Characterization and source analysis of indoor/outdoor culturable airborne bacteria in a municipal wastewater treatment plant

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ABSTRACT
The potential health risks of airborne bacteria emission from a wastewater treatment process have been concerned. However, few studies have investigated the differences in community structure between indoor and outdoor bacteria. In this work, the characterization of airborne bacteria was studied in a municipal wastewater treatment plant in Beijing, China. Two indoor (i.e., fine screen room and sludge dewatering house) and two outdoor (i.e., aeration tank and control site) sampling sites were selected. An Andersen six-stage impactor was used for collecting culturable airborne bacteria in the air, and Illumina MiSeq sequencing was conducted to track the emission source of the culturable airborne bacteria. The results indicate that, compared with the outdoor aeration tank site, the concentrations of culturable airborne bacteria in the indoor fine screen room with poor ventilation were more than ten times higher and the particle size was about twice as large. The community structures of indoor and outdoor culturable airborne bacteria were obviously different. Enterobacteriaceae and opportunistic pathogens were detected in indoor culturable airborne bacteria, with wastewater and sludge dewatering machine identified as the primary sources. Conversely, Enterobacteriaceae and opportunistic pathogens were not detected in outdoor culturable airborne bacteria. Outdoor high wind speed might have resulted in rapid dilution and mixing of culturable airborne bacteria generated from the aeration tank with the ambient air. The results of the present research suggest that covering pollution sources, increasing ventilation rates, and using protective measures for personnel should be implemented to decrease the exposure risk to indoor culturable airborne bacteria.

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INTRODUCTION
Wastewater contains a considerable number of microorganisms, some of them being pathogens that can cause a variety of infectious diseases and allergies (Bibby and Peccia, 2013; Li et al., 2015; Rizzo et al., 2013; Thorn and Beijer, 2004). Through aeration and mechanical agitation, these microorganisms may be released into the air and form airborne bacteria in wastewater treatment plants (WWTPs), which could lead to potential health risks for workers of WWTPs (Divizia et al., 2008; Haas et al., 2010; Korzeniewska, 2011; Li et al., 2016; Sialve et al., 2015). In previous studies, the characteristics of the emission, distribution, and transmission, including the concentrations, particle size, and community compositions of culturable airborne bacteria in

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WWTPs have been extensively investigated (Karra and Katsivela, 2007; Kumaraswamy et al., 2014; Li et al., 2011). Moreover, the Enterobacteriaceae family, adenovirus, and pathogens were also detected (Benami et al., 2016; Korzeniewska et al., 2009; Korzeniewska and Harnisz, 2012; Masciaux et al., 2014). In our surveys, we showed that concentrations of culturable airborne bacteria are apparently higher indoor than outdoor of wastewater treatment facilities (Ding et al., 2015; Han et al., 2013). However, culturable airborne bacteria are not only produced by wastewater treatment processes, but also in the ambient air. Identifying sources of culturable airborne bacteria is essential for evaluating health risks. However, few studies have determined how much of the culturable airborne bacteria in WWTPs derives from wastewater/sludge or wastewater treatment facilities.

In the last decades, high-throughput sequencing (HTS) technologies have developed rapidly (Behzad et al., 2015). It can directly provide a broad profile of airborne microorganisms (Bowers et al., 2012; Cao et al., 2014; Gandolfi et al., 2015; Yoossef et al., 2013), and has been applied to analyze potentially pathogenic bacteria in soil (Hong et al., 2015), wastewater (Kumaraswamy et al., 2014; Lu et al., 2015; Zhang et al., 2012), and urban atmosphere (Bertolini et al., 2013; Cao et al., 2014; Gandolfi et al., 2015).

This work describes the field scale monitoring results of culturable airborne bacteria from a municipal wastewater treatment plant, including the fine screen room (indoor), sludge dewatering house (indoor), aeration tank (outdoor), and a control site of external upwind (outdoor). The concentration and particle size distribution of culturable airborne bacteria at each sampling site were monitored. The pathogens in culturable airborne bacteria, wastewater, sludge, and mixed liquid from aeration tank were analyzed using HTS. The composition of indoor/outdoor culturable airborne bacteria, the relationship of the culturable airborne bacteria with the ambient environment, and the presence of microorganisms in the wastewater treatment process are presented and discussed.

1. Materials and methods

1.1. Sampling site descriptions

Culturable airborne bacteria field sampling was conducted at a WWTP in Beijing, China, treating $6.0 \times 10^5$ m$^3$/day of domestic wastewater based on an anaerobic-anoxic-oxic (A2/O) process. Air samples were collected at four sampling sites (Fig. 1), including the fine screens room (FS), aeration tank (AT), sludge dewatering house (SDH) and an external upwind site of the WWTP used as the control site. FS and SDH are located inside, whereas the other two sampling sites are outside the house. The fine screen is open, and the centrifugal sludge dewatering machine is closed. All samples were taken at a breathing level of 1.5 m.

1.2. Sampling procedures

Four field surveys were performed between 9:00 and 16:30 during work time of the WWTPs on January 16, April 16, July 8, and October 23, 2014, respectively. Samples at FS, AT, SDH, and the control site were simultaneously taken and repeated thrice. In total, 360 air samples were collected and incubated to examine variations of the culturable airborne bacteria under different meteorological conditions. During each air sampling, the wastewater in FS, the activated sludge liquid in AT, and the dewatering sludge in SDH were also sampled.

A Six-stage Viable Andersen Cascade Impactor pumped through a Quick Take 30 Sample Pump (228–9530 K, SKC Gulf Coast Inc., USA) was employed to collect culturable airborne bacteria (Xu and Yao, 2013). Airborne particles were separated into six successive stages (0.65–1.1, 1.1–2.1, 2.1–3.3, 3.3–4.7, 4.7–7.0, and >7.0 μm) in order of decreasing size. Each stage contained a plate perforated by 400 holes with constant aerodynamic cut-size diameter. A 90 mm petri dish containing nutrient agar was placed directly below each stage to capture culturable airborne bacteria.

Air sample was pumped through the sampler by means of a pump at a constant flow of 28.3 L/min for 2 min. Three replicates were taken consecutively at each sampling site. Eighteen air samples with different particle sizes were collected from each location during each sampling operation. A detailed characterization of the sampling was described by Li et al. (2011).

After collection, the air sample was immediately transported to the laboratory, incubated at 30 °C for 48 hr in a constant temperature incubator, and then quantified manually. Positive-hole method was adopted to correct colony counts (Andersen, 1958).

The geometric mean diameter ($d_g$) was calculated by applying the following equation (Yamamoto et al., 2012):

$$d_g = \exp \left( \frac{\sum n_i \ln d_i}{N} \right)$$

where $n_i$ (CFU/m$^3$) is the measured concentration of airborne culturable bacteria in the $i$th stage interval; $d_i$ (μm) is the geometric midpoint of the interval, and $N$ (CFU/m$^3$) is the total concentration of airborne culturable bacteria.

1.3. Microbiological analysis

1.3.1. DNA extraction and PCR amplification

After incubation, the culturable airborne bacteria culture was firstly washed by the 1.5 mL 1× phosphate buffer solution (PBS) and scraped by the coated glass rods. Then, there were 200 μL of the washed-off bacterium solution and the sewage/sludge samples taken for total DNA extraction through a DNA Autoplate using Magnetic System-16 (TanBead, Taiwan), respectively. To validate the sample consistency, a 5 ng DNA aliquot was used as the starting amount for library preparation of each sample. The hypervariable V3 and V4 regions of 16S rRNA were amplified in triplicate by primers of 338F/806R, and PCR products were pooled and purified through the AxyPrep DNA Gel Extraction Kit (AXYGEN) (Hong et al., 2015). Finally, barcode-tagged amplicons from different samples were mixed in equimolar concentrations for MiSeq library construction and sequencing.

1.3.2. Illumina MiSeq sequencing of the culturable airborne bacteria

Purified amplicons were pooled in an equimolar ratio and paired-end sequenced ($2 \times 300$) and sequenced on the Illumina MiSeq platform at Majorbio Bio-Pharm Technology Co., Ltd.
Shanghai, China) following the standard manufacturer’s instructions.

1.3.3. Processing and analysis of sequencing data

Raw FASTQ files were de-multiplexed and quality-filtered using quantitative insights into microbial ecology (QIIME, version 1.17). Paired-end reads were first filtered, and high-quality sequences were clustered by Usearch (version 7.1, http://drive5.com/uparse/) into operational taxonomic units (OTUs) identified with a cutoff of 97% identity. The raw sequence data were deposited in the NCBI short reads archive database (accession number, SRP 096776).

Each representative sequence was taxonomically classified using the RDP classifier with an 80% bootstrap confidence threshold (Wang et al., 2007). The representative bacterial sequences were assigned at different taxonomic levels (from phylum to genus) to the SILVA database (Release 115, http://www.arb-silva.de) (Pruesse et al., 2007).

2. Results and discussion

2.1. Concentration of culturable airborne bacteria

The concentrations of culturable airborne bacteria in the WWTP during four field surveys are illustrated in Fig. 2. The concentrations showed a similar seasonal dependency, with the highest values recorded in July. Relatively large variations were observed between FS (indoor) and AT (outdoor). The concentrations of culturable airborne bacteria were more than ten times higher in FS (9670–46,678 CFU/m³) than in AT (459–4364 CFU/m³). The culturable airborne bacteria level in SDH (1661–5701 CFU/m³) was similar with that in AT, although SDH is located inside a house. The $d_0$ values of culturable airborne bacteria at different sampling sites were variable:

**Fig. 1** – Sampling sites of culturable airborne bacteria on the wastewater treatment plant (WWTP). (FS—fine screen room; AT—aeration tank; SDH—sludge dewatering house; control site—external upwind of the plant).

**Fig. 2** – The concentration of airborne culturable bacteria in wastewater treatment plant (WWTP).
Fig. 3 – Co-occurrence network of bacteria based on OTU levels of samples in July. (AT_Jul-air sample from AT; AT_Jul_W-activated sludge liquid sample from AT; FS_Jul-air sample from FS; FS_Jul_W-wastewater sample from FS; SDH_Jul-air sample from SDH; SDH_Jul_W-dewatering sludge sample from SDH; Control site_Jul-air sample from the control site.)

Fig. 4 – Shared bacterial genera based on operational taxonomic units (OTUs) levels between samples in July. The colour scale reflects the degree of sharing (percent of bacterial genera from the samples on the horizontal axis present in the samples on the vertical axis).
4.32–5.25 μm at FS, 2.09–4.09 μm at AT, and 3.17–4.89 μm at SDH.

These observations are related to the specific meteorological conditions at the sampling sites. At FS, the poor illumination intensity (1.26 W/m²) and air circulation (wind speed 0.02 m/sec) in July leaded to the concentration accumulation and the coarse particle of culturable airborne bacteria. It was noteworthy that the amount of bacteria in influent sewage increased in July. And the high temperature (29.1°C) in July promoted the emission of bacteria from the water into the air. In addition, the high temperature and relative humidity (58.2%) were in favor of the survival of the culturable airborne bacteria. These suitable environmental conditions resulted in the highest bacterial concentration in July. At SDH, the isolation of centrifugal sludge dewatering machine and increasing of air circulation (wind speed 0.12–0.25 m/sec) caused the concentration and particle size of culturable airborne bacteria to be affected by outdoor air. At AT, the high illumination intensity (110–217 W/m²) and wind

Fig. 5 – Heatmap illustrating the top 50 bacteria genera in the control air samples (Control_site_Jul), wastewater samples of FS (FS_Jul_W), activated sludge liquid samples from AT (AT_Jul_W) and dewatering sludge samples from SDH (SDH_Jul_W) in July. Color intensity on the right represents the value of the log_{10} [reads mapped to a genus contig].
speed (1.3–7.1 m/sec) were the main causes of the low concentration and fine particle of culturable airborne bacteria.

2.2. Source analysis of indoor/outdoor culturable airborne bacteria

After quality trimming, 148,262 effective sequences of the 16S rRNA genes were identified from the air, wastewater, and sludge samples of the four sampling sites. Almost all the trimmed sequence lengths ranged from 421 to 460 bp (99.98%) with a median length of 443 bp. A total of 2214 OTUs were obtained, the coverage of each library reaching almost 99%.

To identify the source of culturable airborne bacteria in FS, SDH and AT, a correlation analysis of bacteria in different samples was carried out. Fig. 3 illustrates a co-occurrence network of bacteria in all the air (including control site) and wastewater/sludge samples. In terms of bacterial taxa (OTU levels), indoor and outdoor samples separated from each other, i.e., FS and SDH samples gathered together, while AT and control site samples gathered together. In fact, culturable airborne bacteria in FS, SDH and AT samples were associated with bacteria in wastewater/sludge and control site samples, but their associations vary.

Fig. 4 illustrates the percentage of shared bacteria genera. 40% of OTUs detected in FS were shared with wastewater (28 OTUs), and 31% were shared with control site (22 OTUs). 37% of OTUs detected in SDH were shared with sludge (33 OTUs), and 38% were shared with control site (34 OTUs). Only 7% of OTUs detected in AT were shared with activated sludge (3 OTUs), whereas 71% of OTUs detected in AT were shared with control site. Therefore, wastewater is the main source of culturable airborne bacteria at FS, ambient air is the main source of culturable airborne bacteria at AT, and...
the effects of sludge and ambient air on the composition of culturable airborne bacteria at SDH are equally important.

2.3. Community structure of indoor/outdoor culturable airborne bacteria

Fig. 5 illustrates the diversity and relative abundance of the top 50 bacterial genera in each air sample at the control site, wastewater at FS, activated sludge liquid at AT, and dewatering sludge at SDH. For the air sample from the control site, the dominant genera were Brevundimonas and Bacillus, accounting for 76.22% of the culturable airborne bacteria, while Arcobacter, Enterobacter, Aeromonas, and Pseudomonas were predominant in the wastewater at FS. Bacteroides, Thauera, Zoogloea, and Dechloromonas prevailed in the activated sludge liquid at AT, similar to some extent, to the dominant genera in the dewatering sludge at SDH (Fig. 5). In addition, no Enterobacteriaceae and pathogens were detected in the top 50 bacterial genera of the control sample but they were identified in the top 50 bacterial genera of wastewater and sludge samples.

In earlier studies, family Enterobacteriaceae and opportunistic pathogens, such as Aeromonas hydrophila, A. aquatic, and Pseudomonas aeruginosa, have been identified as the typical microflora in wastewater in WWTPs (Korzeniewska et al., 2009; Korzeniewska and Harnisz, 2012; Podschan and Ullmann, 1998; Parker and Shaw, 2011; Sanders and Sanders, 1997). Fig. 6 indicates that the Enterobacteriaceae and opportunistic pathogens accounted for 27.00% and 10.67% of the culturable airborne bacteria at FS, as well as 35.93% and 6.83% of the culturable airborne bacteria at SDH, respectively. However, these Enterobacteriaceae or opportunistic pathogens were not detected in the culturable airborne bacteria at AT.

Overall, the results illustrated in Figs. 3–6 clearly show that the community structure of indoor and outdoor culturable airborne bacteria are quite different due to distinct sources. Moreover, wastewater and sludge are important sources of Enterobacteriaceae and opportunistic pathogens in the culturable airborne bacteria. Finally, the high outdoor wind speed might have resulted in the rapid dilution and mixing with the ambient air of culturable airborne bacteria generated from the aeration tank.

3. Conclusions

This work focused on a systematic investigation of indoor and outdoor culturable airborne bacteria in a municipal wastewater treatment plant. The community structure of indoor and outdoor culturable airborne bacteria varied greatly, while wastewater, wastewater treatment units, and sludge dewatering machine were identified as the main sources of Enterobacteriaceae and opportunistic pathogens in culturable airborne bacteria. The concentration of indoor culturable airborne bacteria was significantly higher than that of outdoor culturable airborne bacteria. Although covering indoor airborne bacteria sources and increasing air circulation could markedly decrease the concentration of indoor culturable airborne bacteria, the percentage of Enterobacteriaceae and opportunistic pathogens in indoor culturable airborne bacteria did not vary considerably. The concentration and community structure of outdoor culturable airborne bacteria were affected by ambient air, with high wind speed probably resulting in the rapid dilution and mixing of the ambient air with culturable airborne bacteria generated from the wastewater treatment units. Lastly, higher counts of Enterobacteriaceae and opportunistic pathogens in indoor culturable airborne bacteria may lead to increased health risks for workers exposed to them for longer periods of time.

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