Tracking Airborne *Legionella* and *Legionella pneumophila* at a Biological Treatment Plant

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Biological treatment plants are frequently used to degrade organic substances in wastewater from wood refinement processes. Aeration ponds in such plants provide an optimal growth environment for many microorganisms, including *Legionella* species. To investigate whether legionellae could be dispersed as aerosols from the ponds and transported by the wind, the wetted-wall cyclone SASS 2000^{PLUS} and the impactors MAS-100 and STA-204 were used to collect air samples directly above, upwind, and downwind of aeration ponds during a 4-month period. Computational fluid dynamics was used *a priori* to estimate the aerosol paths and to determine suitable air-sampling locations. Several *Legionella* species, including *Legionella pneumophila*, were identified in air samples at the biological treatment plant using microbiological and molecular methods. *L. pneumophila* was identified up to distances of 200 m downwind from the ponds, but, in general, not upwind nor outside the predicted aerosol paths. The highest concentration level of viable legionellae was identified directly above the aeration ponds (3300 CFU/m^3). This level decreased as the distance from the aeration ponds increased. Molecular typing indicated that a single clone of *L. pneumophila* was dispersed from the ponds during the period of the study. Thus, our study demonstrated that aerosols generated at aeration ponds of biological treatment facilities may contain *L. pneumophila,* which then can be transported by the wind to the surroundings. The methods used in this study may be generically applied to trace biological aerosols that may pose a challenge to environmental occupational health.

Introduction

Organic substances present in industrial wastewater originating from, for instance, paper mills must be degraded in order to reduce the impact these substances have on the ecosystem. Borregaard Ind. Ltd., Sarpsborg, Norway, is the world's leading supplier of wood-based chemicals. All wastewater from Borregaard's wood refinement processing is biologically treated according to environmental requirements legislated by the Norwegian Environmental Protection Agency. The biological treatment facility at Borregaard Ind. Ltd. consists of two large aeration ponds containing 30 000 m^3 of liquid. The liquid is kept at approximately 37 °C to promote optimal growth of microorganisms achieving efficient degradation of various organic substances. As about 30 000 m^3 of air is circulated through each pond per hour (20 °C), large amounts of surface evaporation take place. Droplets are aerosolized from the surface of the liquid. These droplets may vary in size, may contain microorganisms originating from the ponds, and may be dispersed and transported by the wind.

The genus *Legionella* comprises bacterial species that are ubiquitous in aqueous environments at temperatures ranging from 5 to 50 °C. *Legionella* bacteria have been identified in potable water systems, hot springs, cooling-tower systems, wastewater treatment plants, chemical industrial plants, water samples taken on ferries and cruise ships, and in hospitals and dental unit waterlines (*1*−*9*). Recently, legionellae have been identified in aeration ponds up to 10^9 CFU/L at Swedish biological treatment facilities (*4, 5*), and $10^8 - 10^{10}$ CFU/L at Borregaard Ind. Ltd. ((*5*), and this study). Airborne legionellae have previously been detected at industrial cooling towers (*6, 10*−*12*), sanitary landfill sites (*13*), wastewater treatment plants (*14, 15*), and evaporate condensers (*16*).

Legionella are Gram-negative coccobacilli requiring soluble iron and L-cysteine for initial isolation and optimal growth. More than fifty species are recognized. A minority of *Legionella* species are pathogenic for humans, the foremost being *Legionella pneumophila*, a causative agent of Legionnaires' disease. *L. pneumophila* comprises at least sixteen different serogroups, with serogroup 1 responsible for more than 90% of the cases of Legionnaires' disease (*17*).

In addition to bacteriological methods, real-time PCR using specific primers and hybridization probes targeting the 16S rRNA and the *mip* (encoding the macrophage infectivity potentiator involved in the virulence of *L. pneumophila*) genes facilitates detection of *Legionella* and *L. pneumophila*, respectively, in environmental samples (*18*−*24*).

The dispersion and transport of aerosols (droplets or solid particles) are determined by complex and mutually dependent physical processes. Outdoor dispersion of aerosols is dominated by the atmospherically driven wind field, and are in general classified according to the horizontal dimension and time periods during the most significant meteorological event. These include (i) macro-scale events characterized by spatial and time scales more than 2000 km and 3 days, respectively, (ii) microscale processes due to spatial scales and temporal variations less than 2 km and 1 h, respectively, and (iii) meso-scale events including spatial and temporal scales of 2−2000 km and 1 h to 3 days, respectively. The microscale processes are the major driving dynamical processes governing the wind field in and over building clusters. These events are generated by atmospheric and local turbulence, street canyon effects, wakes downstream buildings, and at low wind speeds thermal effects are important.

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The most reliable approach to predict microscale processes, such as the wind field at complex industrial sites, is computational fluid dynamics (CFD) (*25*).

In May 2005 an outbreak of legionellosis, caused by *L. pneumophila* serogroup 1, occurred in Sarpsborg/Fredrikstad, Norway. The wet scrubber at Borregaard Ind. Ltd. was identified as the source of the outbreak (*26*). This study indicated that legionella could be dispersed and transported in ambient air at least 10 km from the source. During another outbreak in Pas-de-Calais, France, 2003, it was observed that *L. pneumophila* was transported in air 7 km from the source (*6*).

Little information is available on human exposure to *Legionella* at biological treatment plants. To investigate whether aerosols containing legionellae could be generated from the aeration ponds at Borregaard's biological treatment facility, and further transported and dispersed by wind, a comprehensive experiment using CFD as a planning tool was performed. The transport of water droplets, potentially containing legionellae, dispersed from the aeration ponds was estimated using CFD, and the results were used to select optimal sampling sites for air collection in the vicinity of the ponds. Air sampling was performed by using the wetted-wall cyclone SASS 2000^{PLUS} and the impactors MAS-100 and STA-204. Airborne *Legionella* species, including *L. pneumophila,* were specifically detected using microbiological and molecular analysis. The methods described in this study can be used to track aerosols containing biological agents in various environmental occupational health studies.

Materials and Methods

CFD. The biological treatment plant at Borregaard Ind. Ltd. and its surrounding area cover approximately 1 km × 1 km. This area was geometrically modeled in three dimensions, including detailed topography and major building structures. The computational resolution (i.e., grid size) surrounding the ponds was approximately 0.7 m in the horizontal, vertical, and lateral directions, respectively, whereas the horizontal resolution decreased as the grid size increased away from the aeration ponds. The height of the computational domain was approximately 250 m. The total computational grid consisted of approximately 8.3 million cells and for each wind direction the computational time was about 5 h. Twenty-four different wind directions (evenly separated by 15 degrees) were used to cover the full circle of 360 degrees.

A steady state Reynolds Averaged Navier−Stokes (RANS) approach was used (*25*). The temporal variation of the mesoscale wind field (input for the computation) was significantly longer than the air sampling time in this study. Thus, the external wind conditions were assumed to be steady for each wind direction. Based on historical weather data, a typical wind speed of 2 m/s was used in all twenty-four cases. To simplify the computational modeling, thermal (buoyancy) effects and the environmental fate of the biological aerosols were neglected. The aerosols originating from the aeration ponds were treated as passive tracers following the local wind direction. The aerosol properties were assumed to remain constant during the transport (i.e., coagulation and evaporation were not considered), and the effect of gravity was neglected. The weather forecasts were provided by the Norwegian Meteorological Institute service for the Norwegian Armed Forces.

Air Sampling. Air was collected at ten different dates during September 11 to December 5, 2006, at varying weather conditions (Table 1). Twenty-four sampling sites were selected using CFD modeling at Borregaard Ind. Ltd. (Figure 2). Sampling was performed at various distances (Figure 2) and altitudes (0−64 m above sea level) within the industrial site. Sampling was performed at roofs of buildings on site representing the various altitudes. The wetted-wall cyclone SASS 2000^{PLUS} (Research International) was used for collecting air in 5 mL of PAGE buffer (120 mg of NaCl, 4 mg of $MgSO_4 \cdot 5H_2O$, 4 mg of $CaCl_2 \cdot 2\,H_2O$, 142 mg of Na_2HPO_4, 136 mg of KH_2PO_4 per L of distilled water, pH 6.8 ± 0.2 at 25 °C). No growth of legionellae was observed in the PAGE buffer prior to sampling. The sampling time was 1 or 2 h, corresponding to 19.5 m^3 or 39 m^3 air, respectively (flow rate; 325 L/min). SASS 2000^{PLUS} automatically refills liquid in order to keep the sampling volume constant (5 mL). The SASS 2000^{PLUS} was washed with a 0.5% chlorine solution followed by sterile distilled water with the fan removed. The SASS 2000^{PLUS} air samples were acid- (0.2 M HCl, 0.2 M KCl, pH 2.2 ± 0.2) (5 min) and heat-treated (50 °C, 30 min) prior to microbial growth analysis to select for growth of legionellae. One hundred μL of nondiluted or 10-fold concentrated air samples were plated on GVPC agar selective for *Legionella* spp.

The impactors MAS-100 (flowrate; 100 L/min) (Merck) and STA-204 (flowrate; 30 L/min) (New Brunswick Scientific) were used for collecting airborne *Legionella*-containing particles directly onto GVPC agar for 10 min (1 m^3 air) and 60 min (1,8 m^3 air).

Air sampling was not performed at temperatures below −2 °C.

Bacterial Strains and Growth. The GVPC collection plates were immediately transported to the laboratories and incubated in a humidity chamber at 36 °C up to 10 days. Typical *Legionella* colonies were detected by fluorescence using a 360 nm lamp daily, and a dissecting microscope (20−40 ×) (ground-glass appearance and iridescence). Selected colonies not growing on blood agar, and identified as small Gram negative rods were considered suspected legionellae (*27*), and preserved at −70 °C until further examination.

Liquid samples harvested from the aeration ponds were acid- and heat-treated as described for the air samples before plating on GVPC agar.

The *L. pneumophila* reference strains for serogroup 1−14, ATCC 33152, ATCC 33154, ATCC 33155, ATCC 33156, ATCC 33216, ATCC 33215, ATCC 33823, ATCC 35096, ATCC 35289, ATCC 43283, ATCC 43130, ATCC 43290, ATCC 43736, and ATCC 43703, *L. micdadei* ATCC 33218, *L. maceachernii* ATCC 35300, *L. bozemanii* ATCC 33217, *L. brunensis* ATCC 43878, *L. dumoffi* ATCC 33279, and *L. longbeachae* ATCC 33462, were purchased from the American type Culture Collection (ATCC).

Isolation of Nucleic Acids. Isolation of nucleic acids was performed with the NucliSens Basic kit (BioMèrieux Ltd.) (*28*) according to the manufacturer's recommendation, generally adding 13.5 mL of NucliSENS lysis buffer (5 M guanidin thiocyanate, Tris/HCl, Triton X-100) and 50 μL of silica beads (NucliSENS Isolation Kit) to the collected air sample.

The *Legionella* ATCC reference strains were used to test the specificity of the *mip* primers, and *L. pneumophila* ATCC 33152 was used as positive control in the real-time PCR assays. One colony of these strains was dissolved in 1 mL of PBS buffer (phosphate buffered saline, pH 7.4) and heated at 90 °C for 10 min.

Nucleic acids from the aeration pond liquid samples were isolated with the NucliSens Basic kit (BioMèrieux Ltd.) (*28*). The liquid samples were vortexed for 5 s and 1.5 mL was centrifuged at 1000 rpm for 1 min to sediment the lignin. The supernatant (150 μL) was added to 900 μL of lysis buffer and 50 μL of silica beads. The manufacturer's procedure was then followed.

The isolated nucleic acid extracts were used as templates in the real-time PCR assays.

Real-Time PCR. The LPmip-PT69 (5′ GCATTGGTGC-CGATTTGG) and the LPmip-PT70 (5′ GYTTTGCCATCAAA-

TABLE 1. Air Sampling, Molecular, and Microbiological Analysis of Isolated Airborne *Legionella* at Borregaard's Biological Treatment Facility

date and weather[a]	air collector[b]	sampling site[c]	real-timePCR[d]	CFU/m3[e]	16S rDNA sequencing[g]
11.09.06; cloudy; 4.0 m/s; 200 deg	STA-204	3	nd	15	*L. bozemanii*
		4	nd	1	nd
	MAS-100	3	nd	21	*L. pneumophila*
		4	nd	nd	*L. pneumophila*
	SASS 2000	3	+	111	nd
		4	+	7	nd
21.09.06; overcast; 4.5 m/s; 220 deg	SASS 2000	3	+	nd	nd
		7	+	4	nd
	STA-204	3	nd	118	*L. pneumophila, L. bozemanii*
		7	nd	18	*L. bozemanii*
27.09.06; rain; 3.0 m/s; 190 deg	STA-204	3	nd	>200	*L. bozemanii, L. pneumophila*[g]
		8	nd	47	*L. bozemanii*
		5	nd	0,4	nd
	SASS 2000	3	+	52	nd
		8	+	2	nd
11.10.06; cloudy; 2.5 m/s; 50 deg	SASS 2000	3	+	3300	nd
		11	-	300	nd
	MAS-100	3	nd	14	*L. pneumophila, L. bozemanii*[h]
		11	nd	nd	nd
18.10.06; rain; 1.5−3.5 m/s; 60 deg	SASS 2000	3	+	40	nd
		11	+	25	nd
	MAS-100	3	nd	66	*L. bozemanii, L. pneumophila, L. dumoffii*
		12	nd	94	*L. bozemanni, L. pneumophila, L. oakridgensis*
		11	nd	nd	*L. bozemanii, L. oakridgensis*
25.10.06[f]; sunny; 0 m/s 50−240, deg and 0.5 m/s, 240 deg	SASS 2000	3	+	180	nd
		11	-	0,4	nd
		13	-	nd	nd
		10	+	13	nd
	MAS-100	3	nd	420	*L. bozemanni, L. pneumophila, L. oakridgensis*
		11	nd	nd	nd
		13	nd	nd	nd
		10	nd	26	*L. bozemanni, L. pneumophila, L. oakridgensis*
16.11.06; rain; 4.0 m/s; 180 deg	SASS 2000	1	+	1,7	nd
		7	+	1,7	nd
		16	+	0,5	nd
		15	-	nd	nd
		14	-	nd	nd
		17	-	nd	nd
	MAS-100	1	nd	10	nd
		7	nd	nd	*L. pneumophila*
		16	nd	nd	nd
		15	nd	nd	nd
		14	nd	nd	nd
		17	nd	nd	nd
22.11.06; rain showers; 2.5 m/s; 130 deg	SASS 2000	2	+	440	nd
		18	-	2	nd
		19	+	24	nd
		20	-	30	nd
		21	+	197	nd
		22	+	4	nd
	MAS-100	2	nd	45	nd
		18	nd	22	*L. londiniensis/L. nautarum, L. oakridgensis*
		19	nd	70	*L. pneumophila, L. londiniensis/L. nautarum, L. oakridgensis*
		20	nd	34	*L. londiniensis/L. nautarum, L. oakridgensis*
		21	nd	48	*L. pneumophila, L. londiniensis/L. nautarum, L. oakridgensis*
		22	nd	10	*L. pneumophila, L. oakridgensis*[l]

TABLE 1. Continued

date and weather[a]	air collector[b]	sampling site[c]	real-timePCR[d]	CFU/m[3e]	16S rDNA sequencing[g]
29.11.06; sunny, cloudy; 5.0 m/s; 230 deg	SASS 2000	3	+	93	nd
		25	+	nd	nd
		24	-	0,9	nd
		15	-	2,7	nd
	MAS-100	3	nd	4,6	*L. pneumophila, L. londiniensis/ L. nautarum*
		15	nd	2	*L. pneumophila*
		25	nd	nd	nd
		24	nd	2	nd
		23	nd	nd	*L. pneumophila*
05.12.06; rain; 3.8 m/s; 180 deg	SASS 2000	1	+	79	nd
		20	+	7,2	nd
		14	+	43	nd
		26	-	6,3	nd
	MAS-100	1	nd	30	*L. londiniensis/L. nautarum*
		20	nd	5	*L. londiniensis/L. nautarum*
		14	nd	5	*L. londiniensis/L. nautarum*
		26	nd	3	*L. londiniensis/L. nautarum*
		7	nd	3	*L. londiniensis/L. nautarum*

[a] The wind direction is given as degrees, in which 0 and 360 degrees (deg) represent wind from north and south, respectively. The wind speed and direction were monitored during the sampling campaign. [b] The air collectors are described in Materials and Methods. [c] Locations of sampling sites are shown in Figure 2. [d] *mip* real-time PCR analysis was performed only on the SASS 2000PLUS air samples. [e] CFU reflects the total number of colonies identified as *Legionella* species according to the growth analysis and the drySPOT *Legionella* latex agglutination test. [f] Variable wind conditions occurred. Between 10.30 and 14.00 h the wind direction and speed varied between 50 and 240 deg and 0 and 0.5 m/s, respectively. [g] Species identification was obtained by BLAST analysis of the 16S rDNA sequences.

TCTTTCTGAA) primers, and the specific hybridization probes LPneu-FL (5′ CCACTCATAGCGTCTTGCATGCCTTTA) and LPneu-LC (5′ CCATTGCTTCCGGATTAACATCTATGCC) targeting the *mip* gene (186 bp amplicon), were used for specific detection of *L. pneumophila* (*19*). The real-time PCR assays were performed in 20 μL of real-time PCR assay mixture containing 1 μm primers, 0.2 μm hybridization probes, and 5 μL template, using a LightCycler (Roche Diagnostics) and the LightCycler FastStart DNA MasterPLUS HybProbe hot start reaction mix. The reaction mixture was denatured at 95 °C for 10 min followed by a 50 cycle PCR profile; denaturation at 95 °C for 0 s, annealing at 62 °C for 10 s, and extension at 72 °C for 15 s. Specific amplification was indicated by the threshold-cycle (C_T) value representing the cycle number at which the fluorescence intensity crossed a fixed threshold ten times the standard deviation of the baseline intensity. The amplicons were verified by electrophoresis (Agilent 2100 Bioanalyzer). All real-time PCR assays were performed in triplicate. Distilled sterile water was used as a negative control.

Species Identification. Partial DNA sequencing of the 16S rRNA gene was performed of the *Legionella* isolates for species identification (*29*).

Serotyping of *L. pneumophila* Isolates. The drySPOT *Legionella* latex agglutination test (Oxoid) was used for determining the serogroup of *L. pneumophila* isolates.

Sequence-Based Typing of *L. pneumophila* Isolates. Defined fragments from six genes (*flaA, pilE, asd, mip, mompS*, and *proA*) were sequenced, as described by Gaia et al. (*30*) to identify the sequence type of the isolates.

Results

CFD. Each of the 24 CFD simulations provided a 3-dimensional picture of the aerosol transport originating from the aeration ponds at a given wind direction using a steady state RANS approach. Each result reflected the averaged path lines of aerosols valid for time scales up to a few hours for that particular wind direction (Figure 1). The best-suited regions for air sampling were identified by a combined evaluation of the height and density of the aerosol paths, and the location and height of adjacent building structures (Figure 2).

Air Sampling. Air sampling was performed at 24 different sites at Borregaard Ind. Ltd. (Figure 2). The wetted-wall cyclone collector SASS 2000PLUS was chosen for sampling of air into liquid based on its size, portability, moderate flow rate, and ability to perform 1−2 h sampling retaining the sampling liquid volume. Three SASS 2000PLUS, three STA-204, and one MAS-100 collectors were placed at the selected air sampling sites based on the wind direction and the corresponding CFD simulations (Figures 1 and 2). In general, one SASS 2000PLUS collector and one impactor were positioned directly above, upwind, and downwind of the aeration ponds. Air samples were also collected in regions outside the predicted aerosol transport paths. The SASS 2000PLUS and the impactors collected concentration levels of *Legionella* bacteria up to 3300 CFU/m^3 and 420 CFU/m^3, respectively (Table 1).

Real-Time PCR of Liquid Air Samples. The Lmip-PT69/Lmip-PT70 primers and the corresponding hybridization probes (*19*) were chosen for real-time PCR analysis of air sampled by the SASS 2000PLUS collector. The specificity of the *L. pneumophila* Lmip-PT69/Lmip-PT70 primers and the LPneu-FL/LC640 probes was verified by testing fourteen different *L. pneumophila* strains, representing serogroups 1−14. All real-time PCR analyses resulted in specific, amplified products of the *mip* gene fragment. No amplification was obtained when non-*pneumophila Legionella* species were tested (i.e., *L. micdadei*, *L. maceachernii*, *L. bozemanii*, *L. brunensis*, *L. dumofii*, and *L. longbeachae*).

mip Real-time PCR detected *L. pneumophila* in 22 out of 34 air SASS 2000PLUS samples (excluding samples taken upwind of the aeration ponds) (Table 1), and *mip*-amplicons were detected from all air samples collected directly above the aeration ponds and at twelve different sampling sites downwind of the ponds. In general, air samples collected upwind of the aeration ponds, or outside the predicted aerosol pathways did not contain *L. pneumophila* (no *mip* amplicon), except on one occasion (October 25). The amplification of *mip* generally correlated with the identification of *L. pneumophila* by microbial growth analysis (corresponding impactor samples). In some cases, *mip*-amplicons were ob-

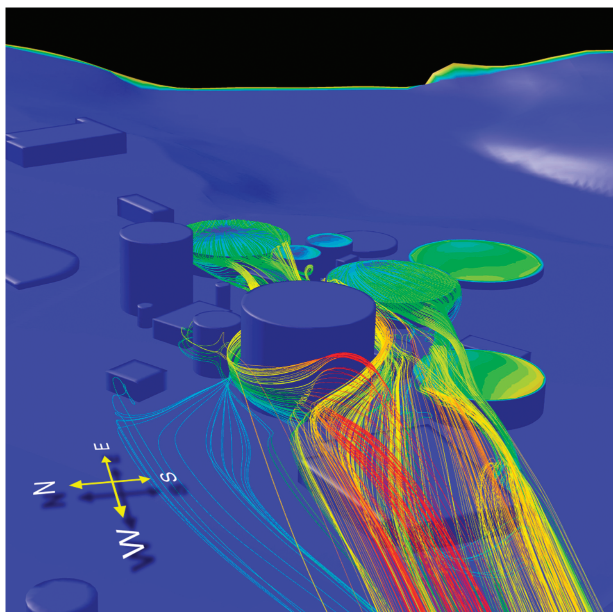

FIGURE 1. Computed paths of particles dispersed from the aeration ponds. The wind direction is from east to west (270 degrees) and wind speed is 2 m/s. Colors indicate altitudes relative to the aeration ponds: blue = low, red = high.

tained where *L. pneumophila* was not identified by growth analysis and 16S rRNA gene sequencing (Table 1).

These results showed that the SASS 2000^PLUS can be used for sampling of viable (culturable) *Legionella* spp., including *L. pneumophila,* and that *mip* can be regarded as an efficient target gene for specific detection of *L. pneumophila*.

Air Samples Collected by Impactors. The species assignment of 169 *Legionella*-like colonies on the GVPC impactor plates was obtained by 16S rDNA sequencing. BLAST analysis showed that these 16S rDNA sequences were either identical or very similar (>97% similarity) to the equivalent sequences deposited in GenBank for six *Legionella* species: *L. pneumophila* (100%), *L. bozemanii* (98−100%), *L. dumofii* (98%), *L. oakridgensis* (99%), *and L. londiniensis* and/or *L. nautarum* (99%) (Table 1). *L. nautarum* and *L. londiniensis* could not be distinguished due to their identical 16S rDNA sequences. *L. pneumophila*, *L. bozemannii*, and *L. dumofii* are classified as pathogenic *Legionella* species. In some samples two or three different species were detected. *L. bozemanii* isolates were found in samples from September 11 to October 25, while *L. pneumophila* was identified at all sampling dates, except for December 5. *L. oakridgensis*, *L. dumofii*, and *L. londiniensis*/*L. nautarum* were sporadically recovered. Sixteen *L. pneumophila* colonies, one from each distinct sample, were characterized by sequence-based typing of 6 gene fragments. All 16 isolates harbored the same sequence profile (6;23;3;28;19;14), defined by the allelic number of the 6 gene fragments analyzed, indicating that all *L. pneumophila* isolates belonged to the same clone. This sequence profile had not been identified previously. These *L. pneumophila* isolates did not react with Oxoid antisera against serogroup 1 or serogroup 2−14. Serogrouping performed at the Health Protection Agency (UK) revealed, however, these isolates as serogroup 4.

Concentration of Airborne Legionellae. All six *Legionella* species, including *L. pneumophila*, were detected in air at various altitudes and distances downwind of the aeration ponds. *mip* Real-time PCR specifically detected *L. pneumophila* at the highest altitude analyzed (64 m above sea level), while microbial growth analysis and 16S rDNA sequencing identified *L. pneumophila* and *L. oakridgensis* at the next highest altitude (50 m above sea level). Different *Legionella* species were detected at various distances downwind of the ponds. *L. londiniensis*/*L. nautarum* and *L. pneumophila* were identified at 180 and 200 m, respectively, downwind of the ponds, by growth analysis and 16S rDNA sequencing. As

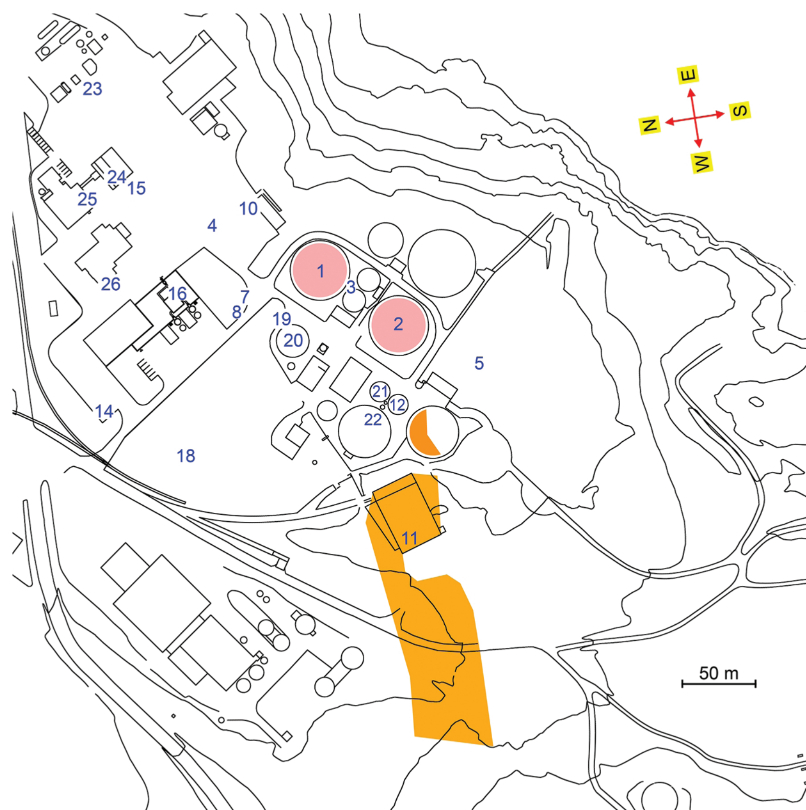

FIGURE 2. Selected air sampling sites surrounding the aeration ponds (in pink) and the recommended and complementary region suitable for air sampling at the same wind direction and speed as for the corresponding Figure 1 (in orange).

expected, the concentration of legionellae was highest directly above the ponds, and decreased as the distance from the ponds increased (Table 1, Figure 2).

Weather Conditions. Several *Legionella* species, including *L. pneumophila,* were identified during various weather conditions (Table 1). The highest and lowest concentrations of legionellae were observed during cloudy weather (3300 CFU/m^3) and rain (1.7 CFU/m^3), respectively. The next lowest concentration was found at a combination of sunny and cloudy weather (4.6 CFU/m^3). Nice warm weather may provide faster drying of aerosols (*31*) containing legionellae, thereby having an impact on the viability of the *Legionella* bacteria. This may explain the reduction in the observed CFU numbers on sunny days. However, it cannot be ruled out that the relative humidity is important for the survival of airborne *Legionella* bacteria. Fisman et al. (*32*) have shown that the incidence of legionellosis correlated with wet humid weather. However, as the concentration of airborne legionellae was not measured in their study, comparisons cannot be made.

Aeration Pond Samples. Liquid samples were harvested from the aeration ponds on the same days air sampling was performed. Microbiological and molecular analyses detected *Legionella* and *L. pneumophila* in all liquid samples. The concentration of legionellae in the ponds varied between 3 $\times$ 10^5 and 8 $\times$ 10^7 CFU/m^3. The concentration of legionellae detected in air directly above the ponds was in a range of 10^2 to 10^7-fold lower compared to that found in the ponds.

Discussion

This study used CFD for optimal experimental planning of air sampling with the aim of detecting airborne *L. pneumophila* at a biological treatment plant. Results showed that CFD was an efficient planning tool and that *L. pneumophila* was detected by culture or by real-time PCR in the aeration ponds, in air directly above the aeration ponds, and up to the farthest sampling point 200 m downwind of the ponds within various vertical distances.

CFD was applied to estimate the drift of water droplets, which could contain *Legionella* bacteria, other pathogenic biological and/or chemical materials, dispersed from the aeration ponds at Borregaard Ind. Ltd. The results were used to determine suitable air-sampling locations having a high probability of encountering aerosols generated from the aeration ponds at various wind directions. Determining these positions was a challenging task due to the densely built-up areas within the industrial plant. This study demonstrated that CFD provides valuable information for planning experimental air-sampling campaigns.

The SASS 2000^{PLUS} proved to be a suitable air collector for sampling *Legionella* bacteria. The need for portable air collectors to be used at sampling sites, at various distances and heights from the ponds at Borregaard Ind. Ltd., was one of the prerequisites for selecting the lightweight SASS 2000^{PLUS} air collector for which the power supply could be provided by batteries. Air was sampled at various locations surrounding the aeration ponds, also upwind of the ponds and independent of the wind direction. Growth analysis and specific *mip* real-time PCR confirmed the lack of presence of *L. pneumophila* in these latter samples, showing that the computed wind fields were suitable in finding optimal sites for sampling airborne *L. pneumophila* at Borregaard Ind. Ltd. As the sampling process may have an impact on the bacterial cell viability, the most suitable air sampler must be selected. In this study, the SASS 2000^{PLUS} collector proved to be suitable for collecting viable and sufficient concentrations of airborne *Legionella* bacterial cells. Air sampling may also be performed by impingement and impaction, whereas most of the literature describes the use of impaction for sampling legionellae. Legionellae have successfully been sampled using a cyclone at a petrochemical plant in the Pas-de-Calais region in France (*6, 12*).

The *mip* gene was found to be a suitable target for detecting *L. pneumophila,* in agreement with previous findings (*19, 23*). PCR detects DNA from viable, viable but nonculturable (VBNC), and dead bacterial cells. Cultivation is, thus, needed to verify the presence of viable legionellae.

The *mip* gene is frequently used as a target for specific detection of *L. pneumophila* (*19*−*23*), but *mip* DNA sequences have been found in *L. micdadei* showing approximately 70% similarity to the *L. pneumophila mip* DNA sequence (*23, 33, 34*). No amplicon was detected for the *L. micdadei* ATCC 33218 strain using the primer/probe set in our study. We strongly believe that the selected *mip* gene region is a suitable target for specific detection of *L. pneumophila,* revealing that *mip* real-time PCR may act as a first level of detection of *L. pneumophila* in the environment. This is supported by the recent findings of Stølhaug and Bergh (*23*) who also used the *mip* gene to discriminate between *Legionella* species.

One or more *Legionella* species were recovered from the impactor collection plates on all ten sampling dates. This confirmed the viability of the bacteria and permitted species identification and further characterization of the *L. pneumophila* isolates. The recovery of different *Legionella* species during the sampling campaign suggested that the dominant *Legionella* population in air may vary significantly, potentially reflecting the population in the aeration ponds. Whether this variation was related to the weather conditions could not be determined in this study. Surprisingly, *L. pneumophila* was detected at all sampling time points, and sequence-based typing showed that a single clone, not detected in previous studies, was identified in nine consecutive samples (Table 1). The *L. pneumophila* isolates from the air samples did not react with the serogroup 1−14 Oxoid reagents. Oxoid forwarded the strain to the Health Protection Agency (UK), who classified it to serogroup 4, suggesting that typing with the commercial reagents must be carefully evaluated. Typing with monoclonal antisera may be an alternative (*35*).

Our results strongly implied that the detected and isolated *Legionella* species, including *L. pneumophila*, from air originated from the aeration ponds at Borregaard's biological treatment plant. To ascertain that the *L. pneumophila* strain isolated from air was identical to the *L. pneumophila* isolated in the ponds, sequence-based typing of the strains from the ponds would have been needed.

The finding of *L. pneumophila* and *Legionella* species in the aeration ponds at Borregaard Ind. Ltd. and in more than 50% of the aeration ponds in Sweden (*4*) addresses whether employees at biological treatment plants may be exposed to such potential respiratory hazards posing an occupational health challenge. These studies indicate that legionellae may be commonly found at biological treatment facilities. However, the most common human pathogen serogroup 1 of *L. pneumophila* was detected in the Swedish paper mills, whereas the serogroups 2−14 dominated (*4*). It is therefore of concern to elaborate on the human exposure and infectious dose of *Legionella.* The infectious dose of inhaled *L. pneumophila* cells causing Legionnaires' disease in humans is not known, but guinea pigs exposed to an inoculum of 10−100 *L. pneumophila* cells, as aerosols, develop asymptomatic infection, disease at about 1000 cells, and death at 10 000 cells (*36*). The Nordic countries are addressing the risk of being exposed to and infected with *Legionella* bacteria at biological treatment facilities. The Norwegian Institute of Public Health is currently investigating whether employees at Borregaard Ind. Ltd. and inhabitants of the region where the outbreak of legionellosis occurred in 2005 have had a higher risk of infection by *Legionella* bacteria compared to

those having residence elsewhere in Norway (*26*). A quantitative microbial risk assessment model for Legionnaires' disease has recently been reported for exposure in whirlpool spa outbreaks (*37*). Future studies may include similar quantitative studies of airborne *Legionella* bacteria at biological treatment facilities.

The aeration ponds contain a variety of microorganisms that degrade organic compounds in industrial wastewater before disposal. Based on the finding of *L. pneumophila* in the aeration ponds as well as in the ambient air, there is no doubt that other microorganisms present in such ponds may also be aerosolized.

The present study has clearly shown that there is a high probability that *Legionella* and *L. pneumophila* detected in air at various positions within the biological treatment plant originate from the aeration ponds. *Legionella* and *L. pneumophila* were detected in liquid samples harvested from the aeration ponds and in the air collected directly above the ponds, as well as downwind of the aeration ponds. More importantly, *L. pneumophila* was not detected upwind of the ponds at different wind directions. This strongly suggests that the source of the detected *L. pneumophila* was the aeration ponds.

The methods described here can be regarded as a general approach for tracing and detecting aerosols containing biological as well as chemical materials that may pose an environmental occupational threat.

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