Relationship between Bacterial Counts and Endotoxin Concentrations in the Air of Wastewater Treatment Plants

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The relationship between bacterial counts and endotoxin concentrations in air samples was studied. Selective EMB medium favored the growth of a larger portion of airborne gram-negative bacteria than LES Endo or MacConkey medium and was a good predictor of the endotoxin levels determined with a chromogenic Limulus assay of the air of wastewater treatment plants. The bacterial counts determined with nonselective media correlated poorly with airborne endotoxin levels; however, R2A medium yielded higher viable bacterial counts than TYG medium. Direct counting by epifluorescence microscopy yielded the highest bacterial counts, but no correlation was obtained between total bacterial counts and endotoxin concentrations.

Inhaled bacteria, especially gram-negative bacteria, which contain endotoxin on their outer cell wall, can cause respiratory diseases and other health effects (3, 15). Accurate and quantitative estimation of exposure is necessary for the examination of health effects caused by endotoxins, but the sampling of airborne bacteria and endotoxins in highly contaminated environments is especially problematic.

In soil and water microbiology, it has been observed that low-nutrient-concentration media yield the highest viable counts of heterotrophic bacteria, although culture media with rich nutrient concentrations have generally been used in aerobiological studies of these microbes (9, 12). Therefore, we compared media with different nutrient contents for the determination of total viable airborne bacterial counts at wastewater treatment plants.

There are many culture media that are selective and that are used for gram-negative bacteria in clinical samples and for the assessment of water quality, but it has not been reported whether they are suitable for airborne bacteria in work environments. We investigated the suitability of three different selective media for airborne gram-negative bacteria at wastewater treatment plants.

Good correlations have been reported between endotoxin concentrations and bacterial counts in environmental samples, such as cotton leaf (4) and seawater (17) samples. This correlation has not been reported for air samples. Therefore, we studied whether there is any relationship between viable airborne bacterial counts measured on different culture media and endotoxin concentrations determined by a chromogenic Limulus assay. In addition, we correlated the total counts obtained by epifluorescence microscopy (11) with the airborne endotoxin concentrations. Direct counting by epifluorescence microscopy reveals nonculturable bacteria as well and therefore may better reflect endotoxin concentrations in the air.

Air sampling. Air samples were taken indoors at 10 wastewater treatment plants that use activated sludge treatment combined with chemical precipitation. Bacterial and endotoxin samples were collected at 1.5 m above the ground within 0 to 1 meter of different phases of the wastewater treatment process, i.e., wastewater pumping, the screen, sand separation, sedimentation basins, biofilters, aeration basins, and sludge treatment. Bacterial and endotoxin samples that were compared were taken simultaneously. Air temperature and relative humidity were recorded.

Viable airborne bacteria were sampled with a six-stage impactor (model 10-800; Andersen Inc.) calibrated at a flow rate of 28.3 liters/min. The sampling times used varied from 1 to 20 min, depending on supposed bacterial concentrations in the air. Total viable bacteria, both gram positive and gram negative, were grown on two nonselective media: nutrient-poor R2A (12) and nutrient-rich TYG (1). The plates were incubated at 20°C for 7 days. EMB (Difco Laboratories, Detroit, Mich.), LES Endo (Difco), and MacConkey (Becton, Dickinson Microbiology Systems, Cockeysville, Md.) agars were used as selective culture media for gram-negative bacteria. The plates were incubated at 37°C for 48 h. All the media contained cycloheximide (0.5 μg/liter; Sigma, Deisenhofen, Germany) as a fungicide (18). After incubation, the colonies were counted and were corrected by the positive-hole correction method (2). The results are expressed as numbers of CFU in 1 m³ of air. Bacterial colonies were differentiated on the basis of colony morphology and Gram staining. Gram-negative bacteria were identified by use of API 20E (for enterobacteria) and API 20NE (for nonenterobacteria) test kits.

Endotoxin and bacterial samples for direct counting by epifluorescence microscopy were taken with sterile glass fiber filters (85/220; Macherey-Nagel, Duren, Germany) in plastic filter holders (Millipore Corp., Bedford, Mass.) by use of suction pumps (model 222-3; SKC) at flow rates of 2 liters/min. Sampling times varied from 0.5 to 2.0 h. After collection, the filters were stored at 4°C for no more than 2 weeks. Each filter was extracted with 10 ml of sterile, nonpyrogenic water in sterile pyrogen-free glassware by horizontal shaking at room temperature for 60 min (8). The extracts were centrifuged at 112 x g for 10 min. A sample of 50 μl of the supernatant fraction was analyzed in duplicate for the presence of endotoxin by use of a chromogenic modification of the Limulus amebocyte lysate test (Coatest endotoxin test kit; Kabi Vitrum Diagnostica, Mölndal, Sweden). The results are expressed as nanograms of endotoxin per cubic meter of air. One nanogram of endotoxin standard concentration was used as a reference.

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from *Escherichia coli* O111:B4 corresponds to 12 endotoxin units.

After removing the samples for the endotoxin analyses, we fixed the bacterial cells in the extracts with 37% formaldehyde (1% [wt/vol]) for the detection of total bacterial counts by epifluorescence microscopy (5, 11). The fixed extracts were centrifuged again at 112 × g for 10 min to separate the possibly remaining glass fibers from the extracts. One milliliter of the supernatant was filtered through a 0.2-μm-pore-size, black polycarbonate membrane (SN 110656; Nuclepore Corp., Pleasanton, Calif.). A Millipore HA membrane (0.45-μm pore size; HAWP 02500) was used below the Nuclepore membrane. The sample was stained with 0.01% (wt/vol) acridine orange (Fluka AG, Buchs, Switzerland) for 2 min. The membrane was mounted on a microscope slide with nonfluorescent Cargille oil type B (Electron Microscopy Sciences, Washington, Pa.) and examined with an epifluorescence microscope at a magnification of ×1,000. The BH2-RFL microscope (Olympus Optical Co., Tokyo, Japan) was equipped with an HBO 100W/2 mercury lamp, an SPlan100/1.25 oil objective, an EY-455 exciter filter, and a dichroic mirror (BDM-500 with an O-515 barrier filter). The bacterial cells that fluoresced either green or orange were counted from so many fields that the total number of cells was over 100.

The statistical significance of the differences in the bacterial counts obtained by different culture media was analyzed by Student's *t* test. Linear regression analysis was used to determine the relationships between bacterial counts and endotoxin concentrations. Logarithmic transformation was used to obtain a normal distribution in the bacterial counts and the endotoxin concentrations.

**Comparison of culture media.** The concentrations of viable bacteria sampled with the Andersen impactor ranged from 10^2 to 10^8 CFU/m^3 (mean, 10^6 CFU/m^3). Of the nonselective culture media, the nutrient-poor R2A medium yielded, on average, 20% higher bacterial counts in the air samples than the nutrient-rich TYG medium (*n* = 68; *P* ≤ 0.01). Of the bacteria grown on R2A medium, 36 to 58% (mean, 47%) were gram negative, while the corresponding proportion grown on TYG medium was 42 to 50% (mean, 46%). It has been shown that R2A is a suitable culture medium for viable counting of aquatic bacteria in potable water samples (12) and of indoor air bacteria (6). Potable water and dwellings are environments in which the nutritive conditions for bacteria are poor. However, R2A medium seems to be a good choice at wastewater treatment plants as well, although the bacterial flora of wastewater is adapted to live in a rich nutrient environment.

The concentrations of viable bacteria obtained with three selective culture media for gram-negative bacteria were compared at two wastewater treatment plants (*n* = 10). The bacterial counts isolated on EMB medium ranged from 10 to 8,700 CFU/m^3 (mean, 2,050 CFU/m^3), those on LES Endo medium ranged from 0 to 1,700 CFU/m^3 (mean, 350 CFU/m^3), and those on MacConkey medium ranged from 0 to 500 CFU/m^3 (mean, 150 CFU/m^3). Because EMB medium clearly yielded the highest bacterial counts (*P* ≤ 0.05) and there was no difference between the percent distribution of gram-negative and gram-positive bacteria on these three media, EMB medium was used for further study at eight wastewater treatment plants. Of the bacteria that grew on EMB medium, 84% on average were gram negative. A reason for the good growth of airborne gram-negative bacteria on EMB medium is probably that EMB medium contains both lactose and sucrose as sources of carbohydrates, whereas the other media studied contain only lactose.

**Total numbers of bacteria compared with viable bacterial counts.** The total numbers of bacteria in extracts of endotoxin samples ranged from 10^3 to 10^9 bacterial cells per m^3, as determined by epifluorescence microscopy. The viable bacterial counts sampled with the Andersen impactor were below 1% of these numbers. There was no statistically significant correlation between viable bacterial counts and total bacterial numbers (Fig. 1). One reason for this result may be a technical problem. The endotoxin filters released glass fibers into the extracts, and some of the fibers may have stayed in the extracts despite the centrifuging. These fibers as well as other nonbacterial particles show fluorescence and may have disturbed the counting of the total bacteria. It has also been noted that organic dust particles comparable in size to microorganisms show high background fluorescence and thus make analysis difficult (11).

**Relationship between concentrations of bacteria and endotoxin.** There was a good correlation (*P* ≤ 0.0001) between the concentration of viable airborne bacteria grown on EMB medium and the endotoxin concentration. Bacterial counts on R2A and TYG media did not correlate as well with the endotoxin concentration (Fig. 2), obviously because there were more gram-positive bacteria on these nonselective media than on EMB medium. We found no correlation between the endotoxin concentration and the total number of airborne bacteria, either. Watson et al. (17) reported a close relationship between total bacterial counts determined by epifluorescence microscopy and endotoxin concentrations determined by the *Limulus* assay for seawater samples. In marine water samples, gram-negative bacteria account for 80 to 95% of the procaryotes (13). In air samples, total bacterial counts also include many gram-positive bacteria, which do not contain endotoxin. Furthermore, bacteria in water samples may remain viable longer than bacteria in air samples after collection on filters. When bacteria are carried by air, they dry out, and their ability to grow decreases. It is predominantly the concentration of cell-wall-dissociated "free" endotoxin that is measured by the *Limulus* assay (16). This endotoxin is released during agitation of a bacterial sample in a liquid environment (7). The free endotoxin is
mainly associated with growing bacteria, not with death and lysis of gram-negative cells (14). A large proportion of bacteria in the air are nonviable or are in a resting state. It is therefore possible that endotoxin concentrations determined by the Limulus assay correlate poorly with total bacterial counts determined by epifluorescence microscopy for air samples.

The present results show that endotoxin levels in the air of wastewater treatment plants can be estimated from bacterial counts by use of a selective medium for gram-negative bacteria, i.e., EMB. In this study, the endotoxin concentrations varied from 0.6 to 310 ng/m³. The proposed occupational exposure limit for endotoxin is 30 ng/m³ as an 8-h time-weighted average (10). This amount equals an airborne bacterial concentration of 7,200 CFU/m³ when bacteria are sampled with the Andersen impactor and grown on EMB agar. On the other hand, the sensitive and rapid chromogenic Limulus assay can be used to estimate the concentration of viable gram-negative bacteria in the air.

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