

Bioaerosols in a Squat Toilet under Various Ventilation Scenarios: Pollution Profile and Self-Developed Plasma Air Purifier Elimination

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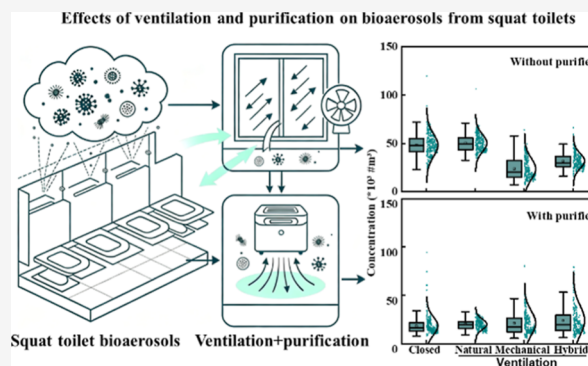
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Supporting Information

ABSTRACT: Squat toilet flushing generates and spreads potential pathogenic bioaerosols that threaten human health, while ventilation and purifiers are potential approaches to alleviate bioaerosol pollution. This study investigated the pollution characteristics of bioaerosols of squat toilet under various ventilation scenarios and their purification by a self-developed plasma air purifier. The culturable bioaerosol concentrations under the natural and mechanical ventilation scenarios were the highest (632 CFU/m³) and lowest (361 CFU/m³), respectively. The purifier's removal efficiencies of culturable bioaerosols were 60.82% and 56.70% under closed and natural ventilation, respectively, which were higher than those of mechanical (52.09%) and hybrid ventilation (38.16%). Particle size distribution analysis showed that the closed treatment increased bioaerosols with a size <7 μm, while the purifier reduced bioaerosol levels, especially for 1.1–3.3 μm bioaerosols. Community analysis showed that ventilation and purification treatments exerted no significant alterations to the fungal community structure but decreased the richness and diversity. Environmental factors influenced fungal community removal more than bacterial communities. The 7-day purification treatment achieved higher removal efficiencies for bioaerosol activity, pathogenic microorganisms, and mobile element-containing microbes than the 17- and 27-day treatments. This research provides a guideline for reducing the pollution and health threats of bioaerosols from toilets under varied ventilation and purification scenarios.

KEYWORDS: toilet bioaerosol, biological particles, community structure and function, ventilation, plasma air purifier purification



1. INTRODUCTION

As toilets are indispensable facilities in indoor buildings, microbe-containing aerosols generated from toilet flushing have long been considered as a potential source of transmission of infectious microorganisms.^{1–3} Thousands of aerosol particles containing bacteria, fungi, or viruses would be released during toilet flushing, which may pollute the air and pose a human health risk.^{4,5} This is because some of the microorganisms in bioaerosols are pathogens.⁶ For instance, *Salmonella enteritidis* can be isolated from air samples collected after flushing.⁷ *Legionella pneumophila*, which can cause a lethal risk of legionellosis, is also found in toilet flush handles, faucets, and sinks.⁸ Many human pathogens such as enteroviruses and respiratory viruses are found in the collected toilet waste.⁹ MS-2 phage and *Escherichia coli* are also found in the pipe system before flushing the toilet.¹⁰ Besides, the particle size of bioaerosols also has a significant impact on human health. The particle size of 99% aerosols generated by toilets is less than 5 μm.¹¹ Such small particles can be persistently airborne, penetrating the lower respiratory tract and potentially inducing respiratory infections or inflammatory responses.¹² Health infection risk exceeds the U.S. EPA benchmark after

bidet toilet flushing from 0 to 15 min.¹³ Therefore, it is crucial to clear the pollution profiles of bioaerosols from the toilet.

Squat toilets are the most widely used toilets in East Asia, and they can release bioaerosols with varying emission characteristics.¹⁴ Except for relative humidity (RH) and temperature, ventilation systems may affect bioaerosol pollution profiles in toilets.^{15,16} So far, numerous studies have reported that bioaerosols are considerably affected by ventilation systems in the indoor environment. For instance, the fresh air system mainly uses mechanical ventilation to control the spread of indoor pollutants, thereby curbing air pollution.¹⁷ The microbial aerosol concentration in office buildings under natural ventilation is higher than that under air-conditioning and mechanical ventilation systems.¹⁸ It is possibly because natural ventilation increases the entry of outdoor microorganisms into buildings or lowers air change

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rates, which makes it less effective at removing and reducing indoor bioaerosol concentrations than mechanical ventilation systems.¹⁹ However, systematic research on the emission characteristics of toilet flushing bioaerosols under various ventilation scenarios is insufficient. Ali et al. found that the concentration of airborne *Staphylococcus aureus* in a bidet toilet under poor ventilation scenarios is higher than that under hybrid ventilation scenarios, and the health risks in mechanical ventilation are lower than those in the natural ventilation scenario.¹³ They also compared bioaerosol emission after flushing squat and bidet toilets and found higher bioaerosol concentrations in squat toilets.¹⁴ However, the emission characteristics of bioaerosols under various ventilation scenarios remain unclear.

The implementation of appropriate cleaning protocols has significantly decreased the likelihood of contamination and the consequent infection risk of bioaerosols. Among them, air purifiers are widely used to improve indoor air quality and reduce indoor particulate matter and microorganisms. For instance, portable high-efficiency particulate air filter (HEPA) air cleaner and hollow fiber membrane can effectively remove airborne bacteria and viruses in enclosed spaces.^{20,21} Mobile indoor air filters equipped with high-performance filter media can reduce 50% of the particle in a classroom within 30 min.²² Air cleaners with HEPA filters can also reduce 35–42% of fungal aerosols in dwellings within 24 min.²³ Jung et al. use a high-temperature, short-time process in a continuous-flow system to inactivate fungal bioaerosols, and >99% of bioaerosols lost their culture ability within 0.2 s at 350 °C.²⁴ Grinshpun et al. use ionic air purifiers to inactivate 92% of *E. coli* in confined indoor spaces within 1 min.²⁵ Fornasaro et al. use a novel UV–C LED aerodynamic device to inactivate *E. coli* (BL21-DE3), resulting in 99% removal efficiency (RE).²⁶ Similarly, Yan et al. achieve an RE of 99.99% for bioaerosols in a simulated cabin within 20 min by a cloud-air-purifying air purifier with UV and disinfectant (ClO₂).²⁷ Li et al. develop a plasma air filtration system, which can filter 91.5% of aerosol particles and inactivate bacteria, fungi, and 99.32% ± 0.15% of the H1N1 virus in diverse environments.²⁸ However, purification efficiency and inhalation risk variations of bioaerosols in squat toilets after treatment by air purifier under ventilation conditions are poorly understood.

Squat toilet flushing generates higher bioaerosol concentrations than bidet toilets due to the squat pan at the floor level and an open flushing mechanism.¹⁴ However, most research is conducted on bioaerosol emission characteristics in bidet toilets after flushing. To address the knowledge gap regarding ventilation-driven dilution and air purifier-based removal of bioaerosols from squat toilets, this study developed a plasma air purifier, investigated characteristics of bioaerosols in a public squat toilet under varied ventilation (via dilution) and purification (via an air purifier and dilution) scenarios, and evaluated the correlation between the environmental factors and bioaerosols using culture-based techniques and amplicon sequencing analysis. In addition, the long-term purification performance of the air purifier under ventilation was also explored based on the concentration, activity, microbial community composition, and function. This study provides data support for the treatment of airborne microorganism emissions from flushing squat toilets.

2. MATERIALS AND METHODS

2.1. Sampling Location and Air Purifier

Experiments to characterize the emission characteristics and purification of toilet-flush-generated bioaerosol were conducted in a medium-sized restroom on a university campus in Guangdong, China. As shown in Figure S1, the overall floor area and spatial volume are approximately 22.1 m² and 61.9 m³, respectively. The door of this public restroom was often kept open. There are three washbasins in the right corner and five squat toilets on the left. All five squat toilets were operational. The doors and panels of squat toilets were 80 in. in height (28 in. from the top to the ceiling), and the per-flushing volume of each toilet was 3 L. A window is situated on the wall near squat toilet 5, and a frequently opened exhaust fan is located on the right side of the window.

A self-developed plasma air purifier was used to purify bioaerosols emitted from the squat toilet (Figure S2). It uses a composite filter screen, comprising a prefilter, a H13 HEPA filter, and a coconut shell-based activated carbon filter (2158 m²/g), and a low-temperature plasma device (voltage: −8 kV) to degrade organic matter on the particulate matter or kill microorganisms at a clean air delivery rate (CADR) of 600 m³/h. It is also equipped with an indoor air online monitor to detect temperature, RH, concentration of formaldehyde, PM_{2.5}, PM₁₀, and total volatile organic compounds (TVOCs). To assess the purification capability of the self-developed plasma air purifier, bioaerosol REs of the plasma air purifier were compared to those of a commercial air purifier (Mijia air purifier Pro), which is suitable for mitigating PM_{2.5} with an indoor area <60 m². It is equipped with three layers of filters (i.e., a primary filter, a high-efficiency filter, and an activated carbon filter) to capture particulate matter at a 500 m³/h CADR.

2.2. Experiment Setup and Sample Collection

To explore the influence of doors, windows, and exhaust fans (volumetric flow rate: 720 m³/h) on purification efficiency of bioaerosol in the toilet, the following eight scenarios were set by opening or closing the window and door, turning on or off the air exhaust, and purifying: (1) closed (closed window and door, turned off air exhaust), (2) natural ventilation (only turned off air exhaust), (3) mechanical ventilation (only closed window and door), (4) hybrid ventilation (opened window and door, turned on air exhaust), (5) natural ventilation and purification, (6) mechanical ventilation and purification, (7) purification, and (8) hybrid ventilation and purification (Table 1). The plasma air purifier is placed near the wall facing squat toilets 3 and operated from 10:00 to 15:00 under different conditions.

The flowchart of bioaerosol sampling and analysis is shown in Figure S3. Specifically, a six-stage Andersen impactor (FA-1, Applied Technical Institute of Liaoyang, China) was used to collect airborne culturable and total microorganisms. The impactor was placed at the center of the toilet room facing toilet 3 to collect unpurified

Table 1. Eight Ventilation and Purification Scenarios^a

groups	doors and windows	exhaust fan	air purifier	exposure scenarios
1	×	×	×	closed
2	✓	×	×	natural ventilation
3	×	✓	×	mechanical ventilation
4	✓	✓	×	hybrid ventilation
5	✓	×	✓	natural ventilation and purification
6	×	✓	✓	mechanical ventilation and purification
7	×	×	✓	purification
8	✓	✓	✓	hybrid ventilation and purification

^aNote: “✓” means open or turned on; “×” means closed and turned off.

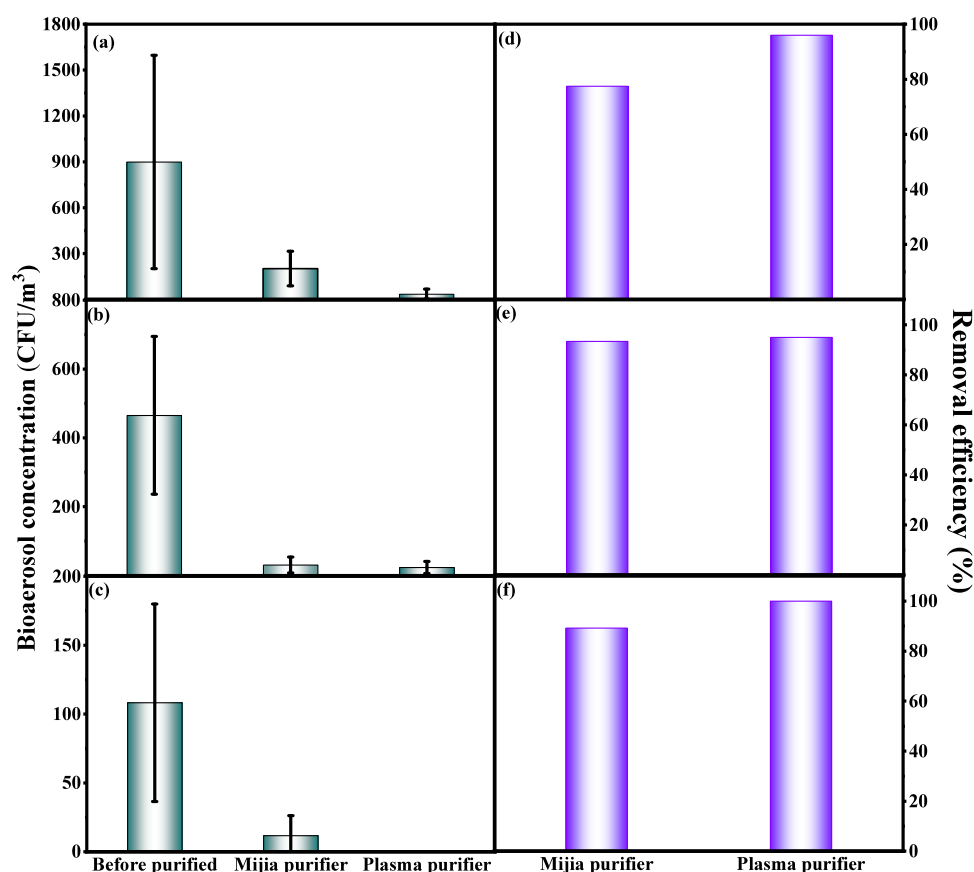


Figure 1. Concentrations and removal efficiencies of airborne culturable bacteria (a,d), fungi (b,e), and pathogenic bacteria (c,f) by self-developed plasma air purifier and commercial Mijia air purifier.

(ventilated-treated) samples, while it was positioned at the outlet of the plasma air purifier to collect air purifier-treated samples. The culturable bacteria, fungi, and pathogens were collected on the nutrient agar plates (with 5 mg/L of nystatin to inhibit the growth of fungi), Rose Bengal Agar (with 0.1 g/L of chloramphenicol to inhibit the growth of bacteria),²⁹ and blood agar (with 5–10% defibrinated blood to provide growth factors),³⁰ respectively, at a flow rate of 28.3 L/min for 5 min. While for the total microorganisms in the air, they were collected on a sterilized mixed cellulose ester microporous membrane at a flow rate of 28.3 L/min for 30 min. The detailed method is described in Text S1 and a previous study.^{31,32} Besides, the real-time concentration of biological and total particles with a 0.5–10 μm size was measured using an HT-Nova BM3001 Biological Aerosol Detector (BM3001, Beijing, China). For each scenario, two rounds of sampling were conducted from February to May 2023 (excluding weekends), and each round of sampling was replicated three times. Since 9:00–10:00 and 14:00–15:00 on weekdays were peak toilet usage periods with a high and similar number of people entering the restroom, we considered samples collected at 9:00–10:00 under normal conditions (open door and window, turn on exhaust fan) prior to implementing the eight scenarios as control samples. While samples collected at 14:00–15:00 after ventilation or purification treatment were designated as treated samples. Each of the eight scenarios has its own corresponding control sample. Before collecting control samples, the toilet was cleaned by removing trash and mopping the floor. After 10:00 am, toilets were subjected to ventilation or purification treatment according to the method in Table 1. The RE of bioaerosols is calculated as one minus the ratio of treated samples' concentration to control samples. To investigate the long-term reduction effect of bioaerosols by air purifiers, the purifier was operated continuously from 8:00 am to 6:00 pm for almost a month under normal conditions (open door and window, turn on exhaust fan). Since the exhaust fan uses motor-driven fan blades to draw air-containing

aerosols from toilets, the bioaerosols will be trapped on the blade surface. To calculate the RE of bioaerosols, the dust samples on the exhaust fan were collected by sterile cotton swabs after 0, 7, 17, and 27 days of purification. Meanwhile, a composite filter screen of the air purifier was also collected and cut into pieces after 27 days of purification. The culturable concentration and activity of microorganisms were analyzed after eluting them from the filter by ultrasonic oscillation.

2.3. Analysis of Microbial Community Structure

The DNA was extracted from the collected filter membrane and cotton swabs using the soil genomic DNA rapid extraction kit (Sangon Biotech, Shanghai, China). The extracted DNA samples were amplified using 338F (5'-ACTCTACGGGAGGCAGAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primer sets to amplify the V3–V4 region of bacterial 16S rRNA.³³ The ITS region of fungi was amplified by ITS1F (5'-CTTGGTCATTTAGAGGATAA-3') and ITS2R (5'-GCTGCGTTCTTCATC-GATGC-3') primer sets.³⁴ Subsequent library preparation and sequencing were completed on the Illumina PE300 platform of Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The detailed methods for DNA extraction, PCR amplification, and sequencing are provided in Text S2.

2.4. Analysis of the Airborne Microbial Activity

Microbial activity of the dust and composite filter samples was analyzed using the fluorescein diacetate (FDA) hydrolysis method,³⁵ with detailed steps in Text S3. SYTO 9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide, were further used to assess the viability of bacteria at different purification times. The viability of bacterial cells was defined as the ratio of the viable bacterial cells to the total bacterial cells (green fluorescence).

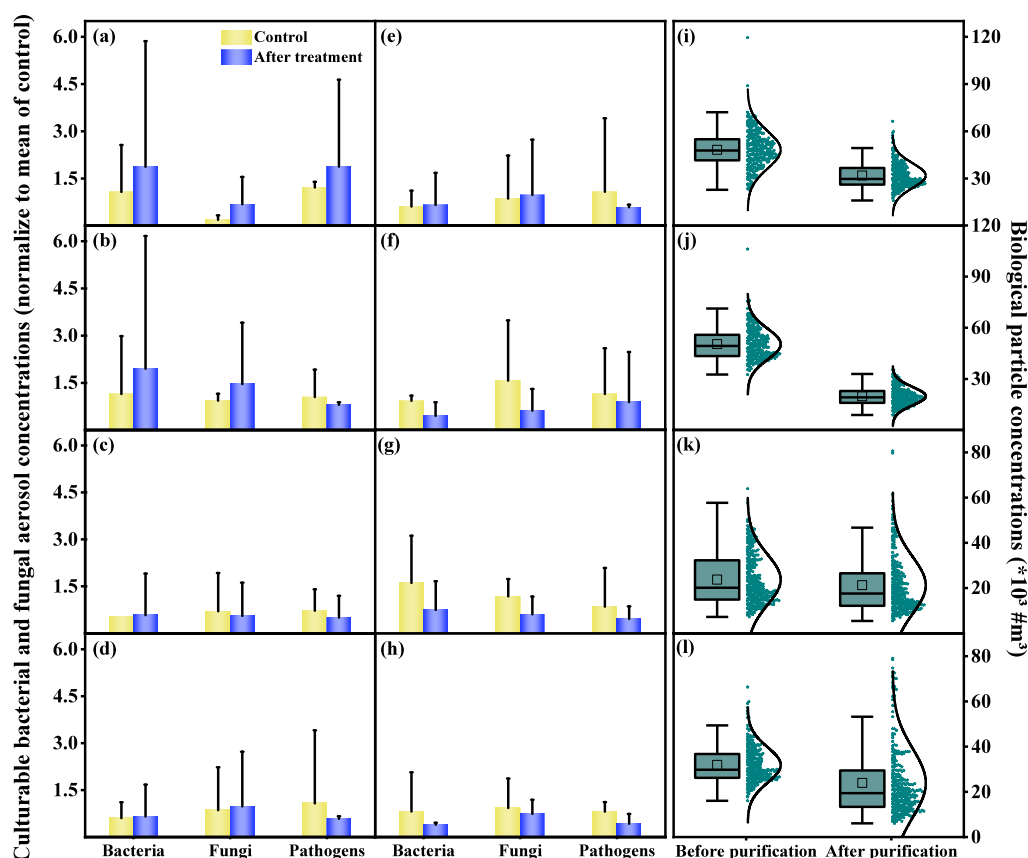


Figure 2. Normalized culturable bacteria, fungi, and pathogenic bacterial concentration, and biological particle concentrations. (a,i) Closed, (b,j) natural ventilation, (c,k) mechanical ventilation, (d,i) hybrid ventilation, (e) purification, (f) purification and natural ventilation, (g) purification and mechanical ventilation, and (h) purification and hybrid ventilation.

2.5. Analysis of the Impact of Environmental Factors on Toilet Bioaerosols

To study the effect of environmental factors on toilet bioaerosols, the concentrations of $PM_{2.5}$, PM_{10} , TVOCs, and formaldehyde were measured using a miniature atmospheric detector (Shaoxing Tengyao Environmental Protection Technology Co., Ltd.). Indoor temperature and RH were measured using an AZ8721 Hygro-Thermometer (Shenzhen YuanHengTong Technology Co., Ltd.). The wind speed was measured using a hot-bulb anemometer (QDF-6, Beijing Cadillac Instrument Equipment Co., Ltd., China). The number of people entering the restroom was also recorded using video observation during sampling periods, and people who stayed in the restroom for less than 15 s were excluded from analysis. This flow of people was used to reflect the number of toilet flushes. The results are presented in Table S1. The Spearman correlation analysis was used to examine the correlation between environmental parameters and dominant bacterial and fungal communities.

2.6. Statistical Analysis

SPSS 27.0 statistical software (International Business Machines Corporation, USA) was used to perform the Spearman correlation analysis. To better account for the variability of control samples, the concentration of culturable microorganisms was divided by the mean concentration of the control samples using data normalization methods. The community structures of different treatment methods (including control, ventilation, and purification) were analyzed by PCoA, and the composition of the microbial community at the genus level was discussed. Subsequently, BugBase phenotype and FUNGuild were used to predict the bacterial and fungal community functions under different treatments, respectively.

3. RESULTS AND DISCUSSION

3.1. Bioaerosol Purification Capability of Self-Developed Plasma Air Purifier

To assess the purification capability of the self-developed air purifier, the removal of bioaerosols by this purifier was compared to that of a commercial one. As illustrated in Figure 1a–c, after 30 min of purification, the concentrations of culturable bacteria, fungi, and pathogenic bacterial aerosols were greatly reduced. The REs of airborne bacteria, fungi, and pathogenic bacteria by the self-developed air purifier reached 95.7%, 95.1%, and 100.0%, respectively, which were higher than the REs of the commercial purifier (79.3%, 93.4%, and 83.3%) (Figure 1d–f), suggesting that the self-developed plasma air purifier has higher purification efficiency than the commercial air purifier. These results may be due to the higher CADR of the self-developed air purifier, since a previous study also showed that an air purifier with superior CADR is more effective for particulate pollution removal in general indoor air.³⁶ Besides, the plasma purifier can generate electrons/ions, reactive oxygen, and nitrogen species to inactivate pathogenic microorganisms,²⁸ resulting in higher inactivation efficiency of pathogenic microbial aerosols than a commercial filter purifier without plasma. Therefore, the self-developed air purifier was selected for the subsequent bioaerosol purification study in real-world squat toilets.

3.2. Culturable and Total Microorganisms

3.2.1. The Effect of Ventilation on Levels of Culturable and Total Microorganisms. Since ventilation

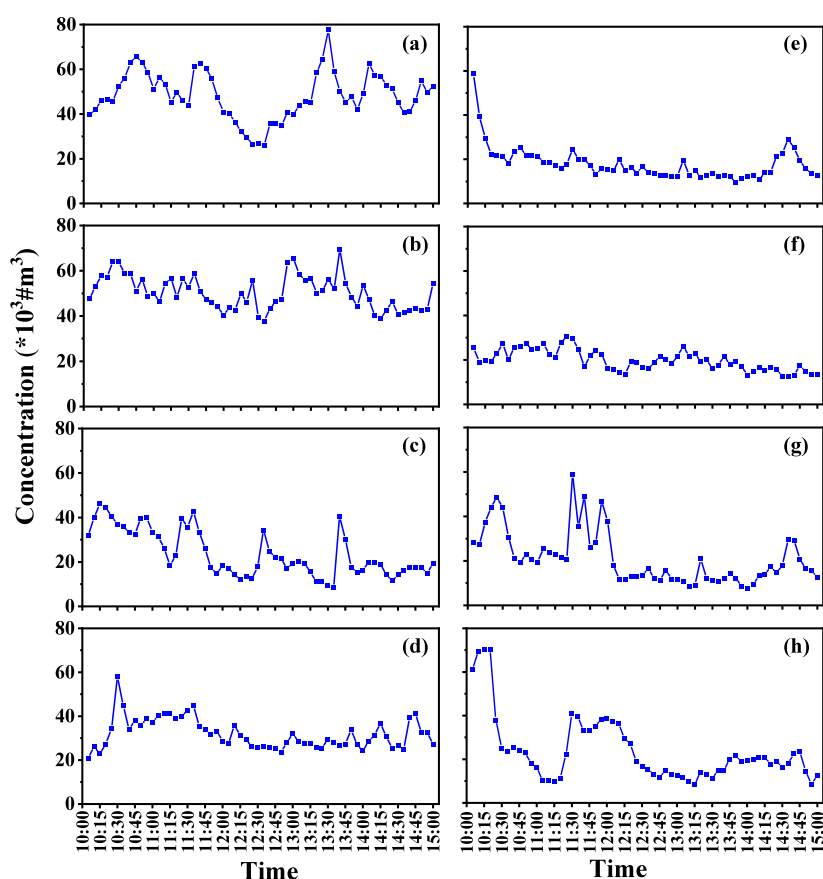


Figure 3. Fifteen minute mean concentrations of biofluorescent particles. (a) Closed, (b) natural ventilation, (c) mechanical ventilation, (d) hybrid ventilation, (e) purification, (f) purification and natural ventilation, (g) purification and mechanical ventilation, and (h) purification and hybrid ventilation.

may influence the air quality of public squat toilets, we evaluated the pollution levels of culturable and total microorganisms under different ventilation scenarios. The average culturable bacterial aerosol concentrations (299 CFU/m^3) in the toilet were higher than those of fungi (163 CFU/m^3), with pathogenic bacteria being the lowest (130 CFU/m^3). The presence of pathogens even at low concentrations poses potential health risks, particularly in enclosed environments like public toilets. After closed treatment, the normalized microbial concentrations increased (Figure 2a), indicating that squat toilet flushing can continually generate bioaerosols and will enrich microorganisms under no ventilation conditions. After natural and hybrid ventilation treatments, pathogenic bacteria decreased, although the normalized concentrations of bacterial and fungal bioaerosols increased (Figure 2b,d). This may be due to the dilution of outdoor bioaerosols since outdoor pathogens were lower than indoor (Figure S4). Notably, under mechanical ventilation, the normalized concentrations of fungi and pathogens decreased (Figure 2c). Overall, ventilation mitigates toilet pathogen concentrations and thereby diminishes associated human health risks. This is consistent with a previous study that the disease burden of *S. aureus* aerosol in the toilets under turned-off air-exhaust fan scenario will be 1.28–1.95 times higher than that under the turned-on air-exhaust fan scenario.¹⁴

Further, the real-time variations of $0.5\text{--}10 \mu\text{m}$ biological particles in the toilet were detected using the Biological Aerosol Detector. Consistent with the culturable microorganisms, the average contents of biological particles under

the closed condition ($5.10 \times 10^4 \text{ #/m}^3$) and natural ventilation modes ($5.09 \times 10^4 \text{ #/m}^3$) were higher than mechanical ($1.87 \times 10^3 \text{ #/m}^3$) and hybrid ventilation ($3.03 \times 10^4 \text{ #/m}^3$) (Figure 2i–l). These indicate that insufficient airflow or inadequate outdoor ventilation would increase the pollution of biological particles.³⁷ The temporal evolution curve showed that biological particle concentrations in natural ventilation and closed conditions were higher than those in hybrid ventilation, with mechanical ventilation as the lowest (Figure 3a–d). These results suggest that mechanical ventilation is favorable for reducing biological particle concentrations since it can ensure a specified level of air exchange.¹³ Specifically, under closed conditions, four distinct peak periods were observed between 10:25–11:00, 11:30–12:00, 13:15–13:45, and 14:00–14:30, suggesting that they might be emitted from flushing toilets. While under the natural and hybrid ventilation, no-to-one peak (10:25–10:40) was detected. Under the mechanical condition, the biological particles suddenly increased at 11:35, 12:35, and 13:40, probably due to the flow of people. This suggests that ventilation reduces the health risks of bioaerosols during the peak toilet-use period.

3.2.2. The Effect of Purification on Culturable and Total Microorganisms. By investigating the purification performance of bioaerosols under different ventilation conditions, we found that the normalized culturable microbial concentrations decreased after purification (Figure 2e–h). Specifically, under closed conditions, the REs of the air purifier for culturable bacteria, fungi, and pathogenic bacteria reached 60.39%, 61.25%, and 46.58%, respectively, which were higher

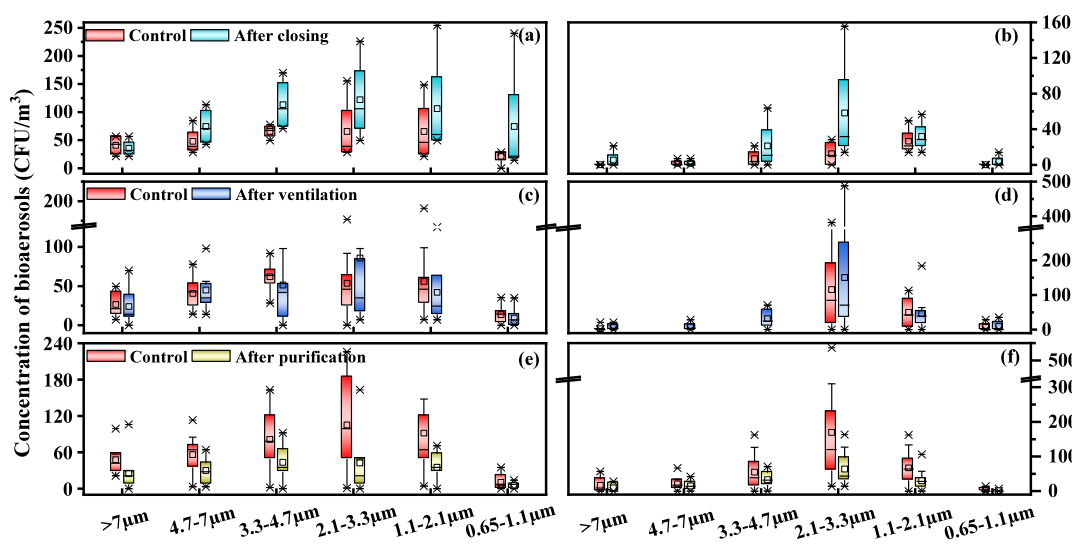


Figure 4. Size distribution of culturable microorganisms after closing, ventilation, and purification treatment. Bacteria (a,c,e) and fungi (b,d,f). “*” and “square symbols” mean the outliers and interquartile range, respectively.

than the other three ventilation scenarios. This result may be influenced by the influx of bioaerosols from outdoors through ventilation.³⁸ Under natural ventilation, the RE of the air purifier for culturable microorganisms was 56.70%, which was higher than those of mechanical (52.09%) and hybrid ventilation (38.16%). In terms of pathogens, the air purifier obtained REs of 46.01% and 47.59% under mechanical and hybrid ventilation conditions, respectively. Both values were higher than under natural ventilation (22.6%), suggesting that mechanical ventilation helps the air purifier reduce pathogenic bacterial concentration. Similar to the culturable bioaerosols, the concentration of biological particles decreased under all ventilation conditions after purification. The levels of purified biological particles under natural ventilation ($1.99 \times 10^4 \text{ \#}/\text{m}^3$) and closed conditions ($1.82 \times 10^4 \text{ \#}/\text{m}^3$) were lower than those of mechanical ($2.12 \times 10^4 \text{ \#}/\text{m}^3$) and hybrid ventilation systems ($2.39 \times 10^4 \text{ \#}/\text{m}^3$), suggesting that mechanical ventilation is not favorable for the removal of bioaerosols by the purifier. No peak was detected under natural and closed conditions during purification (Figure 3e,f); however, two peaks were observed between 10:05–10:40 and 11:20–12:15 (Figure 3g,h) during purification under mechanical and hybrid ventilation. These fluctuations may be attributable to variations in the outdoor bioaerosol concentrations since ventilation will promote the air change rates and increase the infiltration of outdoor bioaerosols.³⁹ All these results suggest that ventilation and purification can both reduce the bioaerosol concentration, and reduced ventilation aids the removal of bioaerosols by the purifier.

3.2.3. Particle Size Distribution of Bioaerosols. Since bioaerosol particle sizes determine their potential for depositing in the human airway, the effects of ventilation and purification on bioaerosol particle size distributions were further studied. As Figure 4a,b shows, the peak proportion of bacterial and fungal aerosol under the control condition was generally within 3.3–4.7, 2.1–3.3, and 1.1–2.1 μm size ranges, and 72.22% and 95.37% of bacterial and fungal aerosol were in the inhalable size range ($<4.7 \mu\text{m}$), respectively. Among them, the proportion of bacterial and fungal aerosols with size $<2.1 \mu\text{m}$ occupied 26.94% and 34.75%, respectively, indicating that most bioaerosols (especially fungal aerosols) can be inhaled

and deposited in the respiratory tract and deeply in the lungs.¹³ The average concentrations of culturable bacterial and fungal aerosols with a size $<7 \mu\text{m}$ increased following closed treatment. Previous study also documented that toilet flushing leads to the dispersion of biological particles with a diameter $<4.7 \mu\text{m}$ into the surrounding indoor atmosphere.⁴⁰ Further, the size distribution of biological particles was consistent with the culturable bioaerosol, and the peak proportion of the bioaerosol particle size distribution was 1–3 and 3–5 μm , suggesting that the biological particles in the toilet were mainly in the range of 1–5 μm . A previous study also showed that size distributions of toilet bioaerosols were bimodal, with 95% of particles $<2 \mu\text{m}$ diameter and $>99\% < 5 \mu\text{m}$.¹¹

Samples collected under the three ventilation scenarios were further classified into the ventilation group, whereas purified samples obtained under closed and three ventilation scenarios were assigned to the purification group. The results revealed that ventilation, especially natural and hybrid ventilation, increased most fungal aerosol levels (except for 1.1–2.1 μm) (Figures 4d and S5). These fungi are probably from the resuspended fugitive dust particles during ventilation.⁴¹ For bacterial aerosol, significant decreases occurred in particle size fractions of >7 , 3.3–4.7, 1.1–2.1, and 0.65–1.1 μm , after ventilation, especially mechanical and hybrid ventilation, indicating that ventilation can effectively remove these bacterial bioaerosols (Figures 4c and S5). In comparison, purification reduced the concentrations of all size bioaerosols, and the optimized purification efficiencies were found for 1.1–3.3 μm bioaerosols (Figure 4e,f). Specifically, under closed and natural ventilation, the purification efficiencies of particle with a size of 2.1–3.3 μm were higher than other sizes, while under mechanical ventilation, more particles with a size of 1.1–2.1 μm were removed (Figure S6a). The results show that the air purifier effectively reduced the concentration of indoor bioaerosols with inhalable particle size, thereby reducing the risk of inhalation. Overall, biological particles were greatly reduced by the air purifier, and their concentrations followed the order closed $>$ ventilation $>$ purification (Figure S7).

3.3. The Airborne Microbial Community Structure, Function, and Its Environmental Factors

3.3.1. Toilet Bioaerosol Community Structure. To further evaluate the effect of three treatment scenarios, including closed, ventilation, and purification, on the diversity of bioaerosols, the Chao and Shannon indices were compared between the control and treated samples. The Chao index reflects richness, while the Shannon index indicates the diversity of microbial communities.⁴² As Figure S8 shows, bacterial aerosol communities were more abundant and more diverse than fungal communities. The richness and diversity of bacteria followed the sequence closed > ventilation > control > purification, indicating that the purifier effectively reduces indoor bacterial aerosol pollution. However, ventilation and closed treatment increase diversity and richness. It may be because outdoor bioaerosols are the main sources of indoor bioaerosol communities, and ventilation will increase the transmission of bioaerosols from outdoors.³⁹ Besides, toilet flushing will generate a mass of bioaerosols due to the turbulence and fluctuation of toilet water.⁴³ For fungi, the order of richness and diversity is control > purification > ventilation/closed. Here, ventilation and purification reduce the indoor fungal aerosol pollution. Ventilation or air cleaning reduces the aerosol concentrations in the size fraction of 0.25–10 μm by more than 60%.^{44,45}

Further examination of the differences in the microbial community between purification and ventilation scenarios using PCA revealed that two principal coordinates, PC1 and PC2, respectively, explained 42.98% and 19.52% of the data changes in bacterial samples. In contrast, fungi accounted for 23.19% and 11.95% of the variations, respectively (Figure S9). Three group samples were clustered together without apparent separation, indicating that ventilation and purification did not change the bacterial and fungal community structures.

Specifically, for bacteria, the most dominant genera in control samples were *Anoxybacillus* (29.9%), followed by *Brevundimonas* (11.2%) (Figure 5). However, after ventilation and purification treatment, the second most dominant bacteria changed from *Brevundimonas* to *Acinetobacter* and *Brevibacillus*, respectively, suggesting that *Brevundimonas* can be removed by ventilation and purification. This is crucial since *Brevundimonas* spp. is an emerging global opportunistic pathogen found in the urine.⁴⁶ In addition, purification also removed *Brevundimonas*, *Roseomonas*, and *Methylobacterium-Methylobacterium*. Whereas ventilation increased the abundance of *Anoxybacillus*, *Acinetobacter*, and *Gordonia* but decreased the abundance of *Brevundimonas* and *Methylobacterium-Methylobacterium*. Notably, the closed treatment significantly increased the relative abundance of *Roseomonas* and *Streptomyces*.

For the pathogenic bacteria, the abundances of *Bartonella* and *unclassified-o-Rhizobiales* were reduced after ventilation and purification. For fungi of the control samples, the two most dominant genera were *Cladosporium* (39.0%) and *Penicillium* (20.4%) (Figure 5b). Closed treatment increased the abundance of *Cladosporium*, *Aspergillus*, and *Fusarium*. After ventilation, the abundance of *Cladosporium*, *Peniophora*, *Talaromyces*, *Aspergillus*, and *Penicillium* decreased, while that of *Schizophyllum*, *Metarhizium*, and *Periconia* increased, suggesting that they may come from outdoors. After purification, *Talaromyces*, *Peniophora*, *g-unclassified-o-Cantharellales*, *Alternaria*, and *Hannaella* were reduced, indicating that they could be removed by the purifier.

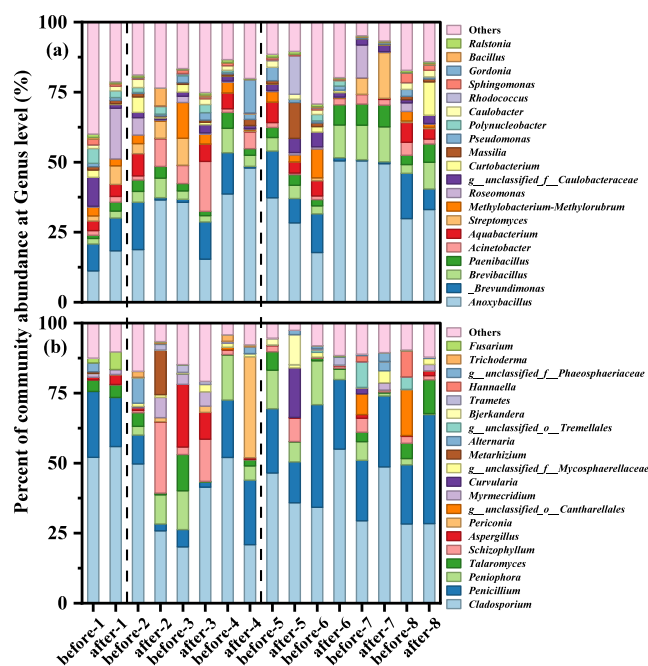


Figure 5. Community composition of airborne bacteria (a) and fungi (b) at the genus level under eight treatment conditions. (1) closed, (2) natural ventilation, (3) mechanical ventilation, (4) hybrid ventilation, (5) purification, (6) purification and natural ventilation, (7) purification and mechanical ventilation, and (8) purification and hybrid ventilation.

Furthermore, the Kruskal–Wallis H test was used to determine the microorganisms with significant differences between the control and treated groups. As Figure S10 shows, *Brevibacillus*, *Paenibacillus*, *Comamonas*, and *Rhodobacter* were statistically different between the control and treated groups. For fungi, *Periconia*, *Pseudopithomyces*, and *Pestalotiopsis* showed statistically significant differences among the three groups of samples. The abundances of these fungi were significantly higher in the ventilation groups than those in the other two groups. The abundances of *Brevibacillus* and *Paenibacillus* were significantly higher in the purified groups than in the other two groups, suggesting that these microorganisms can serve as markers under ventilation and purification treatment.

3.3.2. Function of the Toilet Bioaerosol Communities.

Further, the changes of microbial phenotypes (stress tolerance, mobile elements, Gram-positive, Gram-negative, biofilm formation, potential pathogenicity, aerobic, and facultative anaerobic) were analyzed during ventilation and purification using BugBase phenotype prediction. No significant change was found in phenotypes between the control and ventilation groups, with stress tolerance, biofilm formation, and Gram-negative bacteria as the three dominant phenotypes (Figure S11a). These results indicate that ventilation has a weak effect on reducing the exposure risk of bioaerosols. However, after purification, the abundances of stress tolerance (18.8%), mobile elements (16.2%), and Gram-positive bacteria (14.5%) significantly increased. The results suggest that bacteria with stress tolerance, mobile elements, and thick cell walls are resistant to removal. The possible reason for this is that the Gram-positive *Bacillus subtilis* has higher resistance to cold plasma than Gram-negative bacteria.⁴⁷ It is fortuitous that

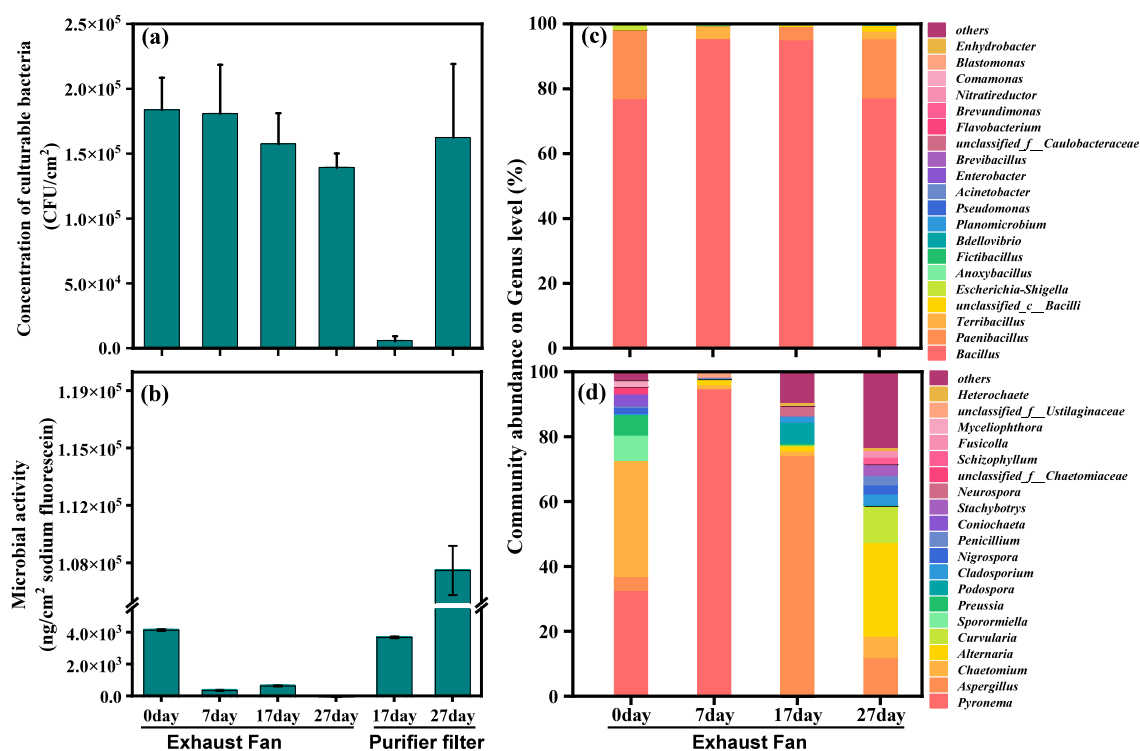


Figure 6. Culturable airborne bacterial concentration (a), total bioaerosol activity (b), and airborne bacterial (c) and fungal (d) community structures on the exhaust fan and purifier filter at different purification times.

the abundance of Gram-negative, aerobic, and form biofilm bacteria decreased.

However, the functional profiles of bacteria predicted by FAPROTAX showed significant metabolic differences ($p < 0.05$) among the three groups of microbiomes. These metabolic functions include methanol oxidation, methylo-trophy, aromatic hydrocarbon degradation, and human pathogens (Figure S11c). After purification, the proportions of human pathogenic bacteria, methanol oxidation, and methylo-trophy were significantly lower than those in the control groups, suggesting that purification reduces the risk of inhaling pathogens.

Functional annotation of fungal data using FUNGuild showed that most fungi were saprotrophs (Figure S11b). Animal pathogen–endophyte–lichen parasite–plant pathogen–wood saprotroph (36.0–42.0%) and undefined sapro-troph (22.5–33.0%) were the two most abundant fungi. Compared to the control group, the abundances of undefined saprotrophs and plant pathogen–wood saprotrophs substantially decreased after ventilation. This observation probably ensued because ventilation reduces the indoor air RH,⁴⁸ which hinders the propagation of saprophytic fungi. After purification, plant pathogen–wood saprotroph was significantly reduced. This is very important since plant-associated microbiomes can enhance pathogen defense, nutrient acquisition, and tolerance of challenging conditions.⁴⁹

3.3.3. Effect of Environmental Factors and Flow of People on Microbial Community. The complex and diverse indoor pollutants (including TVOCs, formaldehyde (HCHO), inhalable PM (PM₁₀), inhalable lung PM (PM_{2.5}), and meteorological parameters) may affect bioaerosol survival.^{50,51} The relationship between different environmental parameters/flow of people and the top 30 microbial communities was analyzed by the Spearman correlation analysis (Figure S12a).

Surprisingly, the flow of people and wind speed did not affect the abundance of bacterial communities; this may be because the flow of people is quite constant, and the wind speed is close to zero during the sampling period. PM_{2.5} and PM₁₀ were negatively related to five bacteria (unclassified f-*Caulobacteraceae*, *Chitinophaga*, *Cloacibacterium*, *Aquabacterium*, and *Ralstonia*), while being positively correlated with two bacteria (*Azospirillum* and *Edaphobaculum*). The correlation of these microorganisms with PM_{2.5} and PM₁₀ was opposite that with RH. For bacteria, RH significantly correlated with up to 15 genera, which is higher than particulate matter (seven genera) and temperature (three genera). Specifically, *Cloacibacterium*, *Paenibacillus*, *Aquabacterium*, *Ralstonia*, *unclassified-f-Caulobacteraceae*, and *Chitinophaga* correlated positively with RH but negatively with PM. The PM level significantly positively affected *Azospirillum* and *Edaphobaculum*, while RH adversely affected these genera. In addition, RH negatively correlated with *Streptomyces*, *Mycobacterium*, *Emericella*, *Polynucleobacter*, and *Legionella*.

For fungi, the effects of temperature on the fungal communities were higher than those on bacterial communities (Figure S12b). This occurred because most indoor fungi grow under lower temperatures (10 °C–35 °C) than bacteria (20 °C–40 °C).⁵² Wind speed is positively related to *Rhodospiridiobolus*. The fungi (including *unclassified-f-peniophoraceae*, *Trametes*, *Bjerkandera*, *Peniophora*, *Rhodospiridiobolus*, *unclassified-o-tremellales*, *unclassified-f-phaeosphaeriaceae*, *Schizophyllum*, and *Sistotrema*) that negatively correlated with temperature were positively correlated with RH, indicating that an environment with high RH and relatively low temperature favors fungal aerosol reproduction. This is because a relatively high RH environment provides sufficient water and nutrients for fungal communities.⁵³ Overall, fungi are more influenced by environmental factors than bacteria; changes in RH,

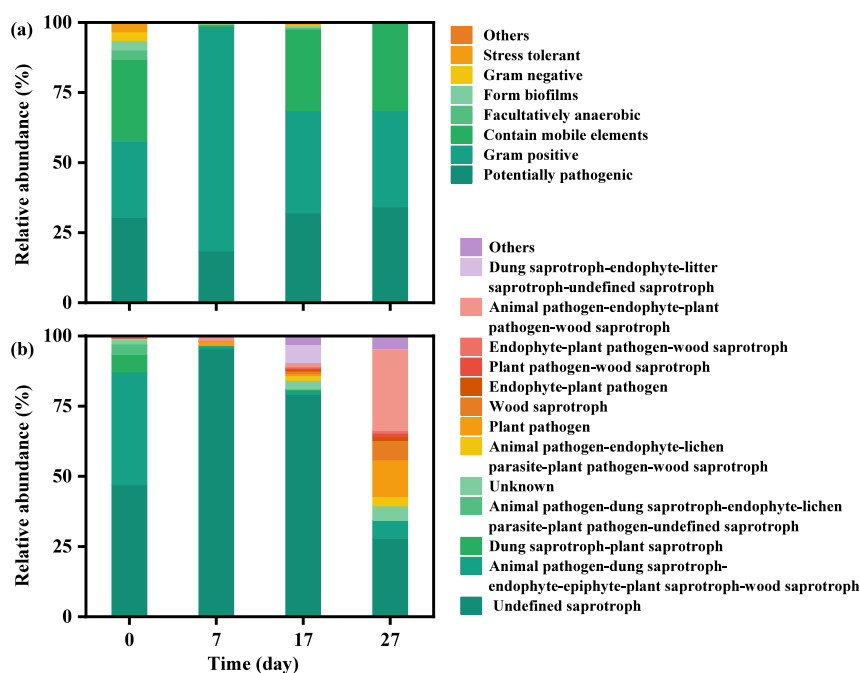


Figure 7. Predicted function of bacterial (a) and fungal (b) aerosol after treatment.

temperature, and particle concentration are helpful for the removal of communities in toilets.

3.4. The Longtime Removal Performance of Bioaerosols by the Purifier

Since the composite filter screen has a service life, to assess the long-term stability of the air purifier, the purifier was operated for 0, 7, 17, and 27 days under normal conditions during the ten working hours. The dust on the exhaust fan and composite filter was collected to evaluate the purification capability of the purifier over a long period of time.

3.4.1. Culturable Microbial Levels and Activity

Reduction. After eluting the microorganisms from the exhaust fan and composite filter, they were inoculated onto the nutrient agar plates and Rose Bengal agar plates to study the bacteria and fungi, respectively. However, only bacteria were successfully grown on the agar plates. Therefore, the characteristics of bacterial aerosol were analyzed in the subsequent experiments, and we found a slow decrease of culturable bacterial aerosol concentration in the first 7 days and then a rapid decrease (Figure 6a). Specifically, as the purification time increased from days 0 to 7, only 1.65% of culturable bacteria were removed. However, further increasing the purification time to 27 days lowered the abundance from $1.81 \times 10^5 \pm 3.76 \times 10^4$ to $1.39 \times 10^5 \pm 1.09 \times 10^4$ CFU/cm², with a purification efficiency of 24.18%. These results suggest that prolonging purification time enhances bioaerosol removal. Notably, after 17 and 27 days of purification, the levels of culturable bacteria in the composite filter reached 5.83×10^3 and 1.63×10^5 CFU/cm², suggesting that it may be a potential source of toilet bioaerosols and that bioaerosol accumulation occurred gradually with the prolongation of purification time.

Furthermore, the activity of bioaerosols was evaluated after purification by the purifier using the FDA hydrolysis and Live–Dead Cell Staining method. Based on the results of Live–Dead Cell Staining, we found that increasing purification time from 0 to 27 days resulted in a gradual decrease in green fluorescence and the appearance of yellow fluorescence (which merges the

green and red fluorescence) (Figure S13). This indicates that the total number of microorganisms decreased and microbial mortality increased as the purification proceeded. Based on the results of FDA hydrolysis, we found that after 27 days of purification, the purification efficiency of the microbial activity reached 99.63% (Figure 6b). Different from culturable bacteria, the microbial activity level decreased sharply within the first 7 days with a reduction efficiency of 90.24% and then slightly improved. This initial sharp decrease may be because most bacteria enter viable but nonculturable state, and they also contribute to the microbial activity.⁵⁴ Overall, the change trend of microbial aerosol activity detected by the two methods was consistent, indicating that the air purifier can effectively reduce the microbial activity level. As for the activity of bioaerosols in the composite filter, we found that the activity was 3.73×10^3 sodium fluorescein after 17 days of purification. However, further prolonging the purification time to 27 days increased the activity to 1.08×10^5 CFU/cm² sodium fluorescein, which was significantly higher than that of the exhaust fan dust on day 0, suggesting that it may pose health risks if the purifier is further used.

3.4.2. Changes of Dominant Microorganisms and Their Predicted Function.

Since we failed to obtain enough DNA concentration from the composite filter to conduct Illumina sequencing, it was excluded from the analysis. According to the Chao and Shannon indices, after 27 days of treatment with the air purifier, bacterial diversity and richness decreased, while fungal diversity and richness increased (Table S2). The abundance of dominant bacterium *Paenibacillus* decreased from 21.16% to 0.06% and 4.16%, and the fungi *Chaetomium* decreased from 35.93% to 1.12% and 1.33% as the purification time increased from 0 to 7 and 17 days (Figure 6c,d). However, prolonging the purification time to day 27 lowered their removal. This observation may be because with the prolongation of the purification time, the accumulated dust on the surface of the exhaust fan gradually increased, which provides growth conditions for these

microorganisms.⁵⁵ In addition, the continuous use of the purifier results in the gradual accumulation of particulates and microbes on the surface of the filter, which in turn reduces the purification capacity. It is worth noting that, after purification, the abundances of *Paenibacillus*, *Chaetomium*, *Sporormiella*, and *Preussia* decreased significantly, which is very important due to their hazardous risks to human health. For example, the diseases and syndromes associated with *Paenibacillus* infection include chronic kidney disease, sickle-cell disease, chronic interstitial nephropathy, and acute lymphoblastic leukemia.⁵⁶

Since the RE of bioaerosols on day 7 is superior to that on other days, a chi-squared test was further used to analyze the difference in microorganisms between days 0 and 7. As Figure S14 shows, *Paenibacillus* and *Bacillus* (*Chaetomium* and *Pyronema*) were the two bacteria (fungi) with significant differences between 0 and 7 days of treated samples. The abundances of *Chaetomium*, *Paenibacillus*, *Sporormiella*, and *Preussia* were higher on day 0 than on day 7, suggesting that they can be the biomarkers of bioaerosol purification.

The BugBase phenotype prediction was further used to analyze the variation in the phenotypes of microorganisms after treatment. As Figure 7 shows, potentially pathogenic, Gram-positive, and mobile element-containing microorganisms were the three dominant phenotypes. The difference was more pronounced on day 7, with a lower abundance of pathogenic and mobile element microorganisms than that of the other three purification times. Besides, the abundances of facultative anaerobic, biofilm-forming, Gram-negative, and stress-tolerant microorganisms also decreased, indicating that the purifier effectively controlled the transmission of such microbes.

4. CONCLUSION

In this study, the characteristics of biological particles from public squat toilets under various ventilation and purification scenarios were evaluated from the aspects of concentration, particle size distribution, and community composition and function. The concentration of bioaerosols under natural ventilation was the highest (632 CFU/m³), followed by closed and hybrid ventilation, with mechanical ventilation as the lowest (361 CFU/m³). The self-developed plasma air purifier reduced the bioaerosol concentrations with higher RE found under closed and natural ventilation conditions. The peak proportion of toilet biological particles was generally in the size range of 1.1–4.7 μm , which could be effectively reduced by an air purifier. After ventilation and purification treatment, the richness and diversity of fungal communities as well as the abundances of *Brevundimonas*, *Bartonella*, and *g-unclassified-o-Rhizobiales* decreased. Temperature (18 °C–28 °C), RH (38–93%), and PM (12–83 $\mu\text{g}/\text{m}^3$) had a significant influence on toilet airborne microorganisms. Long-term stability testing showed that the air purifier's removal performance of bioaerosol activity and community on day 7 was superior to other days. *Paenibacillus* and *Bacillus* (*Chaetomium* and *Pyronema*) were the two bacteria (fungi) with significant differences. Pathogens and mobile element-containing microorganisms were the main phenotypes removed by the purifier.

However, this study has several important limitations that warrant a consideration. The sampling procedure was repeated three times for each experimental scenario, but each experimental scenario was conducted only twice, which may affect the statistical results. Both filtration and plasma technologies were used in a commercial air purifier, but the mechanism of reducing biological aerosols by this purifier was

not investigated. Collectively, these limitations highlight the need for more relevant and technically matched toilet-flushing-related bioaerosol research. Nevertheless, the results suggest that purification and mechanical ventilation can help prevent aerosol accumulation in squat toilets and mitigate the risk of the airborne disease transmission.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsestengg.6c00600>.

Detailed information on analysis of the concentration and activity of bioaerosols, DNA extraction, PCR amplification and sequencing (Texts S1–S3), and advanced supporting analyzing results (Tables S1 and S2 and Figures S1–S14) (PDF)

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Notes

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