological efficiencies that are not statistically different ($p > .05$, LSD test). Each error bar represents one standard deviation from a mean of repeated samples.

TABLE 3
Culturable legionellae detected from the air around cooling towers by various sampling and analytical methods*

Sampler (n_1, n_2) ^a	Sampling time (min)	% positive for <i>Legionella pneumophila</i>					% positive for <i>Legionella</i> spp.				
		BCYE α agar		DGVP agar		Overall	BCYE α agar		DGVP agar		Overall
		N ^b	F/A ^c	N	F/A		N	F/A	N	F/A	
Liquid-based sampler											
BioSampler (6, 5)	30	0	0	0	0	0 (0/22)	17	0	0	0	5 (1/22)
	60	33	17	0	0	14 (3/22)	33	17	40	0	23 (5/22)
MAS-100/L (6, 5)	60	0	0	0	20	5 (1/22)	0	17	0	20	9 (2/22)
SASS 2300 (4, 4)	60	25	25	25	25	25 (4/16)	25	25	25	25	25 (4/16)
	270	50	25	0	25	25 (4/16)	50	50	0	25	31 (5/16)
Filter-based sampler											
IOM (6, 5)	60	17	0	0	0	5 (1/22)	17	17	0	20	14 (3/22)

*Only the sampling methods with at least one legionellae-positive sample are included.

^a n_1 : number of samples on BCYE α agar; n_2 : number of samples on DGVP agar.

^bN: direct plating on agar without filtration and acid treatment.

^cF/A: filtration and acid treatment prior to plating on agar.

significantly greater than that of the BioSampler, MAS-100/L, and SASS 2300 for viable and total *Legionella* spp., regardless of sampling time ($p < .05$).

Biological Efficiency

The BE values for *L. pneumophila* are presented in Figure 3. The BE varied from 27.4% to 80.2% when the sampling time was 15, 30, or 60 min (Figure 3a–c), and no significant differences were found between the samplers ($p > .05$). The only significant differences were revealed at 270-min sampling between the SASS 2300 (19.6%) and IOM (62.6%) and between the SASS 2300 and cassette (61.6%) ($p < .05$; Figure 3d).

For collecting *Legionella* spp. (Figure 4), the mean BE values of the tested samplers determined at 15-, 30-, 60-, and 270-min sampling were 46.7%–58.4%, 29.6%–67.2%, 28.5%–53.9%, and 32.3–62.4%, respectively. No significant differences were observed between the BE values of any two samplers, regardless of sampling time ($p > .05$). Moreover, the sampling time did not significantly affect the BE of any one of the liquid- and filter-based samplers for collecting *L. pneumophila* (Figure 3) and *Legionella* spp. (Figure 4).

Detection of Culturable Legionellae

Culturable legionellae were not detected using the Andersen 1-STG, MAS-100/A, cassette, or AGI-30, regardless of sampling time. Only the BioSampler, MAS-100/L, SASS 2300, and IOM recovered culturable cells from the air after sampling for 30 min or longer (Table 3). The SASS 2300 had the highest positive rate (25%–31%), which was greater than that of the BioSampler (0%–23%), MAS-100/L (5%–9%), and IOM (5%–14%) ($p < .05$). Although the positive rate of culturable legionellae was different among these four sampling methods

($p < .001$), the mean colony count was similar, i.e., 1–2 CFU/plate (data not shown). The CFU number was too low to be used to evaluate sampler performance. However, $1.7 (\pm 0.3) \times 10^4$ CFU/L of culturable legionellae was observed in the CT water (1 L water, filtration and acid treatment, plating on DGVP agar, qPCR identification, data not shown).

DISCUSSION AND CONCLUSION

Viable and Total Legionellae

Compared to other liquid- and filter-based samplers tested in this study, the IOM showed the greatest efficiency for collecting total and viable legionellae, regardless of sampling time. This sampler has also been reported in previous chamber studies to be one of the most appropriate sampling methods to recover total and viable *L. pneumophila* (Chang and Chou 2011b). Similar findings observed in the present and previous studies strongly support that the IOM is the best sampling method for quantifying viable and total legionellae in air. Moreover, the IOM is advantageous because it can be used for both stationary and personal monitoring (Van Droogenbroeck et al. 2009), highlighting the potential that personal exposure to viable and total legionellae can be appropriately quantified by IOM and used to determine the dose–response relationship during LD and PF investigation.

In addition to the IOM, the cassette showed a great efficiency for collecting total and viable legionellae. Such high collection efficiency was also observed in previous chamber evaluations for total *L. pneumophila*, whereas the cassette was significantly less efficient in recovering viable *L. pneumophila* compared with the BioSampler and AGI-30 by a factor of 13 and 17, respectively, after sampling for 60 min (Chang and Chou 2011b). The latter, however, did not occur at the CT sampling (Figure 2c). The

inconsistency between chamber testing and field sampling may be attributed to the difference in the magnitude of dehydration of legionellae cells. Legionellae-containing droplets generated from CTs 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 and Chou 2011a). Considering that dehydration adversely affects the recovery of viable *L. pneumophila* from the PC filter of the cassette (Chang and Chou 2011b), less dehydrated legionellae from CTs may have a better chance of preserving their viability, resulting in an increase in the recovery of viable cells.

Relative to the IOM and cassette, the AGI-30 and BioSampler were less efficient in collecting total and viable legionellae. This may be due to the loss of bacteria in the sampler inlet (Seshadri et al. 2009) and/or the reaerosolization of certain collected cells from the DW (Lin et al. 2000). As for the MAS-100/L, a significantly lower recovery of total *L. pneumophila* was found in previous chamber tests (Chang et al. 2010; Chang and Chou 2011b), which is attributed to the fact that the 50% cut-off diameter (d_{50}) of the MAS-100 (1.7–2.5 μm) is much greater than the d_{ae} of monodispersed *L. pneumophila* (0.7 μm). The present field study also shows that the MAS-100/L presented significantly less recovery of total *L. pneumophila* than the IOM ($p < .05$, Figure 1), and its collection efficiency relative to the IOM (0.002–0.009, Figure 1) was generally lower than what was observed in the chamber testing using monodispersed *L. pneumophila* (0.005–0.02, Chang and Chou 2011b). This finding suggests that an increase in the d_{ae} of *L. pneumophila* by cell aggregation or attachment to larger particles/droplets, if present around CTs, was not great enough to improve legionellae recovery using the MAS-100/L.

To the best of our knowledge, this is the first study that evaluates the performance of the SASS 2300 for legionellae collection. This sampler recovered less viable and total cells than the IOM by approximately two orders of magnitude after sampling for ≥ 30 min. As the d_{50} of the SASS 2300 operated at 325 L/min with 5 mL of collection fluid is between 2 and 2.5 μm (Research International 2011), a portion of legionellae cells in the air of CTs was possibly not captured by the SASS 2300, similar to what was observed for the MAS-100/L.

The BEs of tested samplers were generally statistically comparable for legionellae collection (Figures 3 and 4), suggesting that the stresses that arise from different sampling methods (e.g., impingement/shear forces in liquid and desiccation on filters) do not cause significant differences in terms of the amount of damage to the legionellae membrane. Moreover, no statistical differences between the BE values among various sampling durations support the idea that legionellae that are aerosolized from CTs may maintain their membrane integrity throughout the sampling period. The present findings, however, differ from those previously reported with culture assays. Previous studies showed that when sampling culturable *L. pneumophila* from the air of laboratory chambers, the collection efficiency with filtra-

tion sampling was significantly lower than that with liquid impingement sampling (Li et al. 2003; Deloge-Abarkan et al. 2007; Chang and Chou 2011b), and the collection efficiency was decreased with sampling time for filter- (Chang and Chou 2011b) and liquid-based samplers (Chang et al. 2010). 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, particularly when using filtration sampling, while no significant alternation in cell membrane integrity occurs.

In addition to the samplers shown in Table 1, an AirPort MD8 (MD8, Sartorius) loaded with an 80-mm gelatin filter (3- μm porosity, Sartorius) was also assessed on 3 sampling days. We noticed that a portion of the gelatin filter was dissolved after sampling for 15 and 30 min at a flow rate of 50 L/min. Such phenomenon was found in every MD8 sample and was likely caused by water droplets generated from CTs, possibly resulting in a loss of legionellae collection. The observation of filter dissolution suggests that the MD8/gelatin filter may not be suitable for monitoring legionellae in atmospheres that contain water droplets (e.g., operating CTs and bathrooms after showering).

Culturable Legionellae

The mean concentration of culturable legionellae in the water of the studied CTs was 1.7×10^4 CFU/L, which was slightly greater than that of a CT linked to a LD case (8.8×10^3 CFU/L) (Isozumi et al. 2005). Although the water of the present studied CTs was contaminated with culturable legionellae, only a few colonies were recovered on plates of the air samples, regardless of sampler type, sampling volume, agar type, or sample treatment. A low level of airborne legionellae around a CT has also been reported by Ishimatsu et al. (2001) who revealed $1.2 (\pm 0.3) \times 10^6$ CFU/L of legionellae in CT water but detected only one colony of *L. pneumophila* from air samples that had been collected by an impinger filled with buffered yeast extract broth and operated at 5 L/min for 120 min at 50 cm above the fan of a CT. A similar finding was also observed in shower rooms, where only one colony of *Legionella* spp. was detected in aerosol samples using a MAS-100/A, whereas 4×10^4 CFU/L of *Legionella* spp. were found in hot water (Deloge-Abarkan et al. 2007).

The few CFU counts observed in the present study limit the potential to quantitatively assess the collection efficiency of samplers for culturable legionellae. However, our data show that the SASS 2300 obtained the greatest positive rate of legionellae compared with the other samplers (Table 3, $p < .05$), demonstrating its ability for detecting culturable legionellae. This finding accords with the observation by Blatny et al. (2008) on a similar sampler (SASS 2000) and may be attributed to its high sampling volume (9750–87,750 L) (Table 1) and fluid retention design, which tends to allow more legionellae cells to be trapped in DW. Due to its higher sampling volume and decreased amount of DW (5 mL) compared

with other liquid-based sampling methods (Table 1), the number of culturable legionellae on a plate would be expected to be greater for SASS 2300 samples; however, this was not the case. Low efficiencies for collection of total legionellae by the SASS 2300 (Figure 1b and c) may contribute in part to this phenomenon. Additionally, fungal growth was observed in 25% of the SASS 2300 samples processed with filtration, acid treatment, and DGVP plating (data not shown). This contamination could have interfered with the growth and/or counting of culturable legionellae.

In conclusion, this study shows that the IOM/gelatin filter was the appropriate sampling method to quantify viable and total legionellae in the air around CTs, while the SASS 2300 with DW obtained a greater detection rate of culturable legionellae. These findings should be taken into account when assessing airborne legionellae in fields at regular practice and/or during legionellosis investigation.

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