Legionella and non-*Legionella* bacteria in a biological treatment plant

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Abstract

Legionella pneumophila were previously identified in the aeration ponds (up to 10^{10} CFU/L) of a biological wastewater treatment plant at Borregaard Ind. Ltd., Sarpsborg, Norway and in air samples (up to 3300 CFU/m^3) collected above the aeration ponds. After three outbreaks of Legionaires' disease reported in this area in 2005 and 2008, the aeration ponds of the plant were shut down by the Norwegian authorities in September 2008. The aim of the present work was to analyze the *Legionella* and non-*Legionella* bacterial communities in the aeration ponds before and during the shutdown process and to identify potential human pathogens. The non-*Legionella* bacterial community was investigated in selected samples during the shutdown process by 16S rDNA sequencing of clone libraries (400 clones) and growth analysis. The concentration of *L. pneumophila* and *Pseudomonas* spp. DNA were monitored by quantitative PCR. Results showed a decrease in the concentration of *L. pneumophila* and *Pseudomonas* spp. during the shutdown. This was accompanied by a significant change in the composition of the bacterial community in the aeration ponds. This study demonstrated that several advanced analytical methods are necessary to characterize the bacterial population in complex environments such as the industrial aeration ponds.

Key words: *Legionella pneumophila*, non-*Legionella* bacterial community, molecular cloning, DNA-sequencing, biological treatment plant

Introduction

Legionella pneumophila is the etiological agent of Legionaires' disease and the non-pneumonic legionellosis Pontiac fever. An infection results from inhalation of aerosols containing the bacteria (Cianciotto et al. 2006). In 2005 and 2008 three outbreaks of Legionaire's disease were reported in the Fredrikstad and Sarpsborg communities, Norway (Nygård et al. 2008). In 2005, 56 people were infected and 10 died. Later, an additional 50 people were diagnosed with legionellosis. The aeration ponds of the biological treatment plant at the wood and pulp factory Borregaard Ind. Ltd., a world's leading supplier of lignin-based chemicals, were identified as the main amplifiers and primary disseminators of the outbreak strain of *L. pneumophila* (Olsen et al. 2010). *L. pneumophila* was measured at concentration levels up to 3300 CFU/L in air samples taken directly above the aeration ponds and at lower levels up to 200 m downwind from the ponds (Blatny et al. 2008). Based on these findings the aeration ponds of Borregaard's biological treatment plant were shut down in September 2008. This was a decision taken by the Norwegian Climate and Pollution Agency. Prior to the shutdown in September 2008, the biological treatment plant consisted of two large aeration ponds kept at 36-38 °C for optimal growth of microorganisms to obtain degradation of organic compounds, e. g. lignin, providing an optimal growth environment for *Legionella* species (Allestam et al. 2006; Blatny et al. 2008; Olsen et al. 2010) and other microorganisms including protozoa and Cyanobacteria (Bohach and Snyder 1983; Gilbride and Fulthorpe 2004). An anaerobic step was included prior to the aerobic process to obtain an efficient biological treatment of the wastewater.

In pulp and paper industry both activated sludge and aerated stabilization basins (ASBs) (for review see Pokhrel and Viraraghavan, 2004) are used as treatment systems (Klopping and Foster 2003; Gilbride and Fulthorpe 2004; Mahmood and Paice 2006; Gilbride et al. 2006a). A concern in wastewater treatment might be the presence of microbial pathogens and particularly the aerosolization of these bacteria. The presence of legionellae in wastewater treatment plant is well documented (Allestam et al. 2006; Allestam and Långmark 2007; Huang et al. 2009; Olsen

et al. 2010). Two cases of Legionaires' disease have been reported among employees at two industrial wastewater treatment plants in Finland (Kusnetsov et al. 2010). The understanding of the factors that contribute to survival and growth of *Legionella* in the environment is limited, and only little information is available on the microbial communities present in ecosystems contaminated with *Legionella*. The microbial communities of aerated ponds in pulp and paper industry have traditionally been studied by microbiological and molecular approaches (Liss and Allen 1992; Fulthorpe et al. 1993; Gilbride et al. 2006b). Knowledge about the microbial communities may contribute to highlight factors important for survival or active growth of *L. pneumophila*.

The content of specific groups of bacteria in the aeration ponds at the Norwegian pulp factory Borregaard Ind. Ltd. has previously not been characterized. The aim of the present study was therefore twofold; i) Investigate the concentration of the *Legionella* bacteria in the aeration ponds during the shutdown of the ponds and; ii) Identify non-*Legionella* bacterial members present in the aeration ponds before and during the shutdown process. The concentration of *L. pneumophila* DNA was monitored during the shutdown period by quantitative PCR. Culture-independent methods, i.e. real-time PCR, molecular cloning and 16S rDNA sequencing and microbiological growth methods were used to study the non-*Legionella* bacteria in the pond.

Material and methods

Sample collection

Based on previous findings of *Legionella* in a biological wastewater treatment plant at Borregaard Ind. Ltd., Sarpsborg, Norway (Blatny et al. 2008; Olsen et al. 2010) the Climate and Pollution Agency (under the Ministry of the Environment) initiated a four month long shutdown process of the aeration ponds (September to December 2008) during which a biocide (hypochlorite) was injected into the system on November 17. Prior to the shutdown the activated sludge facility consisted of two large aeration ponds (each 2500 m^2) each containing 30,000 m^3 of liquid kept at 36-38 °C, and 30 000 m^3 of air was pumped through it every hour for

optimal growth of bacteria. From September 4 influx of waste material (substrate for the bacteria) and the airflow into the ponds were stopped. Samples (500 ml) were collected daily from the two aeration ponds (3503 and 3504) from September 4 to 30 2008 and then every week from October 2 until January 19 2009 (Olsen et al. 2010). The samples were collected at the same position and were immediately transported to the laboratory (1.5 hours) and stored at 4 °C on arrival. On October 13, 20, 27 and November 3 the temperature in the aeration pond (3503) was 21 °C, 17 °C, 16 °C and 15 °C, respectively. The pH of the aeration pond varied between was 7.5 and 9 before the shutdown of the plant. After the shutdown of the aeration ponds, authorities acknowledged a limited discharge of wastewater from the anaerobic process to the river Glomma nearby. Samples were therefore harvested from the anaerobic pond and from the outlet of the anaerobic pond on June 8, 2009 using a similar procedure. For cloning experiments and quantitative PCR only samples from the 3503 pond were used. For culture experiments samples from both ponds were used.

Bacterial cultivation

The non-*Legionella* bacterial community of the aeration ponds (September 25, November 3) was investigated by plating on three different agars; Plate Count agar (PCA; Oxoid, Cambridge, UK), Columbia agar with sheep blood PB5008A (Oxoid) and Nutrient Agar (Merck, Darmstadt, Germany). One ml sample harvested from the aeration ponds was inoculated into molten PCA agar and incubated at 22°C for 3 days and 30°C for 2 days. Samples (0.1 ml) plated on blood and nutrient agars were incubated at 37° C and 30°C, respectively, for 24 hours. *Legionella* spp. and *L. pneumophila* were enriched in samples harvested from the aeration ponds samples the following days; 25.11.08, 01.12.08, 08.12.08, 15.12.08, 06.01.09, 19.01.09. The analyses were performed according to the ISO11731 standard. Enrichment of samples harvested between September 3 2008 and November 17 2008 is described in Olsen et al. (2010).

Samples obtained on September 14 and October 2 were heated to 80 °C for 20 min and plated on Brain Heart Infusion (BHI; Acumedia Manufactures Inc: Baltimore, USA) agar plates to

investigate the presence of spore forming organisms surviving heating to 80 °C. Samples from the aeration ponds (September 25 and November 3, 2008) and from the anaerobic degradation process (June 8, 2009) were analyzed for the presence of *Pseudomonas* spp. *Pseudomonas* spp. was isolated by plating 0.1 ml samples on *Pseudomonas* agar base with *Pseudomonas* supplement SR103 (Oxoid) and incubated for 4 days at 37°C.

Cell counting and microscopic investigation

A phase-contrast fluorescence microscope (Zeiss, Germany) was used for microscopic investigations of samples harvested at September 4 and 25, November 3 and December 1. Samples (10 ml) harvested from the aeration pond at September 25 and December 1 were filtered to remove large debris. SYBR GREEN II was added to the filtrate (0.2 µl SYBR Green II/100 µl filtrate). The number of cells was counted in a chamber (Toma) using a phase-contrast fluorescence microscope.

DNA extraction

Samples (50 ml) harvested from the aeration pond at different dates were centrifuged at 1000 x g for 1 min. The supernatant was further centrifuged at 6000 x g for 20 min resulting in a cell pellet in 5 ml of supernatant. This pellet/supernatant was frozen and used for DNA extraction. Briefly, one ml of the thawed suspension was bead-beated using the Mini-Beadbeater-8 (BioSpec products, Bartlesville, OK, USA) for 1 min, and 200 µl of the supernatant was used for total DNA extraction using the DNA isolation kit QIAmp DNA Stool Mini Kit (Qiagen, Valencia CA, USA) as described by the manufacturer. The extracted DNA was used for PCR and cloning experiments.

Standard curve construction and quantitative real-time PCR

L. pneumophila LP357 grown on buffered charcoal yeast extract (BCYE, Oxoid) agar was suspended in phosphate buffered saline (PBS) and DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen) according to the protocol for Gram-negative bacteria and eluted in 100 µl buffer provided with the kit. Average genome size (in bp) of *L. pneumophila* was calculated to 3.4×10^{6} bp from the available genomes NC_002942 and NC_006369 in GenBank (NCBI) and

converted to mass using an average nucleotide phosphate mass (ANPM) of 620 g/mol (38% GC) (double stranded DNA). The concentration of isolated DNA was measured to 351 ng/µl using NanoDrop ND-1000 (Thermo scientific, Wilmington, DE, USA). The number of genomic copies per liter (GU/L) was calculated, a BLASTN search of the Gen Bank indicated one copy of the macrophage infectivity potentiator gene (*mip*) per genome. The standard was diluted to 1.08×10^{14} GU/L before 10-fold serial dilutions were used as DNA standard for preparation of real-time PCR standard curve for detection of the *mip gene* of *L. pneumophila*.

DNA was isolated from a representative *Pseudomonas* sp. isolate from PCA agar. The colony was suspended in PBS and DNA was isolated and measured as described (45.1 ng/µl) using the DNeasy Blood and Tissue kit. The number of 16S rDNA genes per *Pseudomonas* genome varies (usually 4-7) between species and strains (Bodilis et al. 2012). Therefore, the relative concentrations of *Pseudomonas* spp. DNA in the pond for the given days were calculated based on a real-time PCR standard curve prepared from 10-fold serial dilutions the 16S rDNA *Pseudomonas* spp. DNA.

DNA isolated from the pond at the following days; 04.09.08, 07.09.08, 10.09.08, 14.09.08, 25.09.08, 02.10.08, 16.10.08, 03.11.08, 25.11.08, 01.12.08, 15.12.08, 19.01.09; were used for quantitative real-time PCR amplification of *L. pneumophila mip* gene and the 16S rDNA gene of *Pseudomonas* spp. The primer pair *mip-f* /*mip-r* and the FRET probes LPneu-FL/LPneu-LC640, which amplified a 186 bp fragment of the *L. pneumophila mip* gene were used (Wellinghausen et al. 2001). The PA-GS-F /PA-GS-R primers were used for *Pseudomonas* amplification of a 617 bp fragment (Spilker et al. 2004). The PCR product was identified by melting point analysis and confirmed by DNA sequencing. The reaction mixture contained in 20µl; 10µl LightCycler 480 Probe Master mix (Roche Diagnostics, Indianapolis, USA), primers (1 µM), probes (0.16 µM) and 3µl of template DNA. Each sample was run in triplicate on a Light Cycler 480 instrument (Roche Diagnostics) with the following PCR profile: 95°C for 5 min, followed by 50 cycles of 95°C for 5 sec, 62°C for 10 sec and 72°C for 15 sec. A DNA standard curve was generated. The cycle

threshold (Ct) corresponding to the number of cycles at which the reaction becomes exponential, was compared to the standard curve in order to calculate the number of GU of *mip* per liter. For amplification of *L. pneumophila* the amplification efficiency was 1.92 (slope -3.52). The detection range of the PCR was $1x10^6$ to $1x10^{10}$ GU/L (3 to $3x10^4$ GU/PCR reaction). A similar procedure was used for *Pseudomonas* except that a SYBR Green Master mix and an annealing temperature of 58 °C were used and a total of 35 cycles were run. For *Pseudomonas* the amplification efficiency was 1.828 (slope -3.819) with a detection range of 4.5 ng/L to 45 µg/L (13.5 fg to 135 pg/ PCR reaction). The quantities were plotted using the Origin software (Origin Lab Corporation, Northampton, MA, USA).

In general, all PCR reactions were run in triplicates and deionized water was used as negative controls. The isolated DNA contained no PCR inhibitors shown by amplification of *Lamda* phage DNA (Tomaso et al. 2003). The size of the PCR-products was routinely verified by gel electrophoresis (Bio-Analyzer, Angilent Technology, USA).

Clone library construction, DNA-sequencing and phylogenetic analysis

Clone libraries were constructed using the amplified 16S rDNA sequences (9/27f /1492r) (Després et al. 2007) from samples harvested on September 4 and 25, November 3 and December 1. Briefly, PCR was performed using the Qiagen Taq DNA polymerase kit (Qiagen). The reaction mixture contained 10 x buffer (2.5 µl), primers (1 µM), dNTPs (0.25 mM each) $MgCl_2$ (4 mM), 0.5 µl *Taq* DNA polymerase, 3µl of template DNA and H_2O to a final volume of 25µl. The PCR reaction was performed in a SmartCycler II (Cepheid, Sunnyvale, CA, USA). The PCR program consisted of an initial denaturation (95 °C , 5 min) 32 cycle s of denaturation (95 °C, 20 s), annealing (58 °C, 20 s), and extension (72 °C, 80 s) and finally a terminal extension (72°C, 7 min). DNA isolated from ultrapure water using the QIAmp DNA Stool Mini Kit was used as a negative amplification control. The amplicons were purified on a 1% agarose gel followed by DNA extraction using the GenElute Gel extraction kit (Sigma-Aldrich, St. Louis, MO, USA). Purified PCR-product (2 µl) was cloned into pCR 4-TOPO vector and transformed into One shot

Top10 chemically competent *Escherichia coli* cells using TOPO TA Cloning kit as recommended by the manufacturer (Invitrogen, Grand Island, NY, USA). For screening of the white colonies selected from the clone libraries the vector specific M13F and M13R primers were amplified using the Roche LightCycler 480. Correct PCR products were identified by gel electrophoresis, and selected clones were sequenced. The sequence trace files were assembled, trimmed, aligned and manually checked using BioNumerics software 6.0 (Applied Maths, Sint-Martens-Latem, Belgium). Only sequences with read length greater than 1300 were approved. The obtained consensus sequences (a total of 401, about 100 from each day) were analyzed by submitting the 16S rDNA sequences to the Classifier and SeqMatch tools of the Ribosomal Database Project (RDP) webside (http://rdp.cme.msu.edu) (Wang et al. 2007) yielding classification down to genus level, and best hits against the RDP database, respectively. Taxonomic classification was based on DNA sequence similarities > 96-97 %. Differences in the content of sequences affiliated to different groups of bacteria at different days were tested using the Chi-square test. The significance levels was set to $p<0.05$.

Partial 16S rDNA sequencing (Eub f933/r1397) (Iwamoto et al. 2000) of selected colonies from PCA, nutrient, blood, BHI and GVPC agar (BCYE agar containing a glycine, vancomycin, polymixin B, and cyclohexamide supplement, Oxoid) was performed. Single colonies were transferred to 1 ml of H_2O, boiled at 10 min before centrifugation (10,000 x g for 2 min). The supernatant was amplified using the Eub f933/r1397 primers as described above. Sequences of a total of 50 isolated colonies were obtained. The Staden Package (Staden 1996) was used for alignment and edition of the sequences. The MEGA v4.0.2 software was used for construction of consensus sequences (Tamura et al. 2007). Taxonomic classification was based on DNA sequence similarities > 98 % using BLASTN.

The *RNaseP RNA (rnpB)* gene of *Legionella* spp. was amplified from DNA isolated from the aeration pond samples obtained on September 4 and 16, 2008 on the Roche LightCycler 480 as described (Rubin et al. 2005). A clone library was constructed and selected clones were

sequenced and these were analyzed using the Staden Package and MEGA software as described. Taxonomic classification was based on DNA sequence similarities of 90-99 % using BLASTN.

The gene encoding the beta subunit of RNA polymerase (*rpoD*) of *Pseudomonas* was used for sequencing of 62 isolates of *Pseudomonas.* The gene was amplified on the Roche LightCycler 480 as described (Mulet et al. 2009) and subjected to sequencing. The sequences were analyzed using the Staden Package and MEGA software as described. Identification was based on sequence similarities of >97-98 % (BLASTN).

All sequencing was performed at the Eurofins MWG Operon (Ebersber, Germany).

Results

Detection of *Legionella* in the aeration pond

During June to September 2008 the concentration of *L. pneumophila* serogroup 1 (SG1) was detected in the aeration ponds at concentrations from 10^8 to 10^{10} CFU/L (Olsen et al. 2010). In the present work the concentration of *L. pneumophila* DNA was monitored by quantitative PCR of the *mip* gene, a gene target used for specific identification of *L. pneumophila (Wellinghausen et al*. 2001; Blatny et al., 2008). A rapid decrease of the *L. pneumophila* DNA level from 10^9 GU per L to 10^6 GU per L was observed during the first month of the shutdown process (Fig. 1). Later the DNA level was stabilized below the detection limit (10^6 GU/L). After December 31 no *L. pneumophila* DNA was detected using real-time PCR. It is reasonable to believe that such decrease also occurs for other bacteria during the shutdown process. The decrease of the *L. pneumophila* DNA correlated with a decrease of *L. pneumophila* cells from 10^{10} to 10^6 CFU/L (Olsen et al. 2010). At November 17 2008 biocides were added to the system. Plate counting beyond this date, showed low CFU numbers (< 10^5 CFU/L) and after December 15 no legionellae was detected using the standard ISO cultivation method (ISO11731).

The *Legionella rnpB* gene is previously used for differentiation of *Legionella* species (Rubin et al., 2005). The aim of using the *rnpB* gene to construct a clone library from the aeration

pond samples was to identify sequences affiliated with different species of *Legionella* (*L. pneumophila* and non-*L. pneumophila*) since very few *Legionella* clones were identified in the general 16 S rDNA clone libraries. Samples harvested September 4 and 16, 2008 were used for cloning. Sequences affiliated with the *L. pneumophila* (99 % BLASTN), *Legionella oakridgensis* (99 % BLASTN) and *Legionella jordanis* (90 % BLASTN) were identified. In addition non-classified *Legionella* spp. was detected.

Detection of *Pseudomonas* in the aeration pond

Pseudomonas species are common environmental bacteria as well as being a co-cultivator of *Legionella* promoting its growth (Declerck et al. 2005). Therefore the possible decrease in the content of *Pseudomonas* DNA was studied during the shutdown process using real-time PCR. As for legionellae DNA, the concentration level of *Pseudomonas* DNA in the aeration ponds was decreased during the shutdown process (Fig. 1).

Study of the non- legionellae community in the aeration ponds using molecular cloning

Clone libraries were prepared from aeration pond samples harvested throughout the shutdown period (September to December 2008). Samples were harvested September 4 (before shutdown), September 25, November 3, and December 1, 2008. In total, 401 high quality 16S rDNA gene sequences were obtained from the four different clone libraries, and a total of 19 different phyla and 54 different genera were identified, whereas 12 % of the total number of sequences remained non-classified (Table 1). A general trend (Fig. 2) in the bacterial content of the aeration pond was the finding that *Chloroflexi* 16S rDNA sequences were mainly identified in the clone libraries from September 4 and 25 (26-28 % of the total clones). 16S rDNA sequences affiliated with *Proteobacteria* were identified in the clone libraries from all dates, but a higher percentage of these sequences was identified in the clone libraries from November 3 and December 1 (35-42 % of the total clones) compared to September 4 and 25 (20 % of the total clones). This increase was basically explained by an increase of sequences affiliated with the *Alphaproteobacteria*. Sequences affiliated with the phylogenetic group *Actinobacteria* (high

GC content, Gram-positive) constituted 15 % of the total clones at November 3. In general, there was a significant difference in the presence of sequences affiliated with these bacterial groups in the four clone libraries ($p<0.05$). *Bacteroidetes*, TM7 group and *Firmicutes* were detected in all clone libraries. Sequences affiliated with the *Betaproteobacteria Thauera* were mainly identified in the sample harvested on September 4. Not surprisingly, the 16S rDNA sequences identified at November 3 were similar to those identified at December 1 since biocides were injected November 17.

Microscopic investigations of the aeration pond samples harvested September 4 and 25 2008 revealed cell aggregates, filamentous bacteria and single bacteria, while those harvested November 3 and December 1 2008 revealed less aggregates and filaments, and aggregates were smaller and the filaments shorter. Nevertheless, microscopic counting indicated that the total number of cells in the pond was 10^{11} cells per liter both in September and December.

Growth analysis of the non- legionellae community in the aeration ponds

As a supplementary method viable cells in the aeration ponds were studied using general cultivation analysis and their putative identity was revealed by partial 16S rDNA sequencing. Aeration pond samples harvested on September 25 and November 3 2008 were plated on PCA, nutrient and blood agars. In addition, non-*Legionella* species from GVPC plates and heat tolerant bacteria from BHI plates were analyzed. Heat tolerant isolates were identified as the Gram-positive bacteria *Staphylococcus* and *Bacillus*, with a putative (16S rDNA) identification of *Staphylococcus hominis*. In general, the culturable bacteria on the different agars were identified as *Firmicutes* and *Proteobacteria* with a putative identity of *Bacillus* spp. and *Staphylococcus* spp., respectively (Table 2). *Alphaproteobacteria* were identified as *Sphingomonas*, *Paracoccus* and *Brevundimonas,* while *Betaproteobacteria* were identified as *Simplicispira* and *Acidovorax*. Within the *Gammaproteobacteria* the following bacteria were identified: *Legionella*, *Reinheimera*, *Chromatiaceae* (purple sulphur bacteria), *Pseudomonas*, *Shewanella*, *Aeromonas*, *Acinetobacter*

spp. and *Acinetobacter baumani*. The opportunistic pathogens *Aeromonas* and *Acinetobacter* spp. were identified and isolated on blood agar.

A total of 62 viable *Pseudomonas* isolates from the aeration ponds (September 25 and November 3 2008) and from the outlet of the anaerobic pond (June 8, 2009) were putatively identified by sequencing their *rpoD* gene. The *rpoD* gene was chosen for sequencing to improve identification of the 62 *Pseudomonas* isolates (Mulet et al. 2009) since it was challenging to distinguish between different *Pseudomonas* genera using 16S rDNA sequencing. The *Pseudomonas* isolates clustered into three different groups, *Pseudomonas stutzeri* (16 isolates), *Pseudomonas mendocina/alcaliphila/pseudoalcaligenes* (31 isolates) and non-classified *Pseudomonas* spp. (15 isolates).

Discussion

This study has analyzed the community of *Legionella* and non-*Legionella* bacteria in the aerated stabilization basins at Borregaard Ind. Ltd. during the shutdown process in 2008. These ponds were identified as the main amplifiers and disseminators of the *L. pneumoplilia* outbreak species (Olsen et al. 2010). Today, the aeration ponds are non-operational due to restrictions provided by the Norwegian authorities. The decision to close the ponds was based on previous studies showing high concentration of the outbreak strain in the ponds and aerosolization of legionellae from the ponds (Blatny et al. 2008; Olsen et al. 2010). This current study was carried out during a 4 month period; September – December 2008 where biocides were added to the ponds mid November 2008.

The aim of the present study was to investigate the concentration of legionellae DNA during the shutdown period and to identify non-legionellae bacteria present in the pond. A main observation was that the concentration levels of *L. pneumophila* and *Pseudomonas* spp*.* DNA decreased during the shutdown period from September to December 2008, which correlated with the decrease of total *Legionella* and *L. pneumophila* cells from 10^{10} to 10^{6} CFU/L (Olsen et al. 2010). It is reasonable to believe that similar processes are valid for other bacteria present in

the ponds. After the shutdown, the Norwegian Climate and Pollution Agency approved a limited discharge of wastewater from the anaerobic process to the river Glomma. No viable legionellae or other pathogens were identified in the anaerobic degradation plant ten months after initiating the shutdown process. However, viable non-pathogenic *Pseudomonas* was identified in the outlet of the anaerobic pond. These findings support the decision of a limited discharge of wastewater to the river Glomma.

A general observation was that filamentous bacteria, such as the *Chloroflexi,* dominated the clone libraries in September. This finding was supported by the microscopic observation of large aggregates and filaments in the pond. Filamentous bacteria are commonly found in activated sludge plant and are responsible for the bulking sludge problems (Martins et al. 2004) and *Chloroflexi* usually accounts for a large fraction of phylogenetically uncharacterized filaments in activated sludge system (Björnsson et al. 2002). In November, the aggregates were smaller and filaments shorter in length, which agrees with the finding of fewer sequences affiliated with filamentous bacteria in the pond. *Proteobacteria*, particularly the *Alpha*- and *Gammaproteobacteria,* dominated the November library. Nevertheless, the concentration of the *Gammaproteobacteria*, *Legionella* and *Pseudomonas* spp. was reduced, which may be explained by the reduced oxygen level and/or reduced temperature in the pond. The findings of *Proteobacteria*, *Bacteriodetes*, *Acidobacteria, Firmicutes*, *Actinobacteria*, and *Chloroflexi* in the pond was in agreement with previous studies of wastewater treatment systems (Wagner and Loy 2002; Yang et al. 2011). A previous study comparing the bacterial communities in 10 different pulp and paper mill treatment systems performing similar function, showed that the bacterial communities could change and was dependent on external factors e.g. small changes in influx of waste material (Gilbride and Fulthorpe 2004). A survey of the bacterial populations in bleached craft pulp-mill wastewater treatment systems identified *Bacillus*, *Pseudomonas*, and *Xanthobacter* as dominant species among the culturable isolates whereas a 16S rDNA clone library identified other species of *Proteobacteria*, *Ralstonia*, *Alcaligenes*, *Nitrospira*, and *Firmicutes* (Gilbride and Fulthorpe 2004). In this study, bacterial genera such as *Bacillus*,

Pseudomonas, *Legionella*, *Spingomonas*, *Staphylococcus* and *Aeromonas* were identified using culture methods. Viable Gram-positive *Firmicutes* were represented mainly by the class *Bacillus*. *Bacillus* DNA was also identified in pond samples by real-time PCR (*groEL*and the non-hemolytic enterotoxin gene (*nhe*)) in samples harvested October 16 to November 25 (results not shown). It is assumed that *Bacillus* species were most likely present as spores in the aeration ponds at Borregaard Ind. Ltd., which might explain why they were not detected by cloning (Østensvik et al. 2004). This is supported by the present findings of viable *Bacillus* after heat treatment of the pond samples (80 °C, 20 min).

Culture-independent methods are important tools for analysis of bacterial diversity in wastewater systems (for review see Gilbride et al. 2006b) since more than 90 % of the bacteria in such communities is expected to be non-culturable (Amann et al. 1995). However, 16S rDNA clone libraries suffer from methodological constraints as they may skew the distribution of phylotypes in the library relative to the community it was derived from (von Wintzingerode et al. 1997). Therefore, the quantitative distribution of phylotypes may not reflect the real quantitative distribution in the ponds. This is highlighted by the present results that *Legionella* were identified mainly by real-time PCR of the *mip* gene, growth methods and sequence analysis of the *rnpB* clones and not by sequencing of the 16S rDNA clones. On the other hand bacterial groups can be overestimated using growth, exemplified by *Aeromonas* and *Acinetobacter* showing high plating efficiency on nutrient rich agar (Amann, 1995). These studies and our work in general, indicate that several advanced analytical methods are necessary to characterize a bacterial population in complex environments such as wastewater treatment systems.

It is challenging to predict the health risks associated with aerosolization of bacterial pathogens, especially bacteria other than *Legionella*. There are no clinical cases indicating such health risks (Pascual et al. 2001; Blatny et al. 2008, 2011). Still, it cannot be excluded that aerosols generated from these ponds may have a health impact. Previous studies at Borregaard Ind. Ltd. detected aerosol particles of a medium (respirable) size of 3.5 µm above the aeration

ponds and these were estimated to contain 147 leggionellae cells per particle. In addition, 44 taxonomic different bacterial genera were measured above the aeration pond indicating that the aerosols contained a mixture of bacteria including legionellae (Blatny et al. 2011). The following bacterial phyla were identified in the pond and air samples harvested directly above the aeration ponds (Blatny et al. 2011); *Bacillus, Staphylococcus, Sphingomonas, Pseudomonas and Legionella*. *Bacillus* species are opportunistic pathogens and are recognized as potential harmful microbes in pulp and paper industry (Maukonen and Saarela 2009). Other opportunistic bacterial pathogens isolated from liquid samples of the ponds included *Acinetobacter* and *Aeromonas* species, also representing potential pathogens. *P. stutzeri*, an opportunistic pathogen, is ubiquitous in hospital environments, but has a low degree of virulence (Lalucat et al. 2006). *Acinetobacter baumani* is a nosocomial pathogen affecting mainly immunosuppressed patients (Seifert et al. 1995). *S. hominis*, isolated on BHI medium in this study, has been found in infections related to surgery of pacemakers and implantable cardioverter defibrillator (Anselmino et al. 2009).

This study has provided knowledge about the bacterial communities of the aeration ponds at Borregaard Ind. Ltd present before and during the shutdown process. However, identifying single bacterial species or risk factors important for proliferation of legionellae in the ponds is challenging. Likewise no correlation was found between changes in the bacterial community in a cooling tower and proliferation of *L. pneumophila* (Wéry et al. 2008). It is well known that amoebas enhance survival and proliferation of pathogenic *Legionella* species (Wadowsky et al. 1991) and may play a role for growth of legionellae in wastewater treatment plant. We are currently addressing this topic. The results of this study showed that filamentous bacteria dominated the plant before and during the first month after initiation of the shutdown whereas the *Proteobacteria* became the most prominent bacterial group later in the process. This study also clearly demonstrated that several analytical methods are needed for studying the correlation between the legionellae concentration and the content of the non-*Legionella* bacteria during the shutdown of the aeration ponds. Controlling *Legionella* and other bacterial pathogens

in aeration ponds of biological treatment plant is crucial from a public health perspective and necessary for outlining restrictions on the release of wastewater to the neighboring environment. In general, the results of this and similar studies could assist in predicting health risk associated with wastewater treatment plants.

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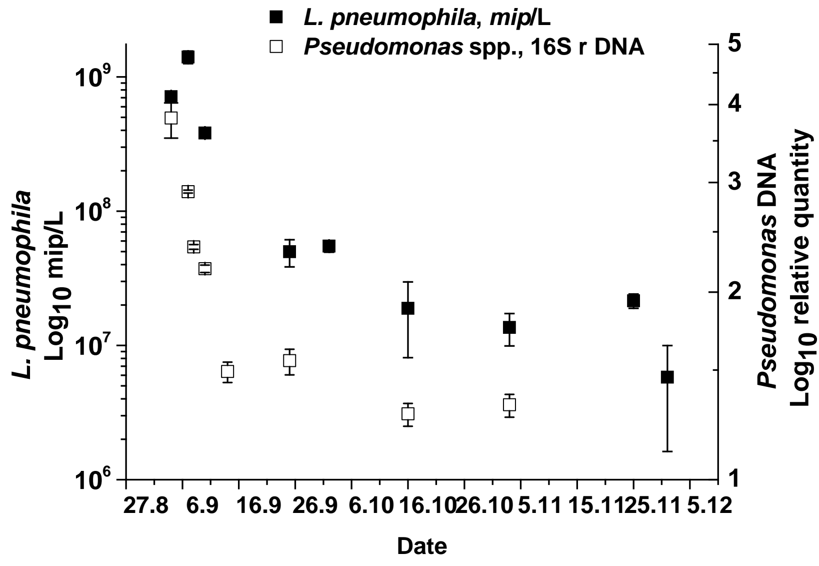
L. pneumophila, mip/L
Pseudomonas spp., 16S r DNA
L. pneumophila
Log10 mip/L
Pseudomonas DNA
Log10 relative quantity
10^9
10^8
10^7
10^6
5
4
3
2
1
27.8
6.9
16.9
26.9
6.10
16.10
26.10
5.11
15.11
25.11
5.12
Date

Figure 1. The decrease of *L. pneumophila DNA and Pseudomonas* DNA in aeration pond samples harvested during September to December 2008 determined by quantitative real-time PCR of the *mip* gene of *L. pneumophila* and the 16S rDNA gene of *Pseudomonas* spp. A standard curve based on a 10-fold serial diluted DNA standard ($1x10^6$ to $1x10^{10}$ GU/L) was used for detection of the *mip gene* of *L. pneumophila*. For *Pseudomonas* a standard curve for relative quantification was based on a 10-fold serial diluted DNA standard from 4.5 ng/L to 45 µg/L.

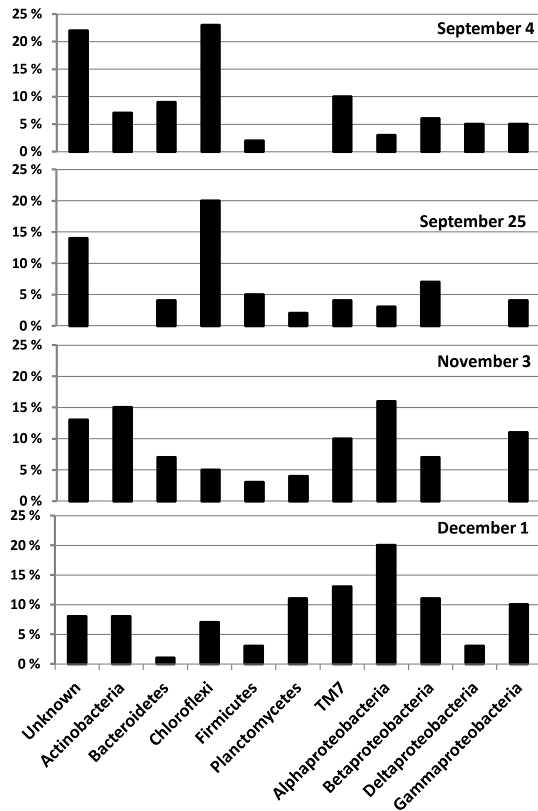

Figure 2. Selected bacterial groups identified from the 16S rDNA clone libraies. Sequences affilated with the phylum *Chloroflexi* was identified mainly in the clone libraries from September 4 and 25 (26-28 % of the total number of clones). The percentage of sequences affiliated with the *Proteobacteria* was increased on November 3 and December 1 (35-42 % of the total number of clones) explained by an increase of sequences affiliated with the *Alpha*- and *Gammaproteobacteria.*

Table 1. 16s rDNA clone libraries from the aeration pond (3503) were prepared from samples harvested on September 4, September 25, November 3 and December 1.

Phylum (class)	Genus	Sep. 4	Sep. 25	Nov. 3	Dec. 1	Total
		n[a]	**n**	**n**	**n**	**n**
Unclassified		**16**	**12**	**14**	**9**	**51**
Acidobacteria			**3**	**1**		**4**
	*GP3**		1			
	*GP6**		2	1		
Actinobacteria		**6**	**3**	**16**	**7**	**32**
	Conexibacter	1		1		
	Mycobacterium			1		
	Nocardia			1		
	Hoyosella	1		6	5	
	Iamia			2		
	Ilumatobacter	1		1		
	Pseudonocardia				1	
	Unclassified	3	3	4	1	
Bacteroidetes		**12**	**5**	**7**	**4**	**28**
	Balneola			1		
	Ferruginibacter		1			
	Flavobacterium				1	
	Haliscomenobacter		1	2	3	
	Lishizhenia		2			
	Persicitalea	1	1			
	Paludibacter			1		
	Unclassified	11		3		
Chloroflexi		**27**	**27**	**4**	**7**	**65**
	Bellilinea		1	1		
	Caldilinea	25	24		3	
	Chloroflexus				2	
Cyanobacteria	*Unclassified*				**1**	**1**
Firmicutes		**3**	**1**	**3**		**7**
	Dehalobacter			1		
	Pelospora	1				
	Weissella		1			
	Unclassified	2		2		
Lentisphaerae	*Unclassified*		**1**			**1**
Nitrospira	*Nitrospira*			**1**		**1**
OD1	*OD1**		**3**	**2**		**5**
OP10	*OP10**		**3**	**1**		**4**
Planctomycetes			**3**	**4**	**12**	**19**
	Gemmata				1	
	Planctomyces		2	1	4	
	Unclassified		1	3	7	
Synergistetes	Thermovirga		**5**		**2**	**7**
Thermotogae	Kosmotoga	**6**	**2**			**8**
TM7	*TM7**	**13**	**4**	**10**	**11**	**38**
Verrucomicrobia			**4**	**1**	**3**	**8**
	Opitutus		1		2	
	Prosthecobacter				1	
	Spartobacteria*		2			
	Verrucomicrobium		1			

	Unclassified			1		
Proteobacteria (total)		**21**	**20**	**40**	**41**	
(Unclassified)			**1**	**2**		**3**
(Alphaproteobacteria)		**4**	**4**	**16**	**17**	**41**
	Defluviicoccus				2	
	Filomicrobium			2		
	Hyphomicrobium			1	5	
	Novosphingobium			1		
	Parvibaculum	1		2		
	Pedomicrobium		1			
	Prosthecomicrobium			1	1	
	Rhodoplanes			2	1	
	Stella				1	
	Unclassified	3	3	7	7	
(Betaproteobacteria)		**8**	**9**	**8**	**11**	**36**
	Nitrosospira			1	3	
	Thauera	8	1	1	2	
	Unclassified		8	6	6	
(Deltaproteobacteria)		**1**			**2**	**3**
	Bdellovibrio	1			1	
	Unclassified				1	
(Gammaproteobacteria)		**8**	**6**	**14**	**11**	**39**
	Aquicella	1	2		1	
	Azomonas	1				
	Legionella		1			
	Rheinheimera		1	2	2	
	Thiofaba	2				
	Thioalkalivibrio				1	
	Unclassified	4	2	12	7	
Sum		**104**	**96**	**104**	**97**	**401**
Number of different phyla		**10**	**16**	**13**	**9**	**19**

[a], number of sequences. Identification of the sequences is based on classification to genus level using the Classifier and SeqMatch tools of the RDP database. Taxonomic classification was based on DNA sequence similarities of > 96 %.

Table 2. Samples harvested on September 25 and November 3 was streaked out on different growth media. Bacterial colonies were sequenced using the 16S rDNA gene and identified by BLASTN source. The following media were used; PCA-, blood-, nutrient- and GVPC-agar (non-*Legionella* species).

Phylum	**Genus**
Alphaproteobacteria	*Sphingomonas*
	Paracoccus
	Brevundimonas
Betaproteobacteria	*Acidovorax*
Gammaproteobacteria	*Pseudomonas*
	Rheinheimera
	Shewanella
	Aeromonas
	Acinetobacter (uncultured)
	Acinetobacter baumanni
	Unclassified
Firmicutes	*Staphylococcus*
	Bacillus
	Chryseobacterium / (Haloanella)
Bacteriodetes	Unclassified

Heat tolerant bacteria were identified on GVPC agar plates and classified as *Staphylococcus* and *Bacillus* by partial 16S rDNA sequencing and BLASTN source. In general, identification is based on sequence similarities of > 98 % (BLASTN). A total of 50 isolates was analyzed.