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Research

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Posted Date: September 18th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-77488/v1

Effects of Bioaerosol Aerodynamic Size on Biodiversity in Three Kindergartens in Taiwan

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Abstract

Background: Human-associated bacteria (HAB) in the microbiome can become airborne bacterial aerosols (bioaerosols). The aerodynamic sizes of bioaerosols demonstrated may significantly affect their behaviors, respiratory deposition and biodiversity.

Methods: The bacterial size, biodiversity and the HAB were evaluated at three kindergartens in central Taiwan in winter and spring. Kindergartens A, B, and C were in urban, semi-urban, and rural areas, respectively. A six-stage viable Andersen cascade impactor was used to collect bioaerosols and to determine their size distributions. A BD Phoenix-100 automated interpretation system was used to identify the species in those bioaerosols. The uniformity transformation was applied to verify the distributions of bacterial concentrations in different sampling rounds.

Results: The results revealed 1,425 colonies (97.6%) that corresponded to 63 species in 29 genera and 35 colonies (2.4%) that were unidentified. The most abundant phylum was Actinobacteria (56.6±22.2%), *Staphylococcus* spp. was found in all sampling rounds, with a range of abundance between 2.0 and 70.0%. In all rounds, the geometric mean diameter of the bioaerosol and the geometric standard deviation of the bioaerosol size ranged from 2.19 to 5.42 μm and from 1.66 to 2.70, respectively. The Shannon diversity (H) and inverse Simpson diversity index (D) of the bioaerosols at each kindergarten were positively correlated with bioaerosol size, as larger bioaerosols had higher values of the biodiversity metrics. The Pearson correlations of H with the urbanization of the area of the kindergarten, temperature, RH and CO_2 concentration were statistically significant ($P < 0.05$), indicating that the environmental variables and biodiversity covaried. The biodiversity in the rural area exceeded that in the urban area. Multiple and stepwise regression revealed that bioaerosol size was statistically significant with H ($P = 0.001$) and D ($P = 0.002$). The study may improve our understanding of the mechanisms and epidemiological spread of airborne infections.

Keywords: kindergarten, human-associated bacteria, bioaerosol, size distribution, biodiversity

Background

In Taiwan, the size distributions of yeasts differ remarkably from those of *Aspergillus* and *Penicillium* spp. [1]. The observed size variation among important bioaerosols reveals that significant variation may exist in their behavior and respiratory deposition. The aerodynamic size of airborne bacterial aerosols (bioaerosols) determines particulate motion, including settling under gravity, resuspension, and transport by moving air, and the location of the deposition of the bioaerosol in the human respiratory tract, and the aerodynamic particle size is defined as the diameter of a sphere of the same particle density having the same terminal velocity in air or some other relevant fluid [2]. Biodiversity may also relate to the size of bioaerosols and be associated with the health of people in the immediate environment. However, the relationship between the biodiversity and size of bioaerosols has seldom been studied. To the best of the authors' knowledge, no study has yet identified a direct relationship of bioaerosol size and biodiversity.

An investigation in Taiwan revealed remarkable seasonal variations of airborne fungus concentrations [1]; they vary significantly across seasons: bioaerosol samples in a subway in Norway were found to be more diverse in spring and summer than in autumn and winter [3]. In Taiwan, indoor ventilation conditions typically vary seasonally for climatic reasons. Therefore, this work involved two consecutive seasons, winter and spring, during which bioaerosols of different sizes were identified. No data on indoor size distributions of bioaerosols in Taiwan, which has a subtropical climate, have previously been reported. In this field investigation, the size and biodiversity characteristics of ambient bacterial particles were evaluated at three kindergartens in central Taiwan in winter and spring.

The skin is the largest human organ, and its microbiome generally exhibits the most direct relationship with the immediate environment, including the built environment (BE) [4]. Recent studies of human-associated bacteria (HAB) have focused on the biodiversity, morphology, and metabolism of a limited group of cultured isolates [5]. HAB in the microbiome of the built environment (MoBE) can attach to airborne dander and sometimes dominate the microbiome communities of bioaerosols. Within the major four phyla of the skin microbiome - Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes [6] - constantly present and plentiful genera on the

human skin or gut include *Brevibacterium*, *Corynebacterium*, *Micrococcus*, *Propionibacterium*, *Staphylococcus*, and *Streptococcus* spp. [7-9]. A study at a kindergarten in Norway identified five HAB [6], *Corynebacterium Micrococcus*, *Propionibacterium Staphylococcus* and *Streptococcus* spp., but not *Brevibacterium* spp. This study follows the study at the kindergarten in Norway in comparing the four phyla and five HAB in three kindergartens in central Taiwan with respect to their abundance.

Methods

Sampling locations

Bacteria were sampled at three kindergartens in central Taiwan. Kindergarten A was located inside an elementary school in an urban area in Taichung city close to an urban four-lane road. Kindergarten B was located in a semi-urban area of Nanto County near a two-lane road. Kindergarten C was close to rice fields and a rural two-lane road in Changhua County. The indoor air at those locations was mixed from outdoor ambient air through windows. To protect the privacy of the children and to avoid disturbing them, size distribution of the bioaerosol neighboring children table was measured. A total of 28 children and two ~ four nursery staff were present in the kindergarten A during sampling. Total 29~30, 19~20 children and one nursery staff were present in the kindergarten B, C during sampling, respectively. In winter, windows in kindergarten A are commonly closed to keep the classrooms warm. The classrooms in kindergartens A and B are about 9.9*9.8*3.6 m^3 and are larger than those in kindergarten C, which are about 6.1*5.8*2.8 m^3. In spring, the windows in kindergarten A are also usually closed to maintain a comfortable environment of recycled air using an air conditioner. In kindergartens B and C, the windows are often left open to allow natural convectional ventilation in the classrooms. Therefore, the average air changes per hour (ACH) in kindergartens B and C exceeded that in kindergarten A.

Sampling frequency

Indoor air in all three kindergartens was sampled once in January and once in April,

corresponding to the winter and spring seasons. The total duration of sampling was 10 min. During sampling, temperature, humidity, and CO_2 concentration were measured.

Bioaerosol sampling and sample analysis

A six-stage viable Andersen cascade impactor (Andersen 6-STG impactor, Thermo Scientific™) was used to perform sampling side-by-side (two neighboring sampling points - two sampling rounds) for 10 min to collect bacterial bioaerosols, and to determine their size distributions; the size classes were 0.65–1.1 (6^{th} STG), 1.1–2.1 (5^{th} STG), 2.1–3.3 (4^{th} STG), 3.3–4.7 (3^{rd} STG), 4.7–7.0 (2^{nd} STG), and >7.0 μm (1^{st} STG) [10, 11]. The sampling flow-rate of the Andersen 6-STG impactor was 28.3 L/min. After sampling, the plates from the impactor, which contained trypticase soy agar (TSA, Difco, Detroit, MI, USA), were incubated at 35°C for 24 h. Then, all colonies were counted and corrected by using positive correction table [10, 11] as to determine the number of colony forming units (CFU) and categorized by bacteria species. The bacteria of the categorized strains were replanted on the TSA with 5% sheep blood. After 24 to 48 hr of cultivation, the growth strains were identified by rapid Gram staining under a microscope and the Gram-positive/-negative bacteria and lactis or bacilli were thus identified.

Automated microbial identification

Microbial identification was performed using a BD Phoenix-100 automated interpretation system (BD Diagnostic Systems, Baltimore, MD). This instrument uses conventional, fluorogenic, and chromogenic substrates and can rapidly identify up to 100 microbes. The system contains 43 dried biochemical substrates and two fluorescent control wells [11]. Stefaniuk et al. [12] found strong agreement between the Phoenix and conventional API test methods, from 100% for Gram-positive cocci to 96.0% for Gram-negative non-fermenters and 93 to 95% for members of the family Enterobacteriaceae [13-15]. Other studies have established that BD Phoenix identifies 91 to 100% of *Staphylococci* [13], 85.9% of *Streptococcus pneumoniae* [16], 89.1% of *Vibrio* spp. [15].

Data processing

Airborne cultured bacterial concentrations (C, CFU/m^3) were calculated using

$$C=\frac{CFU}{Q\times\Delta T} \quad (1)$$

where CFU is the recovered and calibrated number of colony forming units and Q is the sampling flow rate of the bioaerosol sampler (m^3/min). The sampling flow-rate of the Andersen 6-STG impactor was 28.3 L/min. ΔT is the sampling duration (min), which was ten minutes herein.

To determine the particle size distribution (PSD) of bioaerosol, the geometric mean diameter (GMD, μm) and geometric standard deviation (GSD) were calculated as [2]

$$\ln GMD = (\sum n_i \ln d_i) / \sum n_i \quad (2)$$

$$\ln GSD = [\sum n_i (\ln d_i - \ln GMD)^2 / (\sum n_i - 1)]^{0.5} \quad (3)$$

where n_i is the number of colonies in stage i, having a geometric midpoint size of d_i (μm), which is the square root of the product of the upper and lower limits of the interval of diameters in the stage.

Once the bacteria concentration in each Andersen stage had been calculated, the coefficient of variation (CV) was used to determine the variation of bacteria concentration among stages and sampling rounds. CV was obtained as follows.

$$CV = \frac{\sigma}{\mu} \quad (4)$$

where CV is the coefficient of variation; σ is the standard deviation of bacteria concentration in each sampling round; and μ is the mean bacteria concentration in each sampling round. As CV decreased, the variation in bacteria concentration among stages and sampling rounds decreased, indicating that the stages and sampling rounds had more similar bacteria concentrations. Determining whether bacteria concentrations in various stages or sampling rounds are uniform using only the value of CV is difficult. To define uniformity more effectively and to make the index more universal, uniformity was defined as follows [17].

$$U = e^{-CV} \quad (5)$$

where U is the uniformity value of the bacteria concentrations. The exponential of the negative CV value is a transformation that simply captures the uniformity of the bacteria concentration among stages or sampling rounds. According to Eq. 5, the U has a limited variation in the small

range from zero to one. A value closer to one corresponds to a more uniform concentration.

Statistical analysis

All study data were recorded in Microsoft Excel and analyzed using SPSS 18.0 statistical software. Shannon (H) and inverse Simpson (D) diversity index scores were obtained using linear models based on the size of particles in the air samples [11]. Stepwise and multiple regression analysis was used to determine the correlations among environmental factors (concentration of bacteria, temperature, humidity, kindergarten location, season, and kindergarten occupants) and diversity metrics (with $p<0.05$ indicating a significant difference).

Results and discussion

Taxonomic analysis

Following the incubation of 72 samples (6 stage plates*12 sampling rounds) that were obtained in 12 sampling rounds at three kindergartens in central Taiwan, a total of 1,460 colonies, with a median/average of 71/122 colonies per sampling round (min 20, max 725) were recovered. Of the 1,460 colonies, 1,425 (97.6%) corresponded to 63 species in 29 genera and 35 colonies (2.4%) were unidentified.

Phylum-level composition

Analysis of the taxonomic composition at the phylum level revealed that all the identified colonies were Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. The most abundant phylum was Actinobacteria (56.6$\pm$22.2% of colonies), followed by Firmicutes (31.6$\pm$22.3%), Proteobacteria (7.8$\pm$7.2%), and Bacteroidetes (0.2$\pm$0.6%) (Table 1 and Fig. 1). The two most abundant phyla were dominant throughout the 12 sampling rounds; the third phylum, Proteobacteria, was present in only four sampling rounds. Only one colony was classified as Bacteroidetes (*Weeksella virosa*), which appeared in the 3rd - stage plate (bacterial particle size range: 3.1 to 4.7 μm) from sampling round 4 (spring) at kindergarten C. The four phyla in the air

samples (Table 1; Fig. 1) were the four most abundant phyla in the Oslo subway (air samples) [3] and a kindergarten in Oslo (floor dust samples) [6], but in a different order of relative abundance. Bacteroidetes was the least abundant phylum in all cited studies, including the present one. The highest value of abundance of Bacteroidetes in the 12 sampling rounds in this work was as low as 2.0%, while the corresponding average abundances in samples of kindergarten dust and subway air in Oslo were 5.3% and 8.1%, respectively [3, 6].

Firmicutes exhibited a similar abundance (31.6%, Table 1) to the Oslo kindergarten (25.0%) [6], although its variation among the 12 sampling rounds in this study was not small (CV: 22.3/31.6=0.71). Compared with the results of the two aforementioned phyla abundance, Actinomycetes and Proteus differ from the outcomes of the Oslo kindergarten [6]. The proportion of Actinomycetes in this work is higher than that found in samples in Oslo kindergarten [6]. The dominant genus in this study is *Micrococcus* which is belonged to Actinomycetes. The most abundant phyla, Proteobacteria, in Oslo kindergarten were only the third most abundant in this study [6]. However, the samples in Oslo kindergarten [6] were derived from floor dust, and the methods of molecular biological analysis and bacterial culturing were different, consequently, the monitoring results might be different for that reason. Culturable bacteria are only a small proportion of all bacteria and viable bacteria. This fact is a limitation of the present work. However, in this work, microorganisms are most readily identified using culture-based methods. Governmental agencies provide quantitative regulations concerning bioaerosols. These quantitative standards/guidelines are established using culture-based methods and are expressed in units of CFU/m^3. Although this study may underestimate the concentration of viable bacteria, it still provides useful information and comparisons concerning indoor air quality in kindergartens [11, 18, 19].

Three biodiversity metrics for bioaerosols in kindergartens

Three alpha diversity metrics were used in this study - observed number of species (ONS), the Shannon diversity metric, and the inverse Simpson diversity metric, as shown in Fig. 2. The number of species that were identified in each sampling round ranged from eight to 16 (Fig. 2a),

and the results for the three kindergartens were similar. Kindergartens A to C were pooled as 38, 30, and 36 species, respectively, more than the value of ONS in each of the four sampling rounds. It revealed that each round had different bacteria species. The Shannon diversity index in the 12 sampling rounds ranged from 1.09 to 2.19 (Fig. 2b). The values of the three kindergartens across four rounds each were 1.67, 2.70, and 2.85, exceeding the individual value in each sampling round. The 12 values of the inverse Simpson diversity index in the 12 sampling rounds ranged from 2.11 to 6.06 (Fig. 2c). Across the four sampling rounds for each of kindergartens A to C, the overall inverse Simpson diversity index values were 2.78, 8.23, and 9.61, respectively. The combined inverse Simpson diversity index value for kindergarten A was lower than the individual value for sampling round 3, and those for kindergartens B & C were higher than the values in each of the their four sampling rounds.

The biodiversity of swab samples from sink basins at a Neonatal Intensive Care Unit (NICU) in the United States was evaluated using the above three indicators [20]. The value of ONS in this work was lower than the number of operational taxonomic units (OTU) that was measured at US NICU. The average value of the Shannon diversity index from the US NICU monitoring results was ranged from 1.5 to 2.0, similar to that in the current study. The average value of the inverse Simpson diversity index from the US NICU monitoring results was ranged from 3 to 5, which was also similar to that in this study. A comparison of values of the three indicators reveals that even though traditional cultivation methods were used in this work, the value of ONS was less than that of OTU, but the Shannon and inverse Simpson diversity indexes could still provide a fair level of value.

Species-level composition: abundance of five human-associated bacteria (HAB) in bacterial aerosol at kindergartens

The abundance of each of the five HAB that were specified by Nygaard et al. [6] was determined, as shown in Fig. 3. *Staphylococcus* spp. appeared in all sampling rounds, and its range of abundance was between 2.0 to 70.0%. The second most abundant HAB was *Micrococcus* spp., which appeared in most sampling rounds; *Staphylococcus* spp. only appeared in one sampling

round (kindergarten C in winter, sampling round 1) at an abundance of only 2.0%. *Corynebacterium* spp. and *Streptococcus* spp. appeared in five and three sampling rounds, respectively. *Propionibacterium* spp. was not present in any sampling round. Only sampling round 2 in winter in kindergarten A had all of the above four present HAB. In six sampling rounds, three HAB appeared. In four sampling rounds, two appeared.

Propionibacteriaceae did not appear in any sampling rounds in current study as mention before, as it did not appear in skin and MoBE samples from United States Air Force Academy cadets [9]. However, each dust sample from the kindergarten in Oslo, Norway contained *Propionibacterium* spp. and the other four HAB [6]. Like the phylum distributions, the genus distributions in dust from Oslo kindergarten [6] surface and bioaerosol samples in the air in this study were not consistent with each other. It's also because of the identification measurement was different by using 16S rRNA gene sequencing or the BD Phoenix identification system.

Airborne samples were enriched in *Staphylococcus*, consistent with previous observations of skin or MoBE samples [9, 21-24]. However, *Streptococcus* was present in low relative abundance in current study air samples from only two of the 12 sampling rounds, unlike in other skin or MoBE studies [6, 25-27]. In the study of samples from the Oslo subway [3], the most abundant was unidentified (26.51%), and the four present HAB were *Micrococcus* (2nd, 3.97%), *Staphylococcus* (3rd, 3.83%), 8 *Corynebacterium* (8th, 2.13%), and *Streptococcus* (13th, 1.22%). These four HAB were in the 13 most abundant genera in the air samples from the Oslo subway.

Correlations of values of three diversity indicators for bioaerosols from kindergartens with HAB abundance

With HAB abundance as the independent variable and three alpha diversity metrics as the independent variables, a linear regression was performed, and plotted in Fig. 4. No significant statistical correlation existed between HAB abundance and the three alpha diversity metrics. The linear regression R^2 of HAB abundance and against ONS was only 0.021 (p=0.65) (Fig. 4a), indicating almost no correlation between the HAB and ONS of the 12 sampling rounds, and the value of ONS was almost inside the 95% confidence interval curve. The two alpha diversity

metrics were both negatively correlated with HAB (Figs. 4b & c), indicating a higher HAB abundance corresponds to a lower biodiversity, no matter how this relationship between HAB and biodiversity was not statistically significant.

Particle size of bacterial aerosol in kindergartens

An Andersen six-stage sampler collects particles with diameters in the range from 0.65 to 10 μm. Each sampling round yielded a bioaerosol size distribution in terms of GMD and GSD. In 12 sampling rounds, the GMD value ranged from 2.19 to 5.42 μm, and the GSD value ranged from 1.66 to 2.70 (Fig. 5 and Fig. 6). Generally, the aerodynamic size of a single bacteria-cell is within the sixth range (from 0.65 to 1.1 μm). However, the measurements revealed that the sizes of the bacteria were all in the first to fifth stages (bacterial particle size > 1.1 μm), and the sampled GMD values were also often larger than the size of a single bacterial cell, revealing that the sampled bacteria might have been attached to other particles, such as tiny skin flakes that were suspended in the air, or agglomerated due to coagulation [28]. The wide or narrow distribution of the bacterial sizes in the bioaerosols was identified from the GSD. Monodispersed aerosols, in which all airborne particles are of equal size, have a GSD of close to one. In a polydispersed aerosol, particles of various sizes yield a GSD of greater than one. Most aerosols, including those bioaerosols obtained in this work, are polydispersed aerosols [2].

Particle sizes and biodiversity of bioaerosols in kindergartens

The Andersen six-stage sampler was used to collect samples with six bacterial particle size ranges. Figures 5 and 6 present the bacterial particle sizes distribution and the Shannon diversity index in 72 sampling plates in 12 sampling rounds. The Shannon diversity index ranged from zero to 2.095, and the inverse Simpson diversity index ranged from one to 7.200 (in Fig. 8). The highest diversity value appeared in the 2nd stage (size classed from 4.7 to 7.0 μm) in sampling round 2 at kindergarten C in winter (Fig. 5f), but the value of ONS was nine, which was not the highest ONS value among all sampling rounds. The highest value of ONS was 11, which was obtained in sampling round 2 in kindergarten A in winter (Fig. 5d), in the 3rd stage (size from 3.1 to 4.7 μm)

of Andersen six-stage sampler. The Shannon diversity index at kindergarten A was only 1.326, and the inverse Simpson diversity index was only 2.256 (in Fig. 8), mainly because *Micrococcus* spp. was more abundant than the other bacteria species, making both diversity indexes smaller than in sampling round 2 at kindergarten C in winter (Fig. 5f). In contrast, in sampling round 2 at kindergarten C in winter, the bacterial species were relatively widely and distributed more uniformly than in sampling round 2 in kindergarten A in winter (Fig. 5d), and both diversity indexes were higher than in other sampling rounds.

Bacterial aerosol concentration at kindergartens and regulatory compliance

In 11 of the 12 sampling rounds, the bacterial aerosol concentration ranged from 71 to 433 CFU/m^3, meeting the indoor air quality standards (1500 CFU/m^3) of the Taiwan Environmental Protection Administration (TW-EPA). In kindergarten A in winter sampling round 2, a value of 4154 CFU/m^3 was obtained, exceeding the TW-EPA standard possibly because the outdoor temperature in Taiwan is lower in winter than in other seasons, subsequently, doors and windows are often closed, increasing the bacterial concentration. In contrast, in both kindergartens B and C in winter, the windows were not always fully closed, hence dilution ventilation from the outdoors kept the bioaerosol concentration below the TW-EPA limit.

The TW-EPA enacted an indoor air quality management law on November 23, 2011, in Asia, this legislation is pioneering [29]. Article 6 of the law stipulates that eleven types of public and private venue are subject to be officially announced batch by batch following overall consideration by the central competent authority of the premises' crowd capacity, entry and exit capacity, risk of indoor air pollutant hazards and their special needs, their indoor premises shall be considered officially announced premises under this Act, including schools and other places where children congregate for the primary purpose of juvenile education or activities [29]. Consistent with this regulation, no announcement has yet been made concerning public or private kindergartens by the TW-EPA. Therefore, this study refers only to the TW-EPA's indoor air quality standards in advance. Whether the results obtained in winter sampling round 2 at kindergarten A fail to meet the standards, making it subject to the relevant penalties, cannot yet be determined. However, because

it exceeds the upper limit, the kindergarten should consider opening its doors and windows to increase the amount of fresh air from the outside to improve its indoor air quality.

Petrifilm and uniformity analysis

Petrifilm analysis invovles many indicators [6], of which this work is concerned mainly with three indicator: aerobic count (AC), Enterobacteriaceae (ENT) and the putative *Staphylococcus aureus* (STX); Fig. 7 presents results. The highest AC value (2444 CFU/m^3) was obtained in kindergarten A, winter sampling round 2, in the fifth stage (from 1.1 to 2.1 μm) of Andersen six-stage sampler. The concentrations of particles in all stages in the other sampling rounds were lower than 169 CFU/m^3. ENT appeared only in the second to fourth stages (bacterial particle sizes from 2.1 to 7 μm) of Andersen six-stage sampler in kindergarten B in winter sampling round 2. Only one colony was cultured in each plate and its concentration was calculated as 3.5 CFU/m^3. STX was found in every sampling round and appeared in the first to fifth stages (bacterial particle size $\geq$ 1.1 μm) of Andersen six-stage sampler. Only one STX was cultured in the sixth stage (bacterial particle sizes from 0.65 to 1.1 μm) of Andersen six-stage sampler in kindergarten B in winter sampling round 2. The highest concentration of bacteria was 24.73 CFU/m^3, which was found in three samples - the third stage in kindergarten A in winter sampling round 2, and in the third and fourth stages of Andersen six-stage sampler in kindergarten B in winter sampling round 2. The concentration of yeast and mold is another indicator in Petrifilm analysis and these appeared in all sampling rounds (data not shown). Petrifilm analysis is a much-used technique in the food industry to assess the hygienic quality of surfaces [30].

Another indicator, the value of uniformity, was calculated from the bacterial concentrations as reference to bacterial particle size by season, as shown in Fig. 8. The highest uniformity value (0.557) of AC was obtained in kindergarten C in spring. The lowest uniformity value (0.094) was obtained in kindergarten A when the data for winter and spring were combined (4-round). The highest uniformity value (0.501) in STX was obtained in kindergarten C in spring. The lowest uniformity value (0.289) was also obtained in Kindergarten C but in winter. Combining the data for winter and spring yielded the highest uniformity value, except for kindergarten A. In

kindergarten A, the AC value in sampling round 2 (Fig. 7, winter season) were too high to reduce the uniformity value in winter samples and season-combined (4-round) samples. The uniformity values of AC and STX were highest in kindergarten C, probably owing to its rural environment. However, the relationship between uniformity and urbanization warrants further investigation. Uniformity value seems to be a useful index of variation of AC and STX data.

Figure 9 plots the correlation between the biodiversity of the bacterial aerosol from each kindergarten and the bacterial particle size. The Shannon diversity index and inverse Simpson diversity index for each kindergarten were positively correlated with the bacterial particle size to an extent that was statistically significant for kindergartens A and C, respectively. The results herein provide the first direct association between biodiversity and the bacterial particle size of the airborne bacterial microbiomes. The positive relationship revealed that bioaerosols with larger particle size have higher values of the biodiversity metrics. Skin-related HAB attach to dander flakes from human skin that are suspended in air. Larger airborne particles or dander provide more surface area to which bacteria can be attached and are associated with a greater probability of carrying more species than smaller airborne particles; therefore, they are associated with higher values of biodiversity metrics. Since the airborne dander is typically larger than single cells, dander bioaerosols are larger than single cells.

Since the correlation between biodiversity and bacterial particle size was not entirely consistent across the three kindergartens, further analysis using Pearson correlation was performed; it revealed that Shannon diversity index was significantly ($P < 0.05$) related to urbanization (R = 0.749), temperature (R = 0.580), RH (R = -0.806) and CO_2 concentration (R = -0.780), indicating that the environmental variables and biodiversity were positively or negatively correlated. ANOVA was then used to analyze the potential environmental factors. Sampling location (kindergarten A, B and C) was the main independent variable; biodiversity was the dependent variable, and other environmental factors were relevant factors; these were season, temperature, RH, CO_2 concentration, number of occupants in a kindergarten, airborne bacterial concentration (aBC), GMD, and GSD value. ANOVA results revealed that bioaerosols from the classroom environment in kindergartens with different degrees of urbanization exhibited significantly different values of

the Shannon diversity index (P = 0.021) and the inverse Simpson diversity index (P = 0.007). Tukey post-test results revealed that the two diversity indices were significantly higher for kindergarten C than for kindergarten A, with corresponding P values of 0.018 and 0.005. Kindergartens A, B, and C had urban, semi-urban, and rural environments, respectively. Biodiversity in the rural area might have been higher than in an urban area.

Multiple regression and stepwise regression were performed for statistical analysis. The temperature, RH, CO_2 concentration, aBC, and GMD value were independent variables. Table 2 presents the results. The Shannon diversity index was statistically significantly correlated with GMD (P = 0.001). The inverse Simpson diversity index was correlated with RH (P = 0.040) and GMD (P = 0.002). Stepwise regression and multiple regression yielded similar results. Both the Shannon and the inverse Simpson diversity indices revealed RH and GMD as statistically significant variables. In this study, RH ranged from 55% to 75%. Since the relation between biodiversity and RH was negative, a lower RH was associated with higher biodiversity.

Conclusion

The bacterial communities in air in three Taiwanese kindergartens were studied. Three kindergartens had different bacterial particle size distributions and biodiversities in winter and spring. A correlation study revealed a positive correlation between bioaerosol particle size and biodiversity. Identification methods for use in medical laboratories, such as that based on the BD Phoenix system can be used to study microbiomes in air. Airborne HAB abundance varied with time and place, and was negatively correlated with biodiversity. Significant variations in the composition of the bacterial community – and particularly the abundance and proportions of HAB - were observed over time, space (relative humidity) and bioaerosol sizes. This study is the first to use the calculation of uniformity value (U), yielding results consistent with those of Petrifilm analysis. The uniformity value was further defined as the exponential of the negative CV value which was a transformation for easily understanding the uniformity of the bacterial concentrations (AC or STX) as reference to bacterial particle size by season. The benefit of implementing U is

that it has a clear lower limit of zero (smallest uniform) and upper borderline of one (greatest uniform) [17]. Petrifilm analysis was found conveniently to identify areas of poor hygienic quality and associated sizes of bioaerosols. More kindergartens should be tested using similar methods to those used herein. Children in kindergartens may experience greater long-term health effects of exposure to bioaerosol than adults. Further studies could analyze whether the trends observed herein regarding HAB are found in other kindergartens and daycare facilities. This study also reveals the importance of investigating the size of bioaerosols in such environments. Knowledge of the indoor environment of a kindergarten is very valuable for understanding how indoor airborne microorganisms can affect health.

Acknowledgement

We thank employees at the three kindergartens for their assistance and important contributions to this investigation.

Competing interests

The authors declare no competing interests of any kind, including financial.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data that are generated or analyzed during this study can be found in this published article and its supplementary information files.

Funding

No funding was received.

Authors' contributions

C.-Y.L., T.-H.L., and Y.-H.C. planned the work and designed the experiments. C.-Y.L. and T.-H.L. wrote the manuscript. T.-Y.H., and C.-H.H. performed experimental sampling and identified the bacteria. T.-H.L. and C.-Y.L. calculated diversity, size distribution and uniformity. C.-Y.L., T.-H.L., and Y.-H.C. performed statistical analyses. All authors discussed the results and commented on the manuscript.

Table Legends

Table 1 Phylum-level composition of bacterial aerosols (bioaerosols) in kindergartens.

Table 2 Shannon and inverse Simpson diversity indices (n=68).

Figure Legends

Fig. 1 Changes in bacterial composition at phylum level over time in three kindergartens.

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Fig. 5 Concentrations of bioaerosols in winter.

Fig. 6 Concentrations of bioaerosols in spring.

Fig. 7 Heat maps of Petrifilm counts for: **a**. aerobic counts (AC), **b**. Enterobacteriaceae (ENT),

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Table 1 Phylum-level composition of bacterial aerosols (bioaerosols) in kindergartens.

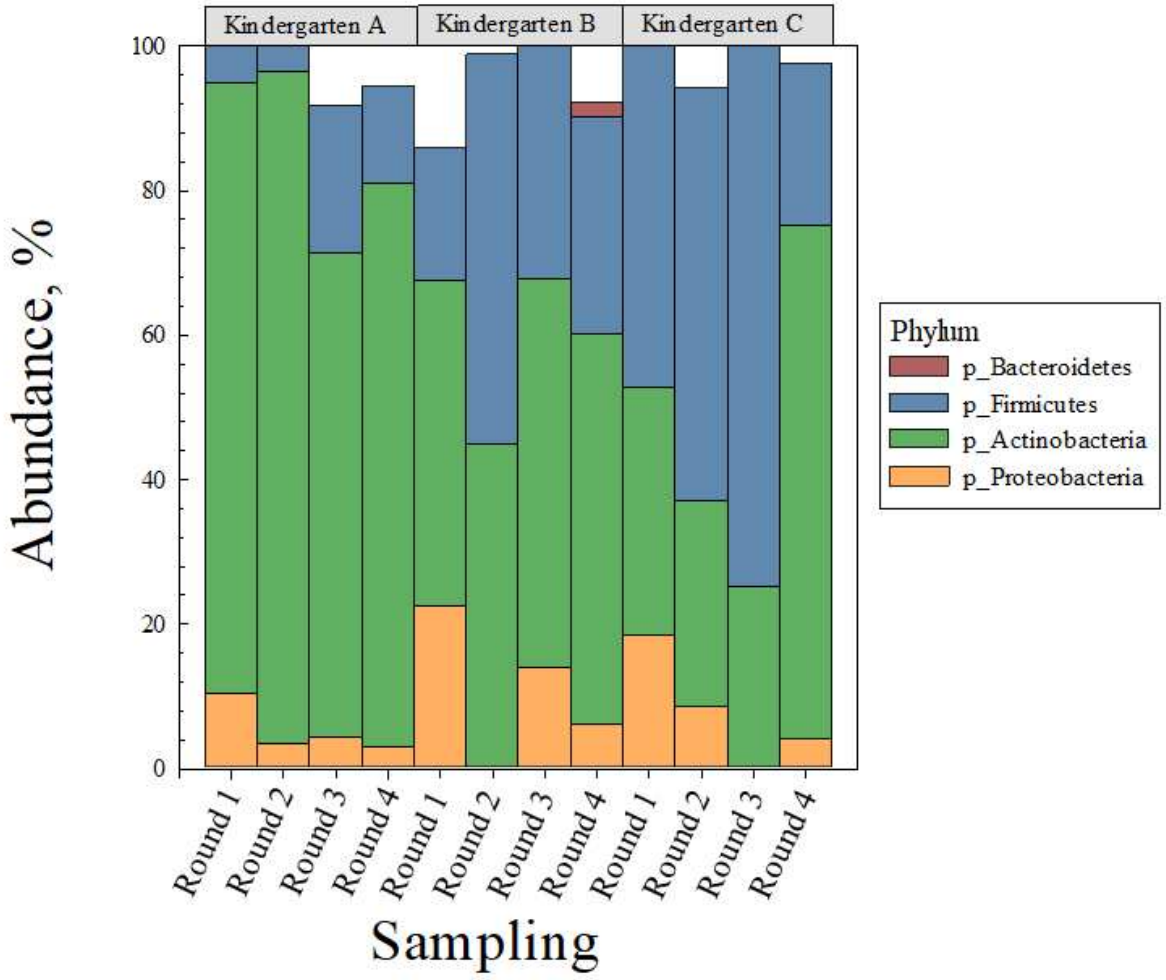
Phylum	Actinobacteria	Bacteroidetes	Firmicutes	Proteobacteria
Sampling rounds (max. 12)	12	1	12	4
Average ± SD (%)	56.6±22.2	0.2±0.6	31.6±22.3	7.8±7.2
Range (%)	25.0 to 93.0	0 to 2.0	3.7 to 75.0	0 to 22.4
Oslo kindergarten dust [6]	27.3	5.3	25.0	41.0
Oslo subway air [3]	42.92	8.1	11.97	23.88

SD: standard deviation.

Table 2 Shannon and inverse Simpson diversity indices (n=68).

Variable	Shannon				inverse Simpson			
	coefficient	SD	*p* value	stepwise	coefficient	SD	*p* value	stepwise
Temperature	0.042	0.065	0.523	-	0.138	0.168	0.414	-
RH	-0.029	0.019	0.134	0.008**	-0.106	0.050	0.040*	<0.001***
CO_2	0.008	11.468	0.999	-	7.35	29.79	0.806	-
aBC	87.2	67.2	0.199	-	124.3	174.6	0.479	-
Bioaerosol size	0.582	0.172	0.001**	0.001**	1.430	0.446	0.002**	0.001**
Full model			0.001**	<0.001***			0.024*	<.001***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



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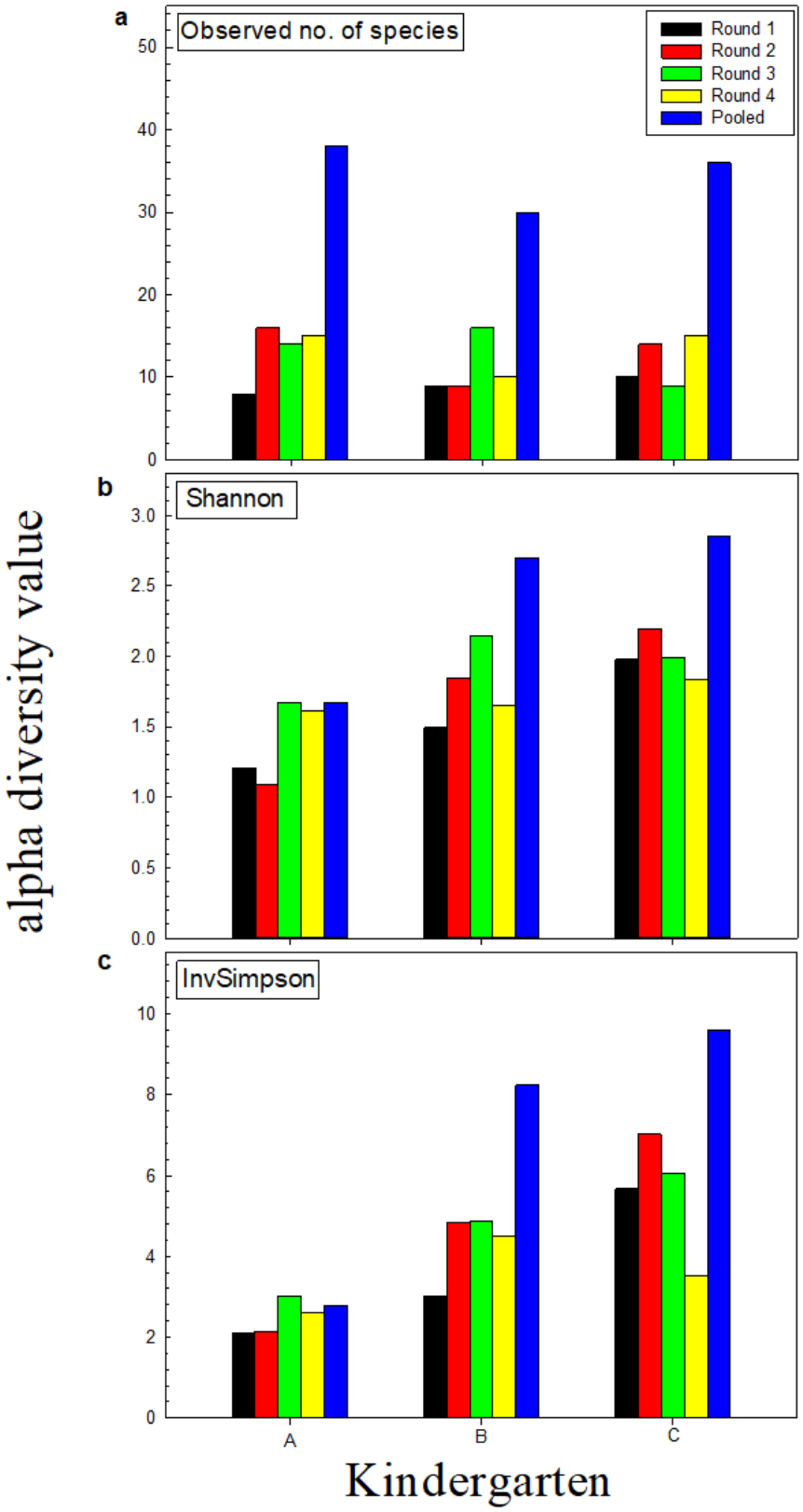


Fig. 2 Incubated colony data was used to calculate **a**. Observed number of species (ONS), **b**. Shannon, and **c**. inverse Simpson diversity metrics for three kindergartens.

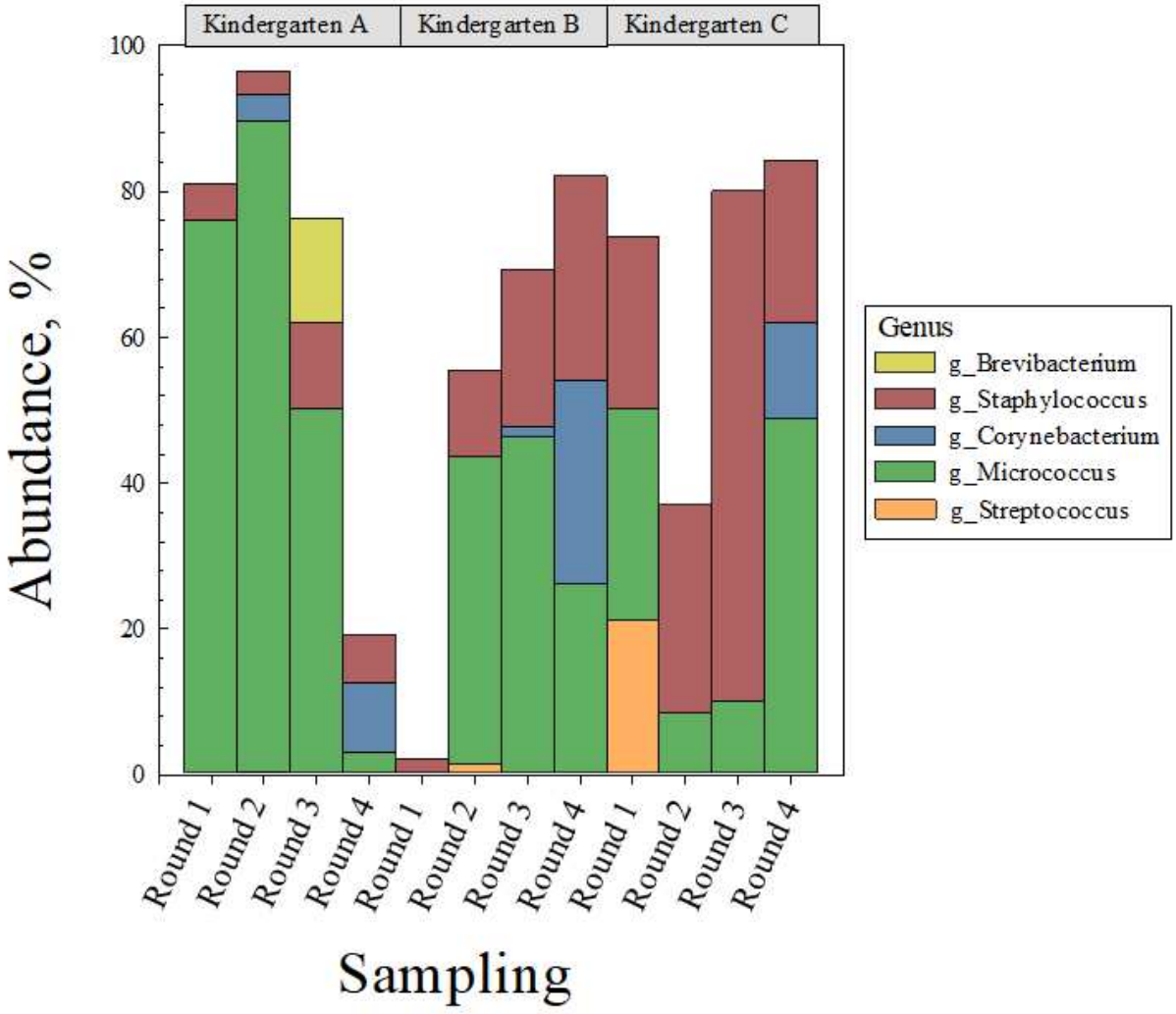
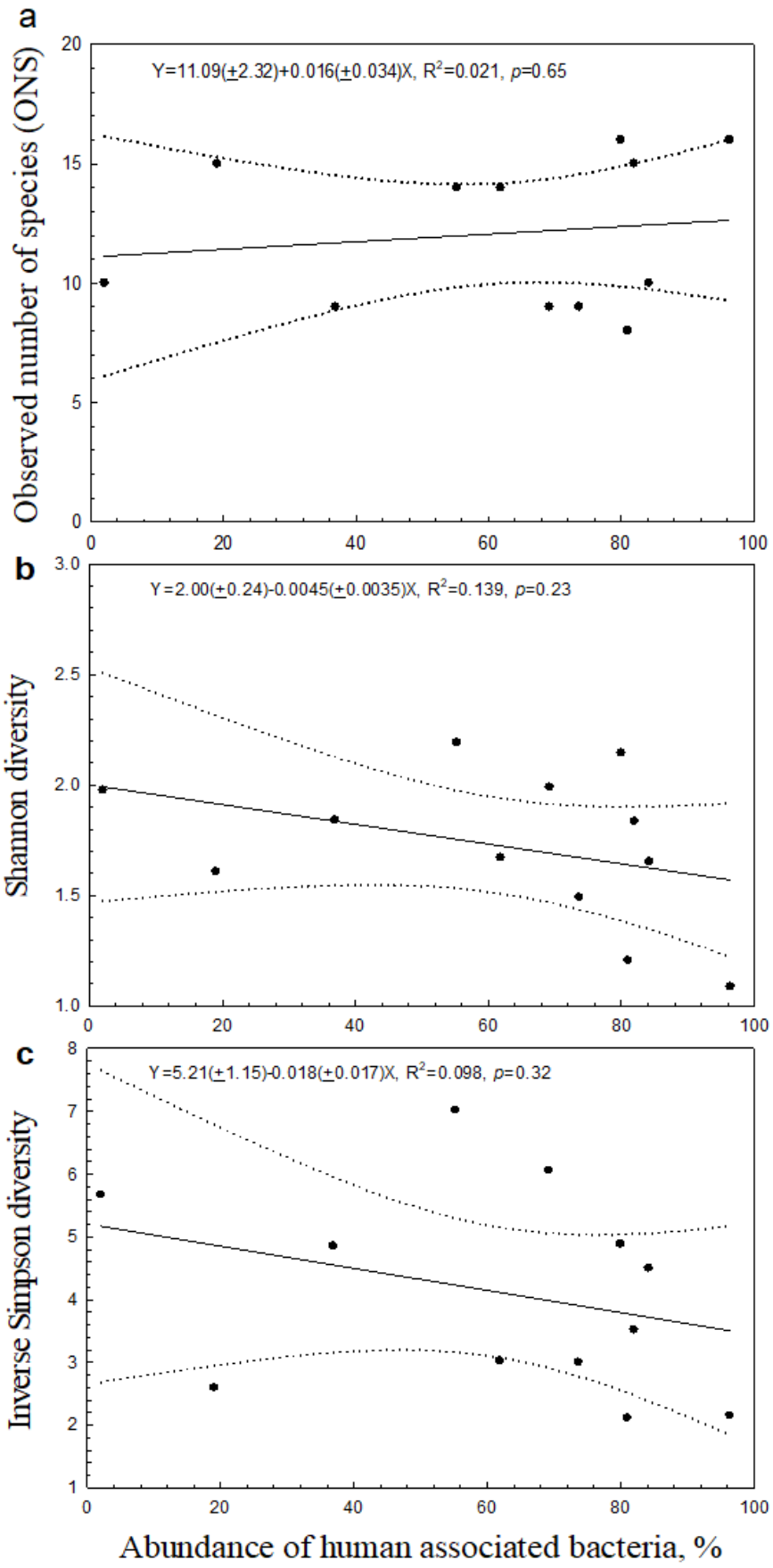


Fig. 3 Changes in relative abundance of selected human-associated bacteria (HAB) over time in three kindergartens.



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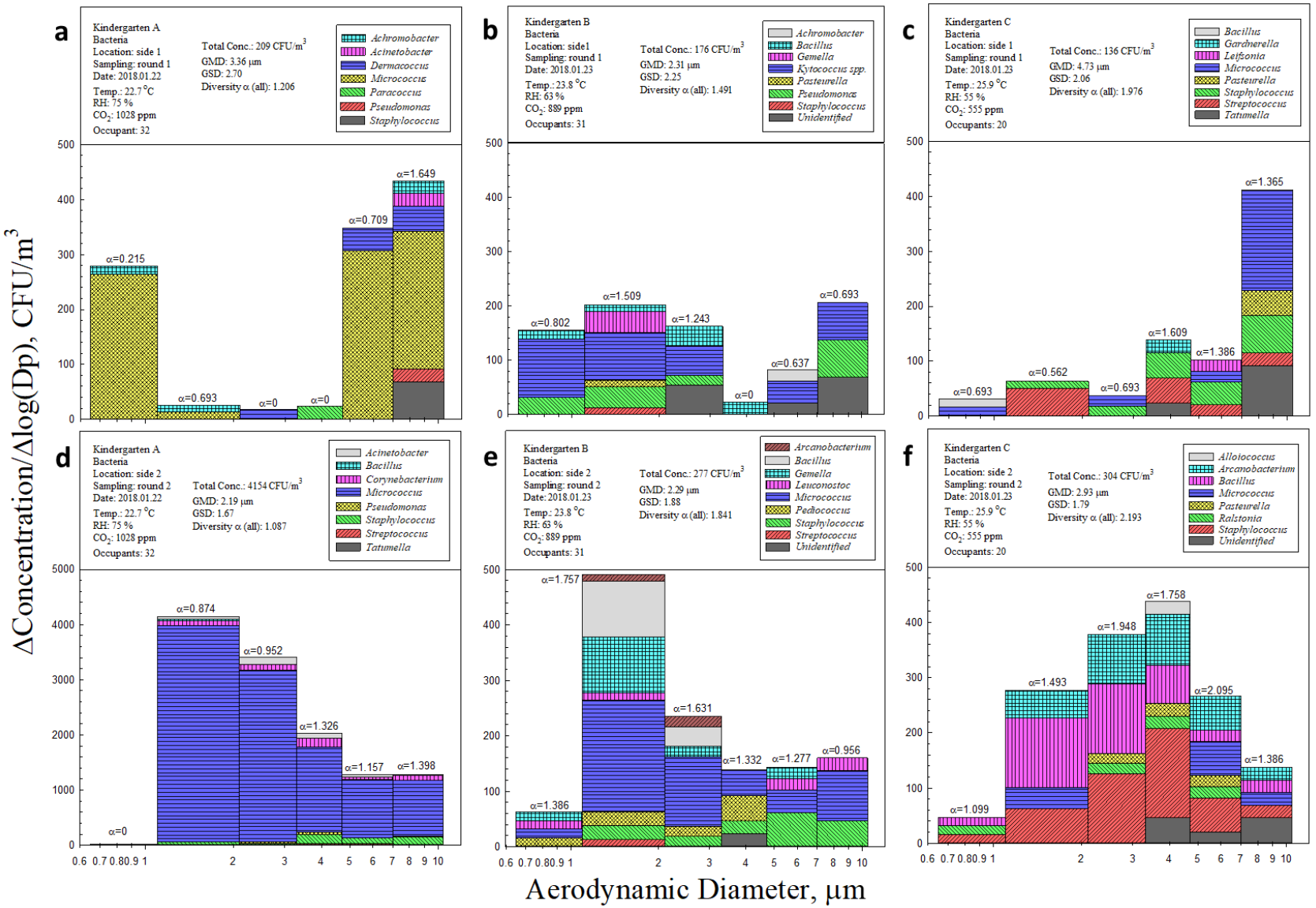


Fig. 5 Concentrations of bioaerosols in winter.

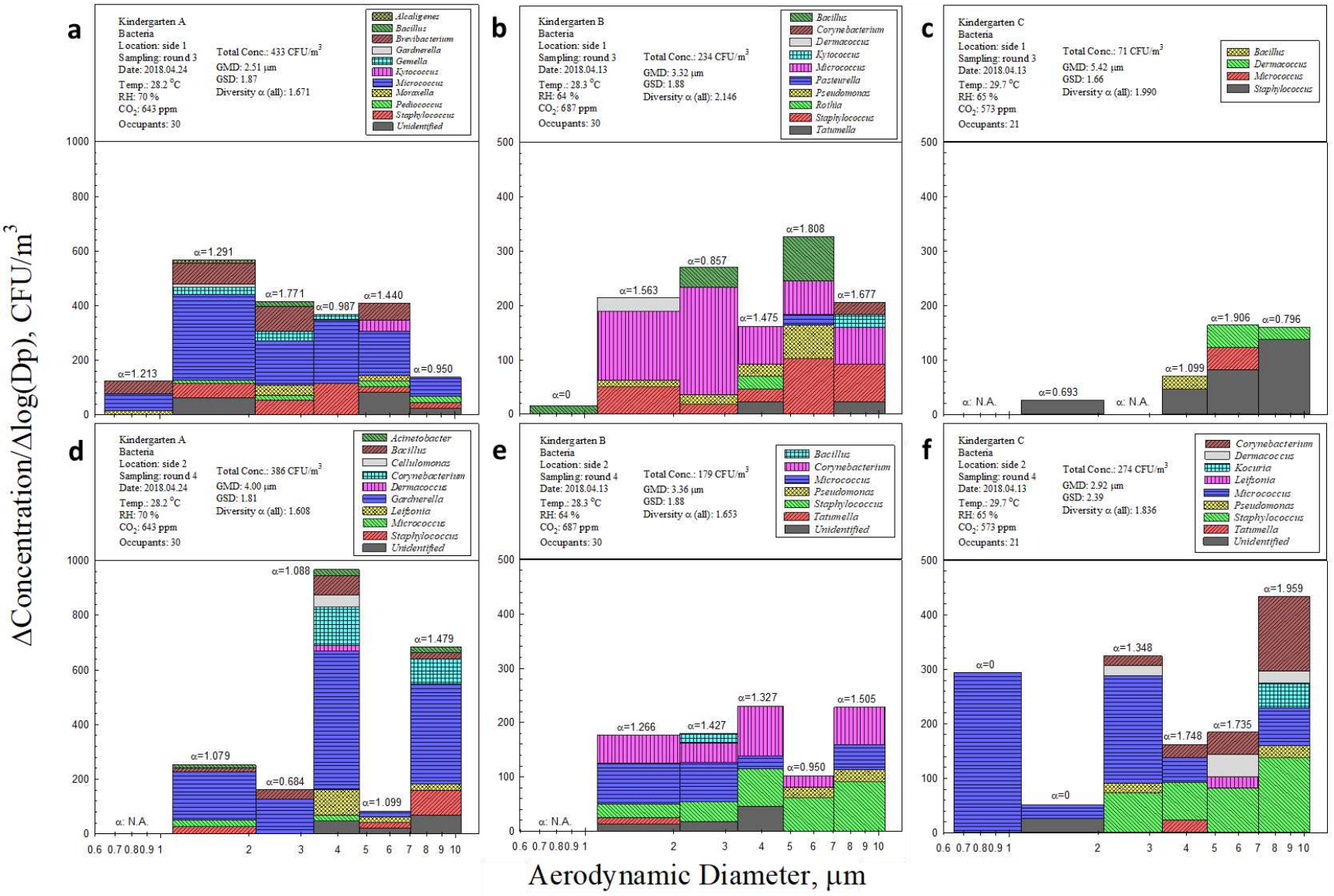
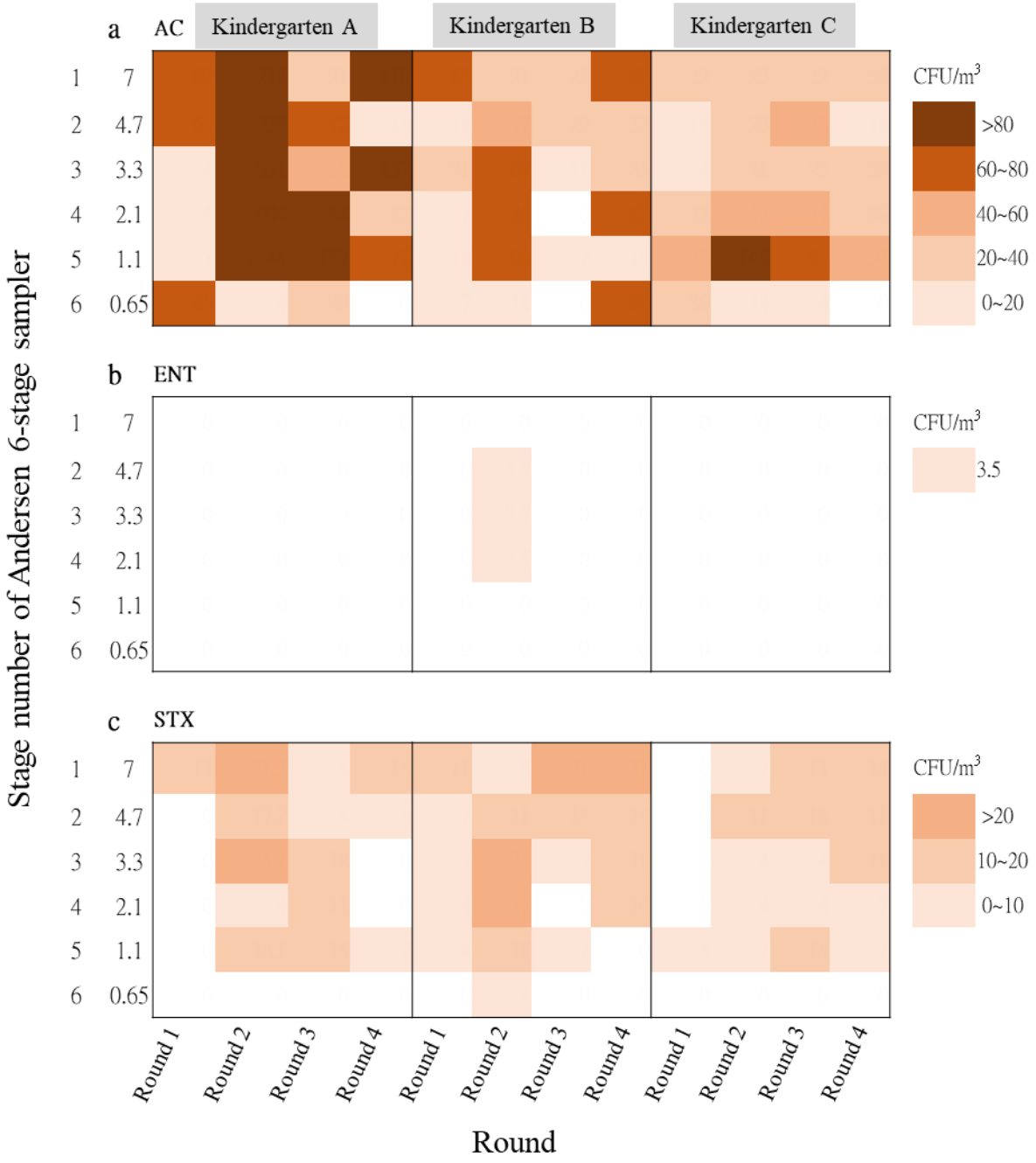


Fig. 6 Concentrations of bioaerosols in spring.



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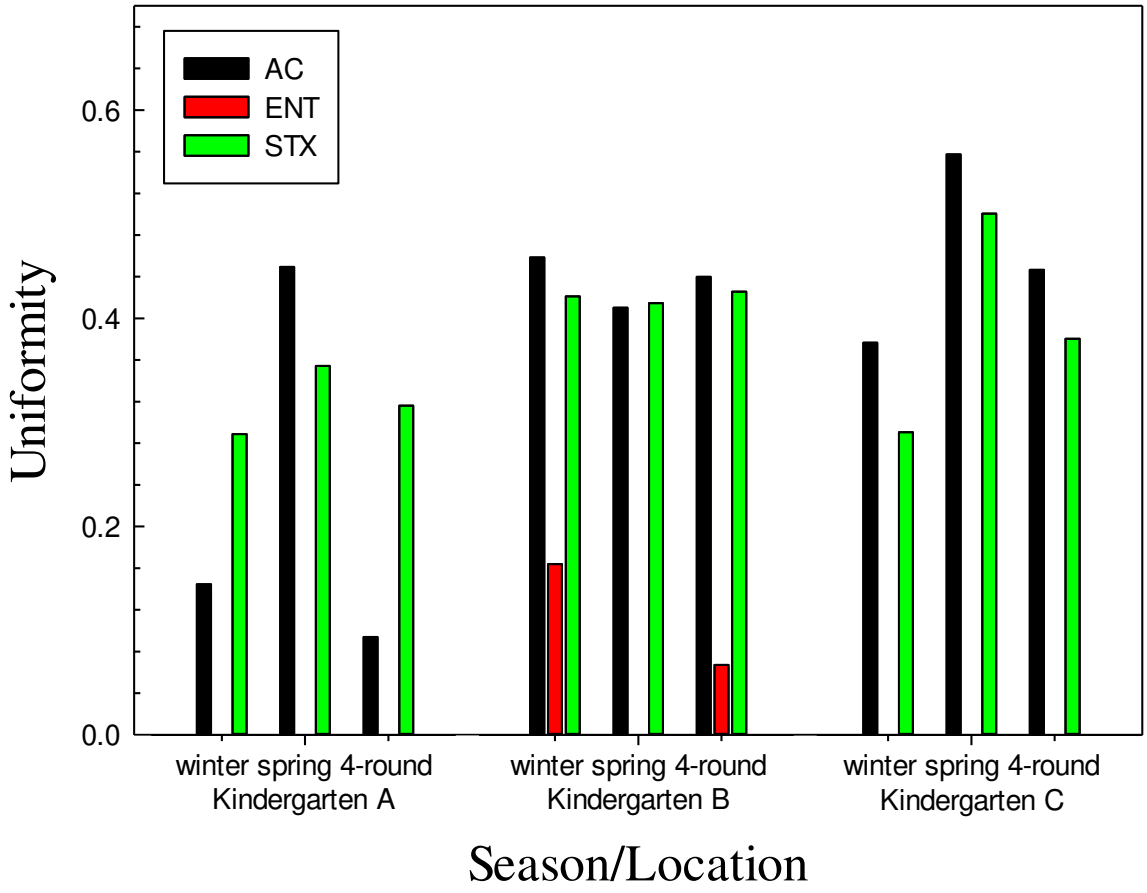


Fig. 8 Uniformity of bacterial concentrations as reference to bacterial particle size in three kindergartens.

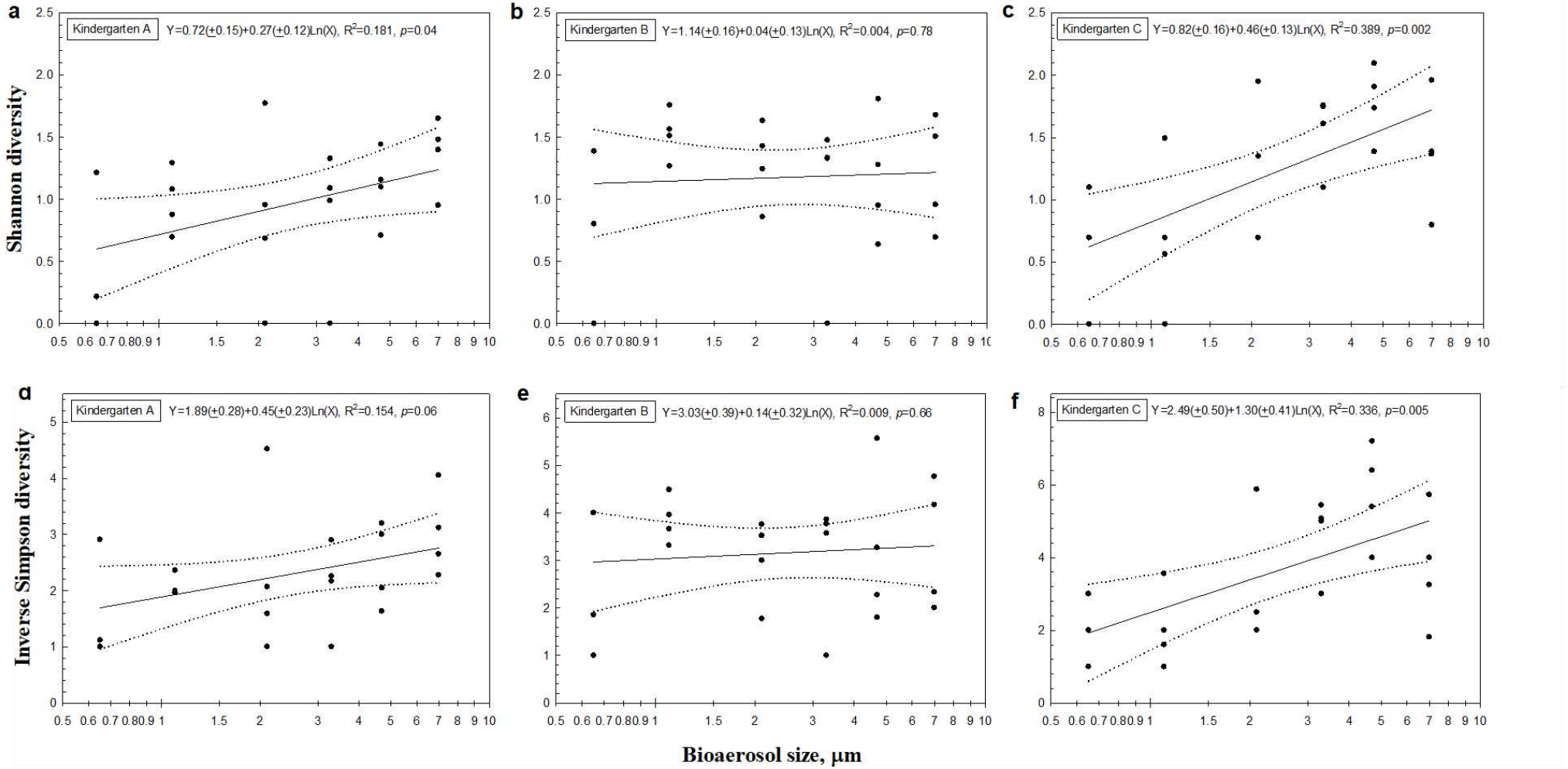


Fig. 9 Relationships between alpha diversity metrics and bacterial particle size. **a**, **b**, and, **c** Shannon diversity metrics for kindergartens A, B, and C; **d**, **e**, and **f** inverse Simpson diversity metrics for kindergartens A, B, and C.

Figures

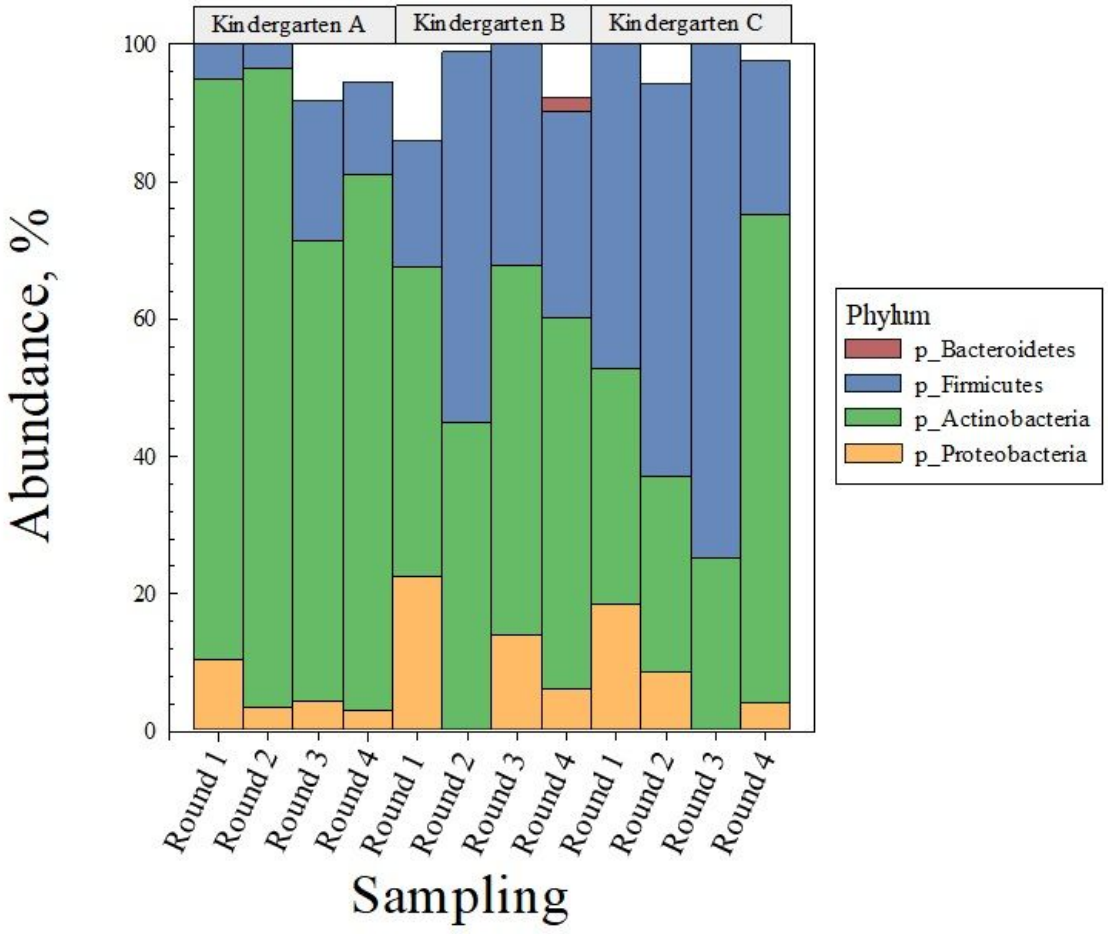
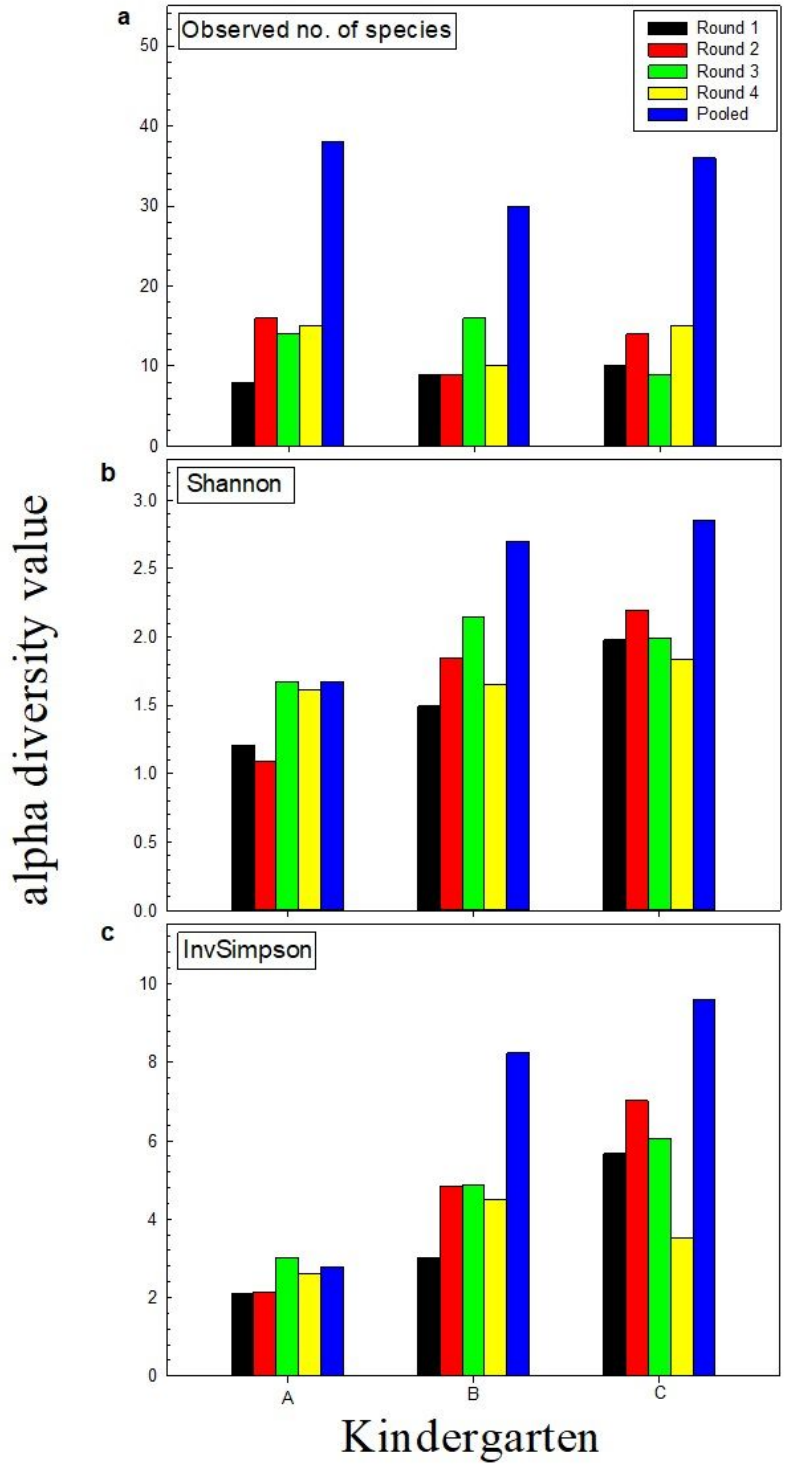


Figure 1

Changes in bacterial composition at phylum level over time in three kindergartens.



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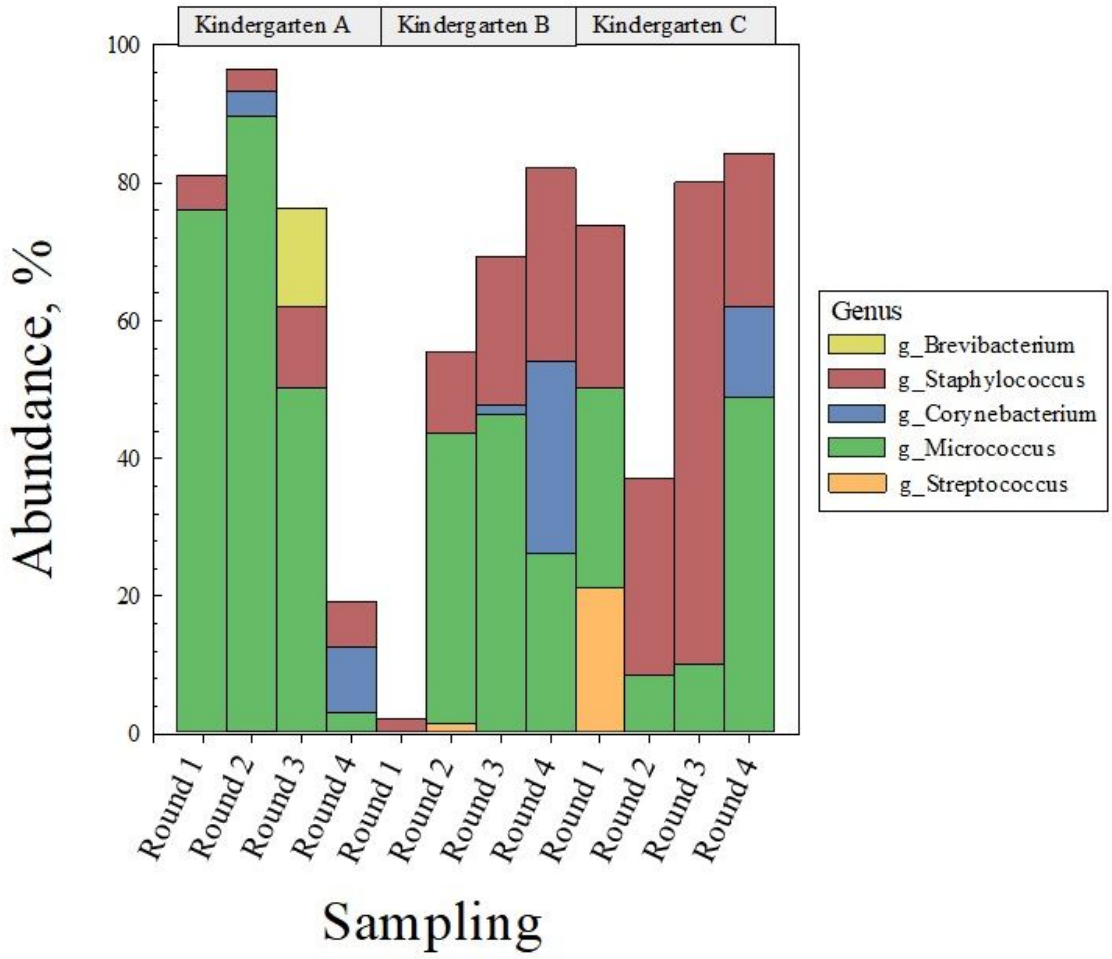

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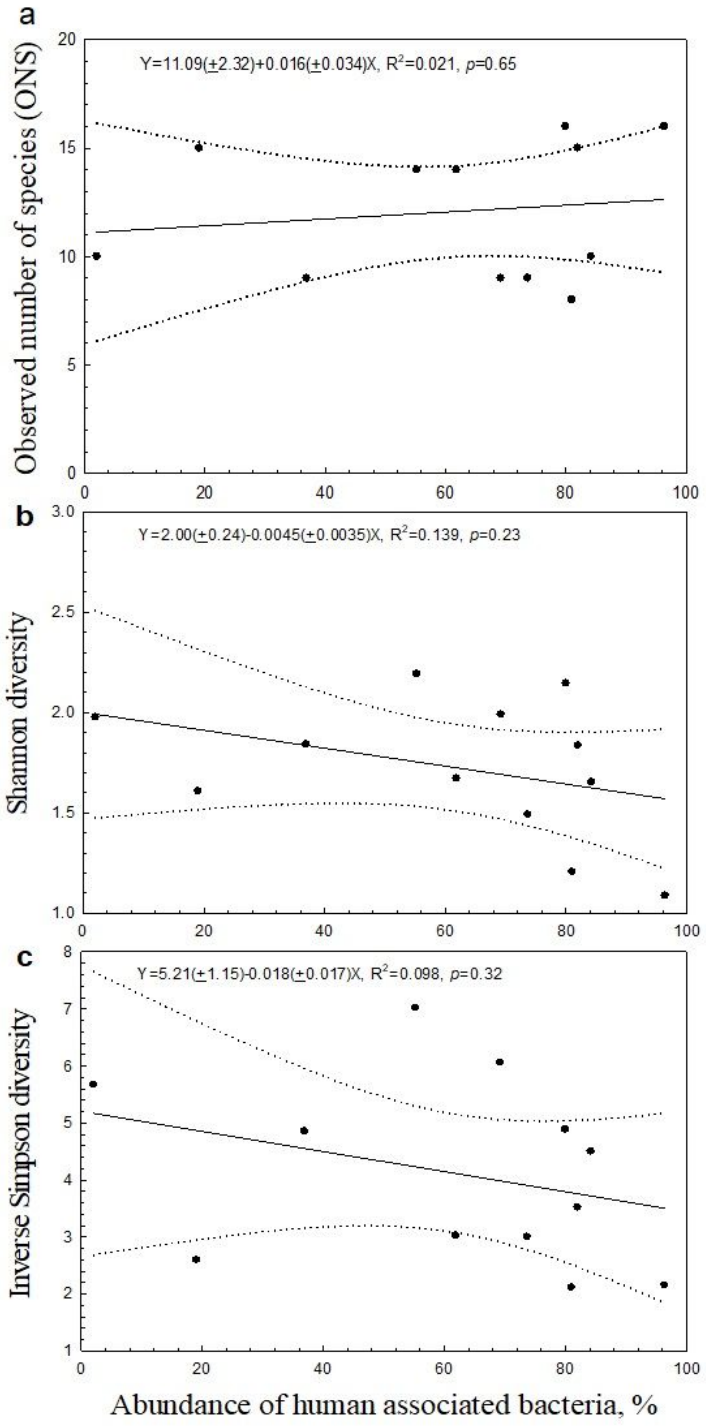

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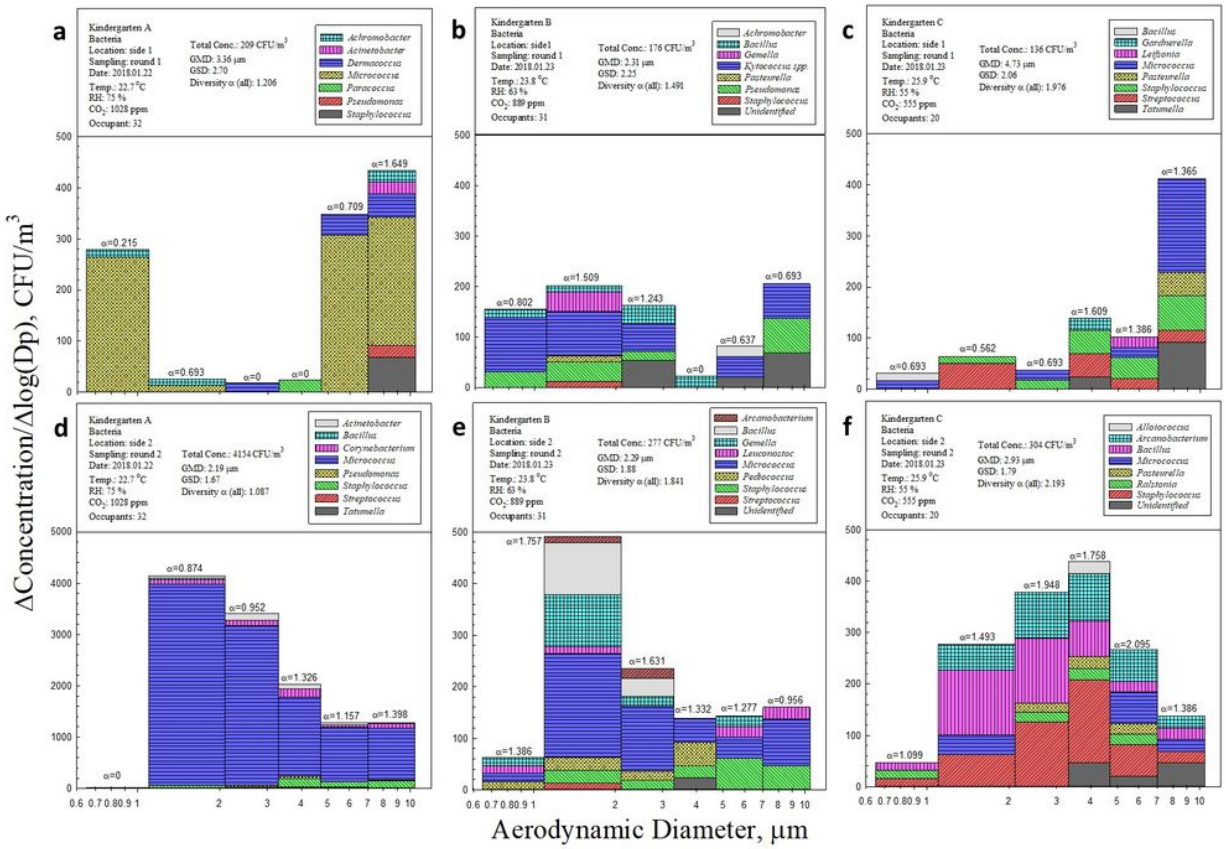
Relationships between three alpha diversity metrics and abundance of human-associated bacteria (HAB). a. Observed number of species (ONS), b. Shannon, and c. inverse Simpson diversity metrics.



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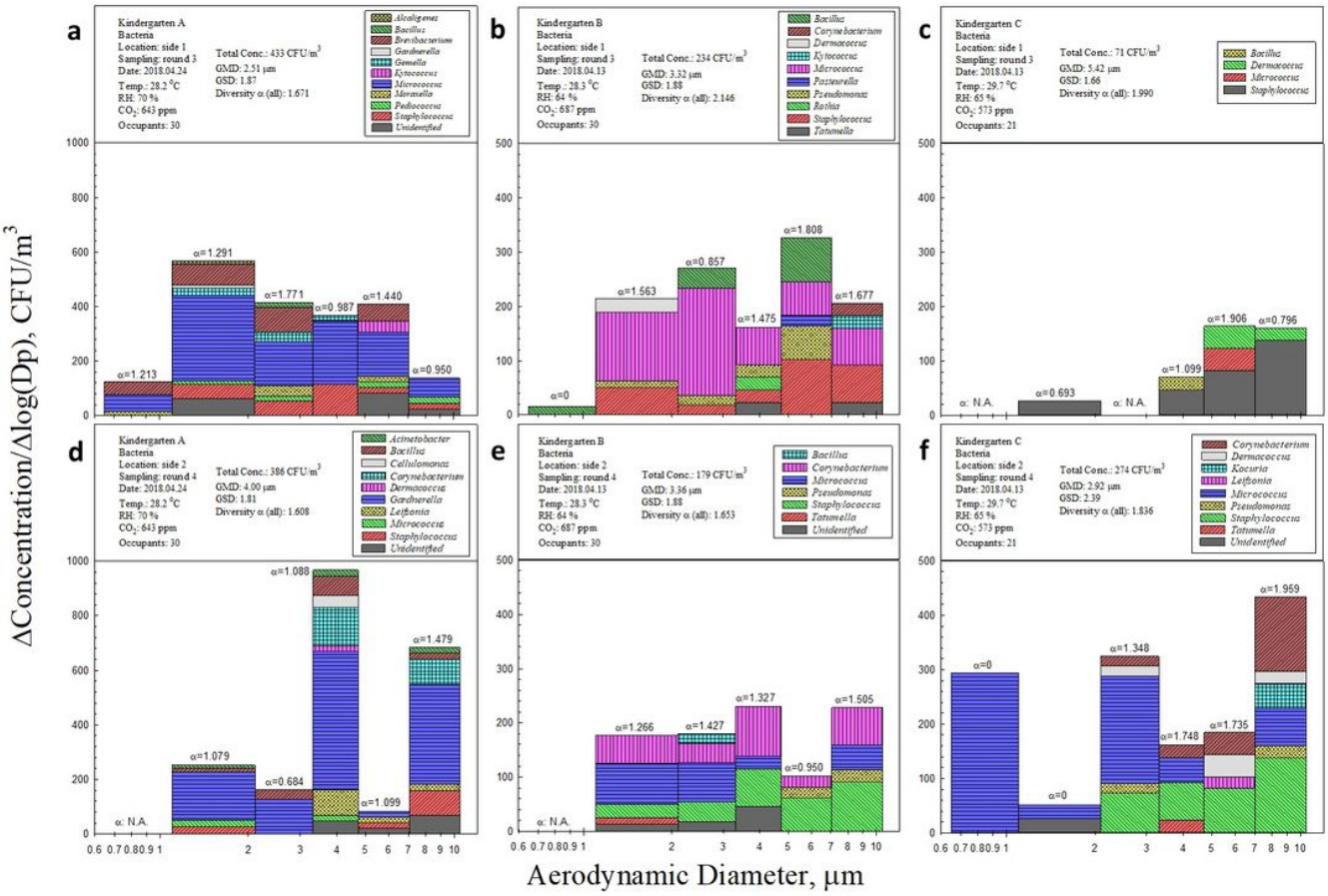

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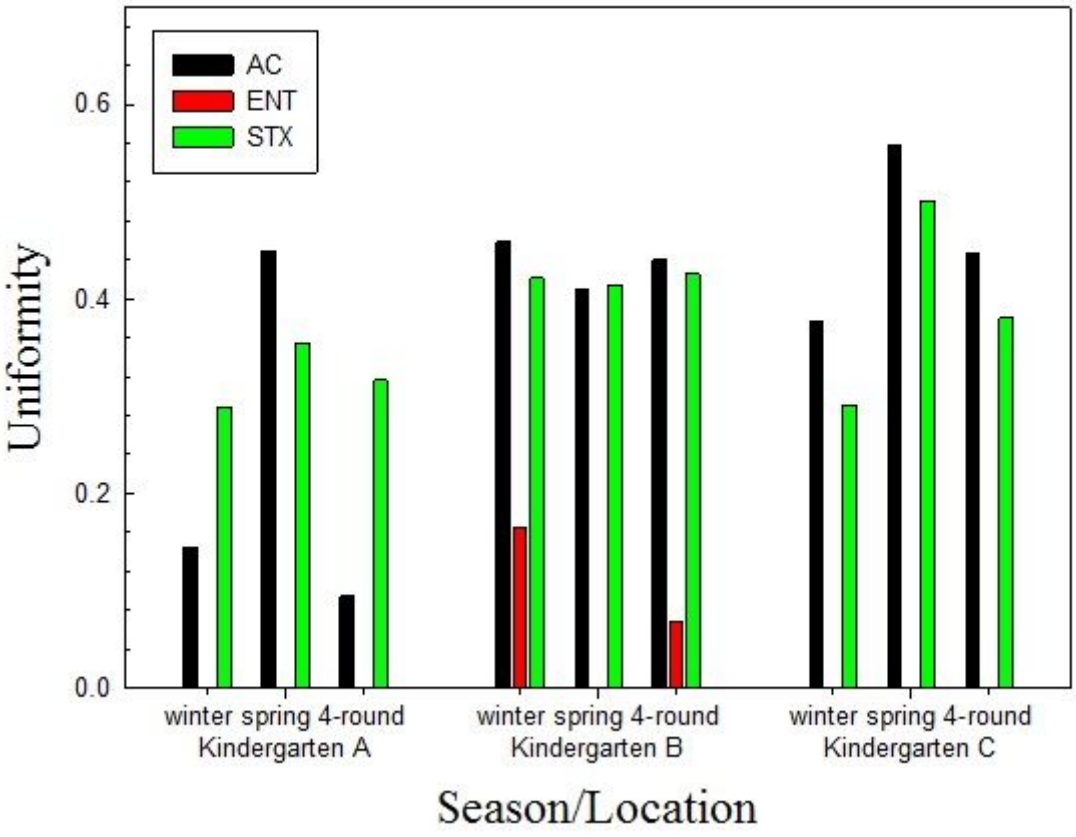

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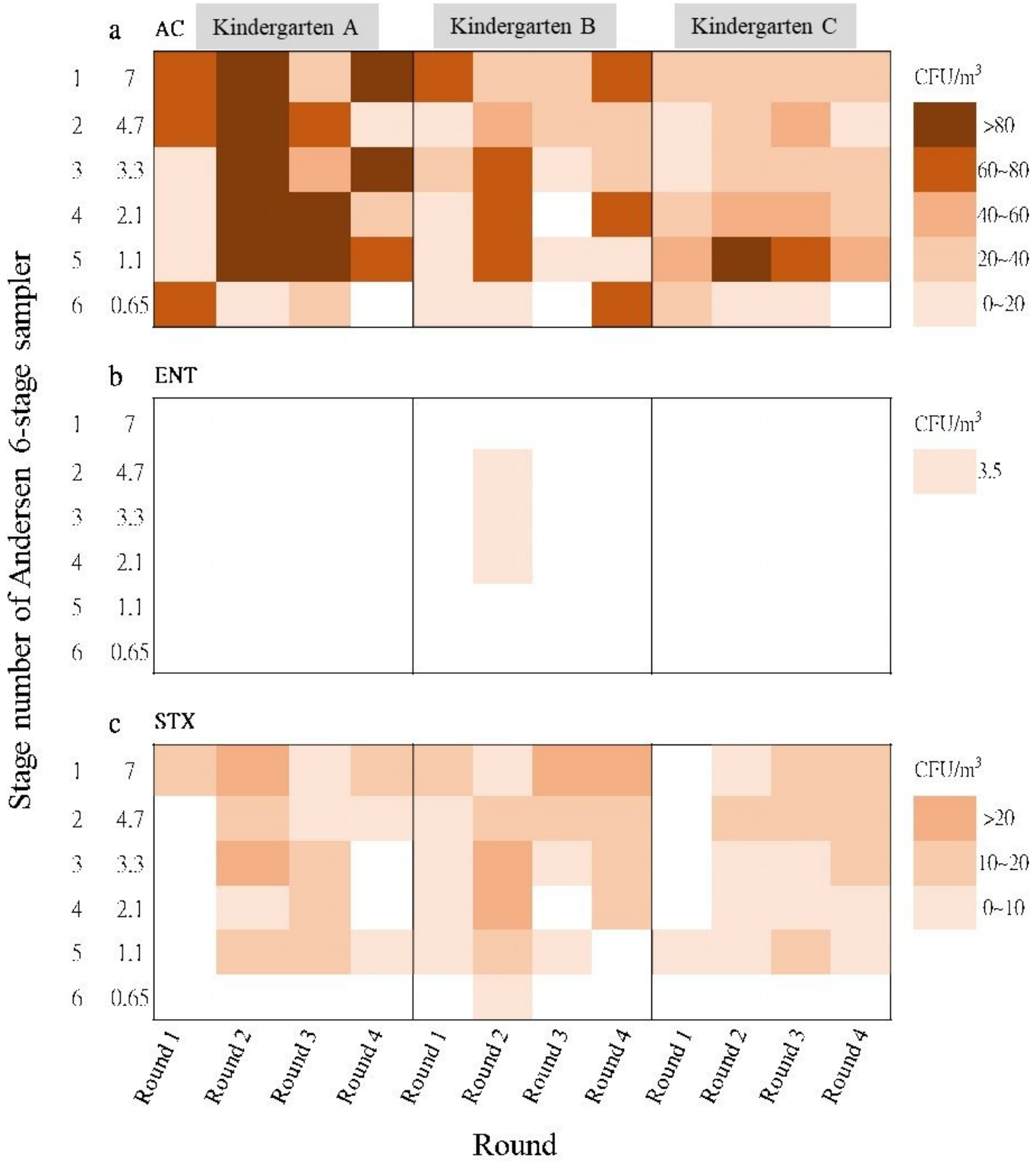

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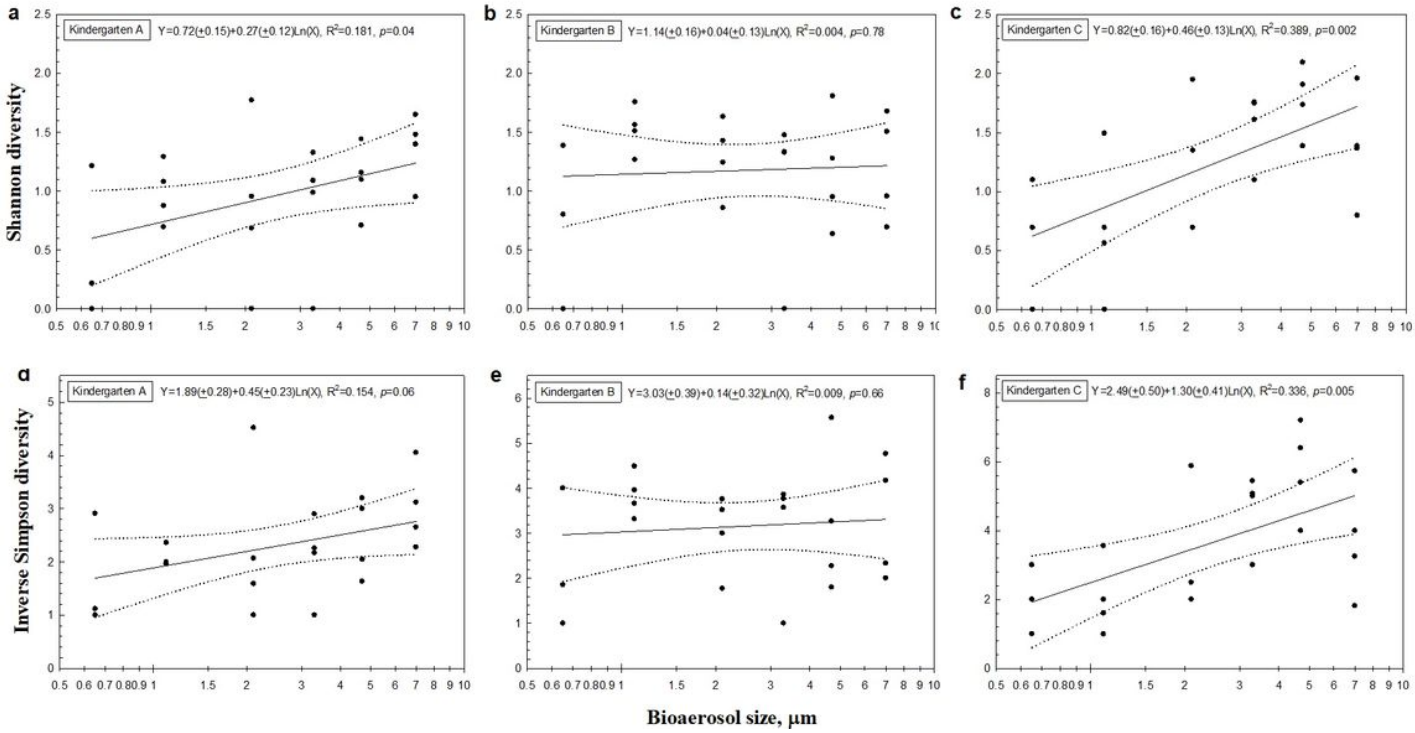

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