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Ciprofloxacin- and azithromycin-resistant bacteria in a wastewater treatment plant

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ABSTRACT

The occurrence of antibiotic-resistant bacteria (ARB) in wastewater treatment plants (WWTPs) has become an occupational and environmental concern. WWTPs are engineered systems that treat wastewater to meet public health standards before release into the environment. The residuals, as either effluent or solids, are then discharged or beneficially recycled into the environment. Since these wastes contain a diverse array of microorganisms, some of which are resistant to commonly used antibiotics, there is a potential for these organisms to spread in the environment via residual recycling and effluent discharge. Human infections with ARB are increasing, and it is not well known how the interaction between humans and the environment plays a role in this process. WWTP workers, who are on the front lines, may come into direct contact with materials containing these microbes. This study aimed to determine the number of ARB present in both air and sewage sludges in a WWTP using nonselective media supplemented with two antibiotics (ciprofloxacin and azithromycin). The densities of total heterotrophic bacteria, ciprofloxacin-resistant bacteria, and azithromycin-resistant bacteria were $7.82 \times 10^5 - 4.7 \times 10^9$, $7.87 \times 10^3 - 1.05 \times 10^8$, and $2.27 \times 10^5 - 1.16 \times 10^9$ CFU/g, respectively. The prevalence [(concentration on medium with antibiotics/concentration on medium without antibiotics) $\times$ 100] of ciprofloxacin-resistant bacteria in treated sludge was twice as low as in digested sludge and approximately three times lower than in raw sludge. For azithromycin, the prevalence of resistant bacteria in treated sludge was about the same in digested and nearly twice lower than in raw sludge. Despite a marked reduction in the mean prevalence of resistant bacteria in dewatered treated sludge for both antibiotics, these differences were not significant. The highest prevalence of antibiotic resistance was observed for azithromycin. Similarly, the prevalence of airborne azithromycin-resistant bacteria inside the belt filter press room (BFPR) was nearly seven times higher than the prevalence of airborne ciprofloxacin-resistant bacteria. These concentrations of ARB were not negligible and may represent an exposure pathway for some workers in WWTPs.

KEYWORDS
Antibiotic; biosolids; prevalence; resistance; sewage sludge

Introduction

Antibiotics, one of the major scientific discoveries of the 20th century, have saved countless lives around the world. However, the overuse and misuse of antibiotics to treat human and animal infections have contributed to bacteria developing antibiotic-resistant mechanisms. Antibiotic resistance is one of the most serious threats to global health and food security (WHO 2015). As potential threats to human health, antibiotic-resistant bacteria (ARB) continue to spread globally (Kim and Aga 2007). According to the Centers for Disease Control and Prevention (CDC), 2.8 million people in the United States are infected with ARB each year, with a mortality rate greater than 35,000 people annually (CDC 2019).

Many antibiotics find their way into wastewater streams for a variety of reasons such as incomplete human metabolism, direct disposal into the waste system, and manufacturing processes. These materials become part of the fabric of the wastewater and can be found in the residuals from wastewater treatment through effluent and solids processing. Wastewater treatment plants (WWTP) are responsible for treating wastes from domestic, industrial, and municipal wastewater or sewage. However, the type of design

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used in each treatment plant and the efficiency of the treatment process can impact the fate of ARB and antibiotic-resistance genes (ARG) in sewage sludges (Mezrioui and Baleux 1994; Iwane et al. 2001; Guardabassi and Dalsgaard 2002; Bouki et al. 2013).

Due to the nature of wastewater processing, WWTPs contribute to the spread of ARB and ARG in the environment (Auerbach et al. 2007; Brooks et al. 2007; Zhang et al. 2009a, 2009b). Understanding the fate of ARB in WWTPs is important in assessing the potential impacts on the environment and worker health. WWTP workers may be exposed to ARB during the treatment process and sludge production as well as during biosolids land application events. The risk of infections from these materials depends on several factors, including the number and type of pathogens present in the sludge, the infective dose of the pathogens, and the susceptibility of the exposed workers (CDC 2002; National Research Council 2002). Health concerns associated with exposure to sludges in WWTP range from acute to chronic diseases (Schlosser et al. 1999; Kusnetsov et al. 2010; Van Hooste et al. 2010). Diseases include respiratory system issues, gastrointestinal illnesses, and skin infections (Schlosser et al. 1999; Kusnetsov et al. 2010; Van Hooste et al. 2010).

Although the prevalence of ARB and ARG in sewage sludge in WWTPs has been documented, workers' exposures to airborne ARB have gained less attention (Kim and Aga 2007; Zhang et al. 2009b; Bouki et al. 2013). This study aimed to quantify the concentrations of ARB in sewage sludge and air samples collected from a WWTP. Sewage sludge samples were collected at various locations during the treatment process, including raw sludge, anaerobically digested material before dewatering, and biosolids samples which consist of dewatered digested material. Air samples were similarly collected outdoors near the pretreatment area and during the dewatering step in the belt filter press room (BFPR), which was determined to represent the worst case regarding the potential aerosolization of the sewage sludge. To our knowledge, this is the first study to combine the analysis of ARB both in different sludge sample types and in air samples from a WWTP.

Materials and methods

Site description

Federal biosolids (treated sewage sludge) regulations pertaining to use and disposal practices, pollutant limits, management practices, and operational standards are contained in the 40 CFR Part 50 (CDC 2002). The studied facility, with several recent and upcoming upgrades, is typical for the region and meets federal biosolids regulations contained in 40 CFR Part 50. Samples were collected from a domestic WWTP in Ohio that serves over 40,000 domestic customers, local hospitals, and several commercial and industrial establishments. The plant treats an average daily flow of 5.5 million gallons a day (MGD) with a permitted capacity of 10 MGD. This activated sludge plant uses anaerobic digestion to produce class-B biosolids. The nutrient-rich biosolids are then land applied on farms as a fertilizer. The wastewater entering the WWTP first undergoes a physical treatment using grit chambers and bar screens to remove large objects such as rags, logs, and glass to prevent these objects from entering the WWTP process. Screened residuals are further processed in the settling tanks, where solids are removed from the effluent. Effluents are further treated with activated sludge aeration, followed by secondary clarification and UV disinfection before discharge into the environment. All incoming flows of sludge (primary sludge and secondary sludge) are combined and pumped into the anaerobic digestion tanks. The mixture is heated to temperatures ranging from 95–98°F and the total retention time in the digestion tanks is roughly 45 days to accelerate the biological conversion of organic solids to methane. The methane gas is captured and used for fuel to provide heat to the process or for other types of heating. The digested sludge is further processed by dewatering and thickened using a belt filter press.

Sample collection and analysis

Three different sewage samples were collected: raw sludge before digestion, digested sludge, and final biosolids (digested dewatered material generated after a belt filter press process). There were five sampling campaigns in this study (11/20/2019, 12/04/2019, 12/19/2019, 1/15/2020, and 2/11/2020). Samples were collected in 1 L sterile polypropylene wide-mouth bottles (Thermo Fisher Scientific, Waltham, MA, USA), transported to the laboratory on ice in coolers, and analyzed within 3 hr of sample collection.

Air samples were collected for 10 min using Andersen 2-stage impactors at a flow rate of 28.3 L/min. Two sampling locations were included in this project (BFPR and outdoors near the BFPR door). The BFPR was selected during a walkthrough as it was identified as the area most likely to represent the worst case for the potential aerosolization of sludge.

Sampling was repeated at two-time intervals. Nutrient agar plates (BD biosciences, Sparks MD) containing 4 μg/mL of azithromycin or ciprofloxacin were used side-by-side to grow ARB. Agar plates without antibiotics were used to estimate the total heterotrophic plate count (HPC) bacteria. Andersen samplers were sterilized using 70% ethanol before sampling at a new sampling site.

When analyzing the sewage sludge and biosolid samples, 300 mL of raw and digested sewage sludge was added to sterile blender jars and blended using the highest setting for 1 min using a Warning blender. Similarly, 30 g of treated biosolids were diluted with 270 mL Butterfields buffer and blended as described above. Samples were then spread plated in triplicate onto nutrient agar supplemented with ciprofloxacin or azithromycin and onto nutrient agar without antibiotics. Cycloheximide (0.5 g/L) was added to the plates to prevent fungal growth. The agar plates were incubated at 28 °C for 2 days before determining colony-forming units (CFU). The concentration was initially expressed as CFU/mL. Final concentrations were converted to CFU/g by dividing the CFU/mL number by the percentage of dry solids present in each sample. The percentage of dry solids for raw sludge, digested sludge, and treated sludge were 7.78%, 1.10%, and 16.3%, respectively.

The concentration of bacteria cultured was expressed as CFU/g in sewage sludge and biosolid samples and as CFU/m^3 in air samples. The lower detection limit (LOD) of air samples was 4 CFU/m^3. The prevalence of antibiotic-resistance bacteria (P), expressed as a percent, is the ratio of the concentration of ARB (C_R) divided by the concentration of heterotrophic plate count bacteria (C_{HPC}) and was calculated using the following equation (1):

$$P = (C_R/C_{HPC}) * 100 \quad (1)$$

where the LOD for the prevalence in the air samples was calculated as: [(4 CFU/m^3)/mean concentration of HPC (168 CFU/m^3)] $\times$ 100 = 0.02%.

Statistical analysis

SAS statistical analysis software was used to perform the data analysis (SAS System for Windows version 9.4, SAS Institute, Cary, North Carolina, USA). A p-value of < 0.05 was considered to be statistically significant. Two one-way ANOVAs were used one for each outcome to compare the concentration and prevalence of ARB between the three bulk sample types. Once ANOVA results indicate statistical significance, a post-hoc Newman-Keuls was automatically performed to see which concentration and prevalence of ARB between the three bulk sample types were different. Finally, a two-sample t-test was used to compare the concentration of airborne-resistant bacteria inside and outside the belt filter press room.

Results

Figure 1 shows the log-transformed concentrations of ciprofloxacin and azithromycin-resistant bacteria as well as the HPC bacteria detected in three different bulk sample types. The mean concentrations of azithromycin-resistant bacteria, ciprofloxacin-resistant bacteria, and total HPC bacteria in raw sludge were 6.07×10^8, 3.08×10^7, and 1.61×10^9 CFU/g,

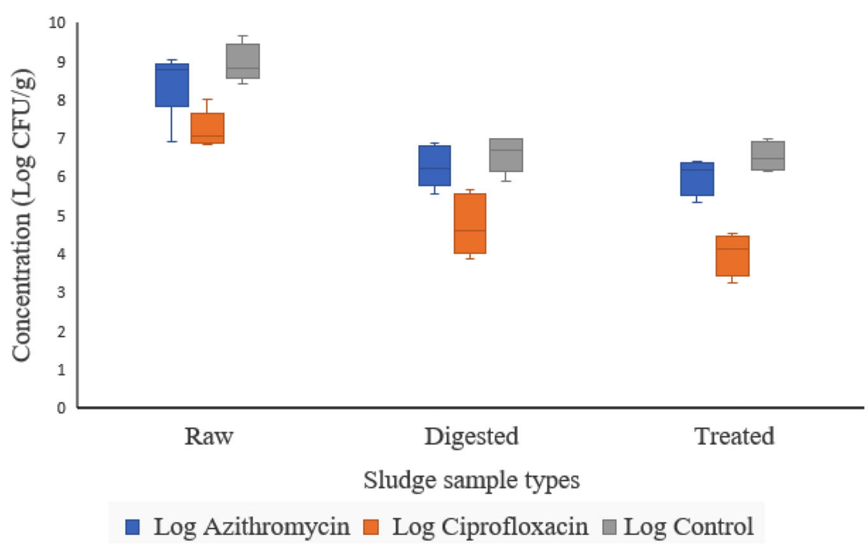

Figure 1. Mean logarithmic concentration of antibiotic-resistant bacteria relative to controls in raw sludge, digested sludge, and treated sludge.

Table 1. Geometric mean prevalence of antibiotic-resistant bacteria in raw sludge, digested sludge, and treated sludge ($n = 14$).

	Geometric mean prevalence* (Geometric standard deviation), %			
Antibiotic	Raw sludge	Digested sludge	Treated sludge	P-value
Azithromycin	30.6 (46)	49.2 (35)	30.3 (21)	0.630
Ciprofloxacin	**1.8** (3)	**1.4** (27)	0.7 (1)	0.501

*Prevalence = (Concentration on medium with antibiotics/concentration on medium without antibiotics) × 100.

respectively. In the digested sludge, the respective mean densities (RMDs) were 3.30×10^6, 1.68×10^5, and 5.62×10^6CFU/g, and in the biosolids, the RMDs were 1.48×10^6, 1.69×10^4, and 4.77×10^6 CFU/g, respectively. It was noted that a statistically significant reduction (p-value = 0.0039) in the concentration of azithromycin-resistant bacteria was achieved in the three sample types as compared to the concentration of ciprofloxacin-resistant bacteria where the reduction was not statistically significant (p-value = 0.1378). A marked reduction in the concentrations of total and resistant bacteria was observed between the raw and the digested sludge and between raw and treated sludge (Figure 1). The concentrations of HPC bacteria and resistant bacteria in raw sludge were 183–337 times higher than in digested sludge and 410–1822 times higher than in treated sludge (Figure 1).

The highest prevalence of antibiotic resistance was observed for azithromycin (Table 1). The prevalence of azithromycin-resistant bacteria in biosolids was about the same in digested and nearly twice as low than raw sludge. For ciprofloxacin, the prevalence of resistant bacteria in the biosolids was twice as low as in digested sludge and nearly three times lower than in raw sludge. Despite the observed reduction in the mean prevalence of resistant bacteria between raw and biosolids and between the raw and the digested sludge, the differences were not statistically significant for either one of the antibiotics.

Figure 2A–C depict the prevalence of ARB in raw, digested, and biosolid samples on different sampling dates. There was a marked difference in the prevalence of ciprofloxacin and azithromycin ARB during all the sampling campaigns. The highest prevalence of antibiotic resistance was consistently observed for azithromycin in all the sludge samples. In raw sludge, the prevalence of azithromycin-resistant bacteria was 54–162 times higher than the prevalence of ciprofloxacin-resistant bacteria for all the sampling campaigns. An exception was the first sampling campaign where the prevalence of ciprofloxacin-resistant bacteria was nearly three times higher. The same trend was observed for digested sludge, where the prevalence of

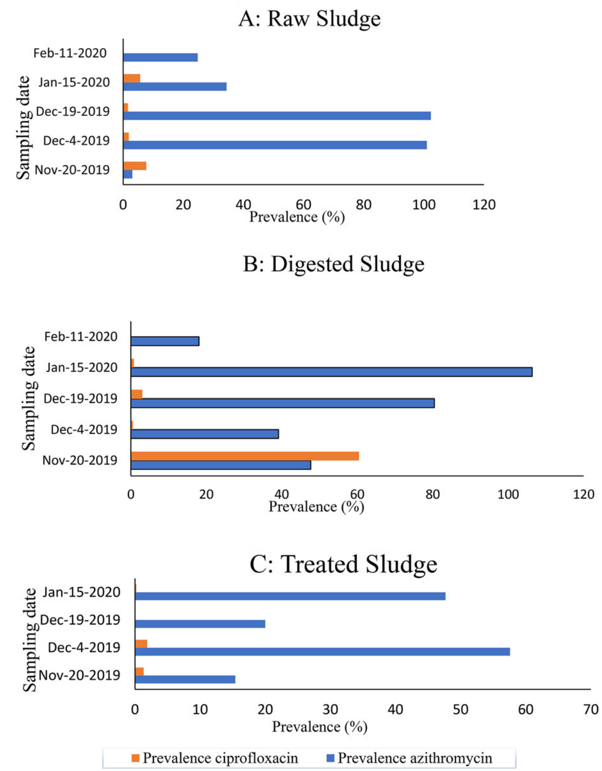

Figure 2. Prevalence of antibiotic-resistant bacteria in raw, digested, and treated sludge samples.

azithromycin-resistant bacteria was 26–223 times higher, except for the first sampling campaign when the prevalence of azithromycin-resistant was lower than the prevalence of ciprofloxacin-resistant bacteria (48% vs. 61%). As for the biosolids, the prevalence of azithromycin-resistant bacteria was 12–1,000 times higher than the prevalence of ciprofloxacin-resistant bacteria during all the sampling campaigns.

Regarding the airborne ARB sampling, HPC bacterial concentrations inside the belt filter press room ranged from 110 to 505 CFU/m^3 with an average concentration of 168 CFU/m^3. ARB concentrations inside and outside the belt filter press are shown in Figure 3. The highest ARB concentration was observed for azithromycin. During the first air sampling campaign (1/15/2020), the concentration of airborne azithromycin-resistant bacteria was 27 times higher inside the BFPR than outside (3,148 vs. 117 CFU/m^3). For the ciprofloxacin-resistant bacteria, the airborne concentration was 205 CFU/m^3 inside the BFPR, whereas it was below the limit of detection outside. As for the second air sampling campaign (2/11/2020), the concentration of airborne ciprofloxacin-resistant bacteria was 31 times higher inside the BFPR than outside (124 vs. 4 CFU/m^3). For the azithromycin-resistant bacteria, an airborne concentration of 438 CFU/m^3 was measured inside the BFPR. The respective

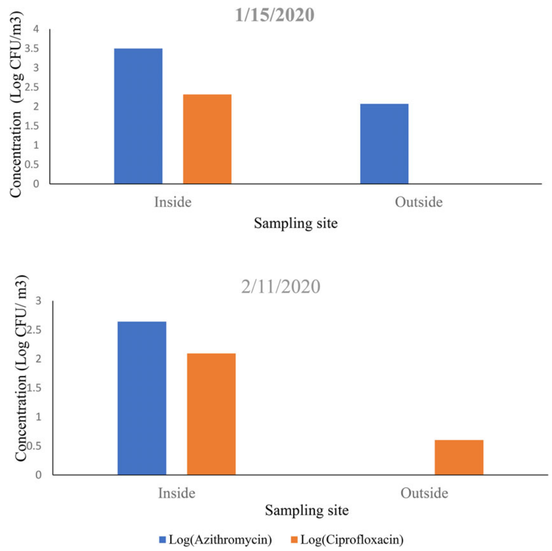

Figure 3. Logarithmic concentrations of airborne antibiotic-resistant bacteria in sampling sites.

Table 2. Geometric mean prevalence of airborne antibiotic-resistant bacteria inside and outside the belt filter press room ($n = 4$).

	Geometric mean prevalence* (Geometric standard deviation), %	
Antibiotics	Inside	Outside
Azithromycin	**498** (1962)	<LOD
Ciprofloxacin	**75** (143)	<LOD

*Prevalence = (CFU on medium with antibiotics/CFU on medium without antibiotics) × 100
LOD for prevalence = 0.02%.

outdoor sample was below the limit of detection. Table 2 shows the prevalence of airborne-resistant bacteria. The highest prevalence of airborne antibiotic resistance was observed inside the BFPR for azithromycin. The prevalence of airborne azithromycin-resistant bacteria inside the BFPR was nearly seven times higher than the airborne ciprofloxacin-resistant bacteria. The prevalence of airborne-resistant bacteria outside was below the limit of detection for both antibiotics.

Discussion

Bioaerosols produced during treatment processes at a WWTP can pose an increased risk of infectious illnesses to workers. Quantifying the concentration and prevalence of ARB from different sources can help in understanding the risk to which workers are exposed. The goal of this study was to quantify the ARB in air and sewage sludge samples throughout effluent and solids processing from a WWTP. The highest prevalence of ARB in the three different sludge samples was observed for azithromycin as compared to ciprofloxacin. These observations were supported by the results obtained with air sampling in the BFPR. The greater prevalence of bacteria resistant to azithromycin over ciprofloxacin could partly be explained by studies showing that a higher percentage of azithromycin compared to ciprofloxacin (75 vs. up to 60%) is excreted from the human body as an unaltered parent compound and can accumulate in the three sewage sludge types (Zuckerman 2000; Girardi et al. 2011; Parnham et al. 2014).

Data concerning raw and treated sludge gives an idea about the reduction efficiencies of the different treatment steps in this WWTP. The treatment process was suitable for reducing the load of HPC and resistant bacteria but was not capable of eliminating all bacteria. Based on our results, the mean concentration of azithromycin-resistant bacteria was close to the mean concentration of HPC bacteria in the digested sludge. This indicates that many of these bacteria are not susceptible to the antibiotic effects of azithromycin. The mean concentrations of azithromycin-resistant bacteria, ciprofloxacin-resistant bacteria, and total HPC in raw sludge found in this study were 20–1,000 times higher than the average bacterial concentration of 1.42×10^6 CFU/g observed by Cyprowski et al. (2018) in sewage samples. The discrepancy can be attributable to the WWTP efficiency in reducing the bacterial concentration and the season of sampling. Our studied facility uses anaerobic digestion, and the study was carried out during the winter season as compared to Cyprowski et al. (2018) study, where the WWTP used anaerobic, hypoxic, and aerobic phases and the sewage and sludge samples were collected during the summer season (Cyprowski et al. 2018).

The prevalence of antibiotic resistance is defined by several authors as the percentage of resistant HPC in the total HPC and calculated as the number of bacteria growing on media containing antibiotics divided by the number of bacteria growing on media without antibiotics (Guardabassi and Dalsgaard 2002; Stürmer et al. 2004; Xi et al. 2009). We are not aware of any prior studies on the azithromycin-resistant bacteria and ciprofloxacin-resistant bacteria in WWTP. However, Guardabassi and Dalsgaard (2002) previously found that the prevalence of ampicillin, gentamicin, and tetracycline-resistant bacteria in raw and treated sewage vary from 0–45.1%. The large variation was found to depend on the treatment plant process, the targeted bacterial population, and the antibiotics used (Guardabassi and Dalsgaard 2002). In the current study, the prevalence of airborne ciprofloxacin-resistant or azithromycin-resistant bacteria inside the BFPR was above 100%. Values greater than 100%

could be due to temporal variation in the concentration of airborne bacteria. The belt filter press was running during the air sampling of antibiotic-resistant bacteria (ARB), which likely increased the variation in the aerosolization of sludge and the emission of bioaerosols inside the room. In the future, simultaneous sampling onto the three different agar media and longer sampling times are recommended. A limitation of the air sampling is the small sample size for airborne ARB. Another limitation of air samples is that the antibiotic-resistant and HPC bacteria were sampled sequentially, not simultaneously.

The average concentration of airborne HPC bacteria in our study was three times higher than the average airborne HPC bacterial concentration of 50 CFU/m^3 reported by Cyprowski et al. (2018). Szyłak-Szydłowski et al. (2016) measured concentrations of airborne bacteria at a bar screen and grit chamber of a wastewater treatment plant and reported concentrations of 1,300 – 11,000 CFU/m^3 (Szyłak-Szydłowski et al. 2016). These results are higher than our airborne HPC bacterial concentrations inside the belt filter press room (Stürmer et al. 2004). The highest airborne concentration of ARB of 3,148 CFU/m^3 was measured for azithromycin inside the belt filter press room. This was twice as high as the highest concentration of airborne HPC bacteria reported by Fathi et al. downwind of the aeration basins (1,373 CFU/m^3) (Fathi et al. 2017).

During this study, workers were observed using safety goggles, safety shoes, liquid-repellent coveralls, and gloves. The PPE worn in this facility is adequate and in accordance with the standard PPE mandate in most WWTP (CDC 2002). Our results suggest the highest risk of exposure from ARB occurs during the dewatering step inside the BFPR. It is recommended that the facility should install engineering control such as a suitable exhaust fan in the BFPR to ensure good air ventilation and airflow changes to dilute and reduce the concentration of ARB in the air. Additionally, further research using a microbial risk assessment approach associated with practical and appropriate methods to reduce the emission of bioaerosols from WWTPs could be useful in the development of a safety plan. Finally, the data presented suggests avoidance of inhalation and dermal exposures during sludge processing would be beneficial to improved worker safety.

Conclusions

The study showed the importance of determining the concentrations and prevalence of ARB at various locations within WWTPs as these represent a potential source of exposure. The concentrations of ARB in the air and during sludge processing at this WWTP are abundant and may cause work-related exposures to these organisms. In addition, the prevalence of azithromycin-resistant bacteria in the three different sewage sludge samples, as well as in air samples, was higher as compared to ciprofloxacin. Finally, this study indicates potential health risks for some workers in WWTPs as these concentrations of ARB were not negligible and may represent an exposure pathway.

Our results suggest that the greatest exposure source to ARB in the studied WWTP occurs inside the BFPR when the treated sludge is being dewatered and thickened. Quantifying the prevalence and concentration of ARB and genes in different sources will improve our understanding of the magnitude of the risk to which workers are exposed.

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Data availability statement

De-identified data will be available upon request and approval by the corresponding author.

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