

Source Bioaerosol Concentration and rRNA Gene-Based Identification of Microorganisms Aerosolized at a Flood Irrigation Wastewater Reuse Site

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Received 19 February 2004/Accepted 2 September 2004

Reuse of partially treated domestic wastewater for agricultural irrigation is a growing practice in arid regions throughout the world. A field sampling campaign to determine bioaerosol concentration, culturability, and identity at various wind speeds was conducted at a flooded wastewater irrigation site in Mexicali, Baja California, Mexico. Direct fluorescent microscopy measurements for total microorganisms, culture-based assays for heterotrophs and gram-negative enteric bacteria, and small-subunit rRNA gene-based cloning were used for microbial characterizations of aerosols and effluent wastewater samples. Bioaerosol results were divided into two wind speed regimens: (i) below 1.9 m/s, average speed 0.5 m/s, and (ii) above 1.9 m/s, average speed 4.5 m/s. Average air-borne concentration of total microorganisms, culturable heterotrophs, and gram-negative enteric bacteria were, respectively, 1.1, 4.2, and 6.2 orders of magnitude greater during the high-wind-speed regimen. Small-subunit rRNA gene clone libraries processed from samples from air and the irrigation effluent wastewater during a high-wind sampling event indicate that the majority of air clone sequences were more than 98% similar to clone sequences retrieved from the effluent wastewater sample. Overall results indicate that wind is a potential aerosolization mechanism of viable wastewater microorganisms at flood irrigation sites.

In the arid border region between Mexico and the United States, a rapidly growing population, an agriculture-based economy, and the low availability of surface and ground water underscore the importance of water reuse (13). The use of effluent wastewater for agricultural irrigation is therefore a growing practice in this region. Throughout the world, an estimated 80% of domestic wastewater produced in developing countries may be reused for irrigation (8). The World Health Organization (34) has defined a coliform guideline for unrestricted irrigation with domestic wastewater, allowing 1,000 fecal coliforms per 100 ml. This standard is difficult to meet in many developing countries, where the wastewater designated for crop irrigation most commonly receives partial treatment and is rarely disinfected (35).

Among workers and occupants of surrounding communities, a lack of disinfection and associated exceeding of indicator organism guidelines have prompted concerns regarding the elevated health risk caused by aerosolization and subsequent transport of effluent wastewater microorganisms (3, 4, 22, 30). Rapid urbanization of many cities along the United States-Mexico border has led to the growth of populations near agricultural land and may exacerbate the risk. In addition to respiratory tract infections, it has been suggested that aerosolized enteric microorganisms common in wastewater may produce intestinal tract infections when particles are deposited in the upper nasal pharynx and later swallowed (28).

Epidemiological studies (16, 26) in agricultural communal

settlements located near fields with wastewater irrigation indicated an increase in the rate of enteric diseases during the irrigation season. This increase was most evident in the youngest population group (0 to 5 years). Negative health impacts linked to wastewater bioaerosols have also been documented in wastewater workers (17), in which a positive correlation between bioaerosol exposure and gastrointestinal infections has been observed. Moreover, workers exposed to air-borne gram-negative enteric bacteria and endotoxins at industrial and municipal wastewater treatment plants reported health issues such as respiratory symptoms, fever, and tiredness (18, 22).

Previous wastewater bioaerosol studies have measured ambient air-borne bacterial concentrations near wastewater systems that have an overt potential for aerosolization. These systems include open activated sludge basins (6, 9, 18) and spray irrigation sites (4, 5, 27, 30, 31). Although wastewater flood irrigation is the more common practice, the effects of wind speed on microorganism aerosolization have not been characterized. Fundamental information to determine the relationship between bioaerosol concentration, identity, and environmental conditions such as wind speed is needed to assess and predict human exposure.

In the present study, a field sampling campaign to characterize bioaerosols collected during periods of both low and high wind speeds and dial solar conditions was conducted at a flooded wastewater irrigation site in northern Mexico. Direct fluorescent microscopy measurements for total air-borne bacteria and culture-based assays were applied to investigate the effect of environmental conditions on the total concentration and culturability of bioaerosols. Phylogenetic identification and comparison of the aerosol and effluent wastewater micro-

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bial communities were made by construction of clone libraries based on sequence analysis of the small-subunit rRNA gene.

MATERIALS AND METHODS

Aerosol and effluent wastewater sampling. Studies were conducted at an agricultural field located in the northern Sonoran Desert on the urban fringe of Mexicali, Baja California, Mexico. The field was seeded for Bermuda grass sod and flood irrigated with nondisinfected effluent from a two-stage anaerobic-facultative lagoon that treated domestic wastewater. The fecal coliform concentration in the effluent wastewater is commonly more than 10^3 CFU/ml (7), and a strong H_2S odor was present near the effluent wastewater. Approximately 80% of the biochemical oxygen demand is removed during lagoon treatment. Flow of effluent wastewater for flood irrigation was started 12 h prior to sampling and was continuous throughout the 3-day sampling campaign performed in November 2002. Standing water at a depth of 1 to 5 cm was always present but never covered the entire field. The fraction of the field covered and the location of standing water varied with time.

Aerosol and effluent wastewater samples were taken five times daily, 7 a.m., 11 a.m., 2 p.m., 4:30 p.m., and midnight. The sampling equipment was located on the east side of the field, and the samplers were directed toward the field. A weather station, Weather Monitor II (Davis Instrument Corporation, Hayward, Calif.), was set 1.5 m high near the sampling site and provided relative humidity, wind speed and direction, and temperature measurements. Solar radiation was measured with a radiometer-photometer, model IL-1700 (International Light, Newburyport, Mass.) with SED005#776 and WBS320#24343 detector and filter specifications, respectively. This filter has a response in the range from 250 to 400 nm (UV-B and UV-A). All meteorological data were recorded every 5 min during each sampling event.

For aerosol sampling, three sterile glass liquid impingers (Biosamplers, SKC Inc., Eighty Four, Pa.) were used simultaneously. Impingers were connected to a one-quarter-horsepower vacuum pump (GAST, Benton Harbor, Mich.) through a galvanized iron manifold and suspended at the breathing zone height, approximately 1.5 m above the ground. The pump operated at a pressure drop greater than 0.5 atm, and the flow rate in each impinger was 12.5 ± 0.5 liters/min. Flow rates were calibrated prior to sampling with a flow meter, Dry Cal DC-Lite (BIOS-Butler). Cells were collected into biosamplers filled to the 20-ml mark with sterile phosphate-buffered saline (30 mM phosphate buffer, 125 mM NaCl, pH 7.2) for 30 min. During sampling, impingers were wrapped with aluminum foil to avoid sunlight inactivation of collected cells.

Aerosol samples for the phylogenetic library were collected with a MicroVIC bioaerosol concentrator (Mesosystems Technology, Kennewick, Wash.). The MicroVIC efficiently collects particles below 10 μ m at a flow rate of 400 liters/min and concentrates the sample into a flow of 12.5 liters/min for liquid impingement. The MicroVIC sampler was operated for 30 min. During each aerosol sampling time, effluent wastewater samples were taken from the irrigated site at a 1-cm depth. Upon collection, all samples were immediately stored at 4°C in the dark (20).

Bacterial culturing and enumeration. Aerosol and effluent wastewater samples were diluted in phosphate-buffered saline, and 0.2 ml of the sample was spread onto medium plates in triplicate in accordance with standard methods (2). R2A agar (Difco Laboratories, Detroit, Mich.) was used to produce heterotrophic plate counts. The plates were incubated aerobically at room temperature for 5 days. MacConkey agar (Difco Laboratories) was used for the select detection of culturable gram-negative enteric bacteria. MacConkey plates were incubated at 37°C for 24 h. Gram-negative enteric bacteria have previously been used as an indicator of domestic wastewater microorganisms (10, 12), and their generally higher concentration in effluent wastewater in comparison to fecal coliforms is useful for bioaerosol measurements, where very low concentrations may exist due to rapid inactivation and dilution in the atmosphere. In all cases, culturable assays were performed within 12 h of collection.

Epifluorescent microscopy was used to enumerate total bacteria (culturable and nonculturable) in accordance with the previously described method for liquid-impinged cells (14). Cells were stained with 4',6'-diamidino-2-phenylindole (DAPI) (Pierce, Rockford, Ill.) at a final concentration of 20 μ g of DAPI/ml, and vortexed for 1 min. Samples were then filtered onto a 25-mm-diameter, 0.2- μ m-pore-size black polycarbonate membrane (Osmonics, Inc., Minnetonka, Minn.) and observed on an Olympus BX51 microscope (Olympus, Melville, N.Y.) at $\times 1,000$ magnification. All direct counts were produced by counting a minimum of eight random fields and more than 400 total cells on each filter. Samples with a coefficient of variance between fields of greater than 20% were reanalyzed.

Total and culturable bioaerosol concentrations for each time point were de-

rived from the average of three spatially separated impinger samples. For statistical comparisons, bioaerosol concentrations were grouped into a low-wind-speed regimen (<1.9 m/s, average wind speed of 0.5 m/s, representing the first half of the sampling campaign) and a high-wind-speed regimen (≥ 1.9 m/s, average speed of 4.5 m/s, representing the second half of the sampling campaign). The 1.9-m/s threshold yielded average wind speeds that were substantially different, and this threshold maximized the data in each wind regimen for statistical comparison. Comparisons of average concentrations between the low- and high-wind-speed regimens were performed with a *t*-test analysis at a specified confidence level.

DNA extraction. For sample concentration and to aid in removal of dissolved PCR-inhibitory compounds, 3 ml of aerosol sample from the MicroVIC sampler, a 0.5-ml sample from the effluent wastewater, and a 5-ml sample of sterile deionized water for a negative control were filtered through a polycarbonate filter (10 mm by 10 mm square, 0.22 μ m pore size; Osmonics, Inc.). To resuspend cells, the filter was placed in a 1.5-ml tube with 150 μ l of extraction buffer I (150 mM disodium EDTA, 225 mM NaCl, 0.05% Tween 80, pH 8.5) (25) and vortexed for 1.5 min at maximum speed. The filters were then removed and discarded.

Cell lysis was initiated by addition of 45 μ l of lysozyme (50 mg/ml) and incubation for 30 min at 37°C. Then 10 μ l of tRNA (100 μ g/ml) (Sigma-Aldrich Co.) was added to aid in subsequent DNA precipitation. Next, 3 μ l of warmed (37°C) 25% sodium dodecyl sulfate and 3 μ l of proteinase K (20 mg/ml) were added to the solution and incubated for an additional 60 min at 37°C. For further chemical and mechanical lysis, 50 μ l of warmed (90°C) extraction buffer II (100 mM EDTA, 400 mM Tris-HCl, 400 mM Na_2PO_4 buffer, 1% cetyltrimethylammonium bromide [CTAB], pH 8.0) (25) and 9 μ l of 25% sodium dodecyl sulfate were added, followed by bead beating with 0.3 g of zirconium-silicon beads (0.1 to 0.5 mm in diameter) for 3 min at 5,000 rpm with a Mini-Beadbeater (BioSpec Products Inc., Bartlesville, Okla.).

Samples were subjected to three cycles of freezing and thawing (-80° C and 65° C). After the final cycle, nucleic acids were isolated with 200 μ l of chloroform-isoamyl alcohol (24:1). The tubes were centrifuged at $15,000 \times g$ for 5 min, and the aqueous top phase was transferred into clean tubes. Nucleic acids were cleaned and concentrated with the Ultraclean soil DNA kit (Mo-Bio, Inc., Solana Beach, Calif.). To precipitate nucleic acids, kit solution S3 was added to the sample at a 3:1 ratio (≈ 900 μ l) and gently mixed. This solution was then loaded into spin filters and cleaned in accordance with kit protocols. DNA was eluted with 30 μ l of nuclease-free water.

PCR, cloning, and sequencing. PCR amplification of the small-subunit rRNA gene was carried out in a 50- μ l volume reaction mixture containing 5 μ l of 10X buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM magnesium acetate), 1 μ l of deoxynucleoside triphosphate mix (0.2 mM each dATP, dCTP, dGTP, and dTTP), 5 μ l of bovine serum albumin (0.1 mg/ml), 4 μ l each of the universal primers 515F (5'-GTG CCA GC[C:A] GCC GCG G-3') and 1392R (5'-ACG GGC GGT GTG TRC-3') (1 ng/ μ l) (19), and 5 μ l of extracted DNA template. Five units of *Taq* DNA polymerase (Eppendorf, Westbury, N.Y.) was added after the initial 5-min hot start. Based on previous optimization experiments, the following thermal cycler (Eppendorf Mastercycler, PerkinElmer Inc., Boston, Mass.) temperature profile was used: hot start for 5 min at 80°C, 95°C for 5 min of initial denaturation, 30 cycles of 95°C for 1.5 min of denaturation, 52°C for 2 min of annealing, and 72°C for 3 min of extension, and terminal extension at 72°C for 10 min. Negative controls from DNA extraction of sterile filtered water were carried through the PCR step, and no amplification of this control was observed.

Amplicons were purified with a QIAquick PCR purification kit (Qiagen Sciences, Valencia, Calif.) and ligated into pCR4 TOPO plasmid vectors (TOPO TA cloning kits, Invitrogen Life Technologies, Carlsbad, Calif.) according to the manufacturer's instructions for maximum plasmid yields. Ligated vectors were transformed into chemically competent *Escherichia coli* cells and grown overnight on Luria-Bertani (LB) agar medium (Mikrobiologie, EM Science, Gibbstown, N.J.) amended with 100 μ g of ampicillin per ml. Prior to plasmid extraction, colonies were screened for inserts by PCR with plasmid-vector specific primers T3 (5'-ATT AAC CCT CAC TAA AGG GA-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3'). Positive clones produced a 1,051-bp PCR product containing the 891-bp small-subunit rRNA insert and the two 80-bp flanking regions of the plasmid vector. For plasmid extraction, positive colonies were selected from plates and individually cultured in LB broth amended with 100 μ g of ampicillin per ml. Plasmids were then extracted from each broth culture with a QIAprep spin miniprep kit (Qiagen Sciences).

Confirmed plasmid inserts were sequenced with a Big Dye sequencing kit (Applied Biosystems, Foster City, Calif.) with 5 μ l (ca. 500 ng) of the purified plasmid DNA with 20 ng of the plasmid-specific T3 and T7 primers. The reaction

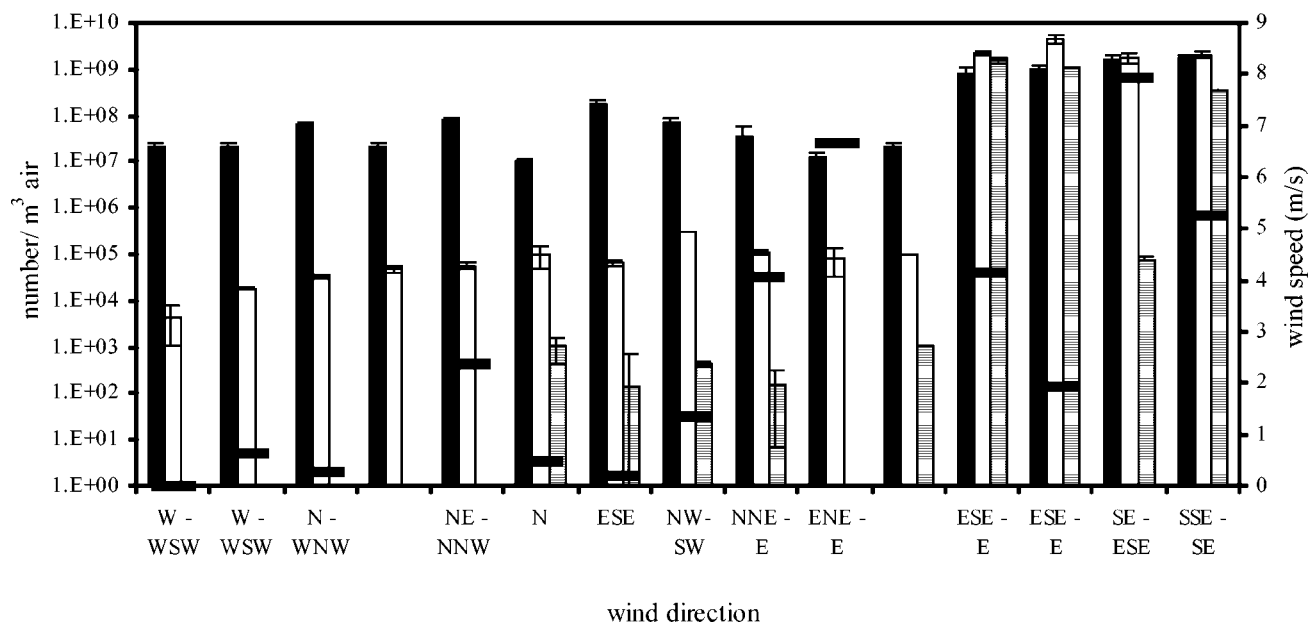


FIG. 1. Average total and culturable air-borne microbial concentrations adjacent to irrigated field at a height of 1.5 m. Bars: stippled, gram-negative enteric bacteria; open, heterotrophic plate counts; and solid, total bacteria. Wind speed is denoted by a dash, and wind direction is listed on the x axis. Error bars denote standard deviation from three separate aerosol samples. Data are presented in chronological order. No statistical difference was found between total and culturable concentrations for the last four samples ($P \leq 0.05$).

mixture was purified by ethanol precipitation with 80 μ l of ethanol-water-3 M sodium acetate (5:1:0.1, pH 5), and then separated and analyzed on an automated ABI Prism 377 DNA sequencer (Applied Biosystems). Retrieved sequences were compared with available sequences with the National Center for Biotechnology Information BLAST program (1). Sequences were aligned with ClustalX software (32). The Phylip software package was used for construction of phylogenetic trees by the neighbor-joining method and for construction of similarity matrices (11, 24).

Nucleotide sequence accession numbers. The Mexicali, Mexico, aerosol- and effluent wastewater-derived sequences have been deposited in the NCBI GenBank database under accession numbers AY532421 to AY532481 (air clones) and AY534152 to AY534194 (wastewater clones). Nucleotide matrix data have also been entered into the TreeBase database under SN1783.

RESULTS

Aerosol microbial concentrations. Bioaerosol results were divided into two wind speed regimens: (i) below 1.9 m/s, average speed 0.5 m/s, and (ii) above 1.9 m/s, average speed 4.5 m/s. The concentrations of total and culturable air-borne microorganisms obtained during the field sampling campaign are presented chronologically in Fig. 1. Wind speed and wind direction are also included in this figure. When grouped into the wind speed regimens, the average concentrations of total bacteria, heterotrophs (heterotrophic plate counts), and culturable gram-negative enteric bacteria were 1.1, 4.2, and 6.2 orders of magnitude greater, respectively, in samples collected during the high-wind-speed regimen than those obtained during a low wind speed (Fig. 2). A *t* test analysis confirmed that the aerosol concentration increased ($P \leq 0.05$ for total bacteria and heterotrophic plate counts and $P \leq 0.13$ for gram-negative enteric bacteria).

Daytime and nighttime total and culturable aerosol concentrations were not different ($P \leq 0.05$) when tested with all of the sampling data or a subset of data controlled for similar wind conditions. All aerosol measurements were taken under

clear conditions. The average daily peak in solar radiation (UV-A and UV-B) was 2.1 ± 10.0 (standard deviation) mW m^{-2} , while minimum nighttime solar radiation averaged $3 \times 10^{-6} \pm 1 \times 10^{-6} \text{ mW m}^{-2}$. During the sample campaign relative humidity levels were stable, averaging $37\% \pm 9\%$ (standard deviation) and ranging from 21% to 51%.

Effluent wastewater microbial concentrations. Total microorganisms, heterotrophic plate counts, and gram-negative enteric bacteria concentrations for the effluent wastewater did not change significantly ($P \leq 0.05$) throughout the sampling campaign. For all samples, the average concentration and stan-

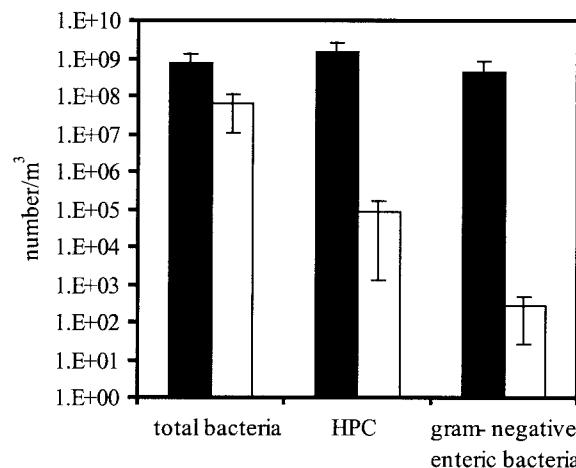


FIG. 2. Average aerosol concentrations of total bacteria, heterotrophic plate counts, and gram-negative enteric bacteria for the high (solid bars) and low (stippled bars) wind speeds. Error bars denote standard error ($n = 7$ for the low-wind-speed regimen, $n = 6$ for the high-wind-speed regimen).

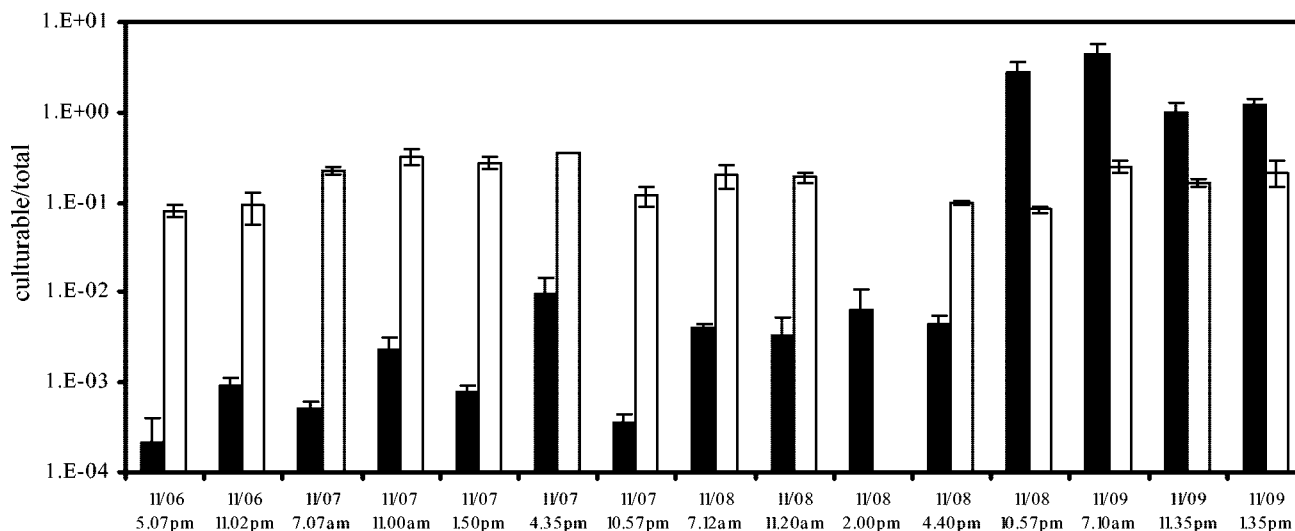


FIG. 3. Ratio of heterotrophic plate counts/total number of microorganisms in aerosol and effluent wastewater. Bars: solid, aerosol ratio; open, effluent wastewater ratio. Error bars denote standard deviation for the ratio of three separate aerosol and three separate wastewater samples. The 8 November 2 p.m. data point for the effluent wastewater sample was not recorded.

standard deviation of total microorganisms, heterotrophic plate counts, and gram-negative enteric bacteria were $1.6 \times 10^8 \pm 1.3 \times 10^7$ number/ml, $2.9 \times 10^7 \pm 4.2 \times 10^6$ CFU/ml, and $2.3 \times 10^7 \pm 4.2 \times 10^5$ CFU/ml, respectively. Figure 3 presents a comparison between the ratio of heterotrophic plate counts to total microorganisms for each effluent wastewater and aerosol sample. This figure demonstrates that changes in the culturability of aerosol samples cannot be attributed to variations in the effluent wastewater culturability.

Phylogenetic analyses of air and wastewater populations.

Aerosol and effluent wastewater populations were further characterized by building phylogenetic clone libraries based on small-subunit rRNA gene sequences. Libraries were constructed from genomic DNA extracted from the final aerosol and effluent wastewater samples. These samples were chosen based on the high total microorganism, heterotrophic plate counts, and gram-negative enteric bacteria aerosol concentrations, as well as a high average wind speed (5.2 m/s). Seventy-seven aerosol clones and 48 effluent wastewater clones were successfully sequenced. Bacteria and eukaryotes were present in both aerosol and effluent wastewater samples (Table 1). The majority (69%) of sequences cloned from the effluent wastewater matched closely with database sequences within the *Proteobacteria* phylum, while sequences cloned from the aerosol samples were dominated (51%) by sequences within the gram-positive phylum.

Several similarities between the aerosol and effluent wastewater populations exist, as depicted in Table 1 for the eukaryotic phylogenetic tree (Fig. 4) and the bacterial phylogenetic tree (Fig. 5). With the exception of one sequence placed in the fungi, all of the eukaryotic cloned sequences correspond to three distinct groups: the red algae (*Rhodophyta* phylum), the *Heteromita* genus, and the *Spumella* genus. Clone sequences derived from both aerosol and effluent wastewater samples were present in each of these groups, and the *Heteromita* sp. and *Spumella* sp. both contained clones from aerosols and effluent wastewater that were more than 98% similar.

The phylogenetic tree for bacteria is shown in Fig. 5. In several clusters, clone sequences retrieved from both the aerosol and effluent wastewater samples are present and are greater than 98% similar (e.g., Air U87 and EWW U05), suggesting that the effluent wastewater is a source for some bacterial bioaerosols. A notable exception is the cluster of gram-positive sequences most closely related to members of the genus *Exiguobacterium* and found only in aerosol samples. The majority of gram-positive clones retrieved, however, were closely related to *Acholplasma* spp., and these clone sequences were found in both aerosol and effluent wastewater samples at more than 99% similarity.

Other clones present in aerosol samples were more than 97% similar to specific genera of the proteobacteria that are commonly identified in domestic wastewater and are culturable on MacConkey agar. These genera include *Aquaspirillum*,

TABLE 1. Phylogenetic distribution of clones

Taxonomic affiliation	% of clones	
	Aerosol	Wastewater
<i>Proteobacteria</i> subgroup		
α	1	10
β	9	38
δ	1	0
γ	12	21
Total	23	69
Gram-positive bacteria (<i>Firmicutes</i>)	51	6
<i>Cytophaga</i> (<i>Bacteroidetes</i>)	13	10
Eukaryota		
Stramenopiles	8	8
Cercozoa	4	6
Rhodophyta	1	1
Total	13	15

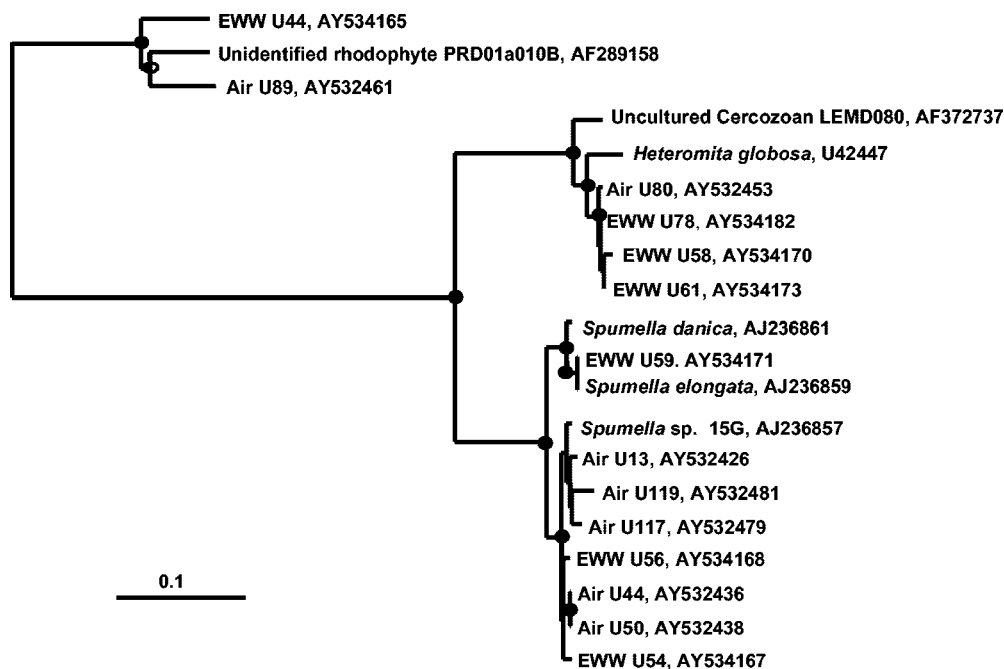


FIG. 4. Eukaryotic phylogenetic tree of 18S rRNA sequences obtained from the air and effluent wastewater (EWW) and related sequences retrieved from GenBank. The tree is based on a comparative analysis of 600 nucleotides. The scale corresponds to 0.1 substitution per nucleotide position, and the tree was rooted with the 16S rRNA sequence from *Methanobacterium congolense* AF233586 (not shown) as an outgroup. Solid circles at nodes indicate >75% bootstrap support, half-shaded circles represent 50% to 75% bootstrap support, and open circles represent less than 50% support from 1,000 resamplings.

Escherichia, *Alcaligenes*, *Shewanella*, *Pseudomonas*, and *Pantoea*.

DISCUSSION

The results obtained during this study suggest that viable wastewater microorganisms can be aerosolized from flood irrigation sites during high-wind events. This conclusion is supported by data derived from the culturable and molecular analysis of effluent wastewater and aerosol samples. At air sampling sites downwind of and adjacent to the flooded fields, total, heterotrophic plate count, and indicator gram-negative enteric microorganism concentrations increased markedly from the low- to the high-wind-speed regimen. The ratio of heterotrophic plate counts to total cells was also higher in the high-wind-speed regimen. In all cases for the high-wind-speed regimen (average speed ≥ 1.9 m/s), samplers were oriented toward the irrigation field and directly into the wind. During the low-wind-speed regimen (average speed < 1.9 m/s), samplers faced toward the irrigated field and both into and away from the wind. In this regimen, however, aerosol concentrations varied by less than one order of magnitude regardless of the wind direction (Fig. 1).

In previous aerosol studies that measured both total and culturable aerosols, Tong and Lighthart (33) similarly detected an increase in the culturable fraction of cells (1:222 to 1:27, heterotrophic plate counts to total) when sampling aerosols above a grass field in which the higher culturability coincided with the highest wind speeds. In addition, the stable culturability results from the effluent wastewater (Fig. 3) demonstrated that changes in bioaerosol concentration and culturability ob-

served in air were not related to changes in temporal wastewater concentration variability. Previous wastewater spray irrigation and open activated sludge basin studies also found that changes in bioaerosol concentrations were not correlated with changes in source concentrations (3, 9, 30).

Finally, while the limited conclusion of higher bioaerosol concentration during the high-wind-speed regimen was supported by the data, uncertainties common in full-scale aerosol research such as variable wind speeds and spatial variation in the wastewater source did not allow for determining a more precise fundamental relationship that describes bioaerosol source concentration as a function of wind speed.

No relationship was observed between aerosol culturable concentrations or culturability ratios and solar radiation levels. This lack of correlation was observed through statistical analysis with either all of the sampling data or analysis restricted to only high- or low-wind-speed regimens. Pilot-scale solar radiation studies on air-borne *E. coli* have demonstrated that at the nearly 50% relative humidity levels observed during this sampling campaign, air-borne inactivation rates are mostly independent of solar radiation, as inactivation is dominated by nonsolar effects (23).

Analysis of the small-subunit rRNA clone library provided valuable information for understanding the aerosolization of wastewater microorganisms under high-wind conditions. Greater than 50% of the clones in the aerosol library were more than 97% similar either to clones found in effluent wastewater or to proteobacteria commonly found in domestic wastewater. However, the standard manner in which aerosol and liquid-phase samples are collected ensures that the popula-

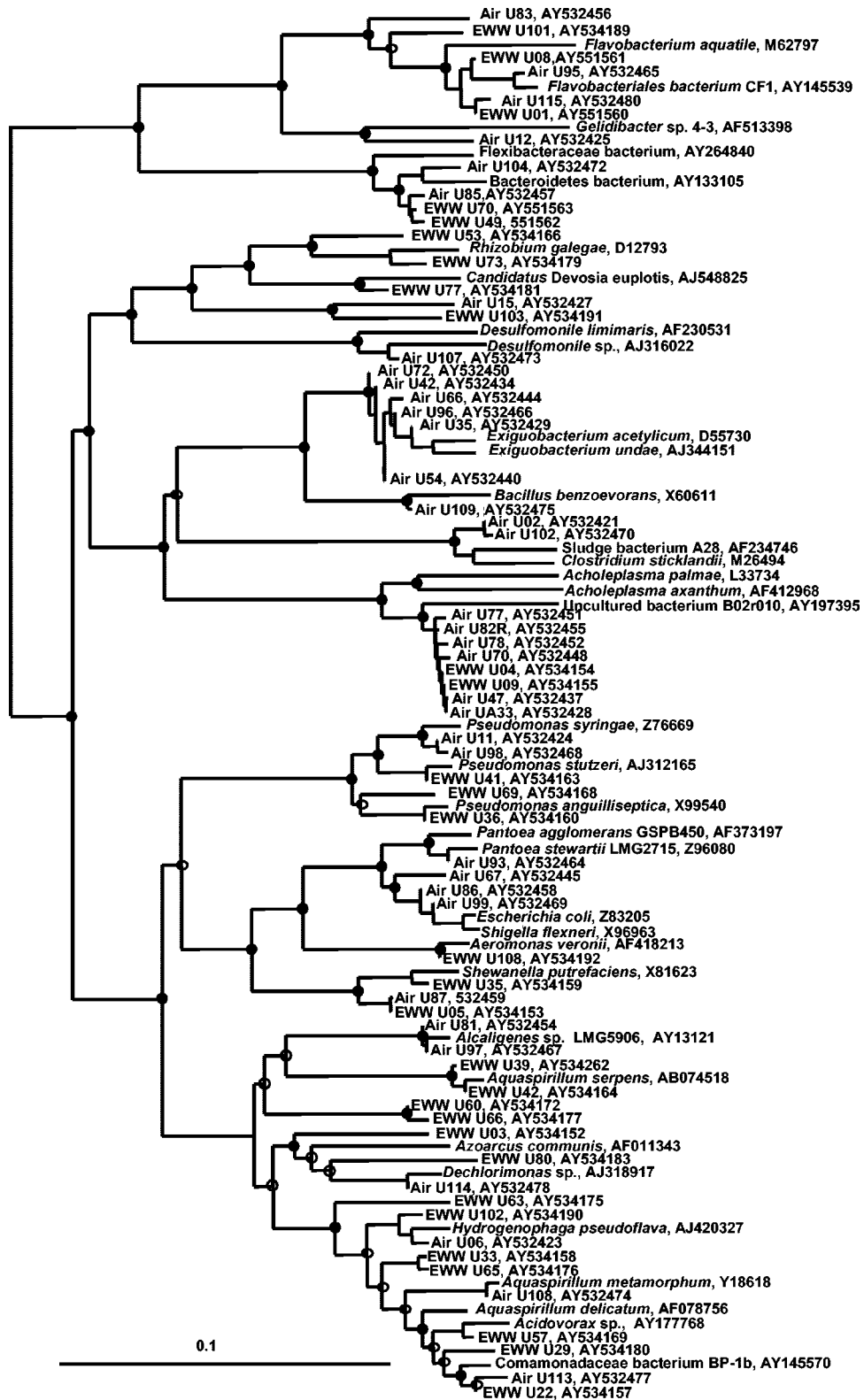


FIG. 5. Prokaryotic phylogenetic tree of 16S rRNA sequences obtained from the air and effluent wastewater (EWW) and related sequences retrieved from GenBank. The tree is based on a comparative analysis of 600 nucleotides. The scale corresponds to 0.1 substitution per nucleotide position, and the tree was rooted with the 16S rRNA sequence from *Methanobacterium congolense* AF233586 (not shown) as an outgroup. Solid circles at nodes indicate >75% bootstrap support, half-shaded circles represent 50% to 75% bootstrap, and open circles represent less than 50% support from 1,000 resamplings.

tions described in this study will generally have differences even if the effluent wastewater is the overwhelming source of bioaerosols.

In aerosol liquid impingement, microbial particles between 1 μm and at least 10 μm in aerodynamic diameter are collected efficiently (15). In this study microscopic analysis showed that while the liquid wastewater samples contained monodispersed cells smaller than 10 μm , particles with associated microorganisms that were greater than 30 μm were also present. When wastewater is aerosolized, smaller microbial particles will remain suspended in air, while larger particles may settle before sampling or may be collected at a different efficiency. Due to these inherent limitations and previously described biases in cloning, such as random error from undersampling the full microbial diversity or unknown vector toxicity (21, 29), tracking of aerosols from effluent wastewater was accomplished by comparing the similarity of individual or groups of aerosol clone sequences to effluent wastewater clone sequences rather than comparing entire populations.

Complex factors such as the physical mixing height of the atmosphere, wind speed, wind-derived aerosol sources, airborne inactivation, and low culturability of environmental bacteria necessitate a combination of culture-based and molecular techniques to characterize environmental sources of bioaerosols. These analyses applied to a sample where both low and high wind speeds were observed indicate that wind may be a substantial mode of aerosolization of viable wastewater microorganisms at flood irrigation sites. While other studies have also linked changes in culturable aerosol concentration near wastewater spray irrigation sites or activated sludge basins with changes in atmospheric stability (4, 9, 30), this work describes the potential of wind as an aerosolization mechanism of culturable microorganisms contained in standing irrigation water. Given the growing need to reuse effluent wastewater for irrigation in water-scarce regions throughout the world, the occurrence of high-wind events poses an increased potential of human exposure for workers and nearby residents through the air-borne route.

ACKNOWLEDGMENTS

This work was sponsored by an Arizona State University Seed Grant and the Fulbright Foundation-Uruguay.

We thank MesoSystems Technology, Inc., for use of the MicroVIC high-volume aerosol sampler.

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