Sampling and identification of *Legionella* spp. at Borregaard Ind. Ltd.

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Sammendrag

Befolkningen i Sarpsborg/Fredrikstad-området opplevde et utbrudd forårsaket av *Legionella pneumophila* serogruppe 1 i mai 2005 hvor 56 personer ble registrert smittet. Skrubberanlegget ved Borregaard i Sarpsborg ble identifisert som sannsynlig smittekilde ved epidemiologiske analyser og genotyping av *L. pneumophil*a bakterier isolert fra pasienter og spredningsanalyser. Tre nye tilfeller av sykdom forårsaket av *L. pneumophila* serogruppe 1 oppstod i november 2005 i samme område, men smittekilden for disse tilfellene er ikke avklart.

Legionellabakterien har blitt påvist ved flere biologiske renseanlegg i Norden, inkludert Borregaard, Sarpsborg. Det er nødvendig å gjøre rede for om *L. pneumophila* generelt er tilstede ved renseanlegget til Borregaard. Et slikt prosjekt ble iverksatt som et samarbeidsprosjekt mellom Forsvarets forskningsinstitutt, Nasjonalt folkehelseinstitutt og Telelab AS, med Borregard som prosjekteier og FFI som prosjektkoordinator. Arbeidet ble utført i perioden 13.06.2006 – 05.12.2006.

Samarbeidsprosjektet har hatt som formål å kartlegge tilstedeværelsen av *Legionella* spp. i luft ved Borregaard og undersøke om legionellabakterien kan spres fra luftebassengene til luft. FFI har, på oppdrag fra Borregaard, utført prøvetaking og genetisk analyse av luftprøver ved renseanlegget på Borregaard, koordinert prosjektarbeidet og utarbeidet en rapport som oppsummerer og beskriver resultatene fra alle samarbeidsaktørene.

Denne rapporten beskriver FFIs arbeid i samarbeidsprosjektet som inkluderer prøvetakingsmetoder, analyse og identifisering av *Legionella* spp. og *L. pneumophila* i luft og væske ved bruk av real-time PCR. En CFD-modell ble benyttet for å estimere hastighetsfeltet i renseanleggets lokale omgivelser og ble brukt som grunnlag for å oppnå optimal plassering av luftprøvetakingsutstyr under gitte vindretninger.

Resultatet fra studien viste at luftprøvetakerne SASS 2000^{PLUS} og MAS-100$^{®}$ var velegnet til luftprøvetaking, og at real-time PCR, med bruk av *L. pneumophila* arts-spesifikke *mip*-primere, identifiserte *L. pneumophila* i luft. *L. pneumophila* ble påvist i væskeprøver fra luftebassengene og fra elven Glomma. Mikrobiologiske og molekylærbiologiske analyser viste tilstedeværelse av *Pseudomonas* spp., *Acinetobacter* spp., *Vibrio* spp. *Shewanella* spp., *Enterococcus* spp., samt en rekke vanlig forekommende miljøbakterier i luftebassengene. Noen av disse bakteriene er opportunistiske human patogener. Dette kan tyde på at varsomhet bør utøves ved håndtering av væskeprøver fra luftebassengene.

Det henvises til FFI rapport 2007/00560 for en oppsummering av resultatene fra alle samarbeidsaktørene.

English summary

During May 2005, 56 inhabitants of Sarpsborg/Fredrikstad developed legionellosis caused by *Legionella pneumophila* serogroup 1, resulting in the death of ten patients. The wet scrubber at Borregaard Ind. Ltd. was identified as the source, In November 2005, three new cases of legionellosis were reported in Sarpsborg/Fredrikstad, but the source for exposure has still not been identified. There are very few studies elaborating the dispersion pattern and the impact of atmospheric conditions on the transmission of *Legionella* in air. This study has focused on the sampling of aerosols containing *L. pneumophila* and identifying this bacterial species by molecular methods. Also, one of the goals of this project was to elaborate the dispersion of *L. pneumophila* from the aeration ponds at Borregaard's biological treatment plants to ambient air.

This project was initiated by Borregaard Ind. Ltd. and involved three collaborating partners; The Norwegian Defence Research Establishment (Forsvarets forskningsinstitutt FFI), the Norwegian Institute of Public Health and Telelab AS. The project owner is Borregaard Ind. Ltd and FFI has been the project coordinator. The work was carried out during 13.06.2006 – 05.12.2006.

This report describes the work performed by FFI. The dispersion of *Legionella* was studied by sampling airborne aerosols above and around the aeration ponds according to selected regions well suited for the aerosol sampling by predictions made by a Computational Fluid Dynamic (CFD) model of the biological treatment plant. The air collector SASS 2000^{PLUS} was suitable for sampling aerosols containing viable *Legionella* cells and *L. pneumophila* was identified by specific real-time PCR using the *mip* primers. Results showed that *L. pneumophila* was, in general, not identified upwind of the aeration ponds. *L. pneumophila* was identified up to 180 m downwind of the aeration pond, strongly indicating that the aeration ponds are a source for generating aerosols of *L. pneumophila.*

L. pneumophila was identified by *mip* real-time PCR in liquid samples harvested from all three aeration ponds at Borregaard Ind. Ltd., and from the Glomma river. It is not known if these air and liquid samples contain the same *L. pneumophila* strain, and if this strain is pathogenic to humans.

Several different bacterial genus and species were identified in the aeration ponds by molecular and microbiological analysis. *Pseudomonas* spp., *Acinetobacter* spp., *Vibrio* spp. *Shewanella* spp., *Enterococcus* spp. and several other bacteria commonly found in the environment were identified in the aeration ponds. Some of these bacterial species are opportunistic human pathogens suggesting that care should be taken when working close to these ponds.

An overall presentation of the work performed from all collaborating partners has been published in the FFI report 2007/00560.

Contents

1 Introduction

1.1 *Legionella* spp.: disease and transmission

Legionella pneumophila is the ethiological agent of Legionnaires' disease, which is an acute pneumonic illness, the nonpneumonic legionellosis known as Pontiac fever, and in some cases of extrapulmonary infections that are more rare occasions. In addition to *L. pneumophila*, more than 49 different *Legionella* species have been described in which 19 species may cause infections in humans. This usually occurs in immonosuppressed patients. The species *L. pneumophila* contains at least 16 serogroups, in which serogroup 1, 4, 6 and 7 cause legionellosis. The incidence of the different clinical isolates of *Legionella* spp. and serogroups does not correlate with that found in the environment where *L. pneumophila* serogroup 1 is not found widespread (Edelstein, 2006, Muder and Yu, 2002). *L. pneumophila* is responsible for about 90 % of community-aquired Legionnaires' disease cases followed by (in chronological order), *L. longbeachae, L. bozemanii, L. micdadei, L. feeleii, L. dumoffii, L. wadsworthii* and *L. anisa* (Muder an Yu, 2002).

The mortality rate of Legionnaires' disease varies, ranging from 1-80%, depending on the health of the patient (Edelstein, 2006). In contrast to Legionnaires' disease, the attack rates of those exposed and developing Pontiac fever are very high (70-90 %). In Norway, about 25 legionellosis cases are diagnosed and reported annually[1]. If pneumonia does not occur and the clinical findings are unspecific it may be difficult to diagnose Pontiac fever. Pontiac fever has recently been suggested as a marker for environmental contamination by *Legionella* to support epidemiological surveillance (Tossa et al., 2006). The incubation period between exposure and onset of symptoms for Legionnaires' disease is usually 2 – 10 days, but even 19 days have been reported (Armstrong, 2006 and references therein).

The most common route for transmission of legionellosis is by inhalation of *L. pneumophila* as aerosols. Even though the infection dose of *L. pneumophila* causing legionellosis is not known, it has been shown that guinea pigs exposed to an inoculum of 10-100 *L. pneumophila* bacterial cells (as aerosols) developed asymptomatic infection, disease at 1000 cells and death at 10 000 cells (Edelstein, 2006). Free-living amoebas support intracellular growth and survival of *Legionella* bacteria thereby acting as reservoars for *L. pneumophila* (Murga et al., 2001, Greub and Raoult, 2004). *Legionella*-infected amoeba cells are found together with other microorganisms in biofilms. Since an amoeba may contain 1000 *L. pneumophila* cells, theoretically, it would be sufficient to inhale one ameoba cell in order to develop legionellosis (Edelstein, 2006, O'Brian and Bhopal, 1993). When low levels of nutrients are present in the environment, *L. pneumophila* may lyse the amoeba cell allowing new amoebal host cells to be infected. It is generally believed that this lifestyle is a way of protecting free-living *Legionella* bacterial cells from harsh environmental conditions and improving its survival rate. Free-living *Legionella* bacterial cells may also enter a low metabolic state, i.e. a viable-but-nonculturable state (VBNC).

[1] www.fhi.no

Legionella bacteria are ubiquitous in aqueous environments (reservoirs, lakes, rivers, biofilms) at temperatures ranging from 5 to 50 ºC. However, growth of this bacterium is restricted to temperatures between 20 to 43 ºC, although *Legionella* has been isolated from environments below 10 ºC and at 60 ºC. It is commonly found in potable water systems (Långmark et al., 2005, Wullings and van der Kooij, 2006), hot springs, cooling-tower systems (Bentham, 2000), wastewater treatment plants and chemical industrial plants (Nguyen et al., 2006). *Legionella* spp. have also been identified in water samples at various locations on ferries and cruise ships (Pastoris et al., 1998, Azara et al., 2006) as well as hospitals and dental unit waterlines (Dutil and et al., 2006). Stagnation of water enhances bacterial growth of biofilm formation that the bacteria may use as shelter or a source of nutrients. It has recently been shown that *L. pneumophila* is able to grow on heat-killed bacterial cells, demonstrating necrotrophic growth of *L. pneumophila* (Temmerman et al., 2006).

1.2 Survival of *Legionella* in air

The survival of *Legionella* in air is believed to depend on biological and physical parameters, as wells as atmospheric conditions. The occurrence of legionellosis seems to be associated with increased relative humidity in the period prior to infection and onset of symptoms (Fisman et al., 2005). It has been shown that the stability of *L. pneumophila* decreases as the relative humidity drops, and that the viability decreases when pH increases (Katz and Hammel, 1987). Bioaerosols are often single units and often associated with particles in the air that may act as carriers for airborne microorganisms. Microorganisms tend to stick to each other in air and form larger particles. The size range of bioaerosols generally varies between 0.02 – 100 µm. Bioaerosols generated from water droplets contain a thin layer of moisture surrounding the microorganisms, and it has been stated that moisture may increase bioaerosol stability (Pillai and Ricke, 2002 and references therein). In general, high temperatures, UV-light and short-wave ionizing radiation have a harmful effect on bioaerosols. Bioaerosols are generally subjected to Brownian motions, but at a particle size range > 1µm, gravitational forces are more prominent (Pillai and Ricke, 2002). Electrical forces may also have an effect on the gravitational settling of bioaerosols, which have an overall net charge. Usually, electrical charge on particles will enhance the growth of very small aerosol particles (Kulmala et al., 2004 and references therein).

1.3 Sampling of *Legionella* spp. as aerosols

Several studies have been outlined in order to sample aerosols and detect *Legionella* spp. in air at various locations, mainly linked to industrial coolings (Nguyen et al., 2006, Ishimatsu et al., 2000, Pascual et al., 2001), sanitary landfill sites (Huang et al., 2002), wastewater treatment plants (Fracchia et al., 2006, Stampi et al., 2000), and evaporate condensers (Breiman et al., 1990). In these studies, impaction has been the most frequent method used. However, sampling of *Legionella* in air may also be performed by impingement. Impaction includes collecting bacteria on the surface of petri dishes containing selective growth medium, requiring viable bacterial cells. In many cases, it is more convenient to collect the bacterial cells in liquid in order to perform molecular techniques, which does not require viable cells. A virtual impactor concentrates and collects the air into an air or liquid sample.

Growth, and thereby phenotypic analysis, is obtained by spreading the liquid solution on agar plates after impingement. In some cases, impingement may have an impact on the viability of the bacterial cells collected. However, this depends on the collector device used. It has been stated that impingers are likely to perform better than impactors (Li et al., 2003, Ishimatsu et al., 2000, Nguyen et al., 2006).

1.4 Molecular techniques for identification of *Legionella* spp.

Various methods for detection and identification of *Legionella* spp. have been elaborated. These include real-time PCR (polymerase chain reaction), FISH (fluorescent in situ hybridization), genotyping, PFGE (puls field gelectrophoresis), AP-PCR (arbitrarily primed PCR), immunological methods using antibodies, cultivation, and fluorescence, among others. Serotyping of *L. pneumophila* is usually obtained by urin antigen testing. The Latex agglutination kits are generally used to identify *L. pneumophila* serogroups 1-15, but they may also detect other environmental *Legionella* spp.. A new agglutination kit designated "Research latex" has been developed in order to distinguish between clinical and environmental *Legionella* spp. such as *L. pneuomophila*, *L. anisa* and *L. taurinensis* (Reyrolle et al., 2006). For review of various detection and identification methods see Pasculle (1992), Fields et al. (2002), and Cianciotto et al. (2006).

Several reports describe the use of general and specific primers and probes for real-time PCR. General targets for *Legionella* spp. are the 23S-5S spacer region (Herpers et al., 2003), 5S rRNA (Hayden et al., 2001), and the 16S rRNA gene (Rantakokko-Jalava and Jalava, 2001, Wellinghausen et al., 2001, Templeton et al., 2003, Joly et al., 2006). For specific real-time PCR identification of *L. pneumophila*, the *mip* gene[2], is frequently used (Ballard et al., 2000, Hayden et al., 2001, Wellinghausen et al., 2001, Templeton et al., 2003, Wilson et al., 2003, Fiume et al., 2005, Khanna et al., 2005, McDonough et al., 2005, Joly et al., 2006, Morozumi et al., 2006). *mip* DNA sequences have been identified in *L. micdadei*, showing up to 70 % DNA identity with the *L. pneumophila mip* DNA sequence (O'Connel et al., 1995, Ratcliff et al., 1997). The *dot* gene[3]

[2] *mip* gene : encodes the macrophage infectivity potentiator involved in the virulence of *L. pneumophila* (Engleberg et al., 1991).
[3] *dot* gene : the defective organelle trafficking gene

has also been used as a specific target for *L. pneumophila* using real-time PCR (Yáñez et al., 2005).

The *rpoB*[4], *rnpB*[5], *mip* and 16S rRNA genes have been used for phylogenetic analyses in order to establish a molecular method for differentiation of *Legionella* species (Hookey et al., 1996, Ko et al., 2002, Rubin et al., 2005). VNTRs (variable number of tandem repeats) have been used for genotyping of *L. pneumophila* (Pourcel et al., 2003) as well as the MLST approach (multi-locus sequence typing) (Maiden et al., 1998, Gaia et al., 2005).

1.5 CFD models

Prediction of dispersion patterns of airborne aerosols in and over building clusters consitutes a particularly challenging task. The physical characteristics of the air flow and the subsequent dispersion processes in these situations are extremely complex. Aside from the interaction between the flow and complex geometries, and with the topographically variation, the situation is characterized by extremely diverse length and time scales, where the length-scales typically range from a few millimeters to several hundred meters. The building structures typically reside deeply inside the atmospheric boundary layer, which is in the order of 300 meters thick, and where turbulent process dominates the momentum exchange and accompanying diffusive processes. Also, meterological conditions may significantly add to the complexity by strong stratification. The drift of water droplets (particle tracking), which may contain pathogenic biological or chemical materials, from cooling-tower installations can be modeled using Computational Fluid Dynamics (CFD) calculations. Such numerical analysis allows for computing wind fields to estimate the particle flux of particles, prediction of plume concentrations and determination of contaminated regions.

Physically-based numerical models are therefore a pre-requisite in order to faithfully predict air flow and dispersion patterns inside, and in the vicinity of, building clusters. These models differs significantly in comparion with the commonly used ”real-time” models that are able to handle dispersion in an approximately flat and unobstructed environment (reviewed in Hosker, 1985). The terminology ”physically-based” numerical models alludes to so-called Computational Fluid Dynamic (CFD) models which are based on solving modeled transport equations governing the dynamics of turbulent statistics and scalar fields. These models are computational expensive and precluded real-time use. However, they are suitable for emergency response exercises, and when applied carefully they are able to predict the complex processes involved.

The CFD methodology used in this study is based on the Reynolds Averaged Navier-Stokes formulation which provide predictions of the statstically averaged wind field (Durbin and Pettersson Reif, 2001). These models have been shown to work reasonable well in these cases and they constitute a good tradeoff between accuracy and computational cost. The outcome of a statistical turbulence model like RANS represents in its most general form an ensemble averaged

[4] *rpoB* gene : the RNA polymerase ß–subunit gene
[5] *rnpB* : the catalytic RNA moiety endoribonuclease P gene (RNaseP)

solution of the velocity and pressure fields. In order to assess the predictive capability of CFD models, benchmark simulations are conducted and compared with detailed experimental results (Lien and Yee, 2004). These simulations usually involve simplified geometrical configurations of building clusters, typically performed in windtunnels or water channels (Yee et al., 2006). In real life, building arrangements in the landscapes are considerable more complex and are therefore also associated with significantly higher uncertainties. Also, variability with respect to turbulence, particle flow and characteristics and temperature in air are concerning factors. A simplifying assumption adopted in the present study is that of isothermal (i.e. constant temperature) conditions. This is not a shortcoming of the CFD aproach per se but has been adopted in the present in order to simplify the approach, mainly motivated by the relatively short duration of the project.

1.6 *Legionella* in biological treatment plants

Biological treatment plants at 43 paper mills in Sweden have been subject for an extensive study during September –October 2005. One worker had developed Legionnaires' disease, in which the isolate was identified as *L. pneumophila* serogroup 1 subtype Benidorm. This strain was also identified in the aeration pond of the treatment plant at the paper mill (Allestam et. al., 2006). *Legionella* is frequently found in biological treatment plants at paper mills, in which *L. pneumophila* serogroup 2-14 is the dominating species (Allestam et. al., 2006). In the study by Allestam et. el (2006), *L. pneumophila* was identified in at least 50% of the aeration ponds in contrast to only 5 % of the cooling-towers analyzed. Other *Legionella* spp. were also identified.

1.7 Borregaard Ind. Ltd.

Borregaard Ind. Ltd is the world's leading supplier of wood-based chemicals and produces high purity specialty cellulose, lignin-based binding and dispersing agents, vanillin products, nutritional omega-3 oils and yeast specialities[6]. Biological treatment plants at paper mills have been introduced to degrade chloro-organic substances that may have an impact on ecosystems. Thus, all wastewater from Borregaard Ind. Ltd. is biologically treated in order to fulfill environmental requirements in reducing the levels of such compounds, including fibre releases, into the river Glomma. Biological treatment plants include the use of large aeration ponds[7] where a large diversity of microorganisms is present to degrade various compounds in the wastewater (figure 1.1). Evaporation clouds are easily seen from these aeration ponds, showing that large amounts of aerosols are generated as about 30 000 Nm^3 per hour air is pumped through the pond that contains 30 000 tons of liquid. The inlet water allows a temperature of about 37 ºC in the pond, which allows optimal growth of the microorganisms present. The generated biosludge is sedimented and subsequently heated (burned), thereby acting as biofuel and reducing the total energy consumption at Borregaard Ind. Ltd.

[6] www.borregaard.no

[7] At Borregaard Ind. Ltd., the diameter and the height of an aeration pond is about 40 m and 12 m, respectively.

During May 2005, 56 inhabitants of Sarpsborg/Fredrikstad developed legionellosis caused by *L. pneumophila* serogroup 1, resulting in the death of ten patients. The majority of these patients were 60 years old and above. The wet scrubber at Borregaard Ind. Ltd. was identified as the source, based on the finding that the *L. pneumophila* strain isolated from the patients were identical to the environmental strain isolated from samples taken from the wet scrubber (Norwegian Public Health Institute). This outbreak indicated that *Legionella* could be dispersed in ambient air at least 10 km from the treatment plants at Borregaard Ind. Ltd.. Similar findings were observed during the outbreak in Pas-de-Calais, France, where a 7 km distance range for transmission of airborne *L. pneumophila* was observed (Nguyen et al., 2006). Three new cases of legionellosis were reported in Sarpsborg/Fredrikstad, November 2005, but the source for exposure has still not been identified. Norway had previously experienced a larger outbreak of legionellosis in 2001, Stavanger, where 26 cases were reported. A cooling-tower system at a city hotel was identified as the source[8].

Figure 1.1. One of the aeration ponds at the biological treatment plant at Borregaard Ind. Ltd. (*Photo: FFI*).

[8] www.fhi.no

1.8 This study

There are few studies elaborating the impact of atmospheric conditions on the transmission of free-living *Legionella* bacterial cells and *Legionella*-infected amoeba cells over a long distance. In general, there is a lack of studies analyzing and evaluating such a dispersion of important pathogenic microorganisms for humans. This study has taken one step further in biological dispersion studies by elaborating the dispersion of *Legionella* spp. and *L. pneumophila* from aeration ponds to ambient air at the biological treatment plant located at Borregaard Ind. Ltd., Sarpsborg.

This project was initiated by Borregaard Ind. Ltd. and involved three partners; The Norwegian Defence Research Establishment (Forsvarets forskningsinstitutt FFI), the Norwegian Institute of Public Health and Telelab AS. The project owner is Borregaard Ind. Ltd.[9]. FFI has been the project coordinator.

The work has been carried out during 13.06.2006 – 05.12.2006. FFI has performed numerical dispersion predictions, air sampling and real-time PCR analyses. The Norwegian Institute of Public Health and Telelab AS have performed microbiological analyses and serogroup typing. The Norwegian Institute of Public Health has also performed genotyping of isolated *Legionella* spp. colonies.

This report describes the work performed by FFI. In some cases, the report compares results obtained by Telelab AS. The authors would like the readers to refer to Blatny et al. (2007) for a review and an overall presentation of the project during 11.09.2006 – 05.12.2006.

We have chosen to collect air into liquid samples and on surfaces (agar plates). Identification of *L. pneumophila* was obtained by specific real-time PCR using the *mip* primers. To optimize the probability for success, we have carried out a comprehensive experiment planning based on computational modeling of aerosol particle transport by wind for particles originating from the aeration ponds at Borregaard's biological treatment plant.

[9] POC at Borregaard : Dr. Viggo Waagen

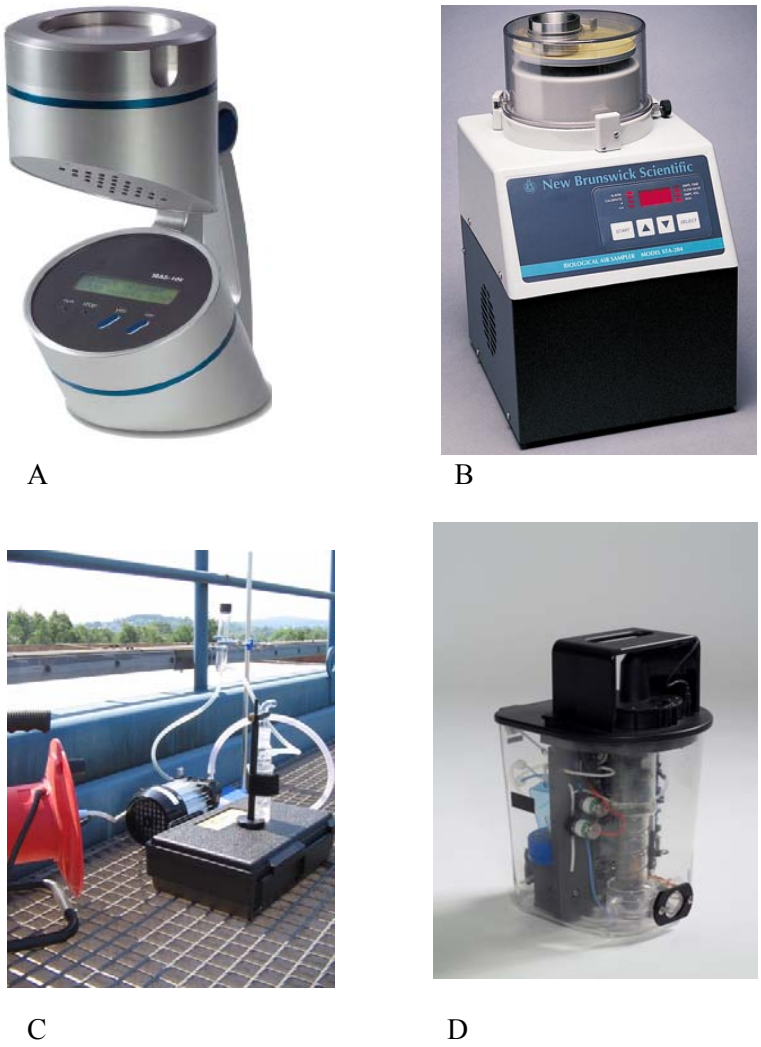
2 Objective

The aim of the study, described in this report, was to collect air and liquid samples from the aeration ponds at Borregaard Ind. Ltd, Sarpsborg, in order to investigate the potential presence of *Legionella pneumophila* in these ponds. Furthermore, the purpose of this project also included finding out whether *Legionella* bacterial cells could be dispersed as aerosols from the aeration ponds at Borregaard's biological treatment plant into the surroundings. The bacterial diversity in the aeration ponds was characterized to a certain extent by molecular and microbiological analysis.

3 Materials and Methods

3.1 Air collection

Air collection was performed by using the liquid impinger SKC BioSampler® (SKC Inc., PA, USA), the cyclone SASS 2000^{PLUS} (Research International, WA, USA) and the impactors MAS-100®Air Sampler (Merck, Darmstadt, Germany) and the STA-204 Microbial Air Sampler (New Brunswick Scientific, NJ, USA) (figure 3.1). In general, air collection was performed upwind, above, and downwind of the aeration ponds at the biological treatment plant. Table 3.1 summarizes the specifications of these air collectors and their use during this study.



A B

C D

Figure 3.1. Air collectors used in this study; MAS-100® (A), STA-204 (B), SKC Biosampler® (C) and SASS 2000^{PLUS} (D).

SKC Biosampler® and SASS 2000^{PLUS}

During sampling, air was collected in a volume of 20 ml (SKC Biosampler®) or 5 ml (SASS 2000^{PLUS}) PAGE buffer (120 mg NaCl, 4 mg $MgSO_4x5H_2O$, 4 mg $CaCl_2x2\ H_2O$, 142 mg Na_2HPO_4, 136 mg KH_2PO_4 per liter destilled water, pH 6.8 ± 0.2 at 25 ºC). The liquid sample was divided in two, in which one half was sent to Telelab AS for microbiological analyses. The remaining half was analyzed by FFI by real-time PCR (section 3.6).

Buffer volume was checked regularly in order to replenish evaporated buffer with sterile water. In contrast to the SKC Biosampler®, the evaporating buffer was replenished automatically in SASS 2000^{PLUS}. From 16.11.06 an onwards, sterile water was used as the collecting fluid and a two fold concentration of PAGE buffer was added to the liquid sample immediately after air sampling with SASS 2000^{PLUS}.

In general, SASS 2000^{PLUS} was washed after use with chlorine solution (10 fold-dilution of a 5 % chlorine solution) and sterile destilled water as the final wash[10]. The cleaning process was performed after removal of the fan of SASS 2000^{PLUS} and with a circulation of the destilled water (i.e. turning on the SASS 2000^{PLUS}). Any growth of *Legionella* spp. in the washing buffer used was verified by plating out the washing buffer on selective growth medium for *Legionella* spp. However, when the air collector was used repeatedly during the same day, cleaning between each sampling was performed three times by flushing the cyclone from the top with fresh buffer and discarding the buffer through the sample port. Buffer from the final washing step was collected and analyzed for the presence of *Legionella* spp. by Telelab AS.

MAS-100® and STA-204

The selective agar growth medium, GVPC, was provided by Telelab AS, for use in MAS-100® and the STA-204 during air (Wierød et al., 2007). GVPC is generally used for enrichment of *Legionella* spp. from the environment. One agar plate from each sample point and from each collector was sent to Telelab AS and the Norwegian Institute of Public Health for microbiological analyses.

[10] Unpublished results by FFI have shown that the washing procedure described here is sufficient for washing SASS 2000^{PLUS} after sampling of *Bacillus globigii* spores.

Table 3.1. Collectors used for air sampling at Borregaard Ind. Ltd. during 13.06.2006 – 05.12.2006.

Air collector		Inlet airflow (l/min)	Sample time (min)	Time period	Total airflow
MAS-100®	impactor	100	10	All sampling dates,except 21.09.06 and 27.09.06	10 000 l = 10 m^3
STA-204	impactor	30	60	11.09.06, 21.09.06, 27.09.06	1 800 l = 1.8 m^3
SKC Biosampler®	impinger	12.5	60 120	13.06.06 – 07.07.06	750 l = 0.75 m^3 1 500 l = 1.5 m^3
SASS 2000^{PLUS}	cyclone virtual impactor	325	60 120	25.10.06 – 05.12.06 11.09.06 – 18.10.06	19 500 l = 19.5 m^3 39 000 l = 39 m^3

3.2 CFD models

The commercial Computational Fluid Dymanics CFD software Fluent was used throughout this study. Basis for the CFD methodology used in this study is the so-called Reynolds Averaged Navier Stokes (RANS) formulation, which is a model that provides a statistical averaged wind field (Durbin and Pettersson Reif, 2001). This is considered to be a suitable technique in this case since a typical air sampling period was 1-2 hours. The computational model consisted of an area 1 km x 1 km surrounding the biological treatment plant that included detailed topography and all major buildings. The wind field was computed up to a height of approximately 250 m above the ground. The resolution varied from approximately 0.7 m in all directions in the most central part of the plant, to about approximately 10 m a few hundred meters away from the aeration ponds. The total computational model of the treatment plant consisted of approximately 8.3 million cells and the computational time for each of the 24 simulations (conducted on 16 parallel processors) was about five hours. The density of the aerosol paths was evaluated for selected wind directions. The best-suited regions for air sampling was selected from a combination of heights and density of the particle paths. Locations with potentially high aerosol flux were selected and a total of 24 air sample maps were constructed.

Constant wind speed was assumed in all 24 simulations (2 m/s), but each simulation was conducted at different directions covering 360 deg (15 deg increment). The dependence on wind speed was considered negligible which could be considered as a viable assumption in the present case. It was further assumed that the incoming boundary layer was fully turbulent and that the general wind speed and direction remained constant. Isothermal weather conditions were also assumed in all cases, i.e. neglecting buoyancy effects. The aerosols originating from the aeration

ponds were assumed to be very small such that they could be treated as passive tracers, following the local wind direction perfectly. Also, the aerosol properties were assumed to remain constant during the transport, i.e. the effects of coagulation and evaporation were not considered.

It should be noted that the wind to some degree varied in direction and strength during each sampling period. As an empirical indication for the wind conditions, a smoke generator was constructed and used. It consisted of a power generator with a specially designed muffler system. Contrary to a standard muffler system, which is designed to reduce noise and to provide cooling of the exhaust gases, the present muffler system was re-designed to become as warm as possible. The muffler consisted of a heat insulated metal tube with an internal greating. As the muffler was heated up, droplets of diesel oil were injected into the muffler where they instantly evaporated to form a thick white smoke. The smoke generator was placed on top of the aeration ponds to visualize the local wind direction at the same time as the air sampling was done. This information was used together with the wind direction derived from the drift of smoke from the chimneys at the biological treatment plant.

3.3 Bacterial strains

The bacterial strains used in this study were obtained from the American Type Collection Center. *L. pneumophila* 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. dumoffii* ATCC 33279 and *L. longbeachae* ATCC 33462.

3.4 Growth of *Legionella* spp.

Legionella spp. was grown on selective growth medium GVPC provided by Telelab AS (Wierød et al., 2007). Verification of *Legionella* spp. colonies was performed by inoculating onto BCYE or sheep blood agar by the Norwegian Public Health Institute (Caugant et al., 2007).

3.5 Isolation of nucleic acids

Isolation of nucleic acids from air and liquid samples were performed with the NucliSens® Basic kit method (BioMèrieux Ltd.) (Boom et al., 1990). Nucleic acids were isolated from 1.5 ml air sample (SASS 2000^{PLUS}, SKC Biosampler®) during time period 13.06.2006 – 18.10.2006. The air sample was added to a mixture of 13.5 ml NucliSENS® lysis buffer (5 M guanidinium thiocyanate, Tris/HCl, Triton X-100) and 50 µl silica beads in a sterile 15 ml tube. The samples were incubated at room temperature in a rotary mixer for 30 min (15 rpm), centrifuged at 2000 g for 3 min in a Sorvall RT 6000D centrifuge. The supernatant was gently discarded, the silica pellet was dissolved in 1ml Wash Buffer (5 M guanidinium thiocyanate, Tris/HCl) and transferred to an eppendorf tube, followed by centrifugation at 13000 g for 1 min (Hättich Micro 20 centrifuge. The washing step was repeated twice with 70% ethanol and once with acetone. The pellet was dried at 56 ^{o}C for 10 min, dissolved in 50 µl sterile water, incubated at 56 ^{o}C for 10

min and centrifuged for 2 min at 13000 g. The supernatant, containing the nucleic acids, was transferred to a new eppendorf tube. During the time period 25.10.2006 – 05.12.2006 nucleic acids were isolated from the entire sample volume (usually 4-5 ml), since the sampling time had been reduced from 2 to 1 hour in this time period. Thus, 5 ml sample was added to 10 ml lysis buffer and 50 µl silica beads.

Liquid samples taken from the aeration ponds were vortexed for 5 sec and 1.5 ml was transferred to an eppendorf tube followed by centrifugation at 100 g for 1 min in order to sediment most of the lignin. 150 µl of the supernatant was transferred to a new eppendorf tube containing 1.35 ml lysis buffer and 50µl silica beads (NucliSENS® Isolation Kit). Nucleic acids were isolated as described above.

Extraction of nucleic acids from river and puddle samples was performed as described for the air samples.

In general, 5 µl of the nucleic acid extract isolated from air and liquid samples was used as template in the real-time PCR assays (section 3.6).

In some cases, nucleic acids were isolated by adding chelex (BioRad) to a final concentration of 5% (BioRad) to a 200 µl air sample (SASS 2000^{PLUS}) or 10 µl liquid sample from aeration ponds. The samples were vortexed 10-15 sec, centrifuged at 12000 g for 10 sec, incubated at 95 for 10 min and centrifuged again at 3000 rpm for 3 min[11] (Pascual et al., 2001). 2 µl was used as template in real-time PCR assays.

3.6 Real-time PCR

Real-time PCR was performed with a LightCycler® (Roche Diagnostics) using LightCycler® FastStart DNA Master^{PLUS} HybProbe hot start reaction mix or Smart Cycler® II Starter System (Cepheid) using the Light Cycler® Faststart DNA Master^{PLUS} SYBR-Green I kit (Roche Applied Science). The primers and probes used in this study are listed in table 3.2.

The detection limit for *L. pneumophila* using the LPmip-f/r primers was investigated using a 10-fold serial dilution of *L. pneumophila* ATCC 33152 in PBS buffer. The concentration of the template for optimization of the real-time PCR conditions (1 µM end concentration of primers, various annealing temperatures) was about a 10-fold higher than that obtained of the detection limit. The optimal annealing temperature was chosen on the basis of the detection limit and primer-dimer content in the end product. The specificity of the amplified products was verified by electrophoresis (Agilent 2100 Bioanalyzer). Primer-dimers were not detected even after 50 cycles. Several dilutions of the template were used as positive controls in each PCR-analysis.

[11] http://people.bu.edu/pbarber/Web%20Protocols/Protocol2.pdf

Table 3.2. Primer and probes used for genus-specific *Legionella* spp. and species-specific *L. pneumophila* real-time PCR analyses, DNA sequencing and DGGE analysis.

Primer/Probe	Sequence 5'→ 3'	Target gene	Reference
Leg-FL	AGTGGCGAAGGCGGCTACCT	16S rRNA	Wellinghausen et al., 2001
Leg-LC	TACTGACACTGAGGCACGAAAGCGT	16S rRNA	Wellinghausen et al., 2001
JFP	AGGGTTGATAGGTTAAGAGC	16S rRNA	Wellinghausen et al., 2001
JRP	CCAACAGCTAGTTGACATCG	16S rRNA	Wellinghausen et al., 2001
LPneu-FL	CCACTCATAGCGTCTTGCATGCCTTTA	mip	Wellinghausen et al., 2001
LPneu-LC	CCATTGCTTCCGGATTAACATCTATGCC	mip	Wellinghausen et al., 2001
LPmip-f	GCATTGGTGCCGATTTGG	mip	Wellinghausen et al., 2001
LPmip-r	GYTTTGCCATCAAATCTTTCTGAA	mip	Wellinghausen et al., 2001
LP3	CAIAGTYGGTCAGGCAAT	rnpB	Rubin et al., 2005
BM1-2	TGTAAAACGACGGCCAGTRTAAGCCGGGTTCTGT	rnpB	Rubin et al., 2005
Eub 933f	(CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGG)GCACAAGCGGTGGAGCATGTGG GC-clamp in parenthesis	16S rRNA	Iwamoto et.al., 2000
Eub 1387r	GCCCGGGAACGTATTCACCG	16S rRNA	Iwamoto et.al., 2000

Real-time PCR assays (*mip* and 16S rRNA gene) were performed in total volume of 20 µl containing 1 µM primers, 0,2 µM hybridization probes and 5 µl template (Lightcycler®). The reaction mixture was denatured at 95°C for 10 min. The PCR temperature profile consisted of 50 cycles, denaturation at 95°C for 0 s, annealing at 62°C for 10 s and extension at 72°C for 15 s. The amplified products were verified by melting point analysis and electrophoresis (Agilent 2100 Bioanalyzer).

For the *rnpB* real-time PCR assay, one single colony was dissolved in 1 ml PBS and incubated at 95°C for 10 minutes. This extract was further used as template. The real-time PCR assay was performed in a total volume of 20 µl containing 2 µl template and 1 µM of each primer (Smart Cycler®). The PCR temperature profile consisted of 35 cycles, denaturation at 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s (modified after Rubin et al., 2005). The amplified products were verified by melting point analysis and electrophoresis (Agilent 2100 Bioanalyzer) and purified prior to DNA sequencing (section 3.7) using the Easy Nucleic Acid Isolation Cycle Pure kit (E.Z.N.A) (Promega).

3.7 DNA sequencing

Amplified PCR products of the *rnpB* and 16S rRNA gene fragments were sequenced with a ABI 310 Genetic Analyser (Applied Biosystems) using a Big Dye Terminator Cycle Sequencing Ready Reaction kit (Abi Prism). Both ssDNA strands were sequenced, and when necessary repeated. The primers LP3/BM1-2 (*rnpB*) and JFP/JRP or Eub 933r/Eub 1387f (16S rRNA) were used for sequencing (table 3.2). The primers Eub 933r/Eub 1387F (without the GC clamp) were used for DNA sequencing of the amplified 16S rRNA gene fragments obtained by DGGE. The sequences were analyzed by BLAST[12] and aligned using ClustalW[13] or Clone.

[12] BLAST : www.ncbi.nlm.nih.gov
[13] ClustalW : www.ebi.ac.uk/clustalw

3.8 Analysis of bacteria in aeration ponds by specific growth analysis

Liquid samples were harvested from B 3503 (15 mL) and B 3504 (15 mL) 18.10.06 and 06.02.07. A liquid sample from B 3501 (anaerobic pond, 10 mL) was taken 18.10.06. Total numbers of colony forming units per ml (cfu/ml) were determined by spread plating of serial dilutions onto non-selective media, Brain Heart Infusion Agar (BHI, Fluka, Sigma). Plates were incubated aerobically at 37°C and anaerobically at 44°C over night. The presence of the commonly found drinking water pathogens *Salmonella* spp., *E. coli*, intestinal enterococci and *Clostridium perfringens* were analyzed by plating the liquid samples on agar plates containing specific growth medium.

Growth of *Salmonella* spp. was analyzed by plating on Brilliant Green Agar (BGA, Oxoid) and Xylose-Lysine-Desoxycholate Agar (XLD, Oxoid), and incubated aerobically at 37ºC for 24±4 hours. Typical colonies of *Salmonella* spp. appears as red/pink opaque colonies surrounded by bright red medium when grown on BGA. Red colonies with black centers, surrounded by pink medium, appears when grown on XLD medium.

E. coli and other coliforms were identified by plating onto Rapid' *E. coli* 2 media (BioRad), and incubated at 37ºC for 24±4 hours. *E. coli* forms pink to lilac colonies, while other coliforms form blue colonies on this medium.

In order to test for the presence of intestinal enterococci the samples were first plated onto mEnterococcus agar (EA, Difco) and incubated at 37ºC for 48±4 hours. Convex colonies with red-redbrown or pink color in the center or through the entire colony, were transferred to Bile Ezculin Azide Agar (BEA, Acumedia) and incubated at 44ºC for 2 hours. Bacterial colonies showing as dark brown or black with dark brown or black surrounding medium were confirmed as intestinal enterococcus.

Growth of *C. perfringens* was analyzed by plating on mCP agar (Sifin) and incubated anaerobically at 44°C for 24±4 hours. Yellow colonies with or without gas bubbles were regarded as presumptive positives. These colonies were confirmed as *C. perfringens* by holding the plate in vapor from 25 % ammonium hydroxide for 30 sec when the former yellow colonies turned pink to red-lilac.

3.9 Denaturating gradient gel electrophoresis DGGE

Denaturating gradient gel electrophoresis (DGGE) is based on electrophoresis of PCR-amplified 16S rRNA gene fragments in polyacrylamide gels (Muyzer et. al, 1993). DNA fragments of the same length are separated due to their different GC/AT-content.

Bacterial DNA was isolated according to the NucliSens® Basic kit method (BioMèrieux Ltd.) and the variable regions 6, 7 and 8 of the 16S rRNA gene were amplified using primers Eub 933f and Eub 1387r containing a GC-clamp (table 3.2). The PCR assay was performed in a total volume of 20 µL and contained 2,5 µL template, 5 µM primers, 2,5 mM $MgCl_2$ and enzym mix according to the Light Cycler® DNA Master^{Plus} SYBR-Green I kit (Roche Applied Science). After denaturation at 95ºC for 5 min, 30 cycles at 95ºC for 30 sec, 55ºC for 30 sec and 72ºC for 30 sec were run (Smart Cycler® II). Loading dye was added to the amplified products and applied onto the DGGE gel. The DGGE analysis was performed on a 8 % wt/vol polyacrylamide gel in 0,5 x TAE-buffer (65ºC) on a Hoefer Scientific SE600 vertical double run system and according to the users manual (Amersham Pharmacia Biotech). 7 M urea and 40 % vol/vol deionized formamide were used as denaturants, and the gel contained a linearly increasen gradient (Sambrook and Russel, 2001). The polymerization was catalyzed by adding TEMED (BioRad) and ammonium persulfate (Sigma) to 0,05 % and 0,1 % respectively.

Electrophoresis was performed at 35 V for 10 min followed by 85 V for 16 hours. The gel was stained with SYBR® Green II Nucleic Acid Gel Stain (Cambrex) for 1 hour in the dark and rinsed thoroughly with deionized water. Photography (Kodak EDAS 290, Eastman Kodak Company, Japan) was obtained by placing the gel on a UV Dual-intensity transilluminator TM-20 (UVP, USA). Selected bands were excised and transferred to tubes containing 20 µL sterile deionized water. The DNA was eluted at 4°C over night and reamplified according to the same PCR conditions as previous, except that the primer Eub 933f used did not contain the GC-clamp (table 3.2). The amplified products were purified using the E.Z.N.A. kit (Promega) prior to DNA sequencing.

4 Results

Detailed information regarding sampling dates, time points, sampling sites, and weather conditions has been completely documented and is filed at Borregaard Ind. Ltd.[14] This journal also states in brief which analysis that has been performed by the collaborating partners. Blatny et al. (2007) gives an overall presentation of the results obtained from the collaborating partners, in which all data during the time period 11.09.2006 – 05.12.2006 has been integrated and compared.

In general, optimal air sampling was performed at various location sites at Borregaard's biological treatment plant (figure 4.1) according to different wind directions based on CFD modeling. The air samples were analyzed by FFI (this report), the Norwegian Institute of Public Health (Caugant et al., 2007) and Telelab AS (Wierød et al., 2007).

[14] POC at Borregaard: Dr. Viggo Waagen

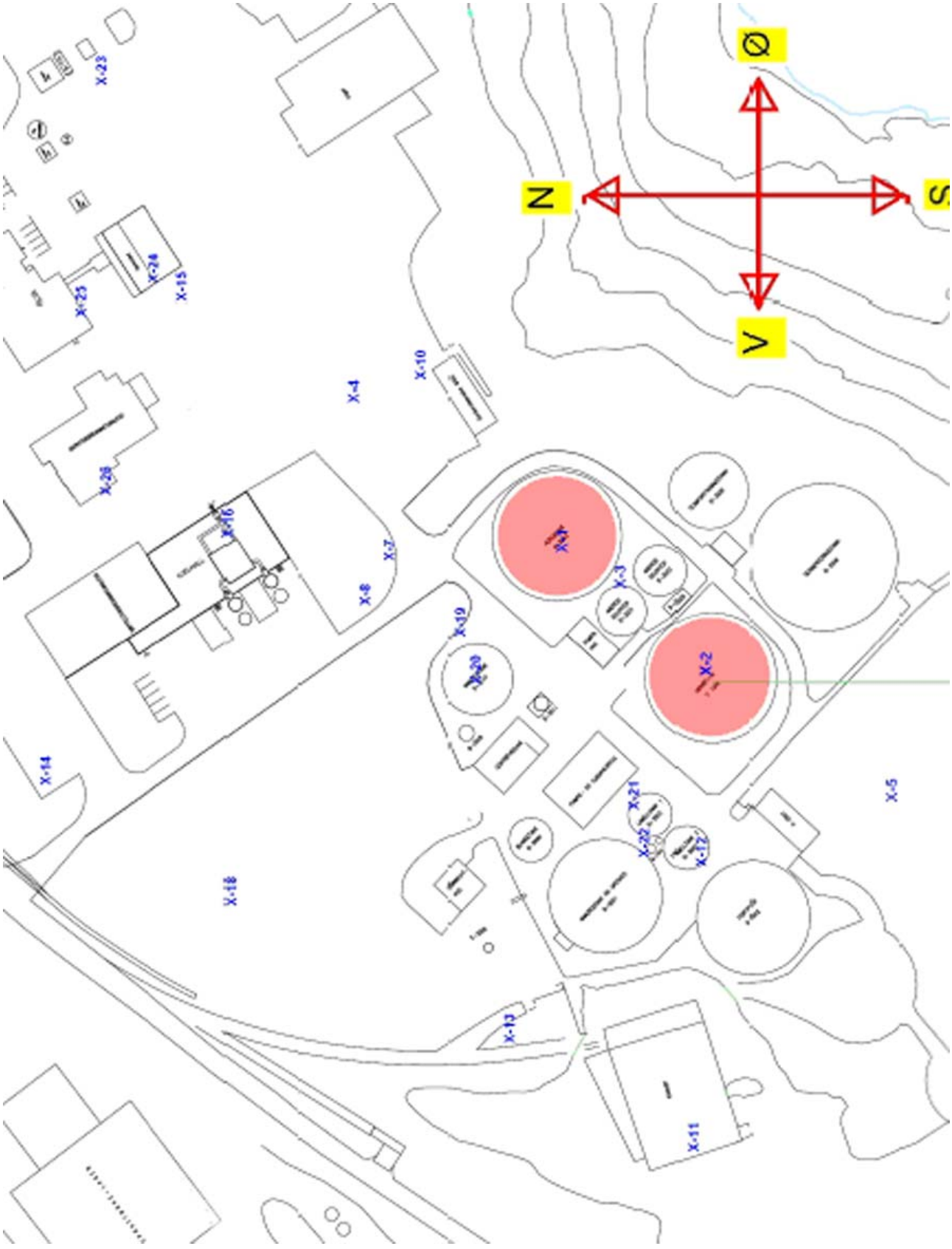


Figure 4.1. Sampling sites (in blue) for air collection at Borregaard Ind. Ltd.

4.1 CFD models

CFD was used for experimental planning, to provide guidelines in finding optimal sites for aerosol sampling equipment depending on general wind conditions. A 1 km x 1 km region[15] surrounding the biological treatment plant was modeled. In general, local wind conditions within large treatment plants vary considerable when comparing to general wind directions due to the complex interactions with buildings and topography. Thus, the local dispersion patterns can become very complex, and care has to be taken when choosing the sampling sites. The computed wind field, together with a particle tracker, was used to identify regions at the biological treatment plant where the flux of particles dispersed from the aeration ponds was expected to be high. At the same time, these regions needed to be physically suited for aerosol sampling (figure 4.2 and 4.3).

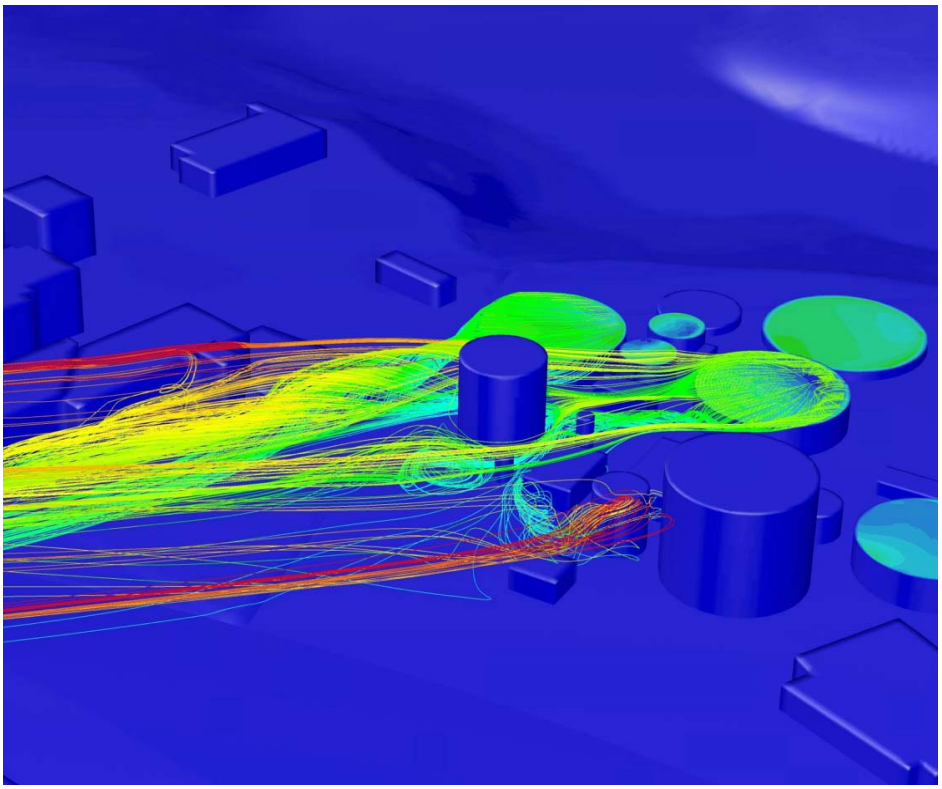


Figure 4.2. Computed particle paths of particles dispersed from aeration ponds at Borregaard Ind. Ltd. The wind direction is from south to north (360 deg) and wind speed at 2 m/s. Figure 4.3 corresponds to the complementary region suitable for air sampling at this wind condition. Colors indicate altitudes relative to the aeration ponds; blue - low, red – high.

[15] The modeled region is 1 km (length) x 1 km (width) x 250 m (height).

24 á priori simulations were conducted for wind directions separated by 15 degrees (deg) (appendix 1 and 2). These served as a basis to construct maps of the plant useful for aerosol sampling. In practical terms, the maps were used to determine the optimal sampling site on that particular day (figure 4.2 and 4.3). One sampling day was generally planned every week during the sampling campaign. The most suitable day with respect to cloud layer thickness, ceiling, temperature, and wind direction and magnitude was selected based on information from local weather forecast made for the region. These forecasts were provided to this study by the meteorological system METOC[16], a component of DNMI's[17] service for the Norwegian Armed Forces. If the forecast predicted poor conditions, regarded as unsuitable for aerosol sampling, the sampling was cancelled.

On the day of a planned measurement, the most suitable pre-computed map over the areas was selected based on the general wind direction. Smoke from chimneys at the Borregaard plant was used as on-site wind direction indicators, as well as a smoke generator. The wind direction was continuously monitored during the sampling period to assure that it did not vary considerable during the sampling period.

An analysis of the SASS 2000^{PLUS} air sampler with respect to ice formation was carried out showing that SASS 2000^{PLUS} should not be operated at temperatures below 1ºC (data not shown). This is consistent with the guidelines to the manufacturer (Research International).

[16] www.metoc.met.no
[17] DNMI : The Norwegian Meteorological Institute

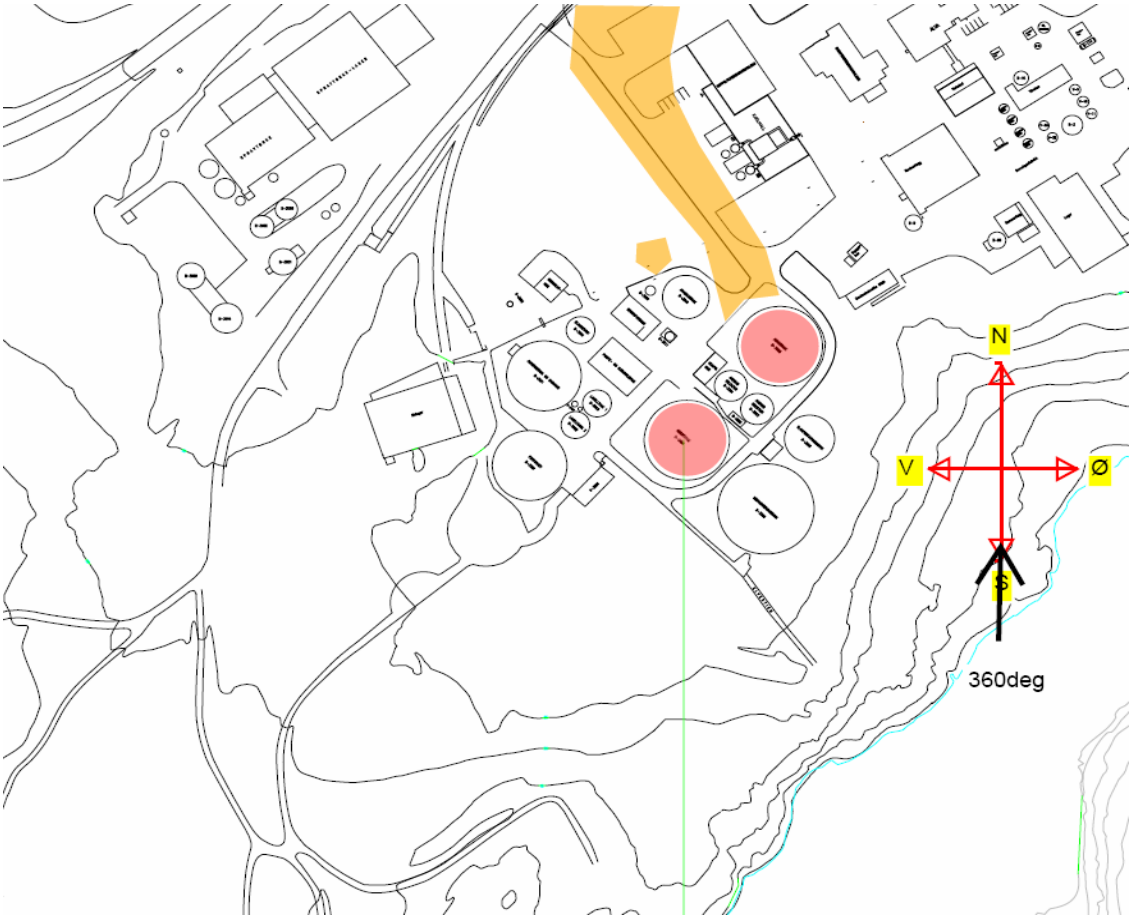


Figure 4.3. The recommended region for air sampling at wind directions from south to north (360 deg) and wind speed at 2 m/s at Borregaard Ind. Ltd. Figure 4.2 corresponds to the complementary particle tracks obtained by CFD.

4.2 Weather conditions

The survival of bacterial cells in air is believed to depend on the meteorological conditions. Convection will occur on a warm day with a clear sky and sunshine due to the heating of the ground. This will lead to a strong mixing of the lower part of the atmosphere. Convective winds will disperse the aerosols over a large volume, leading to strong rarefacation. The sunrays will efficiently dry the aerosols and possibly have an impact on the microorganisms' viability. In some cases, the microorganisms will be killed. The UV radiation from sunlight will also have an adverse impact on the survival of bacterial cells.

Analysis of meteorological data showed humid and cloudy weather prior to the outbreak of legionellosis (onset of symptoms) in Sarpsborg/Fredrikstad, May 2005 (data not shown). The atmosphere was most probably very stable against convection during this type of weather. This study tried to perform air sampling at weather conditions similar to prior to the outbreak, presumed to be ideal for sampling of aerosols containing *Legionella* spp. cells.

4.3 Air collection

The SKC Biosampler® air collector was initially used for air sampling during 13.06.2006 – 07.07.2006, but buffer was significantly evaporated during sampling with SKC Biosampler®. SKC Biosampler® and SASS 2000^{PLUS} collected 0.75 m^3 and 19.5 m^3 air, respectively (a 26-fold difference) during one hour sampling (table 3.1). These findings resulted in choosing SASS 2000^{PLUS} for further sampling. SASS 2000^{PLUS} was cleaned after use and the washing buffer was analyzed for the presence of *Legionella* spp. by Telelab AS. No growth of *Legionella* spp. was observed, except for 21.09.06.

In general, air sampling was performed upwind, above, and downwind of the aeration ponds at certain locations. The sampling time for each air collector is described in table 3.1.

The agar plates used for air collecting with MAS-100® and STA-204 were analyzed by Telelab AS and at the Norwegian Institute for Public Health. Growth of *Legionella* spp. on agar plates collected with STA-204 was compared with that obtained by MAS-100® (Wierød et al., 2007). No significant difference in the total number of cfu/ml was detected. However, in some cases, improved growth of pathogenic *Legionella* spp.[18] on agar plates from STA-204 was observed compared to that obtained with MAS-100®. This might indicate that STA-204 performs air sampling more gently than MAS-100®. However, MAS-100® was chosen for further air collections, due to its "user-friendliness" compared to STA-204.

4.4 Specificity of *mip* real-time PCR

The specificity of the *L. pneumophila* LPmip-f/r primers was analyzed by testing 14 different *L. pneumophila* strains including 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, ATCC 43703. Real-time PCR analyses showed specific PCR-products when using the LPmip-f/r primers together with the LPneu-FL/LC640 probes. No amplification was obtained when other *Legionella* spp. were tested (*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). These results were consistent with the findings by Wellinghausen et al. (2001).

[18] Pathogenic *Legionella* spp. include *L. longbeachae, L. bozemanii, L. dumoffii, L. garmanii, L. jordanis, L. micdadei* and *L. anisa* (Telelab AS).

4.5 Identification of *L. pneumophila* in air

Legionella spp. and *L. pneumophila* were identified in air by real-time PCR using the general 16S rRNA and the specific *mip* primers, respectively. *Legionella* spp. was identified in all air samples collected with SKC Biosampler® and SASS 2000^{PLUS} using the 16S rRNA primer/probe set. Thus, the results in table 4.1 are based on *mip* real-time PCR analysis. *mip* real-time PCR did not identify *L. pneumophila* in air collected with SKC Biosampler®. This is probably due to the very low collection efficiency (table 3.1 and section 4.3). Growth of *Legionella* spp. was obtained in 5 (of 12) sampling series with SKC Biosampler®, in general at low concentrations (< 270 cfu/m^3) (Wierød et al., 2007).

mip was identified in nearly all air samples collected with SASS 2000^{PLUS} (67 %)[19]. This indicated that the air samples contained *L. pneumophila* or *mip* containing *Legionella* spp. Real-time PCR detects DNA from viable, VBNC and dead bacterial cells. Thus, cultivation is needed in order to verify the presence of viable *Legionella* spp. cells. The use of MAS-100® and STA-204 allowed direct analysis of viable *Legionella* spp. cells. Analysis of specific growth of *Legionella* spp. from air collected by SASS 2000^{PLUS} (Telelab AS) showed that *Legionella* bacteria were not killed by this sampling method (Blatny et al., 2007). Results showed that *mip* real-time PCR detection was consistent with growth of *L. pneumophila* on agar plates (Blatny et al., 2007), suggesting that *mip* is an efficient marker for specific identification of *L. pneumophila*. This is consistent with Wellinghausen et al. (2001).

A common observation was that air collected upstream of the aeration ponds did not contain *L. pneumophila* (i.e. *mip* real-time PCR was negative). This is consistent with the finding of Wierød et al. (2007). *L. pneumophila* was identified in all samples harvested from the aeration ponds (section 4.6). These findings suggest that *L. pneumophila* identified in air most probably originates from the aeration ponds. However, the *mip* target was identified in one upstream air sample (no. 230, 25.10.2006).

mip real-time PCR identified *L. pneumophila* at several sampling sites downwind of the aeration pond, in which the far most distance was 180 m (table 4.1, appendix 3.1). *L. pneumophila* was identified by *mip* real-time PCR at various altitudes, even at the highest altitude, site 25, 64 MSL[20] (table 4.1, figure 4.1). *mip* was not identified in a total of ten air samples[21] (no. 168, 224, 225, 260, 261, 300, 302, 335, 337, 370) which is consistent with microbiological analysis (Blatny et al., 2007, Wierød et al., 2007).

mip was identified during various weather conditions, i.e. both at a cloudy and sunny day. This indicates that *L. pneumophila* as aerosols is present during various weather conditions. Growth of *L. pneumophila* was obtained from these samples (Blatny et al., 2007, Wierød et al., 2007),

[19] 23 of 34 samples, not including samples upwind of the aeration ponds
[20] MSL: meters above sea level
[21] Not including air samples from SKC Biosampler®, nor upstream samples.

indicating that effects, such as UV-light, might not have such a large impact on the viability of *L. pneumophila* cells in air as anticipated. A high concentration of airborne bacterial cells may protect some of the *Legionella* cells from the harmful effects of UV-radiation, providing an increased level of viable airborne *Legionella* cells compared to a low concentration level of bacterial cells in air.

These results show that

- ✓ SASS 2000^{PLUS} can be used for efficient sampling of viable *Legionella* spp.
- ✓ *mip* is a suitable genetic marker for identifying *L. pneumophila*
- ✓ *L. pneumophila* is dispersed in air surrounding and close to the aeration ponds at Borregaard's biological treatment plant
- ✓ *L. pneumophila* most probably originates from the aeration ponds

Tabell 4.1. *mip* real-time PCR analysis of air collected at Borregaard, 13.06.2006 – 05.12.2006.

Sample no.[a]	Date	Weather conditions (deg[b], m/s)	Air collector	Sample Site[c]	Distance[d] (m)	Height (MSL[e])	PCR[f]
1	13.06.06	200 deg, 4 m/s	SKC Biosampler®	1	0	35	-
2	13.06.06	200 deg, 4 m/s	SKC Biosampler®	2	0	35	-
19	20.06.06	200 deg, 3 m/s	SKC Biosampler®	1	0	35	-
20	20.06.06	200 deg, 3 m/s	SKC Biosampler®	2	0	35	-
41	27.06.06	Rain, Unstable wind	SKC Biosampler®	1	0	35	-
43	27.06.06	Rain, Unstable wind	SKC Biosampler®	2	0	35	-
45	27.06.06	Rain, Unstable wind	SKC Biosampler®	3	0	35	-
57	04.07.06	Sun, 200 deg, 1.8 m/s	SKC Biosampler®	3	0	35	-
59	04.07.06	Sun, 200 deg, 1.8 m/s	SKC Biosampler®	4	75	26	-
61	04.07.06	Sun, 200 deg, 1.8 m/s	SKC Biosampler®	5 U	65	34	-
63	11.07.06	Rain, 180 deg, 3m/s	SKC Biosampler®	4	75	26	-
65	11.07.06	Rain, 180 deg,3m/s	SKC Biosampler®	3	0	35	-
67	11.07.06	Rain, 180 deg, 3m/s	SKC Biosampler®	5 U	65	34	-
112	11.09.06	Cloudy, 200 deg, 4m/s	SASS 2000PLUS	5 U	65	34	-
114	11.09.06	Cloudy, 200 deg, 4 m/s	SASS 2000PLUS	3	0	35	+
113	11.09.06	Cloudy, 200 deg, 4 m/s	SASS 2000PLUS	4	75	26	+
122	21.09.06	Cloudy, 220 deg, 4,5 m/s	SASS 2000PLUS	5 U	65	34	+[i]/-
123	21.09.06	Cloudy, 220 deg, 4,5 m/s	SASS 2000PLUS	3	0	35	+
124	21.09.06	Cloudy, 220 deg, 4,5 m/s	SASS 2000PLUS	7	55	26	+
148	27.09.06	Rain, 190 deg, 3m/s	SASS 2000PLUS	5 U	65	34	-
149	27.09.06	Rain, 190 deg, 3 m/s	SASS 2000PLUS	3	0	35	+
150	27.09.06	Rain, 190 deg, 3 m/s	SASS 2000PLUS	8	65	26	+
166	11.10.06	Cloudy, 50 deg, 2,5 m/s	SASS 2000PLUS	10 U	65	26	-
167	11.10.06	Cloudy, 50 deg, 2,5 m/s	SASS 2000PLUS	3	0	35	+
168	11.10.06	Cloudy, 50 deg, 2,5 m/s	SASS 2000PLUS	11	145	27,9	-
191[g]	18.10.06	Rain, 60 deg, 1,5-3,5 m/s	SASS 2000PLUS	3	0	35	+
192[g]	18.10.06	Rain, 60 deg, 1,5-3,5 m/s	SASS 2000PLUS	12	55	33,8	ND
193	18.10.06	Rain, 60 deg, 1,5-3,5 m/s	SASS 2000PLUS	11	145	27,9	+
229	25.10.06[h]	Sunny, 50 deg, 0 m/s – 240, 0,5 m/s	SASS 2000PLUS	3	0	35	+
224	25.10.06	Sunny, 50, 0 m/s – 240, 0,5 m/s	SASS 2000PLUS	11	145	27,9	-
225	25.10.06	Sunny, 50 deg, 0 m/s – 240, 0,5 m/s	SASS 2000PLUS	13	125	26	-
230	25.10.06	Sunny, 50 deg, 0 m/s – 240, 0,5 m/s	SASS 2000PLUS	10 U	65	26	+
263	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000PLUS	1	0	35	+
262	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000PLUS	7	55	26	+
264	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000PLUS	16	105	45	+
261	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000PLUS	15	140	28	-
260	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000PLUS	14	180	26	-
265	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000PLUS	17	300	25	-
303	22.11.06	Changing, Rain, 130 deg, 2,5 m/s	SASS 2000PLUS	2	0	35	+
300	22.11.06	Changing, Rain 130 deg, 2,5 m/s	SASS 2000PLUS	18	150	26	-
301	22.11.06	Changing, Rain 130 deg, 2,5 m/s	SASS 2000PLUS	19	45	26	+
302	22.11.06	Changing, Rain 130 deg, 2,5 m/s	SASS 2000PLUS	20	50	47,5	-
304	22.11.06	Changing, Rain 130 deg, 2,5 m/s	SASS 2000PLUS	21	50	33,8	+
305	22.11.06	Changing, Rain 130 deg, 2,5 m/s	SASS 2000PLUS	22	60	50	+
338	29.11.06	Sunny, cloudy 230 deg, 5 m/s	SASS 2000PLUS	3	0	36,5	+
339	29.11.06	Sunny, cloudy 230 deg, 5 m/s	SASS 2000PLUS	25	160	64	+
335	29.11.06	Sunny, cloudy 230 deg, 5 m/s	SASS 2000PLUS	24	150	40,7	-
337	29.11.06	Sunny, cloudy 230 deg, 5 m/s	SASS 2000PLUS	15	140	28	-
372	05.12.06	Rain, 180 deg, 3,8 m/s	SASS 2000PLUS	1	0	35	+
373	05.12.06	Rain, 180 deg, 3,8 m/s	SASS 2000PLUS	20	50	47,5	+
369	05.12.06	Rain, 180 deg, 3,8 m/s	SASS 2000PLUS	14	180	26	+
370	05.12.06	Rain, 180 deg, 3,8 m/s	SASS 2000PLUS	26	145	37	-

[a] The sample number is according to the journal, Borregaard Ind. Ltd.

[b] Wind direction: degrees (deg) according to Borregaard's annotation, wind speed: m/s

[c] The location site is according to the map in figure 4.1. U: air sampling upwind of aeration pond.

[d] The distance (m) is estimated from the nearest aeration pond to the sampling site. 0 m is designated at the nearest aeration pond.

[e] MSL : meters above sea level

[f] Real-time PCR analysis is according to section 3.6.

[g] Technical problems appeared during sampling, thus, sample 192 was discarded.

[h] The wind changed direction during the sampling day. During 10.30h – 14.00h the wind conditions varied between '50 deg, 0 m/s' – '70 deg, 0 m/s' – '100 deg, 0 m/s' – '240 deg, 0 m/s' – '240 deg, 0,5 m/s'.

[i] Very weak amplification signals were obtained with real-time PCR analysis.

4.6 Identification of *Legionella* spp. *and L. pneumophila* in aeration ponds

Real-time PCR using the general JFP/JRP and specific Lmip-f/Lmip-r primers/probe (table 3.2) identified *Legionella* spp. and *L. pneumophila,* respectively, in ponds B 3503 and B 3504 at Borregaard's biological treatment plant (table 4.2).

Table 4.2. Real-time PCR of *Legionella* spp. in liquid samples from ponds B3501, B3503 and B3504.

Pond	Date	Real-time PCR	
		16 S rRNA *Legionella* spp.	*mip* *L. pneumophila*
B3501 (anaerobic)	13.06.2006 – 05.12-2006	+	+
B3503 (aerobic)		+	+
B3504 (aerobic)		+	+

Real-time PCR showed approximately a 10-fold higher amount of *mip*-containing *Legionella* spp. in the aeration tanks during September 2006 compared to the findings in June – August 2006. These findings are consistent with the results obtained by Telelab AS (Wierød et al., 2007) and Smiddskyddsinstitutet, Sweden (*pers. com*. G. Allestam 14.12.2006).

A possible correlation was found between the concentration of *Legionella* (cfu/ml) and the relative concentration indicated by real-time PCR. In general, a low concentration of *Legionella* bacteria (cfu/m^3) was detected at a higher Ct-value[22] by real-time PCR (appendix 4).

[22] The Ct-value represents the PCR cycle at which the fluorescence value crosses a fixed threshold that is 10 times the standard deviation of the baseline intensity.

4.7 Identification of *L. pneumophila* using *rnpB*

Only few markers are available for specific real-time PCR of *L. pneumophila*. In this study, the *mip* gene was chosen, based on literature survey, for specific identification of *L. pneumophila* (section 1.5). A further literature survey suggested also *rnpB* as an alternative marker for specific identification of *L. pneumophila* (Rubin et al., 2005).

Agar plates containing *Legionella spp.*-like colonies were provided by Telelab AS for *mip* real-time PCR analysis. Specific amplification was obtained for 30 colonies analyzed using the *mip* primers/probe set, strongly indicating that these colonies were *L. pneumophila*. Five *mip*-positive colonies were further analyzed by sequencing a fragment of the *rnpB* gene. Successful *rnpB* DNA sequencing was obtained for five colonies. The obtained sequences were aligned using ClustalW (appendix 5) and compared to the nucleotide sequences in GenBank using BLAST. A 100% DNA identity to *L. pneumophila* subsp. *pneumophila* Philadelphia strain ATCC 33152 was found. This supported the finding that the *mip*-positive colonies were *L. pneumophila*.

DNA sequencing of the *rnpB* gene fragment of 15 *Legionella* spp. isolates was also performed. These isolates were harvested directly over the aeration ponds during the summer period (13.06.2006 – 11.07.2006), at a time where the experimental design and microbiological analyses were not optimal. The DNA sequences obtained were compared to nucleotide sequences in GenBank by BLAST[23], and the results are given in table 4.3. An alignment of all *rnpB* sequences are given in appendix 6.

The alignment of the DNA sequences revealed three groups, each containing 2, 5 and 7 DNA sequences, showing 100 % DNA identity to *Fluoribacter bozemanae*[24] strain 11880T, *Legionella maceachernii/ Legionella micdadei*, and *L. londiniensis* strain CCUG 44895T, respectively (table 4.3). The Norwegian Institute of Public Health also identified *L. londiniensis* isolated from agar plates placed directly over the aeration ponds 13.06.2006. However, one *rnpB* DNA sequence (no. B11-A3) showed 100% DNA identity to *L. pneumophila* subsp. *pneumophila* strain Philadelphia. The 16S rRNA gene fragment of the same 15 isolates was sequenced using the JFP/JRP and/or Eub 933r/Eub 1387f primers for comparison and verification.

[23] http://www.ncbi.nlm.nih.gov/

[24] *Fluoribacter bozemanae* is now designated as *Legionella bozemanae*.

Table 4.3. DNA sequencing of the *rnpB* and 16S rRNA gene fragments of 15 *Legionella* spp. isolates. Samples in similar colors represent identical DNA sequences corresponding to appendix 6.

Sample no. [a]	**Date**	**Sampling equipment**	**Sampling site (fig. 4.1)**	**BLAST**[b] **best score, DNA identity to *rnpB***	**BLAST**[b] **best score, DNA identity to the 16S rRNA gene**
B7-A5	13.06.06	MAS-100®	2	*Fluoribacter bozemanae* 100%	*Fluoribacter bozemanae*, 98%
B7-A6	13.06.06	MAS-100®	2	*Legionella maceachernii*/ *Legionella micdadei*, 100%	*Legionella micdadei*, 100%
B11-A3	13.06.06	MAS-100®	1	*Legionella pneumophila* subsp. *pneumophila* strain Philadelphia, 100%	*Legionella pneumophila*, 100 %
B11-A4	13.06.06	MAS-100®	1	*Fluoribacter bozemanae*, 100%	*Fluoribacter bozemanae*, 100%
B1-A1	13.06.06	SKC Biosampler®	1	*Legionella maceachernii*/ *Legionella micdadei*, 100%	*Legionella micdadei*, 99%
B2-A2	13.06.06	SKC Biosample®	2	*Legionella maceachernii*/ *Legionella micdadei*, 100%	*Legionella micdadei*, 100%
B19-A7	20.06.06	SKC Biosampler®	1	*Legionella maceachernii*/ *Legionella micdadei*, 100%	*Legionella maceachernii*, 95%
B19-A8	20.06.06	SKC Biosampler®	1	*Legionella maceachernii*/ *Legionella micdadei*, 100%	*Legionella micdadei*, 100%
B56-B1	04.07.06	SKC Biosampler®	3	*Legionella londiniensis*, 100%	ND[c]
B56-A9	04.07.06	SKC Biosampler®	3	*Legionella londiniensis*, 100%	ND
B64-B2	11.07.06	SKC Biosampler®	3	*Legionella londiniensis*, 100%	ND
B64-B4	11.07.06	SKC Biosampler®	3	*Legionella londiniensis*, 100%	ND
B64-B5	11.07.06	SKC Biosampler®	3	*Legionella londiniensis*, 100%	ND
B64-B6	11.07.06	SKC Biosampler®	3	*Legionella londiniensis*, 100%	ND
B64-B7	11.07.06	SKC Biosampler®	3	*Legionella londiniensis*, 100%	ND

[a] The second column represents various isolates (from Telelab AS)

[b] Best hits: *Fluoribacter bozemanae* strain 11880T, *Legionella maceacherniic* strain CCUG 31116AT, *Legionella micdadei* CCUG 31229T, and *Legionella londiniensis* CCUG 44895T

[c] ND : not determined

4.8 Analyses of samples harvested from puddles and the Glomma river

Water samples were collected from the Glomma river close to the outlet from the biological treatment plant. Samples from the river were collected both upstream and downstream of the outlet. Telelab AS performed air sampling using MAS-100® at various locations along the riverside, by the outlet and upstream/downstream of the outlet. The results obtained by Telelab AS regarding the concentration of *Legionella* spp. are described in Wierød et al. (2007). A general observation was that *Legionella* spp. was not identified in aerosols generated by the waterfall, Sarpsfossen. Neither did the air samples collected along the riverside ranging from Sarpsfossen to Melløs kai contain *Legionella* spp. It cannot be excluded that *Legionella* spp. are present as aerosols at low concentration levels.

mip real-time PCR did not identify *L. pneumophila* in liquid samples harvested at Sarpsfossen (table 4.4). Also, no amplification was obtained from liquid samples at Melløs kai on 25.10.2006, in contrast to a positive amplification using the *mip* primers observed on 11.10.2006. *L. pneumophila* was also identified by *mip* real-time PCR in samples harvested from Glomma by the outlet from the biological treatment plant and at Melløs kai (table 4.4). Telelab AS confirmed these findings showing 10^4- 10^5 cfu/ml *Legionella* spp. by the outlet and 10^2-10^4 cfu/ml *Legionella* spp. at Melløs kai (Wierød et al., 2007). Results indicate that the outlet from the biological treatment plant contribute to an increase in the concentration of *Legionella* spp. to the Glomma river, but it is diluted 100 – 1000-fold when the waterflow enters Melløs kai. The finding of *L. pneumophila* near the outlet at Borregaard Ind. Ltd. is also consistent with the findings by Smiddskyddsinstitutet, Sweden (G. Allestam).

Samples from puddles were harvested 27.09.2006, 11.09.2006 and 25.10.2006. Puddles were present after rain showers. It was assumed that bioaerosols/particles (in air) containing *Legionella* would eventually be found in puddles after rain showers. Samples harvested from three puddles at various locations showed *mip*-positive real-time PCR results, indicating that the stated hypothesis is valid. Amplification was obtained for all samples at various locations using the general 16S rRNA primer set. However, further work is needed in order to elaborate this finding.

Table 4.4. *mip* real-time PCR for detection of *L. pneumophila* in Glomma.

Date	Sample	Upstream of outlet, Sarpsfossen	Outlet, Borregaard Ind. Ltd.	Downstream of outlet, Melløs kai
27.09.06	River	ND	+	ND
11.10.06	River	-	+	+
18.10.06	River	-	+	+
25.10.06	River	-	+	-

4.9 Detection of putative pathogenic bacteria in aeration ponds

Liquid samples from the aeration ponds, B 3501, B 3503 and B 3504, were analyzed for the presence of *Salmonella* spp., *E. coli*, other coliforms, intestinal enterococcus and *C. perfringens* by selective growth analysis (section 3.8). Drinking water is routinely tested for the presence of these bacteria. Liquid samples were harvested twice from the ponds (18.10.06 and 06.02.07). The total concentration (cfu/ml) of aerobic and anaerobic bacteria in the three ponds was estimated (table 4.5). Results showed a 1000-fold higher concentration of aerobic than anaerobic bacterial cells in B 3503/B 3504 versus B 3501, and a 100-fold higher concentration of anaerobic bacterial cells in B 3501 compared to B 3503/B 3504.

Table 4.5. Estimated total concentration (cfu/ml) of aerobic and anaerobic bacteria in ponds B 3501, B 3503 and B 3504 at Borregaard Ind. Ltd.

Pond	cfu/ml[a]		
	Aerobic growth 18.10.2006	Aerobic growth 06.02.2007	Anaerobic growth 18.10.2006
B 3501[b]	$<1 \times 10^3$	ND	$3{,}8 \times 10^5$
B 3503	$3{,}8 \times 10^6$	$4{,}75 \times 10^6$	$4{,}0 \times 10^3$
B 3504	$6{,}5 \times 10^6$	$5{,}1 \times 10^6$	$1{,}0 \times 10^3$

[a] Spread plating of serial dilutions of bacterial cultures was performed four days after the samples were harvested at the treatment plants. Thus, the cfu/ml figures might be underestimated.

[b] B 3501 is an anaerobic pond

Results showed growth of non-typical *Salmonella* spp. on BGA and XLD media (table 4.6, appendix 7). Specific real-time PCR[25] verified that these colonies were not *Salmonella* spp. (data not shown). DNA sequencing[26] of the 16S rRNA gene fragment (section 3.7) of single (restreaked) colonies showed that the majority of these colonies belonged to the bacterial species *Pseudomonas*. Also, some of the colonies were identified as *Vibrio* spp. and *Shewanella* spp., by DNA sequencing of the 16S rRNA gene fragment.

E. coli was not identified in any of the ponds by the cultivation methods used (table 4.6). However, putative coliform bacteria showing growth on RAPID was identified as *Vibrio* spp. by DNA sequencing of the 16S rRNA gene fragment.

Growth of *C. perfringens*-like colonies from the liquid samples appeared on mCP agar plates (table 4.6, appendix 7). Specific real-time PCR[27] analysis (data not shown) did not verify the presence of *C. perfringens*. Interestingly, DNA sequencing of the 16S rRNA gene fragment identified the colonies as *Enterococcus* spp. Intestinal enterococci was detected in both aeration

[25] *invA* gene as target for real-time PCR analysis
[26] DNA sequencing was performed on the amplified 16S rRNA gene fragments using the Eub 933r and Eub 1387F primers (section 3.7)
[27] *cpa* gene as target for real-time PCR analysis

ponds. The presence of *Enterococcus* spp. showing growth on mEA/BEA agar plates was confirmed by DNA sequencing of the 16S rRNA gene fragment.

Some of the identified bacterial species are opportunistic human pathogens suggesting that care should be taken when handling liquid samples from the aeration ponds.

Table 4.6. Bacterial species identified in ponds B 3501, B 3503 and B 3504 at Borregaard Ind. Ltd. 18.10.2006 and 06.02.2007. The basis for

Growth medium[a]	**Date**	**Microorganism selective growth**	**B 3503 (aerobic)**	**B 3504 (aerobic)**	**B 3501 (anaerobic)**	**16S rRNA seq.**[c]
BHI	18.10.06	Non selective	Overgrowth			ND
BHI	18.10.06	Non selective	Overgrowth			ND
BHI (anaerobic)	18.10.06	Non selective	Colorless/white colonies, various morphological shapes			ND
BGA	18.10.06	*Salmonella* spp.	Growth, non typical *Salmonella* spp.[b]			ND
BGA	06.02.07	*Salmonella* spp.	Growth, non typical *Salmonella* spp.			*Pseudomonas*[28] spp. *Vibrio*[29] spp. *Shewanella*[30] spp.
XLD	18.10.06	*Salmonella* spp.	Growth, non typical *Salmonella* spp.			ND
XLD	06.02.07	*Salmonella* spp.	Growth, non typical *Salmonella* spp.			*Pseudomonas* spp. *Acinetobacter*[31] spp.
Rapid	18.10.06	*E. coli* / coliforms	*E. coli:* 0 cfu/mL Coliforms: 0 cfu/mL	*E. coli:* 0 cfu/mL Coliforms: 125 cfu/mL	*E. coli:* 0 cfu/mL Coliforms: 0 cfu/mL	ND
Rapid	06.02.07	*E. coli* / coliforms	*E. coli*: 0 cfu/mL Coliforms: 125 cfu/mL	*E. coli*: 0 cfu/mL Coliforms: 12,5 cfu/mL	ND[b]	*Vibrio* spp.
mCP	18.10.06	*C. perfringens*	100 cfu/mL	50 cfu/mL	Non-suspicious colonies	ND
mCP	06.02.07	*C. perfringens*	12,5 cfu/mL	12,5 cfu/mL	ND	*Enterococcus*[32] spp.
mEA/BEA	18.10.06	Intestinal *Enterococcus*	50 cfu/mL	50 cfu/mL	Non-suspicious colonies	ND
mEA/BEA	06.02.07	Intestinal *Enterococcus*	37,5 cfu/mL	50 cfu/mL	ND	*Enterococcus* spp.

[a] See section 3.8.

[b] ND; not determined

[c] See section 3.7.

[28] *Pseudomonas* spp. are generally obligate aerobes, Gram-negative bacteria commonly found in the environment. Some species are opportunistic human pathogens.

[29] *Vibrio* spp. are Gram-negative bacteria, facultative anaerobes, commonly found in saltwater. Some species are pathogenic, exemplified by *V. cholerae*.

[30] *Shewanella* spp. are often used for bioremediation purposes during clean-up of contaminated environments.

[31] *Acinetobacter* spp. are Gram-negative encapsulated aerobic bacteria commonly found in soil and water, in which some are opportunistic human pathogens.

[32] *Enterococcus* spp. are facultative anaerobic Gram-positive bacteria. Some species may cause important clinical infections, while some are commonly found in the intestines of humans.

4.10 Bacterial diversity in aeration ponds

DGGE was used to analyze the bacterial diversity in aeration ponds B 3503 and B 3504, since the microorganisms in these ponds have not previously been identified. DGGE is a molecular technique avoiding the use of cultivation methods and a tool for analyzing culturable and unculturable bacteria.

Liquid samples were harvested from both ponds on seven different time points (26.06.2006, 04.07.2006, 11.07.2006, 11.09.2006, 21.09.2006, 27.09.2006 and 11.10.2006). Figure 4.2 shows the obtained diversity of fragments, theoretically representing various bacterial species based on their variable regions 6, 7 and 8 of the 16S rRNA gene, from three samples (similar results for the entire sampling period is given in appendix 8). DNA sequencing of fragments 1-24 (figure 4.4) showed a variety of uncultured bacteria commonly found in the environment (table 4.7). The DNA sequences of the amplified 16S rRNA gene fragments were compared to the nucleotide sequences available in GenBank by BLAST analysis. Hits included Alpha-, Delta-, and Gammaprotebacteria, Bacteriodetes, Spirochaetales, *Clostridium*[33] spp., *Pseudomonas* spp, *Rhizobium*[34] spp., *Burkholderia*[35] spp., *Shewanella* spp., *Flexibacter*[36] spp., and *Pelobacter* spp.

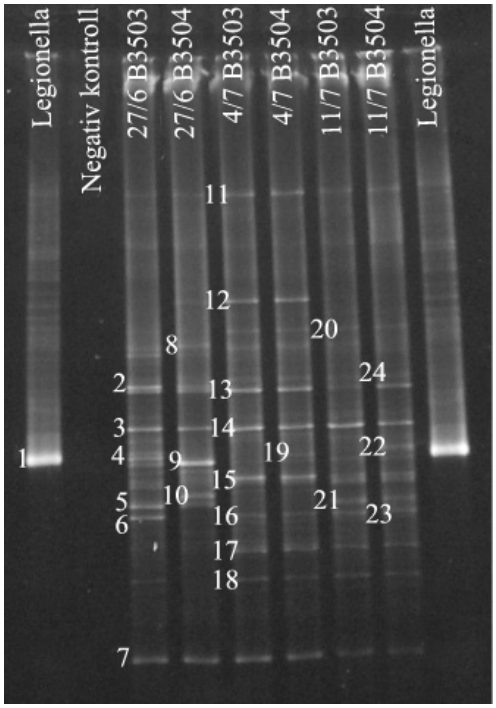


Figure 4.4. DGGE analysis of the bacterial diversity in ponds B 3503 and B 3504 at 26.06.2006, 04.07.2006 and 11.07.2006. The fragments 1-24 represent different bacterial species based on their 16S rRNA gene.

[33] *Clostridium* spp. are Gram-positive bacteria, which are able to sporulate, commonly found in soil. Some species may cause infections in humans.
[34] *Rhizobium* spp. are Gram-negative, nitrogen-fixing bacteria found in roots, or rhizosphere, of other types of plants where they cause the formation of nodules.
[35] *Burkholderia* spp. are generally obligate aerobes, Gram-negative bacteria that are capable of both pathogenic characteristics and degrading PCBs, and are commonly found in soil and groundwater.
[36] *Flexibacter* spp. are chemoorganotrophic, Gram-negative bacteria and known fish pathogens.

There seems to be a rather constant distribution level of fragments throughout the time period (figure 4.4 and appendix 8). However, some fragments are present at all sampling dates, while a few are present in only one of the ponds, or at one of the time points. Changes in the presence of fragments might reflect different environmental conditions in the ponds, different concentration levels of the bacterial species and/or that the method used is not suitable in obtaining in-depth studies of the bacterial diversity in these aeration ponds.

It was of interest to analyze the presence of *Legionella* spp. by DGGE. The *L. pneumophila* strain ATCC 33152 was used as a positive control. However, *Legionella* spp. was not found in this experiment. This finding could be due to low concentrations of *Legionella* spp. present in the ponds, or that the conditions used were not suitable for detecting *Legionella* spp. by DGGE. Further work is needed to elaborate these observations.

Table 4.7. DNA sequencing of fragments 1-24 in figure 4.4 obtained by DGGE analysis of ponds B 3503 and B 3504.

Band	**Results obtained by BLAST analysis (best hits) of sequenced 16S rRNA gene fragments from DGGE (figure 4.4).**
1	Positive control. *Legionella pneumophila*. Philadalphia 1 ATCC 3315
2	Uncultured bacteria. In particular, Gammaproteobacteria from marine environment
3	Uncultured environmental bacteria
4	ND[a]
5	Uncultured *flavobacterium*, 87% DNA identity. Uncultured *Bacteriodetes* isolated from marine environment
6	ND[a]
7	Uncultured bacteria. 92 % DNA identity, mainly Alfaproteobacteria.
8	Uncultured bacteria, *Burkholderia* spp., *Rhizobium* spp., *Pseudomonas* spp. (50 best hits), 100 % DNA identity
9	Uncultured bacteria and several *Pseudomonas* spp. 31 hits with 100 % DNA identity
10	*Pseudomonas* spp. and uncultured bacteria, 31 hits with 99 % DNA identity
11	Uncultured bacteria, 94 % DNA identity. Some *Bacteriodetes* spp.
12	Uncultured soil bacteria, 94 % DNA identity, *Bacteriodetes* and *Flexibacter* spp.
13	Uncultured bacteria, *Clostridium* spp. and soil bacteria, 100 % DNA identity
14	Uncultured bacteria, best hit 99 % DNA identity
15	Uncultured Alfaproteobacteria , 98 % DNA identity
16	Uncultured bacteria, *Spirochaetales* spp., 100 % DNA identity
17	Uncultured bacteria, best hit 96 % DNA identity, Gamma and Delta proteobacteria,. *Pelobacter spp*.
18	Uncultured bacteria, best hit 98 % DNA identity, *Spirochaeta* spp. (97 % DNA identity)
19	Uncultured bacteria, best hit 98 % DNA identity, *Shewanella* spp. (*S. morhuae* and *S. putrefaciens,* 98 % DNA identity)
20	ND
21	ND
22	Uncultured bacteria, best hit 98 % DNA identity. *Shewanella* spp. (98 % DNA identity)
23	ND
24	ND

[a] ND, due to inadequate DNA sequences or no reamplification of excised bands

5 Conclusion

The results from this work have provided insight into the dispersion of airborne aerosols containing *Legionella* spp. at Borregaard's biological treatment plant.

The following results were obtained in this study:

- ✓ *L. pneumophila* was identified in air at Borregaard's biological treatment plant using real-time PCR (the *mip* gene as marker)
- ✓ The experimental design using the CFD software Fluent, for modeling the statistical averaged wind field and the aerosol particle flux, was suitable in finding optimal sampling regions during different wind conditions
- ✓ The air collector SASS 2000^{PLUS} and MAS-100$^{®}$ were suitable for sampling aerosols containing viable *Legionella* spp. cells, including *L. pneumophila*
- ✓ *L. pneumophila* was generally identified directly above and downwind of the aeration ponds, but not upwind of the aeration ponds
- ✓ *L. pneumophila* was identified by real-time PCR (*mip*) up to 180 m from the aeration ponds
- ✓ *L. pneumophila* was identified in liquid samples harvested from the aeration ponds and the Glomma river close to Borregaard Ind. Ltd. by specific real-time PCR (*mip*)
- ✓ The bacterial species *Pseudomonas* spp., *Acinetobacter* spp., *Vibrio* spp. *Shewanella* spp., *Enterococcus* spp. and several other bacteria commonly found in the environment were identified in the aeration ponds

These results strongly indicate that the aeration ponds are a source for generating aerosols of *L. pneumophila*, and that *Legionella* spp. cells may be dispersed downwind of the ponds at least up to a distance of 180 m.

It is not known if the *L. pneumophila* strain identified in air at Borregaard Ind. Ltd. is genetically identical to that found in the liquid samples harvested from the aeration ponds, and if this strain is pathogenic to humans. The finding of opportunistic human pathogens in the aeration ponds suggest that care should be taken when handling the liquid suspension in the ponds.

6 Discussion

This report describes the identification of *L. pneumophila* in air by specific real-time PCR at Borregaard's biological treatment plant. Results showed that *L. pneumophila* was identified over the aeration ponds and up to a distance 180 m downwind of the ponds.

Identification of *L. pneumophila* in air

The Norwegian Public Health Institute and Telelab AS have confirmed our finding of *L. pneumophila* at Borregaard Ind Ltd. Interestingly, this strain seems not to belong to serogroup 1-14 (Caugant et al., 2007, Wierød et al., 2007). However, some preliminary results indicate that this strain might belong to serogroup 4 (Wierød et al., 2007). Caugant et al. (2007) showed that all isolates of *L. pneumophila* belonged to the same sequence type using multi-locus sequence typing (MLST) analysis. However, it is not known whether the isolated *L. pneumophila* strain from air is identical to that found in the aeration ponds or in the Glomma river. Further studies are needed to elaborate this, as well as analyzing the pathogenicity, if any, of this strain. A further approach in characterization of the isolated *L. pneumophila* strain would be genomic sequencing, which would primarily facilitate *in silico* studies of its genome.

In addition to *L. pneumophila*, several other *Legionella* spp. were identified from the air samples; *L. bozemanii, L. dumoffii, L. oakridgenesis, L. londiniensis* and *L. nautarum* (Caugant et al., 2007, Wierød et al., 2007).

The results obtained in this study strongly indicate that the aeration ponds at Borregaard are a source for dispersion of aerosols containing *L. pneumophila*. This is supported by the finding that *L. pneumophila* was generally not identified in air samples upwind of the ponds. Air sampling was performed by the waterfall close to Sarpsfossen, located upstream of the biological treatment plant, using MAS-100® (Telelab AS). *Legionella* spp. was not identified by growth analysis (preliminary results, Telelab AS). However, *Legionella* spp. aerosols may be generated at concentrations too low to be detected.

Air sampling conducted at higher locations and far more distant to the aeration ponds is needed to confirm whether the isolated *L. pneumophila* strain, or other *Legionella* spp., are transported over a long distance.

The *mip* gene, encoding the macrophage infectivity potentiator involved in the virulence of *L. pneumophila* (Engleberg et al., 1991), was used for specific identification of *L. pneumophila* (Ballard et al., 2000, Hayden et al., 2001, Wellinghausen et al., 2001, Templeton et al., 2003, Wilson et al., 2003, Fiume et al., 2005, Khanna et al., 2005, McDonough et al., 2005, Joly et al., 2006, Morozumi et al., 2006). Previously published results have shown that *mip* is specific for *L. pneumophila*, but *mip* DNA sequences have been found in *L. micdadei* showing approximately 70% DNA similarity with the *mip* DNA sequence from *L. pneumophila* (O'Connel et al., 1995, Ratcliff et al., 1997). This degree of similarity will not affect the specificity in identification of *L.*

pneumophila with the *mip* primers and probe selected for this study. An alignment of the *mip* DNA sequence from *L. micdadei* and *L. pneumophila* is shown in appendix 9. If *L. micdadei* would be present, Telelab AS would have detected this species as one of the group members of "the pathogenic *Legionella* spp.". This group includes the seven species *L. longbeachae, L. bozemanii, L. dumoffii, L. garmanii, L. jordanis, L. micdadei* and *L. anisa*. However, it should be noted that BLAST analysis revealed the finding of one *L. micdadei* strain PAVIA 16 isolated in Italy[37] showing a high degree of DNA similarity to *mip L. pneumophila* (appendix 10). Literature survey has not revealed other references describing the origin of the PAVIA strain. The DNA sequence of the *mip* primers and probe used in this study are identical to the corresponding DNA sequence of the PAVIA isolate, as well as to *L. pneumophila* ATCC 33215 (the Philadelphia strain). Thus, it cannot be ruled out that the *mip* real-time PCR analysis in this study have identified *mip*-containing *Legionella* spp., i.e. not only *L. pneumophila*. However, there is a lack of information regarding the classification of the PAVIA strain.

Preliminary studies in this project suggest that *rnpB* may be used for identification of *L. pneumophila* (section 4.7). DNA sequencing of the *rnpB* gene has been used to distinguish different *Legionella* spp., but it is not suitable for discriminating between *L. micdadei* and *L. maceachernii*. This can be done using *mip* (Rubin et al., 2005).

SASS 2000^{PLUS} has proven to be an efficient air collector for sampling *Legionella* spp., also including viable *L. pneumophila* cells. The reduction of sampling time from two hours to one hour resulted in a somewhat increase in the Ct-value from the real-time PCR analysis (i.e. decrease of target DNA concentration), suggesting that at least one hour should be used for sampling. FFI is currently analyzing the collection efficiency and the survival rate of viable bacterial cells sampled by SASS 2000^{PLUS}. Preliminary results indicate that SASS 2000^{PLUS} provides a high survival rate of bacterial cells. The SASS 2000^{PLUS} collector was cleaned each day after use. This was time-consuming and sometimes tedious since the fan of the air collector needed to be dismantled in order to obtain a good decontamination process (FFI unpublished results, data not shown). The SKC Biosampler® is known to perform careful sampling of microorganisms in air, which was one of the reasons in elaborating the use of this air collector for sampling of *Legionella* spp. Its low collection efficiency is not suitable for sampling microorganisms outdoor that are usually found in low concentrations, and the SKC Biosampler® was therefore not used for further sampling. The results obtained in this study showed that *L. pneumophila* was not found in samples collected with SKC Biosampler® directly over the aeration ponds (table 4.1), in contrast to that obtained using SASS 2000^{PLUS}.

[37] *L. micdadei* PAVIA 16, locus number AJ496274, www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=22553049

Evaluation of the CFD dispersion model

The outcome of a statistical turbulence RANS model represents an averaged solution of the velocity and pressure field. In statistical steady configurations, the average can be viewed as a time average. However, it is important to note that it is not physically consistent to compare the results from one full scale realization (i.e. the results from one full scale dispersion experiment) with such model predictions. ”Real life” is characterized by large variability both in time and space. Thus, in order to compare, and evaluate the outcome of the RANS model, several thousand experiments must be performed at similar weather conditions and an ensemble average must be produced. Such an analysis is not possible in the majority of full scale experiments.

The evaluation of turbulence models used for dispersion applications are usually conducted by using small scale wind- or water-tunnel experiments. The model used in this study is the so-called $k - \omega$ turbulence model which has been validated for many different applications (Durbin and Pettersson Reif, 2001). This model probably constitutes the most secure choice with respect to being reliable within this category of turbulence models, and it constitutes a relatively good compromise between accuracy and computational cost.

In this study, isothermal conditions have been assumed for simplicity and of particular importance for this work, i.e. the effects of buoyancy have not been included. By adopting this assumption, it is not possible to express convection, which results from the ground heating. It should be noted that the aeration ponds contain about 30 000 tons warm liquid, leading to the formation of a local convection cell. This results in a larger aerosol dilution than expressed by the model. The model used will therefore overestimate the aerosol flux. It was expected that the vertical disperison of the aerosols in reality was higher than in the predictions made in the model. To compensate for this, the aerosol samplers were positioned as high as possible above the terrain or at the top of buildings where possible.

Another simplifying assumption that might affect the results is that the aerosols are considered as passive tracers, thus inherently assuming that the particles were perfectly advected by the wind. This is a good approximation for small particles (< 10 microns), while this is erroneous for larger particles. The effect of coalescence, interactions with water droplets, evaporization, condenzation, and drying has not been modeled. In general, aerosols ranging in size from 1-3 microns may easily be deposited in the lungs, while particles larger in size may be settled in the head regions.

Even when considering the above mentioned simplifications, the present model represents a viable approach that satisfies the purpose of this study rather well. Together with the fact that small aerosols are regarded as more hazardous for humans compared to larger ones, our simplified CFD model for aerosol transport is a sufficient tool for this kind of experimental planning, as demonstrated in this study.

Bacterial diversity in aeration ponds

Analysis showed a high degree of bacterial diversity in the aeration ponds (table 4.6 and 4.7). Many of the bacterial species are commonly found in the environment. *Shewanella* spp. was detected in the ponds, which is not a surprise since this species may be used for bioremediation purposes. The initial microbial diversity and concentration at the start of the biological treatment plant process is not known, which would be of interest in order to evaluate any change in the bacterial diversity over time. Samples harvested from the aeration ponds were analyzed for the presence of the drinking waterborne pathogens *Salmonella* spp., *C. perfringens*, *E. coli*/coliform bacteria and intestinal *Enterococcus* spp. These bacteria are generally used as marker organisms for analyzing drinking water quality. None of these bacterial species were identified, except for *Enterococcus* spp. The growth of *Pseudomonas* spp., *Vibrio* spp. and *Acinetobacter* spp. were identified on specific growth medium for *Salmonella* spp. According to the manufacturer, BGA medium may promote growth of *Pseudomonas* spp. The results obtained showed that the specific growth analysis were not consistent with the molecular analysis (i.e. real-time PCR and DNA sequencing). However, some of the bacterial species identified may cause infections in humans suggesting that care should be taken when working near these ponds and when handling liquid samples harvested from the ponds.

Legionella spp. was not detected by DGGE, but it cannot be ruled out that the conditions used were not optimal for this analysis. *Legionella* spp. has been identified in the ponds both by real-time PCR and by specific growth analysis. An increased insight into the conditions used (such as temperature, pH, salinity, organic and inorganic compounds, chemical factors) during the biological degradation process would provide valuable information in order to evaluate if these factors contribute in enhancing growth of *Legionella* spp. in the ponds. *L. pneumophila* survives in salt solutions up to 3 % NaCl at 4-20ºC, but not at 30 – 37ºC (Heller et al., 1998). *Legionella* spp. may colonize biofilms in the environment and it has been stated that swab sampling facilitates the detection of *Legionella* spp. in biofilms (Rogers et al., 1994). The mechanism of *Legionella* bacteria to colonize and release from biofilms is not known. Free-living amoebas may act as reservoars and support intracellular growth and survival of *Legionella* cells (Murga et al., 2001, Greub and Raoult, 2004). Studies by Fields and Lucas (2006) indicate that *L. pneumophila* may persist in biofilms in the absence of amoeba, which will have implications for transmission to humans. Thus, it would be of interest to analyze the presence of amoebae in the aeration ponds.

Borregaard Ind. Ltd.

Borregaard Ind. Ltd. is taking part in a Nordic collaboration project where one of the aims is to analyze the presence of *Legionella* bacteria at various paper mills. Different studies are ongoing, including characterization of various biological treatment plant processes at both new and older paper mills. It is not known if the finding of *L. pneumophila* as aerosols at Borregaard Ind. Ltd. is generally valid for other paper mills and therefore, similar studies, as described in this report, should be performed at other mills.

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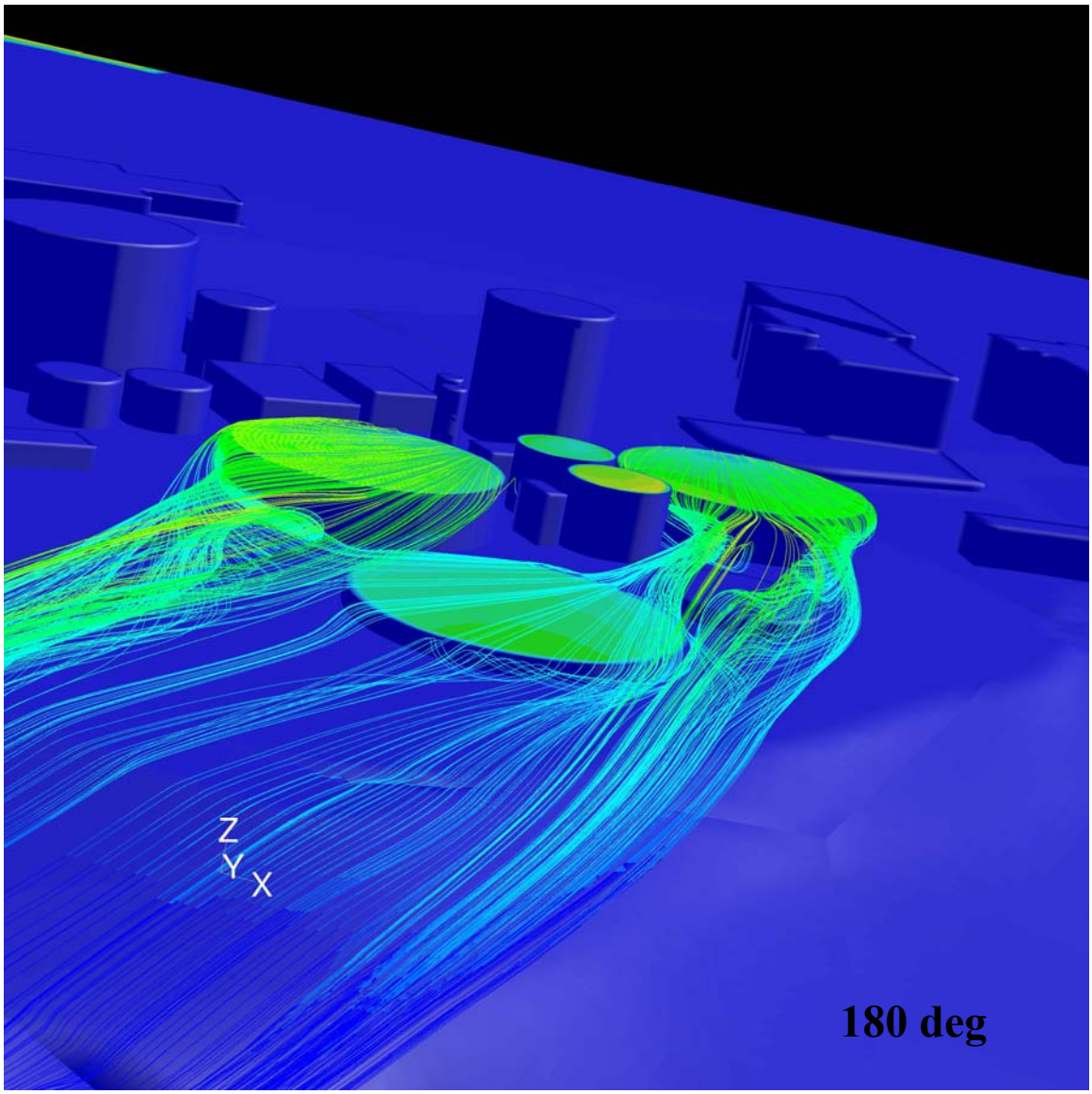
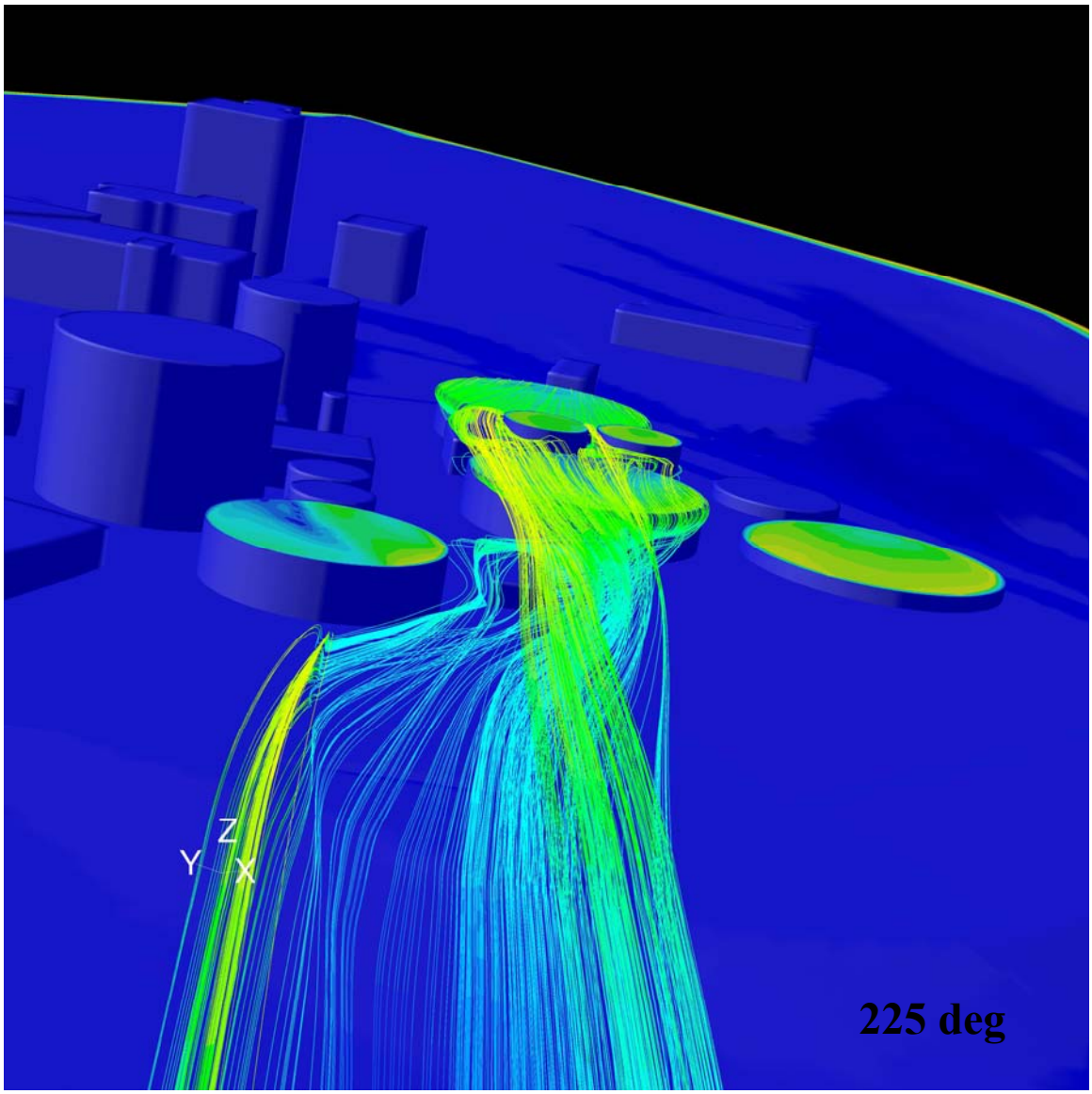
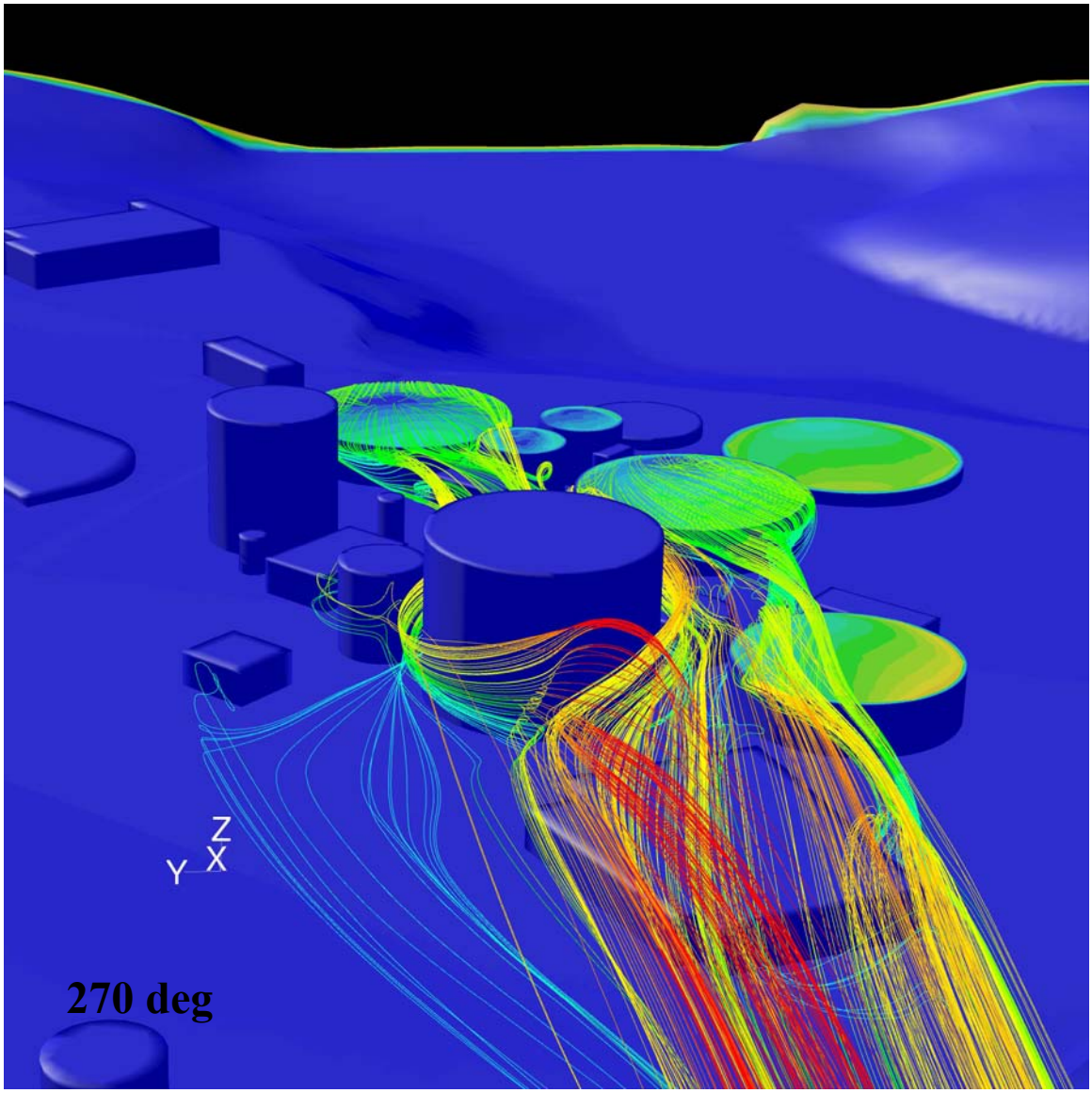
9 Appendix

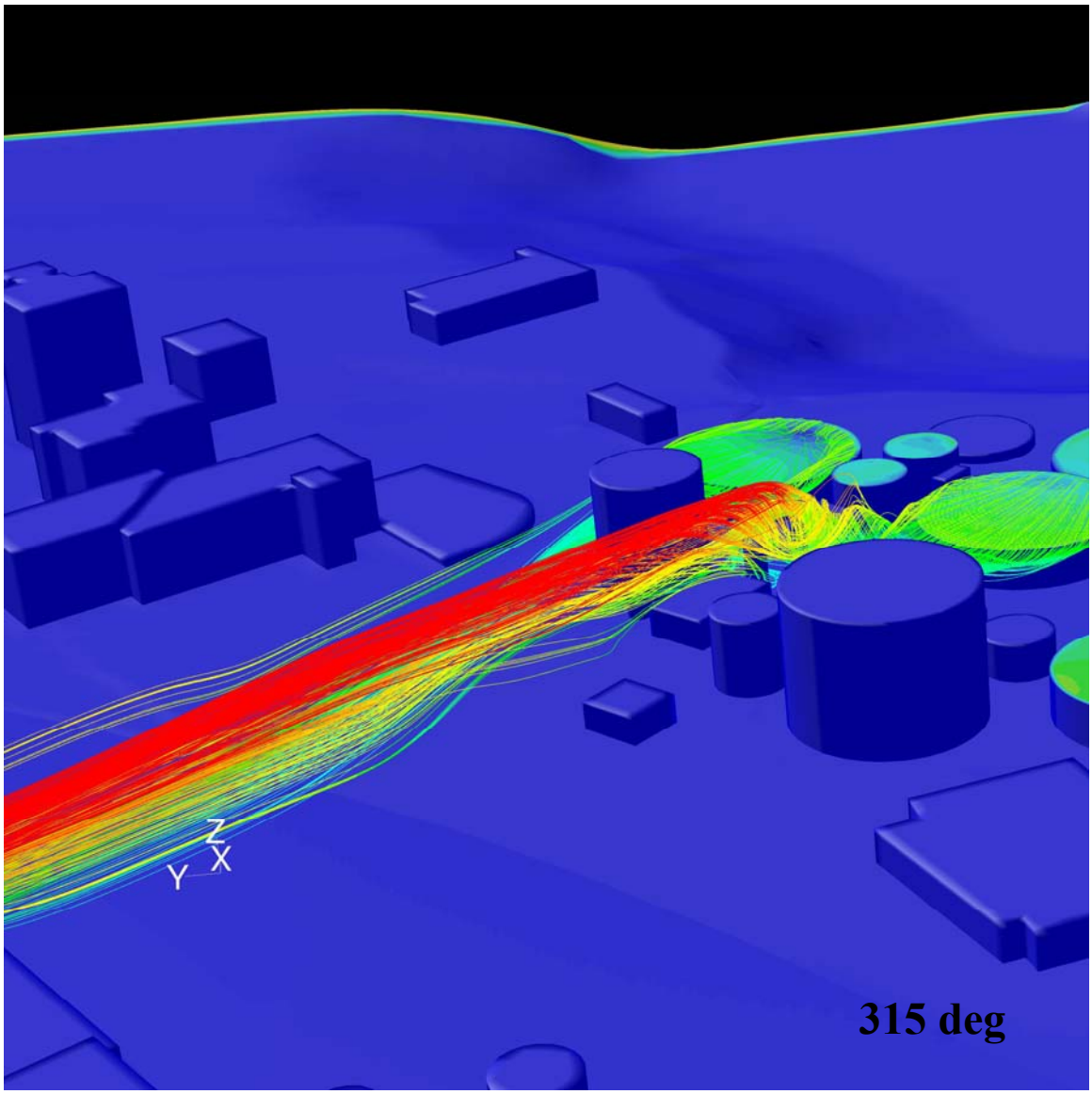
A.1 Appendix 1

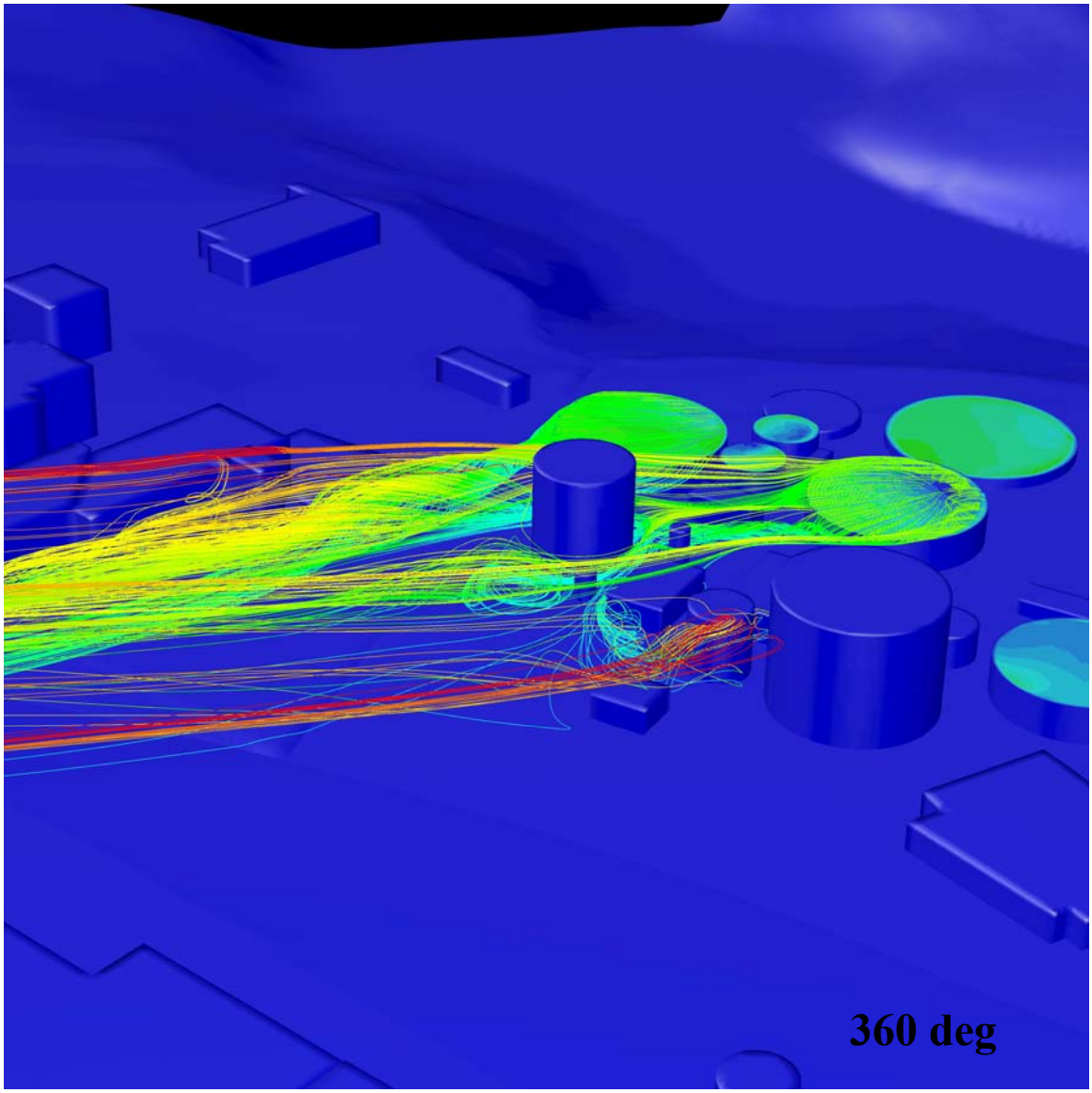
Computed particle paths for particles dispersed from aeration ponds at Borregaard Ind. Ltd. at various wind directions. See also appendix 2 for the corresponding regions optimal for air sampling. Colors indicate altitudes relative to the aeration ponds; blue - low, red - high.




Z
Y
X
225 deg

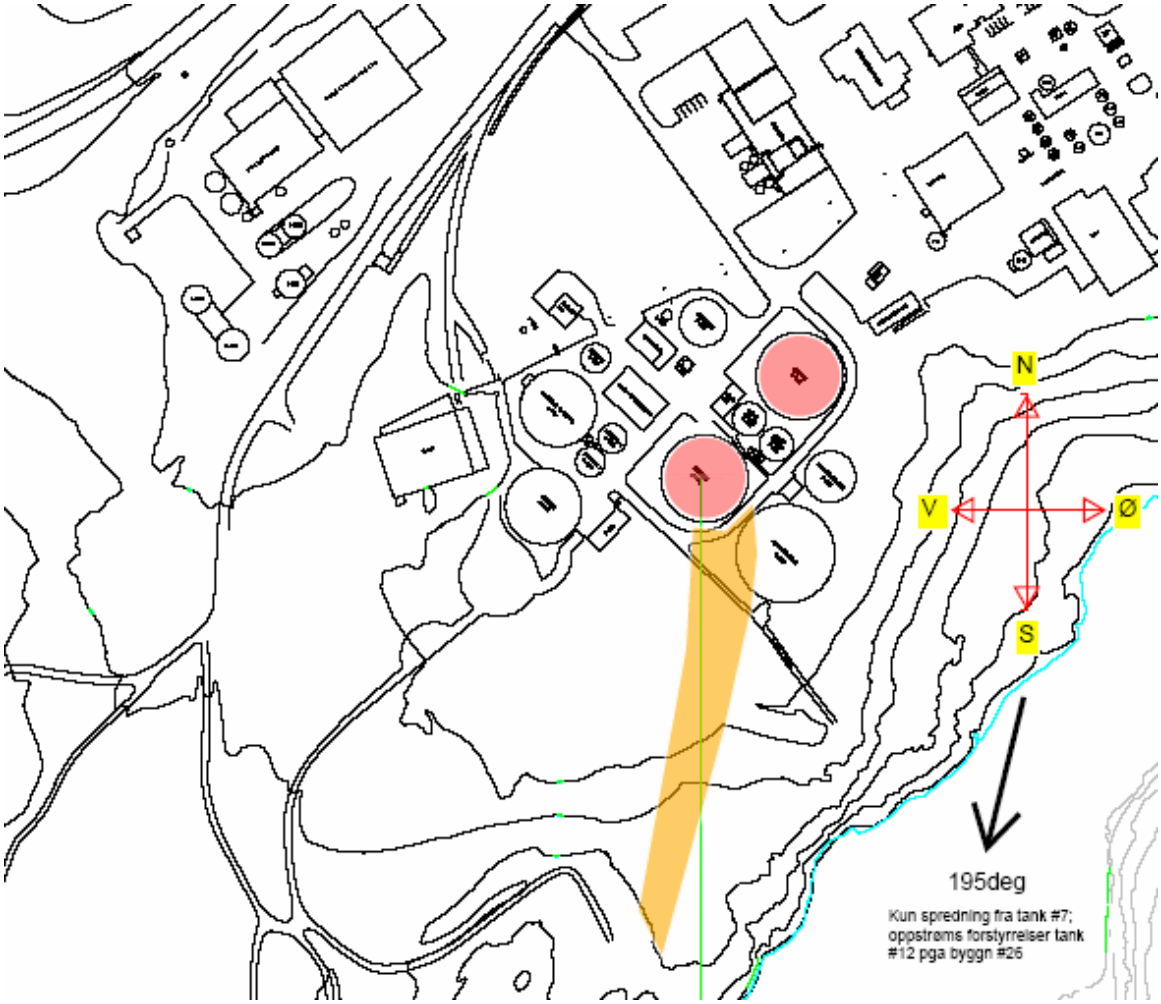

Z
X
Y
270 deg


Z
X
Y
315 deg


360 deg

A.2 Appendix 2

Recommended regions for air sampling at Borregaard Ind. Ltd according to various wind directions. 24 á priori simulations were conducted for 24 different wind directions using the CFD tool Fluent.


N
V
Ø
S
195deg
Kun spredning fra tank #7;
oppstrøms forstyrrelser tank
#12 pga byggn #26


N
V
Ø
S
210deg


N
V
Ø
S
225deg

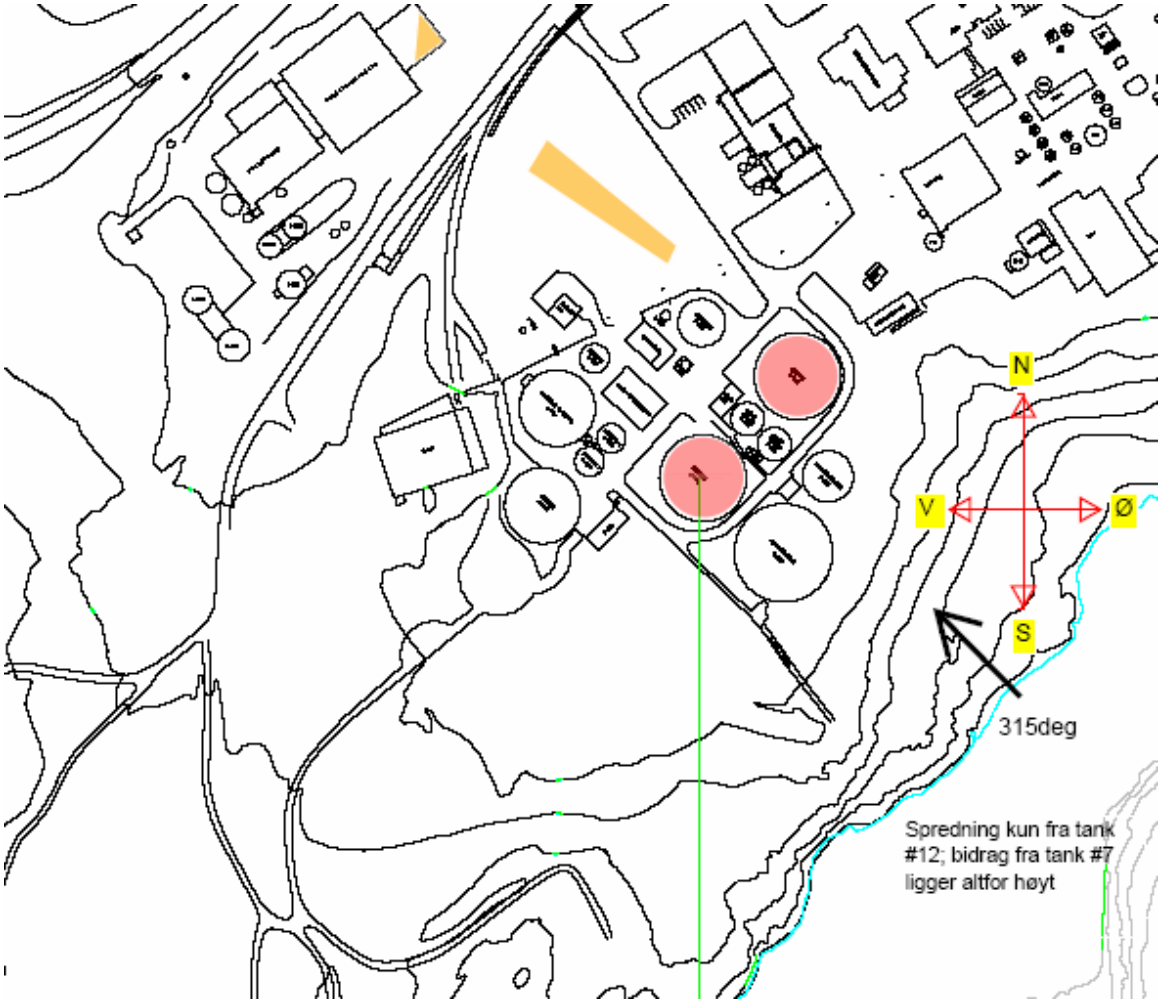

N
V
Ø
S
240deg

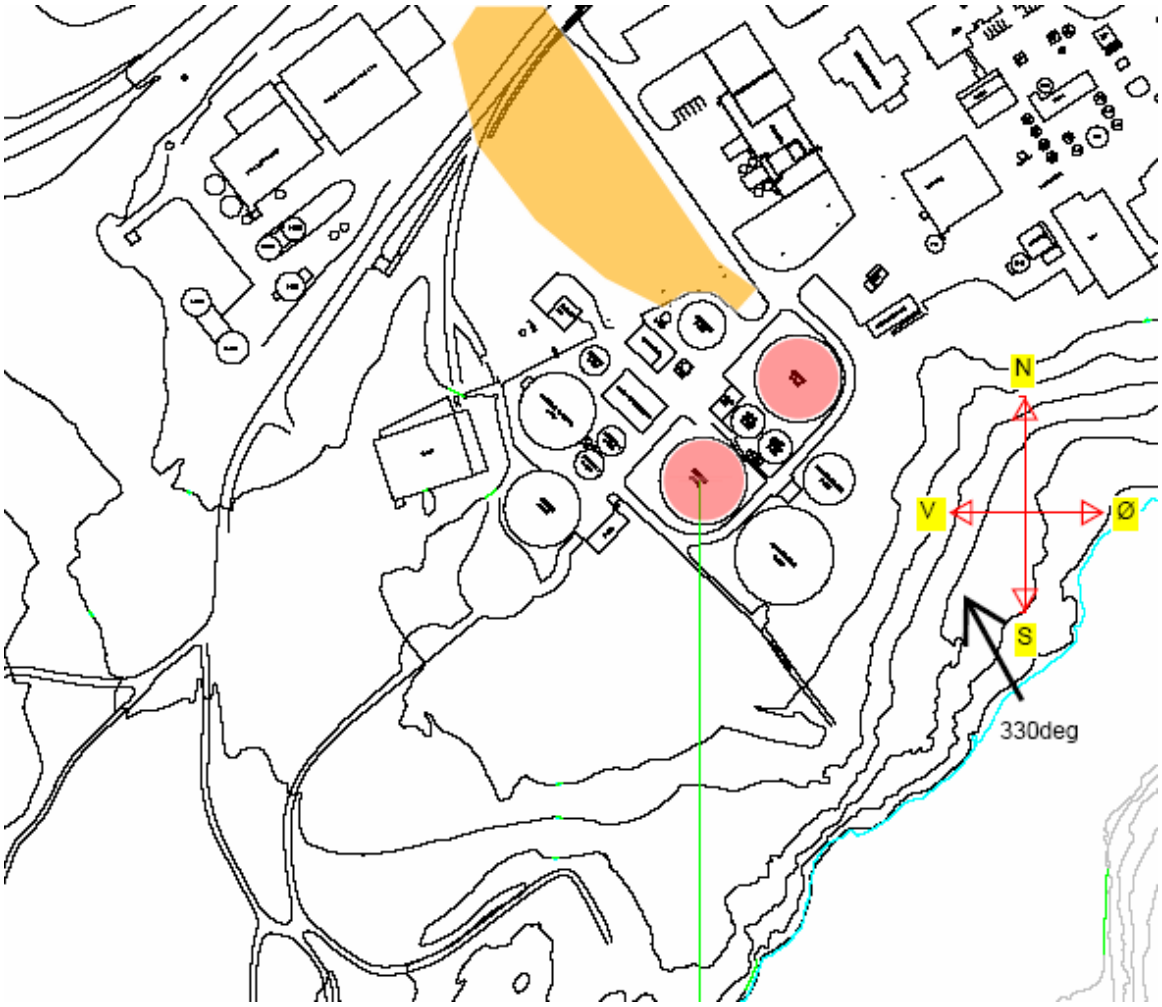

N
V
Ø
S
255deg

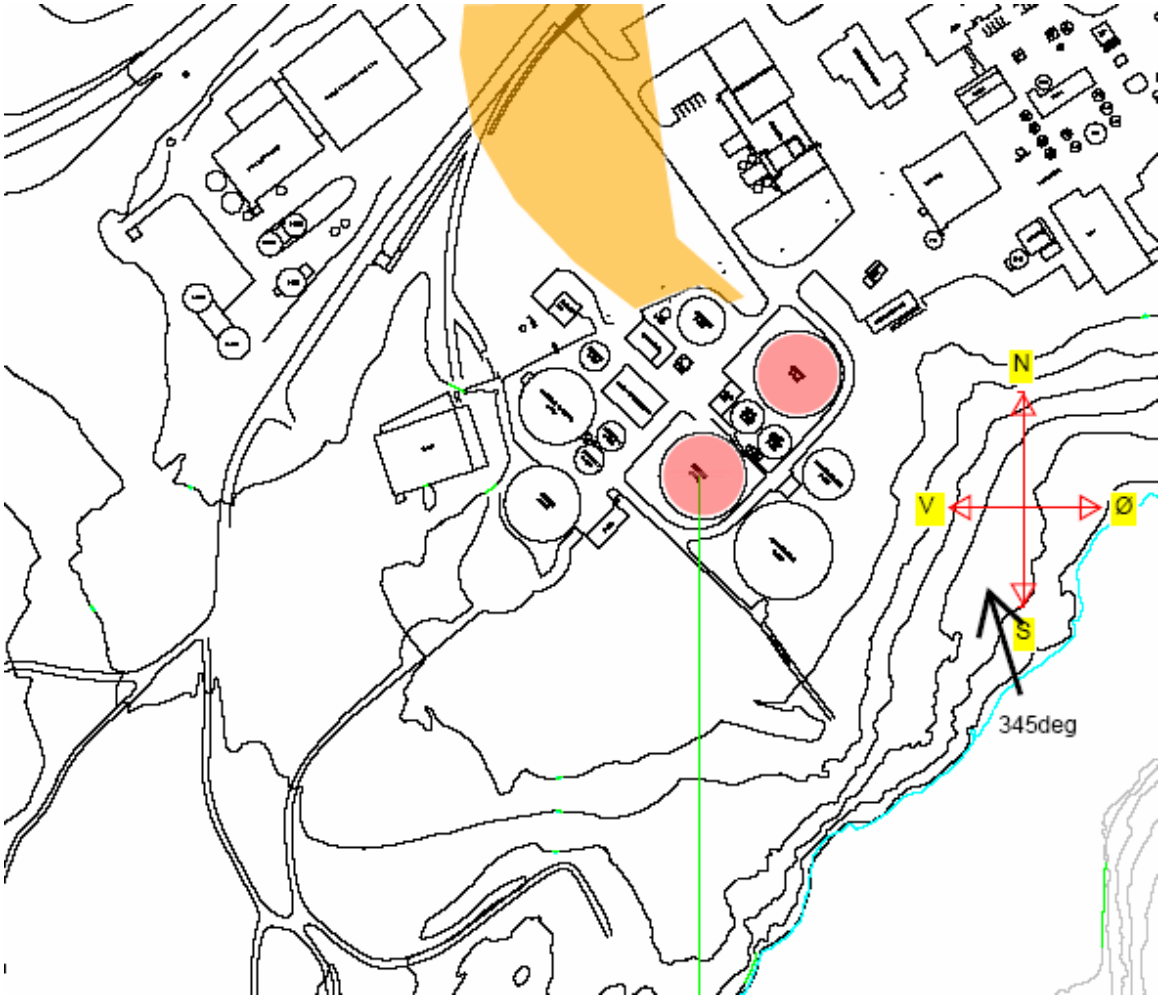

N
V
Ø
S
270deg

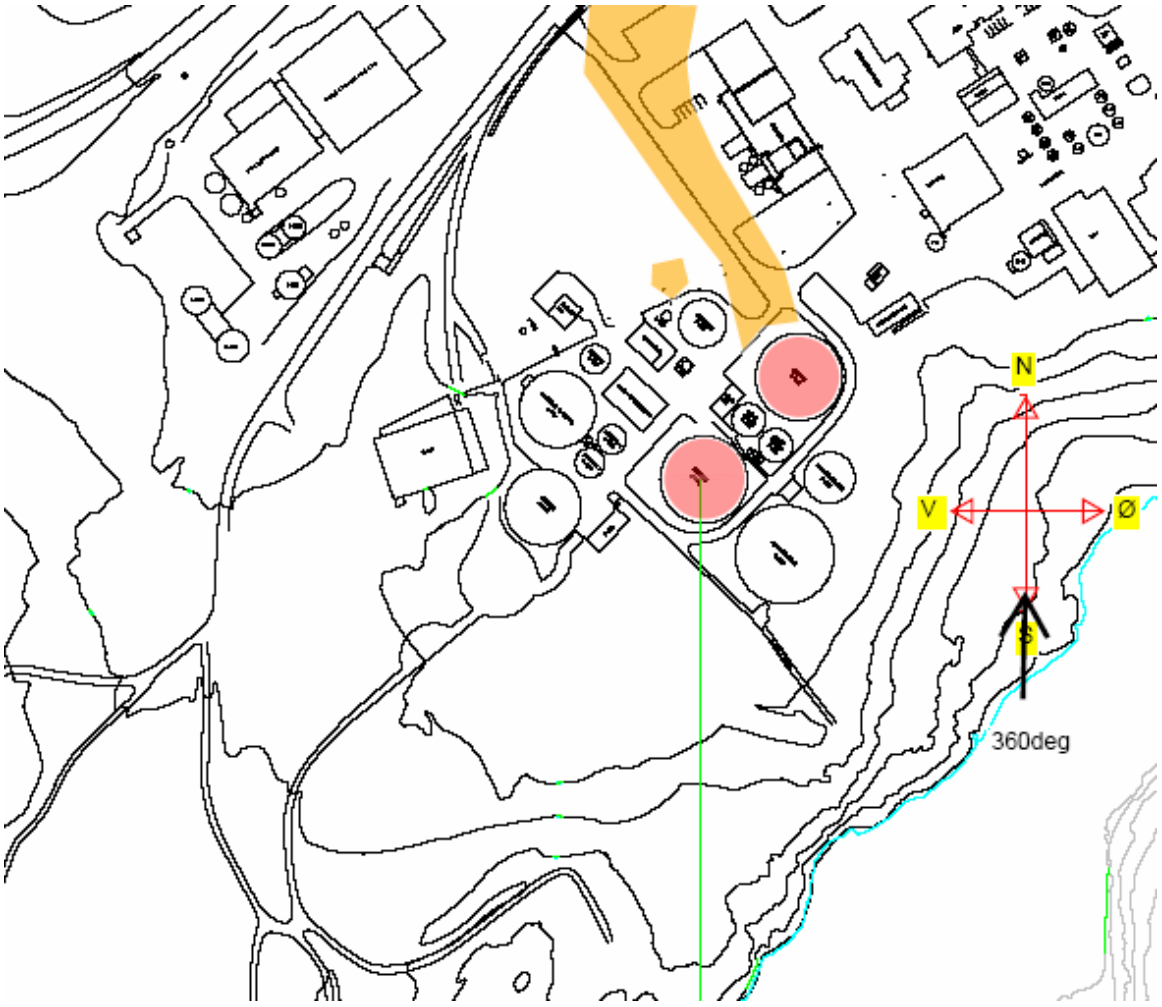

N
V
Ø
S

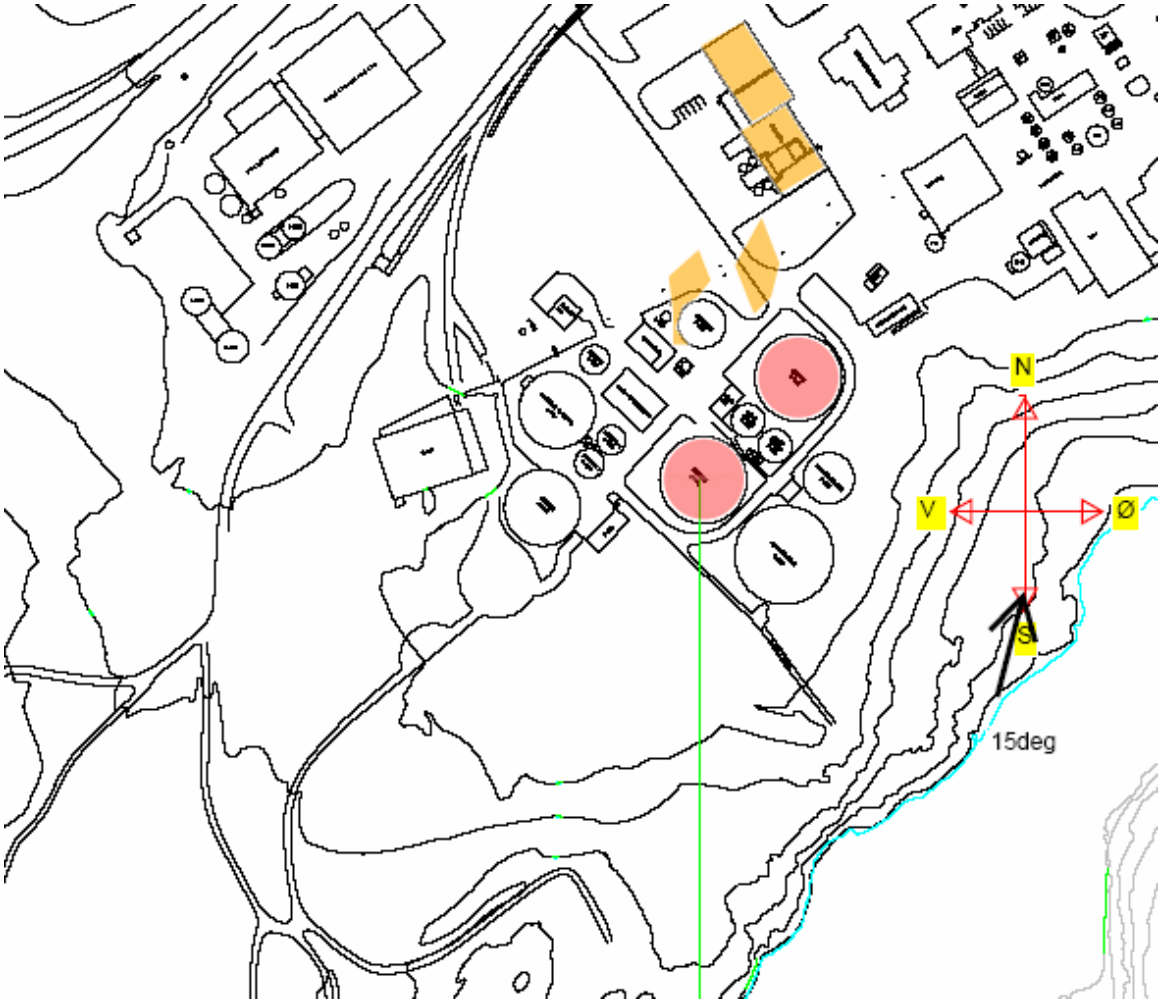

N
V
Ø
S
300deg

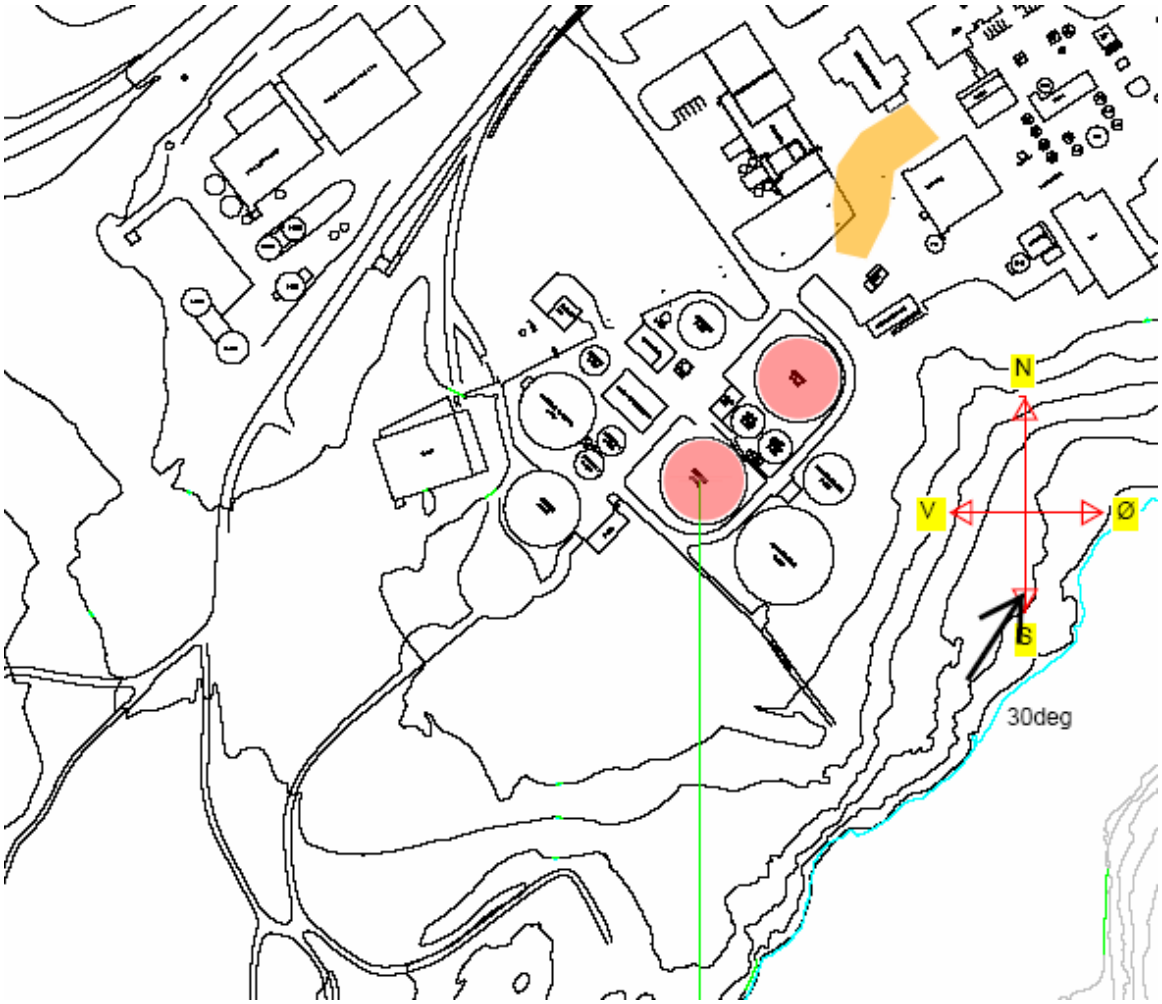

N
V
Ø
S
315deg
Spredning kun fra tank
#12; bidrag fra tank #7
ligger altfor høyt


N
V
Ø
S
330deg


N
V
Ø
S
345deg


N
V
Ø
S
360deg


N
V
Ø
S
15deg


N
V
Ø
S
30deg


N
V
Ø
S
45deg


N
V
Ø
S
60deg


N
V
Ø
S
75deg


N
V
Ø
S
90deg


N
V
Ø
S
105deg


N
V
Ø
S
120deg


N
V
Ø
S
135deg


N
V
Ø
S
150deg


N
V
Ø
S
165deg

A.3 Appendix 3

mip real-time PCR analysis of air collected with SKC Biosampler® and SASS 2000^{PLUS} during 13.06.2006 – 05.12.2006 as a function of the distance from the aeration ponds.

Sample No.[a]	Date	Weather conditions (deg[b], m/s)	Air collector	Sample Site[c]	Distance[d] (m)	Height (MSL[e])	PCR[f]
1	13.06.06	200 deg, 4 m/s	SKC Biosampler®	1	0	35	-
2	13.06.06	200 deg, 4 m/s	SKC Biosampler®	2	0	35	-
19	20.06.06	200 deg, 3 m/s	SKC Biosampler®	1	0	35	-
20	20.06.06	200 deg, 3 m/s	SKC Biosampler®	2	0	35	-
41	27.06.06	Rain, Unstable wind	SKC Biosampler®	1	0	35	-
43	27.06.06	Rain, Unstable wind	SKC Biosampler®	2	0	35	-
45	27.06.06	Rain, Unstable wind	SKC Biosampler®	3	0	35	-
57	04.07.06	Sun, 200 deg, 1.8 m/s	SKC Biosampler®	3	0	35	-
65	11.07.06	Rain, 180 deg, 3 m/s	SKC Biosampler®	3	0	35	-
114	11.09.06	Cloudy, 200 deg, 4 m/s	SASS 2000^{PLUS}	3	0	35	+
123	21.09.06	Cloudy, 220 deg, 4,5 m/s	SASS 2000^{PLUS}	3	0	35	+
149	27.09.06	Rain, 190 deg, 3 m/s	SASS 2000^{PLUS}	3	0	35	+
167	11.10.06	Cloudy, 50 deg, 2,5 m/s	SASS 2000^{PLUS}	3	0	35	+
191[g]	18.10.06	Rain, 60 deg, 1,5-3,5 m/s	SASS 2000^{PLUS}	3	0	35	+
229	25.10.06[h]	Sunny, 50 deg, 0 m/s – 240 deg, 0,5 m/s	SASS 2000^{PLUS}	3	0	35	+
263	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000^{PLUS}	1	0	35	+
303	22.11.06	Changing, Rain, 130 deg, 2,5 m/s	SASS 2000^{PLUS}	2	0	35	+
338	29.11.06	Sunny, cloudy 230 deg, 5 m/s	SASS 2000^{PLUS}	3	0	36,5	+
372	05.12.06	Rain, 180 deg, 3,8 m/s	SASS 2000^{PLUS}	1	0	35	+
301	22.11.06	Changing, Rain 130 deg, 2,5 m/s	SASS 2000^{PLUS}	19	45	26	+
302	22.11.06	Changing, Rain 130 deg, 2,5 m/s	SASS 2000^{PLUS}	20	50	47,5	-
304	22.11.06	Changing, Rain 130 deg, 2,5 m/s	SASS 2000^{PLUS}	21	50	33,8	+
373	05.12.06	Rain, 180 deg, 3,8 m/s	SASS 2000^{PLUS}	20	50	47,5	+
124	21.09.06	Cloudy, 220 deg, 4,5 m/s	SASS 2000^{PLUS}	7	55	26	+
192[g]	18.10.06	Rain, 60 deg, 1,5-3,5 m/s	SASS 2000^{PLUS}	12	55	33,8	ND
262	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000^{PLUS}	7	55	26	+
305	22.11.06	Changing, Rain 130 deg, 2,5 m/s	SASS 2000^{PLUS}	22	60	50	+
61	04.07.06	Sun, 200 deg, 1.8 m/s	SKC Biosampler®	5 U	65	34	-
67	11.07.06	Rain, 180 deg, 3 m/s	SKC Biosampler®	5 U	65	34	-
112	11.09.06	Cloudy, 200 deg, 4m/s	SASS 2000^{PLUS}	5 U	65	34	-
122	21.09.06	Cloudy, 220 deg, 4,5 m/s	SASS 2000^{PLUS}	5 U	65	34	+[i]/-
148	27.09.06	Rain, 190 deg, 3 m/s	SASS 2000^{PLUS}	5 U	65	34	-
150	27.09.06	Rain, 190 deg, 3 m/s	SASS 2000^{PLUS}	8	65	26	+
166	11.10.06	Cloudy, 50 deg, 2,5 m/s	SASS 2000^{PLUS}	10 U	65	26	-
230	25.10.06	Sunny, 50 deg, 0 m/s – 240 deg, 0,5 m/s	SASS 2000^{PLUS}	10 U	65	26	+
59	04.07.06	Sun, 200 deg, 1.8 m/s	SKC Biosampler®	4	75	26	-
63	11.07.06	Rain, 180 deg, 3m/s	SKC Biosampler®	4	75	26	-
113	11.09.06	Cloudy, 200 deg, 4 m/s	SASS 2000^{PLUS}	4	75	26	+
264	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000^{PLUS}	16	105	45	+
225	25.10.06	Sunny, 50 deg, 0 m/s – 240 deg, 0,5 m/s	SASS 2000^{PLUS}	13	125	26	-
261	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000^{PLUS}	15	140	28	-
337	29.11.06	Sunny, cloudy 230 deg, 5 m/s	SASS 2000^{PLUS}	15	140	28	-
168	11.10.06	Cloudy, 50 deg, 2,5 m/s	SASS 2000^{PLUS}	11	145	27,9	-
193	18.10.06	Rain, 60 deg, 1,5-3,5 m/s	SASS 2000^{PLUS}	11	145	27,9	+
224	25.10.06	Sunny, 50 deg, 0 m/s – 240 deg, 0,5 m/s	SASS 2000^{PLUS}	11	145	27,9	-
370	05.12.06	Rain, 180 deg, 3,8 m/s	SASS 2000^{PLUS}	26	145	37	-
300	22.11.06	Changing, Rain 130 deg, 2,5 m/s	SASS 2000^{PLUS}	18	150	26	-
335	29.11.06	Sunny, cloudy	SASS 2000^{PLUS}	24	150	40,7	-

		230 deg, 5 m/s					
339	29.11.06	Sunny, cloudy 230 deg, 5 m/s	SASS 2000^{PLUS}	25	160	64	+
260	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000^{PLUS}	14	180	26	-
369	05.12.06	Rain, 180 deg, 3,8 m/s	SASS 2000^{PLUS}	14	180	26	+
265	16.11.06	Rain, 180 deg, 4 m/s	SASS 2000^{PLUS}	17	300	25	-

[a] The sample number is according to the journal, Borregaard Ind. Ltd.

[b] Wind direction: degrees (deg) according to Borregaard's annotation, wind speed: m/s

[c] The location site is according to the map in figure 4.1. U: air sampling upwind of aeration pond.

[d] The distance (m) is estimated from the nearest aeration pond to the sampling site. 0 m is designated at the nearest aeration pond.

[e] MSL : meters above sea level

[f] Real-time PCR analysis is according to section 3.6.

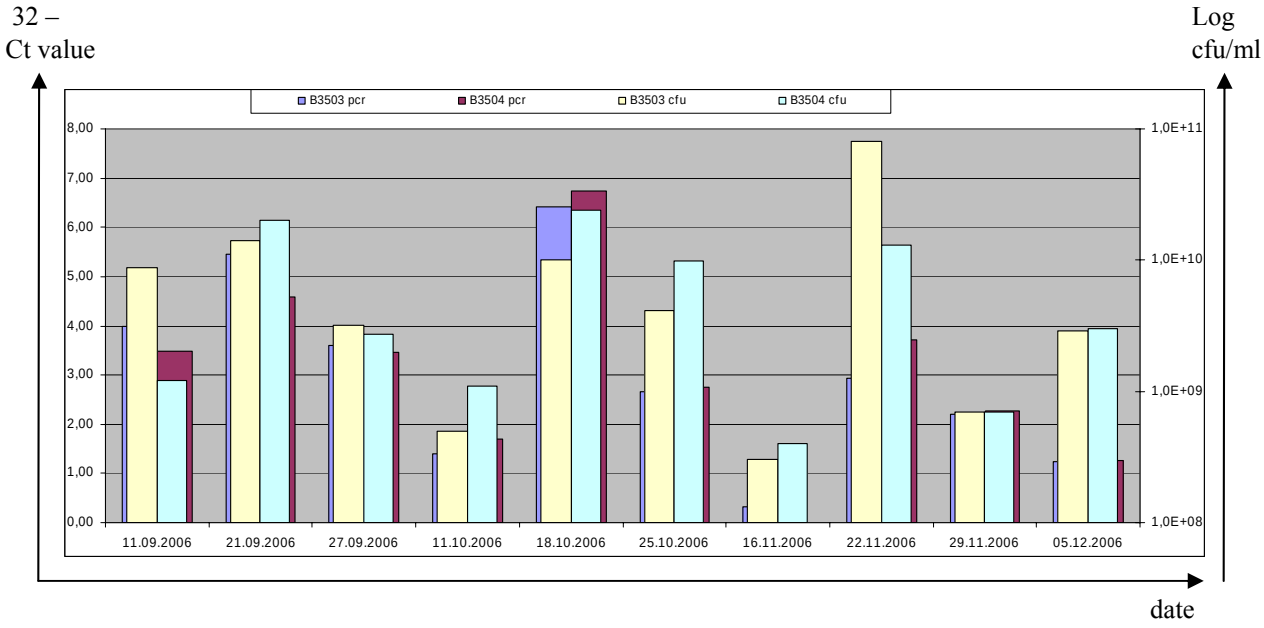
[g] Technical problems appeared during sampling, thus, sample 192 was discarded.

[h] The wind changed direction during the sampling day. During 10.30h – 14.00h the wind conditions varied between '50 deg, 0 m/s' – '70 deg, 0 m/s' – '100 deg, 0 m/s' – '240 deg, 0 m/s' – '240 deg, 0,5 m/s'.

[i] Very weak amplification signals were obtained with real-time PCR analysis.

A.4 Appendix 4

Correlation of *mip* real-time PCR (32 – Ct value)[38] and concentration of bacterial cells (cfu/ml). The cfu/ml values were kindly provided by Telelab AS.



[38] (32 – Ct value) represents the relative ratio between the Ct values, in which Ct = 32 was the highest obtained Ct value.

A.5 Appendix 5

Five *mip*-positive *Legionella*-like colonies were analyzed by sequencing the *rnpB* fragment. An alignment using ClustalW was used. BLAST analysis showed 100 % DNA identity to *L. pneumophila* subsp. *pneumophila* Philadelphia strain.

CLUSTAL W (1.83) multiple sequence alignment

```
RNPb7-f   -GGCCAGTATAAGCCGGGTTCTGTCGTGGGCAACCATTCATCTGGGACATGCGTCACCAC 59
RNPb1-    --GCCAGTATAAGCCGGGTTCTGTCGTGGGCAACCATTCATCTGGGACATGCGTCACCAC 58
RNPb2     -------------------TCTGTCGTGGGCAACCATTCATCTGGGACATGCGTCACCAC 41
RNPb11    CGGCCAGTATAAGCCGGGTTCTGTCGTGGGCAACCATTCATCTGGGACATGCGTCACCAC 60
RNPb8-    ----------------------------------------------ACATGCGTCACCAC 14
                                                         **************

RNPb7-f   ATGCCTCAAGCGACCTACCCGAATCCCGTATGGGCCATACGTTAGGACCTTGCAGTCAAA 119
RNPb1-f   ATGCCTCAAGCGACCTACCCGAATCCCGTATGGGCCATACGTTAGGACCTTGCAGTCAAA 118
RNPb2-r   ATGCCTCAAGCGACCTACCCGAATCCCGTATGGGCCATACGTTAGGACCTTGCAGTCAAA 101
RNPb11-r  ATGCCTCAAGCGACCTACCCGAATCCCGTATGGGCCATACGTTAGGACCTTGCAGTCAAA 120
RNPb8-r   ATGCCTCAAGCGACCTACCCGAATCCCGTATGGGCCATACGTTAGGACCTTGCAGTCAAA 74
          ************************************************************

RNPb7-f   TGGATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTACCAATCG 179
RNPb1-f   TGGATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTACCAATCG 178
RNPb2-r   TGGATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTACCAATCG 161
RNPb11-r  TGGATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTACCAATCG 180
RNPb8-r   TGGATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTACCAATCG 134
          ************************************************************

RNPb7-f   CGCGGTGCGCTCTTACCGCACCATTTCACCCTTACCTACGATTCCCAAAGGAAAAGTGGG 239
RNPb1-f   CGCGGTGCGCTCTTACCGCACCATTTCACCCTTACCTACGATTCCCAAAGGAAAAGTGGG 238
RNPb2-r   CGCGGTGCGCTCTTACCGCACCATTTCACCCTTACCTACGATTCCCAAAGGAAAAGTGGG 221
RNPb11-r  CGCGGTGCGCTCTTACCGCACCATTTCACCCTTACCTACGATTCCCAAAGGAAAAGTGGG 240
RNPb8-r   CGCGGTGCGCTCTTACCGCACCATTTCACCCTTACCTACGATTCCCAAAGGAAAAGTGGG 194
          ************************************************************

RNPb7-f   CGGTATATTTTCTGTGGCACTTTCCGTAGGCTCACACCTCCCAGGAGTTACCTGGCACTC 299
RNPb1-f   CGGTATATTTTCTGTGGCACTTTCCGTAGGCTCACACCTCCCAGGAGTTACCTGGCACTC 298
RNPb2-r   CGGTATATTTTCTGTGGCACTTTCCGTAGGCTCACACCTCCCAGGAGTTACCTGGCACTC 281
RNPb11-r  CGGTATATTTTCTGTGGCACTTTCCGTAGGCTCACACCTCCCAGGAGTTACCTGGCACTC 300
RNPb8-r   CGGTATATTTTCTGTGGCACTTTCCGTAGGCTCACACCTCCCAGGAGTTACCTGGCACTC 254
          ************************************************************

RNPb7-f   TGCCCTATGGAGCCCGGACTTTCCTCCCCTTGCTGTTAGGCAAAGAGCGATTGCCTG 356
RNPb1-f   TGCCCTATGGAGCCCGGACTTTCCTCC---------------------------- 325
RNPb2-r   TGCCCTATGGAGCCCGGACTTTCCTCCCCTTGCTGTT------------------- 318
RNPb11-r  TGCCCTATGGAGCCCGGACTTTCCTCCCCTTGCTGTTAGGCAAAGAGC--------- 348
RNPb8-r   TGCCCTATGGAGCCCGGACTTTCCTCCCCTTGCTGTTAGGCAAAGAGC--------- 302
          *************************
```

A.6 Appendix 6

Alignment of 15 *rnpB* DNA sequences (Clustal W) from *Legionella* spp. isolates provided by Telelab AS.

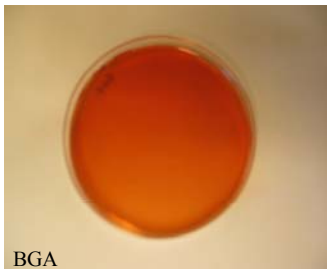
CLUSTAL W (1.83) multiple sequence alignment

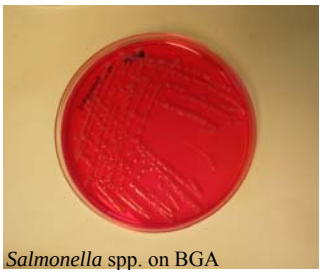
```
rnpB-B7-A5_F      ------------------------------GGACAATCATTCATCTGGGACATGCGTCA
rnpB-B11-A4_F     ACGACGGCCAGTATAAGCCGGGTTCTGTCATGGACAATCATTCATCTGGGACATGCGTCA
rnpB-B1-A1_F      --CGCGGCCAGTATAAGCCGGGTTCTGTCGTGGACAATCATTCATCTGGGACATGCGTCA
rnpB-B19-A8-F     ----CGGCCAGTATAAGCCGGGTTCTGTCGTGGACAATCATTCATCTGGGACATGCGTCA
rnpB-B2-A2_F      --------------AGCCGGGTTCTGTCGTGGACAATCATTCATCTGGGACATGCGTCA
rnpB-B19-A7-F     ----CGGCCAGTATAAGCCGGGTTCTGTCGTGGACAATCATTCATCTGGGACATGCGTCA
rnpB-B7-A6_F      ---ACGGCCAGTATAAGCCGGGTTCTGTCATGGACAATCATTCATCTGGGACATGGGTCA
rnpB-B64-B4-F     --GACGGCCAGTATAAGCCGGGTTCTGTCGCGGACAATCATTCATCTGGGACAAACGTCA
rnpB-B64-B5-F     --GACGGCCAGTATAAGCCGGGTTCTGTCGCGGACAATCATTCATCTGGGACAAACGTCA
rnpB-B64-B2-F     ---ACGGCCAGTATAAGCCGGGTTCTGTCGCGGACAATCATTCATCTGGGACAAACGTCA
rnpB-B64-B7_F     ----CGGCCAGTATAAGCCGGGTTCTGTCGCGGACAATCATTCATCTGGGACAAACGTCA
rnpB-B64-B6-F     ---ACGGCCAGTATAAGCCGGGTTCTGTCGCGGACAATCATTCATCTGGGACAAACGTCA
rnpB-B56-B1-F     ---ACGGCCAGTATAAGCCGGGTTCTGTCGCGGACAATCATTCATCTGGGACAAACGTCA
rnpB-B56-A9_F     -GCACGGCCAGTATAAGCCGGGTTCTGTCGCGGACAATCATTCATCTGGGACAAACGTCA
rnpB-B11-A3_R     --GACGGCCAGTATAAGCCGGGTTCTGTCGTGGGCAACCATTCATCTGGGACATGCGTCA
                                            **  ** ***************  *****

rnpB-B7-A5_F      CCACATGCCTCAAGCGACCTACCCGAATCCCATGCGGGTCACACGTAATGACTTTTCAGC
rnpB-B11-A4_F     CCACATGCCTCAAGCGACCTACCCGAATCCCATGCGGGTCACACGTAATGACTTTTCAGC
rnpB-B1-A1_F      CCACATGCCTCAAGCAACCTACCCGAATCCCGTACGGGCCATACGCTCTTACTAAAAAGT
rnpB-B19-A8-F     CCACATGCCTCAAGCAACCTACCCGAATCCCGTACGGGCCATACGCTCTTACTAAAAAGT
rnpB-B2-A2_F      CCACATGCCTCAAGCAACCTACCCGAATCCCGTACGGGCCATACGCTCTTACTAAAAAGT
rnpB-B19-A7-F     CCACATGCCTCAAGCAACCTACCCGAATCCCGTACGGGCCATACGCTCTTACTAAAAAGT
rnpB-B7-A6_F      CCACATGCCTCAAGCAACCTACCCGAATCCCGTACGGGCCATACGCTCTTACTAAAAAGT
rnpB-B64-B4-F     CCGTTTGCCTCAAGCGACCTACCCGAATCCCACATGGGCCATGTG---TTGCAATAAATT
rnpB-B64-B5-F     CCGTTTGCCTCAAGCGACCTACCCGAATCCCACATGGGCCATGTG---TTGCAATAAATT
rnpB-B64-B2-F     CCGTTTGCCTCAAGCGACCTACCCGAATCCCACATGGGCCATGTG---TTGCAATAAATT
rnpB-B64-B7_F     CCGTTTGCCTCAAGCGACCTACCCGAATCCCACATGGGCCATGTG---TTGCAATAAATT
rnpB-B64-B6-F     CCGTTTGCCTCAAGCGACCTACCCGAATCCCACATGGGCCATGTG---TTGCAATAAATT
rnpB-B56-B1-F     CCGTTTGCCTCAAGCGACCTACCCGAATCCCACATGGGCCATGTG---TTGCAATAAATT
rnpB-B56-A9_F     CCGTTTGCCTCAAGCGACCTACCCGAATCCCACATGGGCCATGTG---TTGCAATAAATT
rnpB-B11-A3_R     CCACATGCCTCAAGCGACCTACCCGAATCCCGTATGGGCCATACGTTAGGACCTTGCAGT
                  **   ********** ***************  * *** **   *      *

rnpB-B7-A5_F      CAAATGG-ATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGCCTGTTAC
rnpB-B11-A4_F     CAAATGG-ATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGCCTGTTAC
rnpB-B1-A1_F      -AAGTGGGATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTAC
rnpB-B19-A8-F     -AAGTGGGATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTAC
rnpB-B2-A2_F      -AAGTGGGATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTAC
rnpB-B19-A7-F     -AAGTGGGATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTAC
rnpB-B7-A6_F      -AAGTGGGATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTAC
rnpB-B64-B4-F     GCAATGG-ATTCCTATTTGGTCTTGCTCCAAGCGGGGTTTTCCATGCCATTGCTGTTAC
rnpB-B64-B5-F     GCAATGG-ATTCCTATTTGGTCTTGCTCCAAGCGGGGTTTTCCATGCCATTGCTGTTAC
rnpB-B64-B2-F     GCAATGG-ATTCCTATTTGGTCTTGCTCCAAGCGGGGTTTTCCATGCCATTGCTGTTAC
rnpB-B64-B7_F     GCAATGG-ATTCCTATTTGGTCTTGCTCCAAGCGGGGTTTTCCATGCCATTGCTGTTAC
rnpB-B64-B6-F     GCAATGG-ATTCCTATTTGGTCTTGCTCCAAGCGGGGTTTTCCATGCCATTGCTGTTAC
rnpB-B56-B1-F     GCAATGG-ATTCCTATTTGGTCTTGCTCCAAGCGGGGTTTTCCATGCCATTGCTGTTAC
rnpB-B56-A9_F     GCAATGG-ATTCCTATTTGGTCTTGCTCCAAGCGGGGTTTTCCATGCCATTGCTGTTAC
rnpB-B11-A3_R     CAAATGG-ATTCCTATTTGGTCTTGCTCCGAGTGGGGTTTTCCCTGCCACGACTGTTAC
                    * *** ********************* ** ********** *****   ***** **
```

```
rnpB-B7-A5_F      CAGCCGCGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCTCT--------------
rnpB-B11-A4_F     CAGCCGCGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCTCT--------------
rnpB-B1-A1_F      CAATCGCGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCTGT------TAAAAAT-
rnpB-B19-A8-F     CAATCGCGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCTGT------TAAAAAT-
rnpB-B2-A2_F      CAATCGCGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCTGT------TAAAAAT-
rnpB-B19-A7-F     CAATCGCGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCTGT------TAAAAAT-
rnpB-B7-A6_F      CAATCGCGCGGTGCGCTCTTACCGCACCATTTCTACCCTTACCTGT------TAAAAAT- 230
rnpB-B64-B4-F     CAGCAATGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCCAC------CCAAGATT 226
rnpB-B64-B5-F     CAGCAATGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCCAC------CCAAGATT 226
rnpB-B64-B2-F     CAGCAATGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCCAC------CCAAGATT 225
rnpB-B64-B7_F     CAGCAATGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCCAC------CCAAGATT 224
rnpB-B64-B6-F     CAGCAATGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCCAC------CCAAGATT 225
rnpB-B56-B1-F     CAGCAATGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCCAC------CCAAGATT 225
rnpB-B56-A9_F     CAGCAATGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCCAC------CCAAGATT 227
rnpB-B11-A3_R     CAATCGCGCGGTGCGCTCTTACCGCACCATTTC-ACCCTTACCTACGATTCCCAAAGGAA 146
                  **     ************************* *********

rnpB-B7-A5_F      -AAGAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGTTCACACCTCCCAGGTGTTACCT 251
rnpB-B11-A4_F     -AAGAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGTTCACACCTCCCAGGTGTTACCT 282
rnpB-B1-A1_F      -AACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTTACGCCTCCCAGGTGTTACCT 287
rnpB-B19-A8-F     -AACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTTACGCCTCCCAGGTGTTACCT 285
rnpB-B2-A2_F      -AACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTTACGCCTCCCAGGTGTTACCT 274
rnpB-B19-A7-F     -AACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTTACGCCTCCCAGGTGTTACCT 285
rnpB-B7-A6_F      -AACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTTACGCCTCCCAGGTGTTACCT 289
rnpB-B64-B4-F     GGACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTCGCGCCTCCCAGGTGTTACCT 286
rnpB-B64-B5-F     GGACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTCGCGCCTCCCAGGTGTTACCT 286
rnpB-B64-B2-F     GGACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTCGCGCCTCCCAGGTGTTACCT 285
rnpB-B64-B7_F     GGACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTCGCGCCTCCCAGGTGTTACCT 284
rnpB-B64-B6-F     GGACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTCGCGCCTCCCAGGTGTTACCT 285
rnpB-B56-B1-F     GGACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTCGCGCCTCCCAGGTGTTACCT 285
rnpB-B56-A9_F     GGACAGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTCGCGCCTCCCAGGTGTTACCT 287
rnpB-B11-A3_R     AAGTGGGCGGTATATTTTCTGTGGCACTTTCCGTAGGCTCACACCTCCCAGGAGTTACCT 206
                       ******************************** *  * ********* *******

rnpB-B7-A5_F      GGCACTCTACCCTATGGAGCCCGGACTTTCCAC--------------------------- 284
rnpB-B11-A4_      GGCACTCTACCCTATGGAG------------------------------------------ 301
rnpB-B1-A1_F      GGCACTCTGCCCTGCGGAGCCCGGACTTTCCTCCCTTTGTTTTGCACAA----------- 336
rnpB-B19-A8-F     GGCACTCTGCCCTGCGGAGCCCGGACTTTCCTCCCTTTGTTTTGCC-------------- 331
rnpB-B2-A2_F      GGCACTCTGCCCTGCGGAGCCCGGACTTTCCTCC--------------------------- 308
rnpB-B19-A7-F     GGCACTCTGCCCTGCGGAGCCCGGACTTTCCTCCCTTTGTTTTGCACAAAGAGCGATTGC 345
rnpB-B7-A6_F      GGCACTCTGCCCTGCGGAGCCCGGACT---------------------------------- 316
rnpB-B64-B4-F     GGCGCTTTGCCCTGTGGAGCCCGGACTTTCCTCTCTCCGCGAATTGCGAAAAGCGATTGC 346
rnpB-B64-B5-F     GGCGCTTTGCCCTGTGGAGCCCGGACTTTCC------------------------------ 317
rnpB-B64-B2-F     GGCGCTTTGCCCTGTGGAGCCCGGACTTTCCTCTCTCCGCGAATTGC------------- 332
rnpB-B64-B7_F     GGCGCTTTGCCCTGTGGAGCCCGGACTTTCCTCTCTCCGCGAATTGCGAA---------- 334
rnpB-B64-B6-F     GGCGCTTTGCCCTGTGGAGCCCGGACTTTCCTCTCTCCGCGAATTGCGAAAAGCGATTGC 345
rnpB-B56-B1-F     GGCGCTTTGCCCTGTGGAGCCCGGACTTTCCTCTCTCCGCGAATTGCGAAAAGCGATTGC 345
rnpB-B56-A9_F     GGCGCTTTGCCCTGTGGAGCCCGGACTTTCCTCTCTCCGCGAATTGCGAAAAGCGAATTG 347
rnpB-B11-A3_R     GGCACTCTGCCCTATGGAGCCCGGACTTTCCTCCCCTTGCTGTTAGGCAAAGAGCGATTG 266
                  *** ** * ****  ****

A1rnpB-B7-A5_F        -----------
B2rnpB-B11-A4_F       -----------
B6rnpB-B1-A1_F        -----------
B2rnpB-B19-A8-F       -----------
C1rnpB-B2-A2_F        -----------
A9rnpB-B19-A7-F       CTGACCAAACT 356
A5rnpB-B7-A6_F        -----------
B6rnpB-B64-B4-F       CTGAC------ 351
B10rnpB-B64-B5-F      -----------
C1rnpB-B64-B2-F       -----------
C5rnpB-B64-B7_F       -----------
A5rnpB-B64-B6-F       CTGA------- 349
C5rnpB-B56-B1-F       CTGACC----- 351
B10rnpB-B56-A9_F      CC--------- 349
A11rnpB-B11-A3_R      CC--------- 268
```

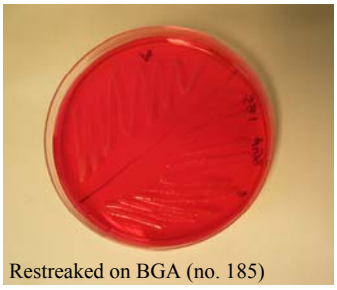
A.7 Appendix 7

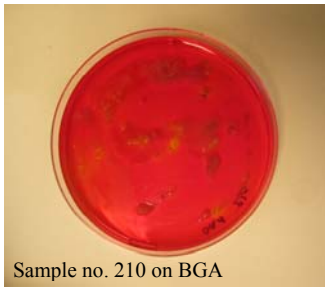
Detection of *Salmonella*-like spp., coliforms (non-*E. coli*), intestinal enterococcus and *C. perfringens* in the ponds B 3501, B 3503 and B 3504 using specific growth analysis.
See section 3.8 and 4.9.

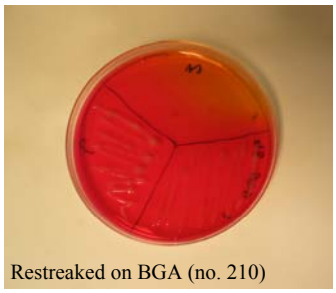

BGA

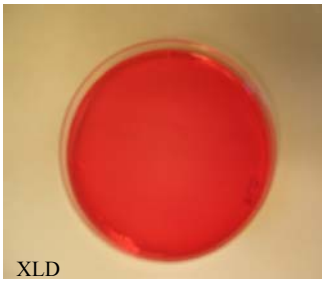

Salmonella spp. on BGA

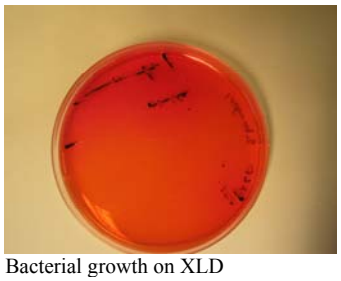

Sample no. 185 on BGA

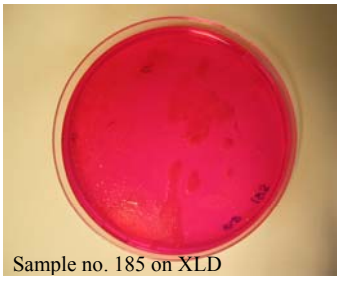

Restreaked on BGA (no. 185)


Sample no. 210 on BGA

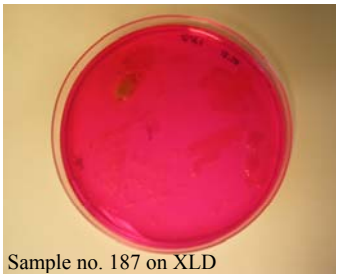

Restreaked on BGA (no. 210)


XLD

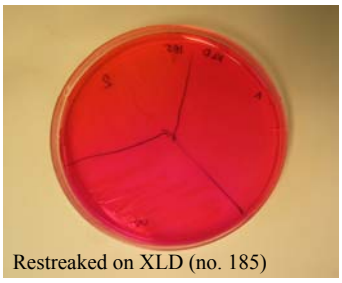

Bacterial growth on XLD



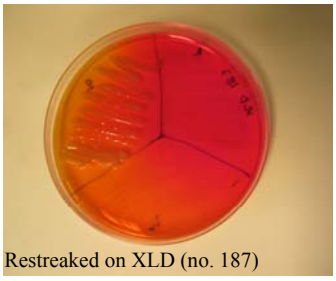
Sample no. 185 on XLD



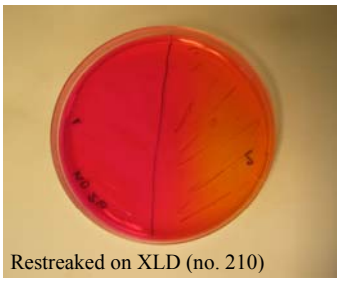
Sample no. 187 on XLD



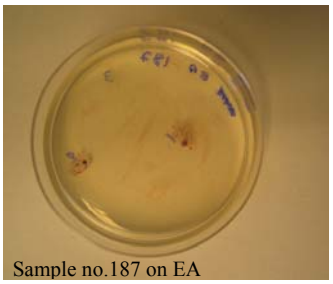
Restreaked on XLD (no. 185)



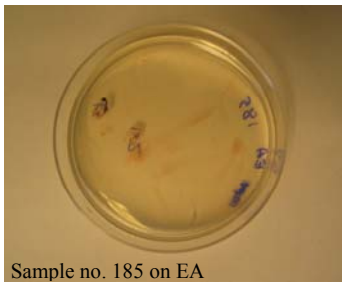
Restreaked on XLD (no. 187)



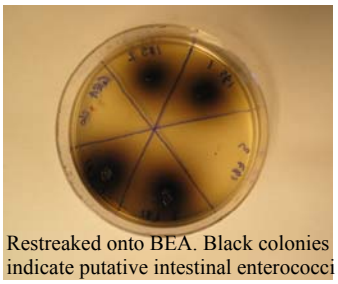
Restreaked on XLD (no. 210)



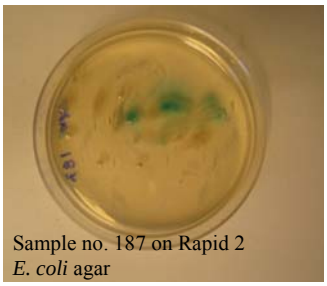
Sample no.187 on EA



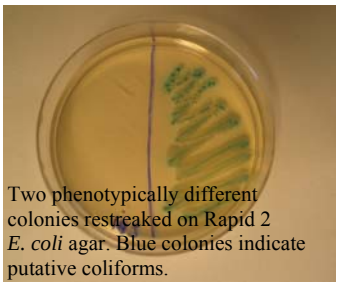
Sample no. 185 on EA



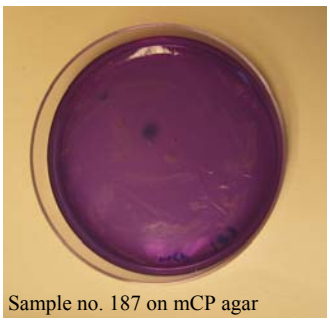
Restreaked onto BEA. Black colonies indicate putative intestinal enterococci



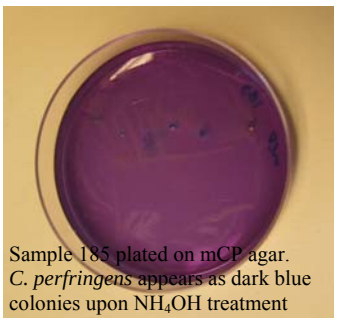
Sample no. 187 on Rapid 2 *E. coli* agar



Two phenotypically different colonies restreaked on Rapid 2 *E. coli* agar. Blue colonies indicate putative coliforms.



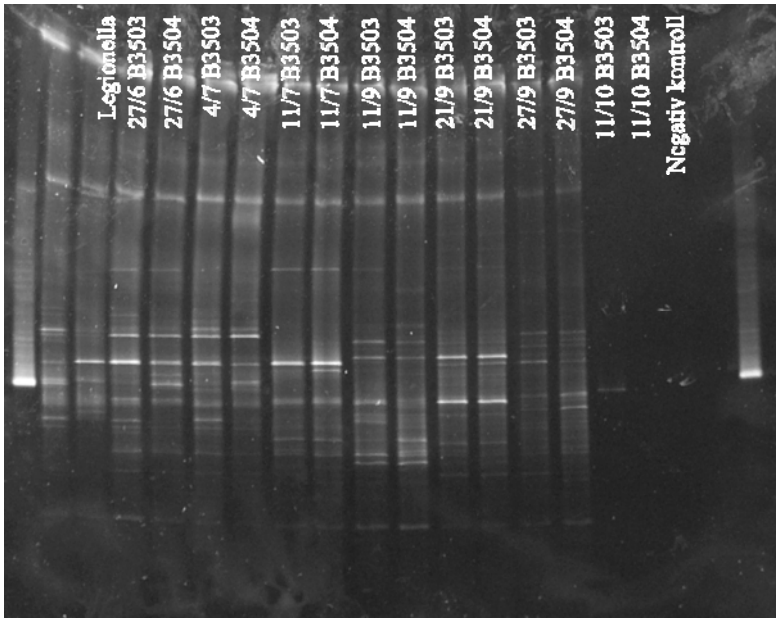
Sample no. 187 on mCP agar



Sample 185 plated on mCP agar. *C. perfringens* appears as dark blue colonies upon NH_4OH treatment

A.8 Appendix 8

DGGE of liquid samples from ponds B 3503 and B 3504 harvested 27.06.2006, 04.07.2006, 11.07.2006, 11.09.2006, 21.09.2006. *L. pneumophila* ATCC 33215 was used as a positive control. See section 3.9 and 4.10 for details regarding the experiment and the results obtained, respectively.



A.9 Appendix 9

ClustalW alignment of *mip* DNA sequence from *L. micdadei* ATCC 33218 and *L. pneumophila* ATCC 33215.

```
ATCC33218       CTGAAAAGACAAAAGGGGATTGTTTATGAAGATGAGATTGGTCGCTGCAGCTGCCATGGG 720
ATCC33215       -------------------------ATGAAGATGAAATTGGTGACTGCAGCTGTTATGGG 35
                                          ********** ******  *********  *****

ATCC33218       TTTGGCAATGTCAACGACAATAGCTGCAACCGCTACAACTGATGCGACAACTTCTGCACC 780
ATCC33215       GCTTGCAATGTCAACAGCAATGGCTGCAACCGATGCCAC---------------------- 74
                  * ***********  **** ********** * * **

ATCC33218       AGGAACATCATTGACTACAGACACAGAAAAGCTCTCATACAGCATTGGTGCTGATTTGGG 840
ATCC33215       ------ATCATTAGCTACAGACAAGGATAAGTTGTCTTATAGCATTGGTGCCGATTTGGG 128
                      ******  *********  ** *** * ** ** *********** ********

ATCC33218       TAAGAATTTTAAAAAGCAGGGAATAGAAATTAGTCCTGCTGCTATGGCAAAAGGTTTACA 900
ATCC33215       GAAGAATTTTAAAAATCAAGGCATAGATGTTAATCCGGAAGCAATGGCTAAAGGCATGCA 188
                 ************** ** ** *****  *** *** *  ** ***** *****  * **

ATCC33218       AGATGGAATGAGCGGCGGCCAATTGTTGCTGACCGACGACCAGATGAAGGATGTGCTAAA 960
ATCC33215       AGACGCTATGAGTGGCGCTCAATTGGCTTTAACCGAACAGCAAATGAAAGACGTTCTTAA 248
                *** *  ***** ****  ******    * *****  * ** ***** ** ** ** **

ATCC33218       TAAGTTTCAAAAAGATCTAATGATGAAACGCAGCGCAGAATTCAATAAGAAAGCTGAAGA 1020
ATCC33215       CAAGTTTCAGAAAGATTTGATGGCTAAGCGTACTGCTGAATTCAATAAGAAAGCGGATGA 308
                 ******** ****** * ***   ** ** *  ** ***************** ** **

ATCC33218       GAATAAGTCGAAAGGAGAAGCTTTCCTTAACGAAAATAAATCAAAAGAAGGTGTTGTTAG 1080
ATCC33215       AAATAAAGTAAAAGGGGAAGCCTTTTTAACTGAAAACAAAAACAAGCCAGGCGTTGTTGT 368
                 *****    ***** ***** **  * *  ***** ***   **   *** ******

ATCC33218       TTTACCTAGCGGTTTGCAGTATAAGATCCTTGAACGAGGCGATGGTGCTAAACCGACCAA 1140
ATCC33215       ATTGCCAAGTGGTTTGCAATACAAAGTAATCAATTCTGGAAATGGTGTTAAACCCGGAAA 428
                 ** ** ** ******** ** **  *  *  *    **  ****** ******    **

ATCC33218       GGATGACGTCGTTACTGTGGAATACACCGGCAAGCTGATTGACGGTCAGGTTTTCGACAG 1200
ATCC33215       ATCGGATACAGTCACTGTCGAATATACTGGTCGTCTGATTGATGGTACCGTTTTTGACAG 488
                    **    ** ***** ***** ** **    ******** ***   ***** *****

ATCC33218       TACTGAAAAGACAGGCAAACCTGCAACCTTTAAAGTTTCTCAAGTTATTCCAGGTTGGAC 1260
ATCC33215       TACCGAAAAAACTGGTAAGCCAGCAACGTTCCAGGTTTCACAAGTTATCCCTGGATGGAC 548
                *** ***** ** ** ** ** ***** **  * ***** ******** ** ** *****

ATCC33218       TGAAGCACTGCAATTAATGCCAGCAGGTTCTACTTGGGAAGTGTATATCCCTTCCAATCT 1320
ATCC33215       AGAAGCTTTGCAATTGATGCCAGCTGGATCAACTTGGGAAATTTATGTTCCCTCAGGTCT 608
                 *****  ******* ******** ** ** ********* * *** * ** **   ***

ATCC33218       GGCTTATGGCCCACGTAGCGTTGGCGGCCCAATTGGACCTAATGAAACTTTAATTTTCAA 1380
ATCC33215       TGCATATGGCCCACGTAGCGTTGGCGGACCTATTGGCCCAAATGAAACTTTAATATTTAA 668
                ** ********************** ** ***** ** ************** ** **

ATCC33218       AATTCATCTGATTTCAGTGAAGAAATCTGACGCGTAAAATGCGTTTTATCCCAGTCGCTC 1440
ATCC33215       AATTCACTTAATTTCAGTGAAAAAATC---ATCTTAA---------------------- 702
                ******  * ********** *****     * ***
```

A.10 Appendix 10

ClustalW alignment of *mip* DNA sequence from *L. micdadei* PAVIA AJ496274 and *L. pneumophila* ATCC 33215.

The *mip* primers and probe are shown in red and blue, respectively (see also table 3.2).

```
ATCC33215       ATGAAGATGAAATTGGTGACTGCAGCTGTTATGGGGCTTGCAATGTCAACAGCAATGGCT 60
AJ496274        ATGAAGATGAAATTGGTGACTGCGGCTGTTATGGGGCTTGCAATGTCAACAGCAATGGCT 60
                ********************** ************************************

ATCC33215       GCAACCGATGCCACATCATTAGCTACAGACAAGGATAAGTTGTCTTATAGCATTGGTGCC 120
AJ496274        GCAACCGATGCCACATCATTAGCTACAGACAAGGATAAGTTGTCTTATAGCATTGGTGCC 120
                ************************************************************

ATCC33215       GATTTGGGGAAGAATTTTAAAAATCAAGGCATAGATGTTAATCCGGAAGCAATGGCTAAA 180
AJ496274        GATTTGGGGAAGAATTTTAAAAATCAAGGCATAGATGTTAATCCGGAAGCAATGGCTAAA 180
                ************************************************************

ATCC33215       GGCATGCAAGACGCTATGAGTGGCGCTCAATTGGCTTTAACCGAACAGCAAATGAAAGAC 240
AJ496274        GGCATGCAAGACGCTATGAGTGGCGCTCAATTGGCTTTAACCGAACAGCAAATGAAAGAC 240
                ************************************************************

ATCC33215       GTTCTTAACAAGTTTCAGAAAGATTTGATGGCTAAGCGTACTGCTGAATTCAATAAGAAA 300
AJ496274        GTTCTTAACAAGTTTCAGAAAGATTTGATGGCAAAGCGTACTGCTGAATTCAATAAGAAA 300
                ******************************** ***************************

ATCC33215       GCGGATGAAAATAAAGTAAAAGGGGAAGCCTTTTTAACTGAAAACAAAAACAAGCCAGGC 360
AJ496274        GCGGATGAAAATAAAGTAAAAGGGGAAGCCTTTTTAACTGAAAACAAAAACAAGCCAGGC 360
                ************************************************************

ATCC33215       GTTGTTGTATTGCCAAGTGGTTTGCAATACAAAGTAATCAATTCTGGAAATGGTGTTAAA 420
AJ496274        GTTGTTGTATTGCCAAGTGGTTTGCAATACAAAGTAATCAATGCTGGAAATGGTGTTAAA 420
                ****************************************** ******************

ATCC33215       CCCGGAAAATCGGATACAGTCACTGTCGAATATACTGGTCGTCTGATTGATGGTACCGTT 480
AJ496274        CCCGGAAAATCGGATACAGTCACTGTCGAATACACTGGTCGTCTGATTGATGGTACCGTT 480
                ******************************** ***************************

ATCC33215       TTTGACAGTACCGAAAAAACTGGTAAGCCAGCAACGTTCCAGGTTTCACAAGTTATCCCT 540
AJ496274        TTTGACAGTACCGAAAAAACTGGTAAGCCAGCAACTTTTCAGGTTTCACAAGTTATCCCA 540
                ********************************** ** ********************

ATCC33215       GGATGGACAGAAGCTTTGCAATTGATGCCAGCTGGATCAACTTGGGAAATTTATGTTCCC 600
AJ496274        GGATGGACAGAAGCTTTGCAATTGATGCCAGCTGGATCAACTTGGGAAATTTATGTTCCC 600
                ************************************************************

ATCC33215       TCAGGTCTTGCATATGGCCCACGTAGCGTTGGCGGACCTATTGGCCCAAATGAAACTTTA 660
AJ496274        TCAGGTCTTGCATATGGCCCACGTAGCGTTGGCGGACCTATTGGCCCAAATGAAACTTTA 660
                ************************************************************

ATCC33215       ATATTTAAAATTCACTTAATTTCAGTGAAAAAATCATCTTAA------------------ 702
AJ496274        ATATTTAAAATTCACTTAATTTCAGTGAAAAAATCATCTTAAGTTTTTTTGAATTAAAGT 720
                ****************************************
```