Detection of *Legionella* by cultivation and quantitative real-time polymerase chain reaction in biological waste water treatment plants in Norway

Vidar Lund, Wenche Fonahn, Jens Erik Pettersen, Dominique A. Caugant, Eirik Ask and Åse Nysaeter

ABSTRACT

Cases of Legionnaires' disease associated with biological treatment plants (BTPs) have been reported in six countries between 1997 and 2010. However, knowledge about the occurrence of *Legionella* in BTPs is scarce. Hence, we undertook a qualitative and quantitative screening for *Legionella* in BTPs treating waste water from municipalities and industries in Norway, to assess the transmission potential of *Legionella* from these installations. Thirty-three plants from different industries were sampled four times within 1 year. By cultivation, 21 (16%) of 130 analyses were positive for *Legionella* species and 12 (9%) of 130 analyses were positive for *Legionella pneumophila.* By quantitative real-time polymerase chain reaction (PCR), 433 (99%) of 437 analyses were positive for *Legionella* species and 218 (46%) of 470 analyses were positive for *L. pneumophila.* This survey indicates that PCR could be the preferable method for detection of *Legionella* in samples from BTPs. Sequence types of *L. pneumophila* associated with outbreaks in Norway were not identified from the BTPs. We showed that a waste water treatment plant with an aeration basin can produce high concentrations of *Legionella*. Therefore, these plants should be considered as a possible source of community-acquired *Legionella* infections.

Key words | biological waste water treatment plants, cultivation, *Legionella*, quantitative real-time PCR

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INTRODUCTION

Three outbreaks of Legionnaires' disease associated with waste water treatment plants have been registered in Norway in the past decade, all in the south-eastern part of the country (Blatny *et al.* 2008; Nygård *et al.* 2008). Outbreaks associated with aerated biological waste water treatment plants (BTPs) have also been reported elsewhere, especially from the Nordic countries in association with the pulp and paper industry (Allestam & Långmark 2007; Eurosurveillance 2010; Kusnetsov *et al.* 2010). Additionally, outbreaks of Pontiac fever associated with the food processing industry have occurred, with five persons infected after working with sludge in a treatment plant in Denmark in 1997 (Gregersen *et al.* 1999) and 15 cases of Pontiac fever transmitted by use of a high-pressure hose with treated waste water from a sugar production factory in the USA in 2000 (Castor *et al.* 2005). Two *Legionella* outbreaks from the chemical industry have also been reported, one from Pas-de-Calais in France in 2003/2004 with 84 reported cases including 18 deaths (Tran Minh *et al.* 2006) and a minor outbreak in Finland in 2007 with five cases of Pontiac fever (Ruotsalainen *et al.* 2008) and three diagnosed and one probable case from the leather industry in Sweden in 2008 (local doctor, personal communication). Overall a total of 233 cases of Legionnaires' disease from six countries have been reported associated with BTPs between 1997 and 2010. However, knowledge about the occurrence of

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Legionella in BTPs is scarce. Hence, we undertook a qualitative and quantitative screening of *Legionella* from BTPs treating waste water from municipalities and industries in Norway, to assess the transmission potential of *Legionella* from these installations.

MATERIALS AND METHODS

Characteristics of waste water treatment plants

Our investigation included 33 treatment plants from different industries: eight municipal sewage treatment (MST) plants, eight from dairies, nine from petrochemical industries, four from wood processing industries and four from other kinds of industry. Other kinds of industry included one factory for production of chemicals for wood processing and the oil industry, one company producing silicon wafers for solar panels, one factory producing marine omega-3-derived pharmaceuticals and one factory producing potato chips. There were considerable differences in construction and size among the plants, ranging from open ponds with a surface area of 1,000 m^2 to completely covered indoor units of 10–50 m^2. Key data for each plant, such as detention time and oxygen concentration in the aeration ponds, sludge age, sludge concentration and sludge load were also collected, but the quality of the information was not sufficient for statistical analysis of the correlation between different plant parameters and *Legionella* content. Most plants were 'activated sludge' units with free floating sludge, and the others were mainly based on the 'moving bed' principle, with the bacteria in a fixed biofilm on plastic particles circulating in the waste water plant.

Municipal sewage

All eight treatment plants had both biological and chemical treatment processes, but for some of the plants the chemical step did not affect the water quality at the sampling point, either because the samples were taken before the chemical treatment step or because of no re-circulation of chemically treated water back to the biological treatment step. Five plants had nitrogen removal by de-nitrification. Typical sewage temperatures varied between 7 and 15 °C depending on the season. pH values were between 4.9 and 7.9. Average detention time in the aeration ponds was between 1 and 6 hours.

Dairies

All eight treatment plants had only biological treatment, and all except one were activated sludge units. Typical sewage temperatures were between 20 and 30 °C. pH values were between 5.3 and 8.0. Average detention time in the activated sludge ponds was between 24 and 74 hours.

Petrochemical industry (PI)

The nine plants had different kinds of biological treatment processes, and some had additional chemical treatment. Typical sewage temperatures were between 25 and 35 °C. pH values were between 6.5 and 9.1. Average detention time in the aeration ponds was between 11 and 72 hours.

Wood processing industry (WPI)

The four plants had biological treatment based on 'activated sludge' reactors. One plant pre-treated part of the sewage anaerobically before the aerobic step. Typical sewage temperatures were between 35 and 40 °C. pH values were between 7.0 and 7.8. Average detention time in the aeration ponds was between 20 and 68 hours.

Other industries

The factory for production of chemicals for wood processing and the oil industry, the factory for producing wafers and the factory producing potato chips all used 'activated sludge' reactors. The factory producing omega-3-derived pharmaceuticals used a 'moving bed' reactor. Sewage temperatures were below 20 °C in the sewage from the chips-producing factory. The sewage temperatures in the other plants ranged from 20 °C up to about 35 °C.

An overview of the temperatures in the aeration ponds from different industries is illustrated in Figure 1.

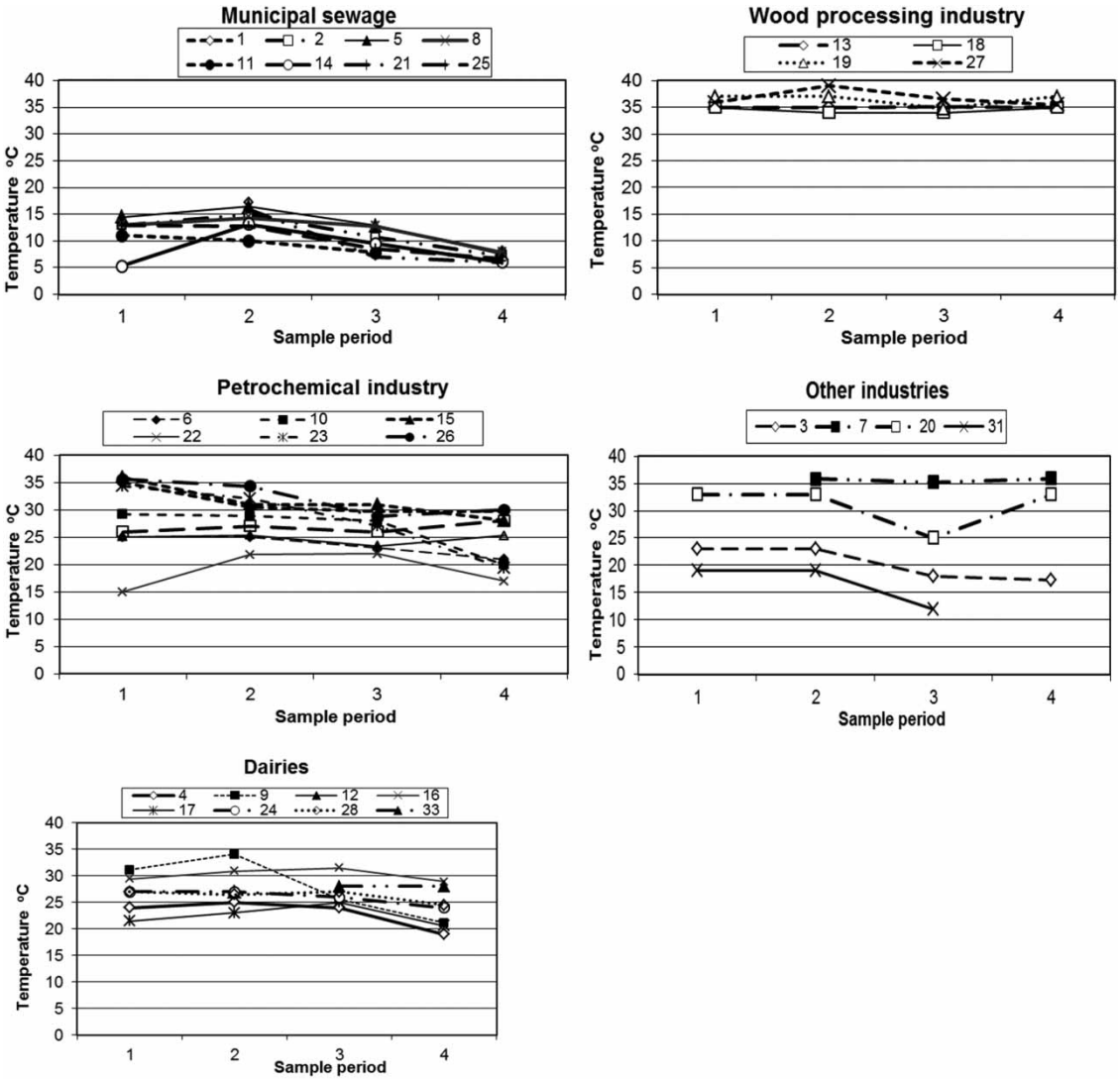

Figure 1 | Temperatures in aeration ponds for different industries. Sample periods: 1: June 2009, 2: August/September 2009, 3: November 2009, 4: April 2010.

Sampling

Each plant was sampled four times during 1 year, in June 2009, August/September 2009, November 2009 and April 2010, to include seasonal variations. All samples of waste water including floating biofilm/sludge were taken 0.5–1 m below the surface from the aerated ponds under normal operation. Each sample was taken from the same spot in the aerated ponds. Two 1-litre samples were taken each time. Samples were sent in Styropore boxes equipped with cooling elements, by express mail overnight to the laboratories for microbial analyses, and prepared for analyses within 24 hours. Samples were analysed for *Legionella* by cultivation at Unilabs Laboratoriemedisin AS, *Legionella* spp. and *Legionella pneumophila* were analysed by quantitative real-time polymerase chain reaction (PCR) at the Norwegian Institute of Public Health (NIPH).

Cultivation method

Legionella was cultured according to ISO 11731:1998. Samples were acid and heat treated (as described in ISO 11731:1998) and serially diluted in Ringer's solution before plating on GVPC agar for environmental samples (Oxoid:

Legionella CYE Agar Base CM0655 with growth supplement SR010C (L-cysteine and α-ketoglutarate) and selective supplement SR0152E (vancomycin and cycloheximide)). If confluent growth of background flora occurred in less diluted samples the limit of detection was calculated from the lowest dilution where colony separation was sufficient to allow the characteristic colony morphology of *Legionella* to be observed. Colonies were further examined under long-wave ultraviolet illumination (wavelength 365 nm). Three presumptive colonies (or, where morphologically distinct putative *Legionella* colonies were present, three of each) were selected for further examination. Confirmed *Legionella* were identified as *L. pneumophila* serogroup 1, *L. pneumophila* serogroup 2–14 or pathogenic *Legionella* (*L. longbeachae, L. bozemanii, L. dumoffii, L. gormanii, L. jordanis, L. micdadei, L. anisa*) with *Legionella* latex test (Oxoid, Basingstoke, UK). Isolates were stored frozen at −70 °C in brain-heart infusion broth containing 10% glycerol until transport to NIPH for species identification and genotyping.

Quantitative real-time PCR

At NIPH, the samples were preserved the same day or the day after arrival. Two parallels of 1,500 μL each were pipetted from the sample into Eppendorf tubes. The tubes were centrifuged for 10 minutes at 13,000 rpm, the supernatants removed and the pellets re-suspended in 400 μL TE-buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8). The tubes were stored at −20 °C until DNA extraction.

DNA was extracted by using a cetyl trimethyl ammonium bromide method as described by Nederbragt *et al.* (2008).

The quantitative real-time PCR was performed by using the *L. pneumophila* and *Legionella* spp. TaqMan® Quantification Kits (Labaqua, Spain). Except for the DNA extraction step, the samples were analysed according to the user guides for the kits, and run on Applied Biosystems 7500 Fast real-time PCR system.

For each sample the two separate DNA extracts were tested twice for *L. pneumophila* and twice for *Legionella* spp., i.e. four parallels for each sample. The parallels were always run on different plates.

A standard curve was prepared by serial dilution of the positive control (1×10^6 GU (genome units)/μL) from the kit, and was included in each plate run with six different positive controls analysed by triplets in the range of 10^6–10^1 GU. The thermo cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min × 42 cycles.

A sample was considered positive for *Legionella* whenever the fluorescence emitted by the probe was above the threshold. The CT-value was correlated to the standard curve to calculate the concentration of the sample. The samples were analysed in the presence of IPC (internal positive control). When both the *Legionella* and the IPC fluorescence were below the threshold value, the amplification reaction was assumed inhibited. The samples were then diluted 1:10 or 1:100 to reduce the concentration of inhibitors. However, some samples did not exhibit valid amplification reactions after dilution, and thus no results could be obtained. In addition, some of the quantification results showed obviously divergent values, mostly due to misshaped amplification curves, and the results were then omitted.

Identification of *Legionella* species

Isolates cultured at Unilabs Laboratoriemedisin AS were subjected to 16S rDNA sequencing to confirm species assignment using the method described by Handal *et al.* (2004).

Sequence-based typing

L. pneumophila isolates were genotyped by sequencing 7 gene fragments, as described (Gaia *et al.* 2005; Ratzow *et al.* 2007). Sequence types (STs) were assigned using the database http://www.ewgli.org/.

RESULTS AND DISCUSSION

Quantification of *Legionella* species and *L. pneumophila*

Results from the analyses of *Legionella* species and *L. pneumophila* are summarized in Table 1. For both methods the *Legionella* species analyses also includes *L. pneumophila*.

Table 1 | *Legionella* species and *L. pneumophila* analyses from five categories of biological treatment plants

Plant	***Legionella* species By cultivation (CFU/L)[a] Number and % of positive analyses**	**By quantitative real-time PCR (GU/L)[a] Number and % of positive analyses**	***L. pneumophila* By cultivation (CFU/L)[a] Number and % of positive analyses**	**By quantitative real-time PCR (GU/L)[a] Number and % of positive analyses**
	Concentration range	**Concentration range**	**Concentration range**	**Concentration range**
MSTs 8 plants	1 (3%)	125 (98%)	1 (3%)	37 (30%)
	nd–10^5	nd–10^{10}	nd–10^5	nd–10^6
Dairy industry 8 plants	0 (0%)	102 (100%)	0 (0%)	34 (33%)
	nd	10^4–10^9	nd	nd–10^7
PI 9 plants	13 (36%)	118 (99%)	5 (14%)	80 (65%)
	nd–10^9	nd–10^{15}	nd–10^7	nd–10^8
Wood processing industry 4 plants	7 (44%)	34 (100%)	6 (38%)	40 (65%)
	nd–10^8	10^3–10^8	nd–10^8	nd–10^9
OI 4 plants	0 (0%)	57 (100%)	0 (0%)	27 (46%)
	nd	10^3–10^{10}	nd	nd–10^9
Total	21 (16%) $n_{tot} = 130$	433 (99%) $n_{tot} = 437$	12 (9%) $n_{tot} = 130$	218 (46%) $n_{tot} = 470$

nd = not detected.

[a]The range of the lower detection limit for cultivation is 10^4–10^7 CFU/L, and for PCR 10^3–10^4 GU/L.

By cultivation, 21 (16%) of 130 analyses were positive for *Legionella* species and 12 (9%) of 130 analyses were positive for *L. pneumophila*. By quantitative real-time PCR, 433 (99%) of 437 analyses were positive for *Legionella* species and 218 (46%) of 470 analyses were positive for *L. pneumophila*. These results indicated that quantitative real-time PCR is a better tool for detection and quantification of *Legionella* in BTPs than the conventional cultivation methods.

In MST plants, *Legionella* species were detected in 3% of the cultivation analyses, and in 98% of the PCR analyses, demonstrating the higher sensitivity of the PCR method, and that inhibition of the PCR was not a major problem with this kind of sample.

No positive analyses of *Legionella* species were found in the dairy industry plants or other industry (OI) plants by cultivation. By PCR 100% of the samples were positive. However, inhibitors of the PCR analyses were seen in a number of samples from three of the dairy industry plants.

In the treatment plants from petrochemical industries, *Legionella* species were detected in 36% of the analyses by cultivation, and 99% by PCR, in some samples also in extremely high, but reproducible concentrations (10^{15} GU/L). The factors contributing to these favourable growth conditions are not known, and would need a closer investigation. *L. pneumophila* was detected in 14% of the cultivation analyses and in 65% by PCR analyses. The PCR was inhibited in several analyses from two of the plants.

All treatment plants from petrochemical industries positive for *Legionella* by cultivation-treated waste water from industries producing natural gas from the North Sea and liquid natural gas (LNG).

In treatment plants from wood processing industries, *Legionella* species were detected in 44% of the analyses by cultivation and in 100% by PCR. However, a significant number of the PCR analyses were inhibited in samples from two of the plants.

In Figures 2–6, a more detailed presentation of the results from cultivation and quantitative real-time PCR for *L. pneumophila* are shown. The diagrams show results for each group of plants, and the four seasonal samples for each

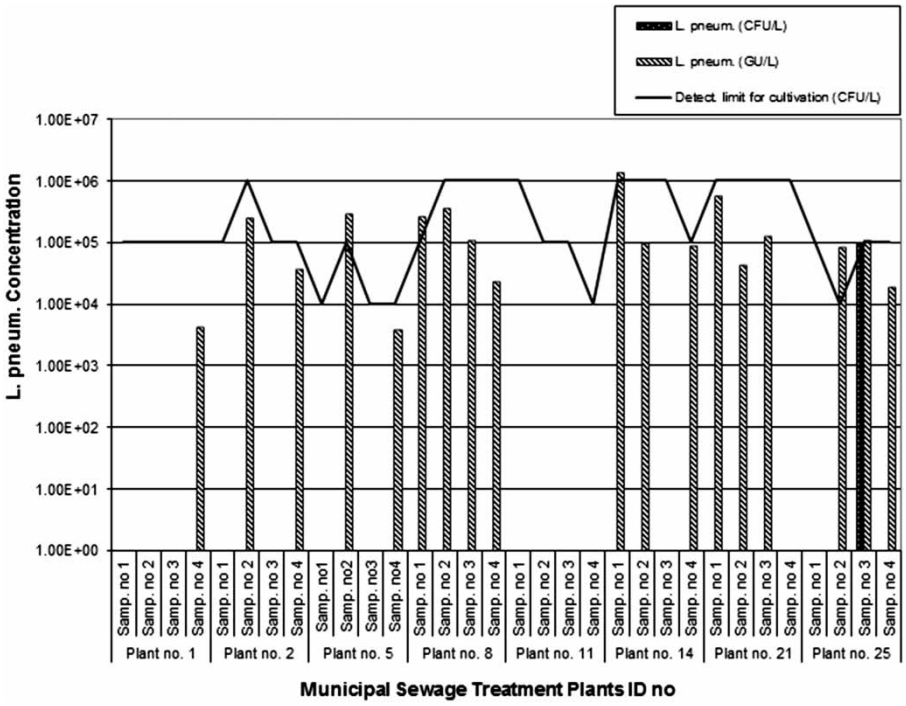

Figure 2 | Concentrations of *Legionella pneumophila* detected by cultivation (CFU/L) and real-time PCR (GU/L) for MSTs plants.

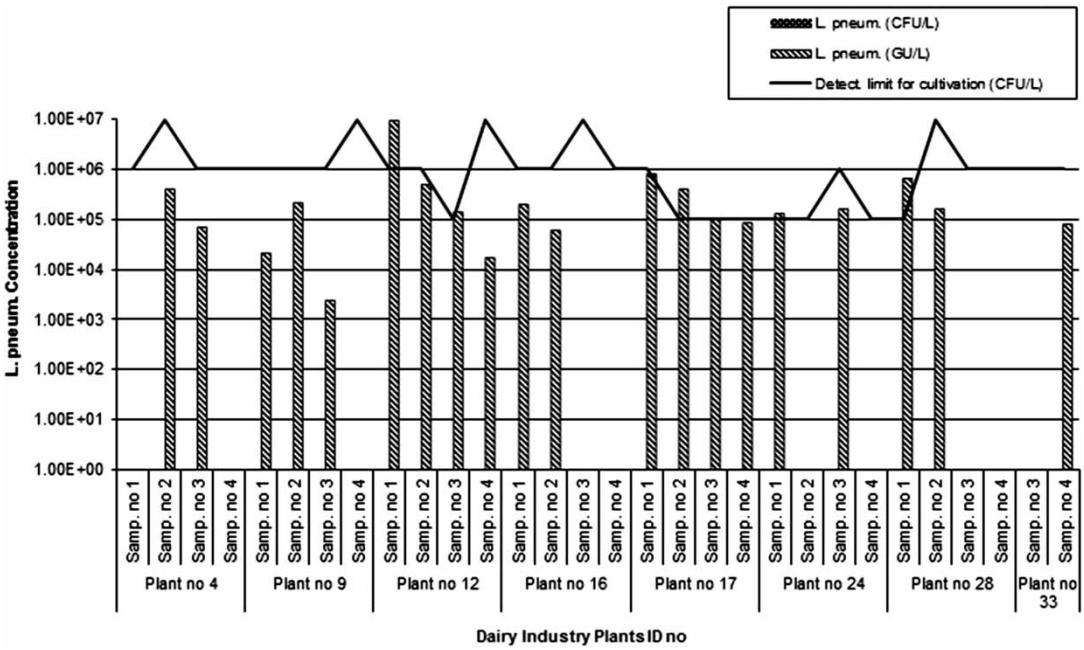

Figure 3 | Concentrations of *Legionella pneumophila* detected by cultivation (CFU/L) and real-time PCR (GU/L) for dairy industry plants.

plant are arranged in succeeding order corresponding to the sampling no. 1–4. Quantitative real-time PCR results are calculated on the basis of four parallels. In case of inhibition or typical outliers, the number of valid parallels is less than four. Results below detection limit (not detected) are designated as 'zero' and are included in the calculation. Based on the

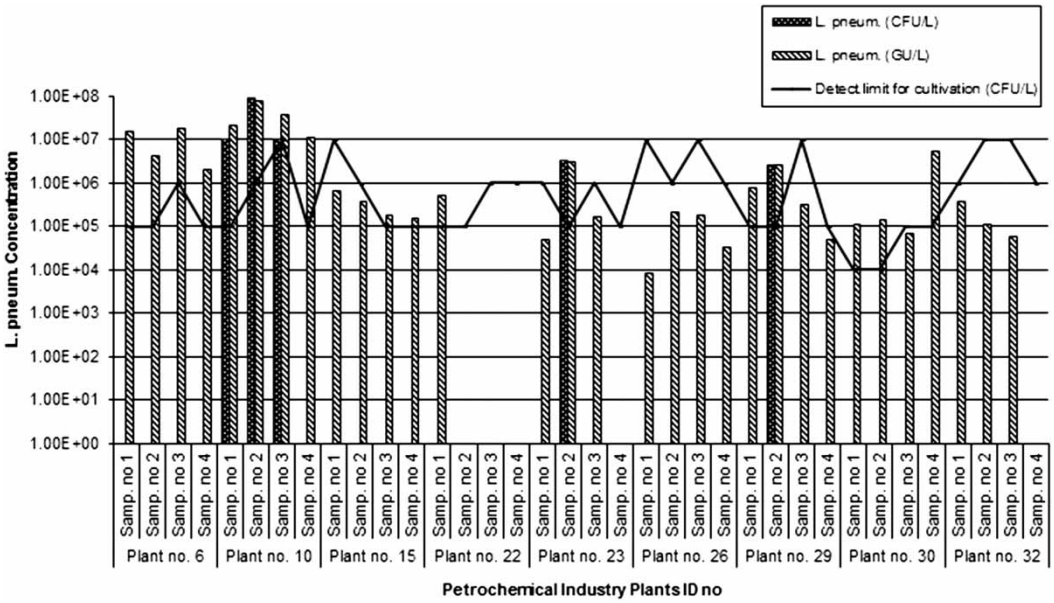

Figure 4 | Concentrations of *Legionella pneumophila* detected by cultivation (CFU/L) and real-time PCR (GU/L) for petrochemical industry plants.

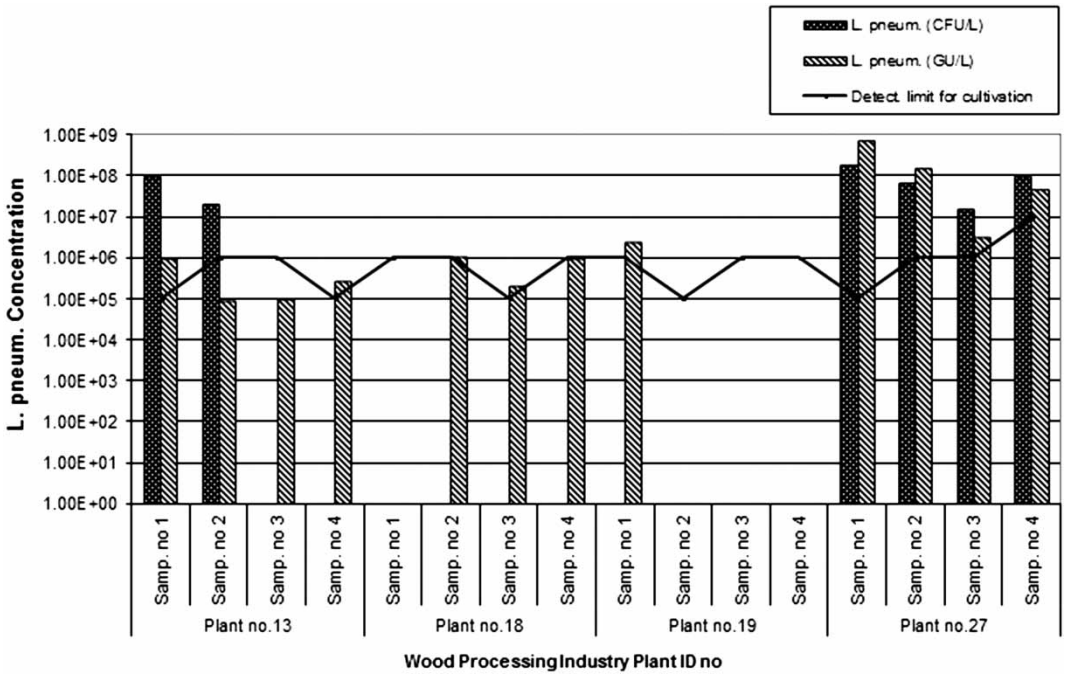

Figure 5 | Concentration of *Legionella pneumophila* detected by cultivation (CFU/L) and real-time PCR (GU/L) for wood processing industry plants.

experimental data, the detection limits for quantitative real-time PCR are in the range of 10^3–10^4 GU/L.

The cultivation results are based on one estimated value (CFU/L), and the results are always given together with the experimental detection limit (CFU/L) for each sample. Based on the limited results from this screening, there was no detectable trend for seasonal variation of the *Legionella* concentration in any of the plants.

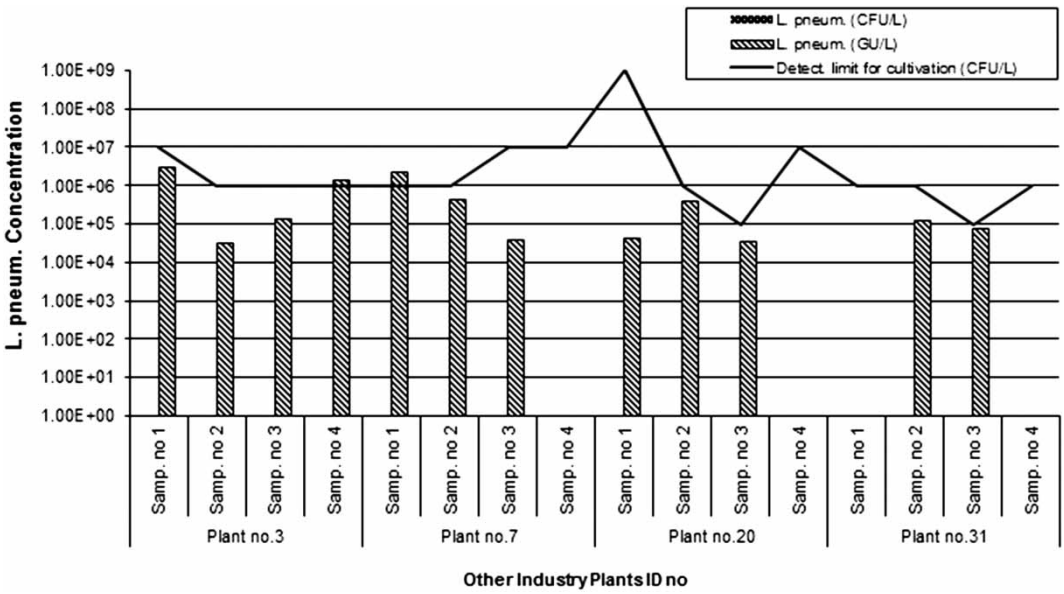

Figure 6 | Concentration of *Legionella pneumophila* detected by cultivation (CFU/L) and real-time PCR (GU/L) for other industry plants. No samples were positive by cultivation.

MSTs plants showed a very low occurrence of *L. pneumophila* by cultivation; the bacteria were detected only once at a low concentration, 10^5 CFU/L, in one plant (Figure 2). However, *L. pneumophila* was detected by real-time PCR at least once at seven of the eight plants, in the range 10^3–10^6 GU/L, and *Legionella* species were detected in all the MST plants, with average concentrations in the range of 10^6–10^9 GU/L. Concentrations as high as 10^{10} GU/L were detected, which was not expected because the mean water temperature in these plants was below 20 °C, which is far below the optimal growth temperature for *Legionella*. Little information exists about *Legionella* in MSTs. A Finnish survey of 17 MSTs isolated *Legionella* from only one plant (Kusnetsov *et al.* 2009). In a Dutch study *Legionella* was detected by PCR in all five plants examined, but *Legionella* was not detected in any of the plants by cultivation (detection limit 10^4–10^5 CFU/L). The authors were also able to detect *Legionella* and *L. pneumophila* in air samples from three of the five plants by PCR, but at very low concentrations (0.56–56 GU/m^3 air). They concluded that the risk for *Legionella* infection among the workers at these treatment plants was low even though they found a high risk of infection from other pathogenic bacteria, especially when doing service or cleaning of these systems (Medema *et al.* 2004). Because of the low water temperatures we did not expect to find any positive samples, so we advised the companies to take more samples over a certain period to give a better background for evaluating the potential risk associated with these systems. The inlet water should also be sampled to be able to evaluate the *Legionella* content in the incoming waste water. We advise the use of suitable respiratory protection equipment when working in exposed areas in the vicinity of the treatment plants.

None of the dairy industry plants (DI) showed detectable concentrations of *L. pneumophil*a by cultivation. However, at all eight plants, relatively low concentrations of *L. pneumophila* were detected by real-time PCR one to four times, in the range 10^3–10^7 GU/L (Figure 3). Since the mean temperature of the water in these plants is about 25 °C, which is known to be favourable for *Legionella* growth, we would expect to find higher concentrations of *Legionella* in these plants. Thus, there might be components in the waste water that are inhibiting the growth of *Legionella*, or the high number of *Lactobacillus* and other related bacteria present in these waste waters because of the milk content suppresses *Legionella* growth. Another reason for negative cultivation results is that the bacterial content in most of the samples is below the detection limit for the cultivation method used.

Of the nine PI plants, *L. pneumophila* was detected by cultivation in three of them, one to three times, in the

range of 10^6–10^7 CFU/L. *L. pneumophila* was detected in all the plants one to four times in the range of 10^4–10^7 GU/L by real-time PCR (Figure 4). One important factor for the high concentration of *Legionella* is that the water temperature in these aerated biological treatment ponds is above 25 °C, which is favourable for *Legionella* growth. The plants with the lowest concentrations of *Legionella* treated waste water from the production of polyethylene or ethylene/propylene, which is different from the other petrochemical industries which refined oil and produced natural gas from the North Sea.

As expected, *Legionella* was detected in most of the WPI plants (Figure 5). Most of the isolates were *L. pneumophila*, but *L. bozemanii* and *L. feeleii* were also detected. In two of the four WPI plants high concentrations, in the range of 10^7–10^8 CFU/L of *L. pneumophila*, were detected by cultivation. *L. pneumophila* was detected in all four plants one to four times in the range of 10^5–10^8 GU/L by real-time PCR. Even though *Legionella* bacteria were not detected in all samples, the results confirm what has been found in other studies, that high concentrations of *Legionella* are frequent in WPI plants (Allestam & Långmark 2007; Lindeberg 2007).

In 2005 there was a large outbreak of Legionnaires' disease associated with an aeration pond at a biological treatment plant at a WPI in Norway. *Legionella*-containing aerosols were spread over a distance of 10 kilometres, resulting in 103 cases of Legionnaires' disease and ten deaths (Nygård *et al.* 2008; Wedege *et al.* 2009). The factory closed the aeration ponds and proceeded to build a bigger anaerobic treatment process to treat their waste water. Further, air samplings in Norway and France in the vicinity of aerated sludge basins revealed that viable *Legionella* bacteria can be isolated at least 200–300 m downwind (Mathieu *et al.* 2006; Blatny *et al.* 2008). It is evident that a waste water treatment plant with an aeration basin can produce *Legionella*-containing aerosols. Therefore, these plants should be considered as a possible source of community-acquired *Legionella* infections, directly or indirectly via for instance air-scrubbers or cooling towers.

L. pneumophila was not detected by cultivation in any of the four plants from OI (Figure 6). Concentrations in the range of 10^4–10^6 GU/L were detected by real-time PCR two to four times in each of the plants. As can be seen from Figure 6 the total *L. pneumophila* content in these samples, displayed by the PCR results, in most cases is below the detection limit for the cultivation method used.

Table 2 shows the overall PCR results for *Legionella* species and *L. pneumophila* for each plant. For each plant [except MST (25) and DI (33)] there were in total 16 analyses for *L. pneumophila* and 16 analyses for *Legionella* species. The number of valid results for each plant, denoted n_{LP} or n_{Lspp}, demonstrates that inhibition factors varied. This is most likely due to the various and very complex matrixes that are analysed in this survey. Analyses from the MST plants and plants from the category 'OI' show that very few of the total analyses are inhibited: 5.3 and 11%, respectively. From the other plant categories, analyses are more frequently inhibited: 45% of the analyses from the WPI, 26% of the analyses from the PI and 20% of the analyses from the dairy industry (DI) were inhibited. No further control tests were done to determine the source or type of inhibitors.

However, the analyses of *L. pneumophila* were to a much lesser degree inhibited. The inhibition percentage was in the range of 0.8% (MTBs) to 9.7% (PIs). There are no obvious explanations for the lack of correlation between the analyses of *L. pneumophila* and *Legionella* species. The differences are pronounced in the analyses from WPIs and PIs. When using quantitative real-time PCR for analysing complex and polluted samples, the factors inhibiting the PCR-process should be carefully examined. It will also be useful to check different kits and different DNA extraction methods, to compare results and validate the method for the specific use.

Average values were calculated by setting the 'not detected' (nd) results to zero. Thus, the distribution of the results around the average value cannot be determined, and standard deviation is not adequate. Instead, minimum and maximum values are presented in the table to indicate the range.

The more frequent detection of *Legionella*, and the higher concentration levels recorded by PCR versus cultivation, are probably due to several factors. The PCR detects both viable and non-viable *Legionella*, the detection limit is far below the detection limit of culture-based methods, the PCR method detects a larger number of *Legionella* species, and inhibition factors for cultivation and PCR are

Table 2 | *Legionella* species (L.spp) and *Legionella pneumophila* (LP) results from the quantitative real-time PCR analyses of biological treatment plants connected to MST plants, dairy industry (DI), petrochemical industry (PI), wood processing industry (WPI) and other industry (OI). Plant ID number is given in brackets

Plant (ID)	n_{Lspp}	L.spp GU/L average	L.spp GU/L min	L.spp GU/L max	n_{LP}	LP GU/L average	LP GU/L min.	LP GU/L max.
MST (1)	16	4.70E + 07	2.00E + 06	4.40E + 08	16	2.7E + 02	nd	4.30E + 03
MST (2)	16	9.10E + 07	9.90E + 05	4.70E + 08	14	3.8E + 04	nd	3.20E + 05
MST (5)	15	4.10E + 07	2.70E + 06	1.50E + 08	14	4.2E + 04	nd	3.40E + 05
MST (8)	16	1.30E + 08	1.20E + 06	1.20E + 09	16	9.50E + 04	nd	1.0E + 06
MST (11)	16	7.90E + 06	nd	2.10E + 07	15	nd	nd	nd
MST (14)	13	1.60E + 08	2.1E + 06	1.70E + 09	16	1.90E + 05	nd	2.60E + 06
MST (21)	13	1.20E + 08	1.00E + 06	5.60E + 08	15	1.50E + 05	nd	1.60E + 06
MST (25)[a]	20	1.40E + 09	7.50E + 06	2.70E + 10	18	4.20E + 04	nd	2.30E + 05
DI (4)	16	1.30E + 07	1.60E + 05	7.10E + 07	16	3.00E + 04	nd	4.10E + 05
DI (9)	16	3.40E + 06	1.70E + 05	2.90E + 07	14	1.70E + 04	nd	2.20E + 05
DI (12)	7	1.20E + 08	2.50E + 04	7.60E + 08	10	3.10E + 06	nd	2.60E + 07
DI (16)	16	6.00E + 08	1.20E + 05	2.70E + 09	16	1.60E + 04	nd	2.00E + 05
DI (17)	9	1.40E + 08	1.70E + 07	3.50E + 08	16	3.50E + 05	nd	2.70E + 06
DI (24)	15	1.70E + 07	2.00E + 05	4.90E + 07	15	1.10E + 04	nd	1.70E + 05
DI (28)	16	7.90E + 06	5.00E + 04	6.00E + 07	9	2.60E + 05	nd	1.80E + 06
DI (33)[b]	7	7.20E + 06	.4.40E + 05	3.00E + 07	6	1.40E + 04	nd	8.40E + 04
PI (6)	16	2.40E + 14	5.80E + 12	3.30E + 15	14	1.20E + 07	6.90E + 05	5.30E + 07
PI (10)	12	8.10E + 07	1.50E + 05	3.20E + 08	16	3.70E + 07	3.00E + 06	1.60E + 08
PI (15)	15	1.50E + 08	1.50E + 07	1.40E + 09	15	2.90E + 05	nd	1.70E + 06
PI (22)	7	3.90E + 05	1.30E + 04	1.70E + 06	10	5.20E + 04	nd	5.20E + 05
PI (23)	15	4.00E + 07	7.20E + 06	1.90E + 08	16	8.10E + 05	nd	7.40E + 06
PI (26)	9	1.30E + 06	nd	9.50E + 06	8	1.00E + 05	8.50E + 03	2.00E + 05
PI (29)	15	2.20E + 09	2.60E + 06	1.20E + 10	15	7.30E + 05	nd	5.60E + 06
PI (30)	16	1.20E + 09	2.30E + 06	1.40E + 10	15	3.90E + 05	nd	5.40E + 06
PI (32)	14	8.80E + 08	1.70E + 06	1.10E + 10	14	5.50E + 04	nd	3.60E + 05
WPI (13)	5	3.70E + 07	2.00E + 04	1.90E + 08	14	2.80E + 05	1.10E + 04	2.00E + 06
WPI (18)	11	2.80E + 08	4.50E + 03	8.50E + 08	16	3.50E + 05	nd	3.30E + 06
WPI (19)	14	7.30E + 07	2.30E + 04	7.20E + 08	16	7.50E + 05	nd	9.10E + 06
WPI (27)	4	1.70E + 08	2.50E + 05	5.90E + 08	16	2.30E + 08	1.10E + 04	1.40E + 09
OI (3)	13	5.40E + 09	2.3E + 04	7.5E + 10	13	7.90 E + 07	Nd	1.10E + 09
OI (7)	16	1.20E + 06	1.20E + 04	3.300E + 06	16	7.90E + 05	Nd	7.90E + 06
OI (20)	15	5.20E + 07	8.20E + 03	2.80E + 08	15	1.00E + 05	Nd	9.70E + 05
OI (31)	13	7.60E + 09	1.50E + 04	8.90E + 10	15	2.00E + 04	Nd	1.20E + 05

nd = not detected.
[a]One extra sample.
[b]Sampled twice.

not identical. The cultivation method will probably not reflect the correct composition of *Legionella* species and *L. pneumophila* as they may occur in mixed samples. The PCR method specifically identifies and quantifies *L. pneumophila* and *Legionella* species in a mixture, and our results show that the concentration of *Legionella* species (which includes *L. pneumophila*) is always higher or similar to the *L. pneumophila* concentration.

Table 3 | *Legionella* species identification by 16S rDNA sequencing in the biological treatment plants. Percentage similarity to the closest sequence in GenBank is given in parentheses

Plant (ID)	Sample 1	Sample 2	Sample 3	Sample 4
MST (25)			*L. pneumophila*	
PI (6)	*L. gormanii* (97%)	*L. gormanii* (97%)	*L. gormanii* (97%)	
		L. parisiensis (98%)		
PI (10)	*L. pneumophila*	*L. pneumophila*	*L. pneumophila*	
PI (15)	*L. longbeachae* (99%)	*L. longbeachae* (99%)	*L. longbeachae* (99%)	
PI (23)		*L. pneumophila*		
PI (29)		*L. pneumophila*		
PI (32)	*Legionella* sp.	*Legionella* sp.		
WPI (13)	*L. feeleii* (99%)	*L. pneumophila*		
WPI (19)			*L. bozemanii* (99%)	
WPI (27)	*L. pneumophila*	*L. pneumophila*	*L. pneumophila*	*L. pneumophila*

Characterization of *Legionella* isolates

A total of 59 isolates recovered from the water samples were characterized by 16S rDNA sequencing. Of these, 51 were confirmed as belonging to the genus *Legionella* (Table 3). In most cases, the dominant *Legionella* species found in multiple samples collected at a plant was the same, except for WPI (13) where isolates genetically close to *L. feeleii* in sample 1 were replaced by *L. pneumophila* in sample 2.

Sequence-based typing of the 29 *L. pneumophila* isolates recovered from six plants was performed and revealed eight sequence types (STs): ST39 in WPI (13), ST59 in PI (29), ST68 in PI (10) and MST (25), ST70 in PI (29), ST345 in PI (10), ST576 in WPI (27), as well as two STs not assigned a number because of the lack of the *neuA* gene. PI (10) harboured ST345 in samples 1 and 2 but shifted to ST68 in sample 3. ST68 was also found in MST (25) in sample 3. PI (29) harboured two distinct STs in sample 2 (ST70 and ST59), which were serogroup 1 and 2–14, respectively. In WPI (27) ST576 was recovered in all samples, together with a *neuA*-negative variant of ST576 in samples 1 and 4. While all ST576 isolates were serogroup 1, the *neuA*-negative variants of ST576 were serogroup 2–14. All these STs, except ST576 and its *neuA*-negative variant had been identified outside Norway, and were represented by both clinical and environmental isolates, but at a low frequency in the EWGLI database (<1.5% each). Thus, apparently none of these strains has been the cause of large outbreaks. The three STs, ST40, ST15 and ST462 responsible for outbreaks in Norway in 2001, 2005 and 2008, respectively (Wedege *et al.* 2013), were not identified from the BTPs.

CONCLUSIONS

This survey indicates that PCR could be the preferable method for detection and quantification of *Legionella* in samples from BTPs. The samples included in this survey represent various highly complex and polluted matrixes, and more studies are recommended to evaluate the method in a systematic way under controlled conditions, especially related to different matrix composition, and how this affects the inhibition of the PCR analysis.

Based on the limited results from this screening, there was no detectable trend for seasonal variations of the *Legionella* concentration in any of the BTPs. All but two identified STs had previously been identified outside Norway, and were represented by both clinical and environmental isolates, but at a low frequency in the EWGLI database. Thus, apparently none of these strains has been associated with large outbreaks.

It is evident from our results that a waste water treatment plant with an aeration basin can produce high concentrations of *Legionella*. Therefore, these plants should be considered as a possible source of community-acquired *Legionella* infections, directly or indirectly via air-scrubbers or cooling towers.

Based on these findings, we advise the industries to carry out a risk assessment of the exposure from these plants, for the workers and for exposure to the public. This should also include analysis of *Legionella* from the inlet and the outlet water from a BTP, as well as air samples. As a general precaution we recommend the use of suitable respiratory protection equipment for technical staff when working in the vicinity of biological treatment plants unless low exposure risk can be documented.

REFERENCES

Allestam, G. & Långmark, J. 2007 *Legionella* i bioreningsanläggningar – Kartleggning och riskbedömning 2005–2007. Smittskyddsinstitutets rapportserie nr 3: s 12.

Blatny, J. M., Reif, B. A. P., Skogan, G., Andreassen, O., Høiby, E. A., Ask, E., Waagen, V., Aanonsen, D., Aaberge, I. S. & Caugant, D. A. 2008 Tracking airborne *Legionella* and *Legionella pneumophila* at the biological treatment plant. *Environ. Sci. Technol.* **42** (19), 7360–7367.

Castor, M. L., Wagstrom, E. A., Danila, R. N., Smith, K. E., Naimi, T. S. & Besser, J. M. 2005 An outbreak of Pontiac fever with respiratory distress among workers performing high-pressure cleaning at a sugar-beet processing plant. *J. Infect. Dis.* **191**, 1530–1537.

Eurosurveillance 2010 Outbreak of Legionella in Domsjø, Sweden. **15** (43), 28 October 2010.

Gaia, V., Fry, N. K., Afshar, B., Lück, P. C., Meugnier, H., Etienne, J., Peduzzi, R. & Harrison, T. G. 2005 Consensus sequence-based scheme for epidemiological typing of clinical and environmental isolates of *Legionella pneumophila*. *J. Clin. Microbiol.* **43**, 2047–2052.

Gregersen, P., Grunnet, K., Andersen, B. H. & Madsen, H. 1999 Pontiac fever at a sewage treatment plant in food industry. *Scand. J. Work. Environ. Health* **25** (3), 291–295.

Handal, T., Olsen, I., Walker, C. B. & Caugant, D. A. 2004 Beta-lactamase production and antimicrobial susceptibility of subgingival bacteria from refractory periodontitis. *Oral Microbiol. Immunol.* **19** (5), 303–308.

International Standards Organisation 1998 *Water quality – detection and enumeration of Legionella*. ISO 11731:1998E.

Kusnetsov, J., Airaksinen, P., Pitkänen, T., Kauppinen, A., Vepsäläinen, A., Miettinen, I. & Torvinen, E. 2009 Occurrence of Legionella and other pathogenic microbes in communal waste water treatment plants with low water temperature. Abstract book, Legionella 2009, 13–17 October, Institut Pasteur, Paris.

Kusnetsov, J., Neuvonen, L.-K., Korpio, T., Uldum, S. A. & Mentula, S. 2010 Two Legionnaires' disease cases associated with industrial waste water treatment plants: a case report. *BMC Infect. Disease* **10**, 343.

Lindeberg, O. 2007 Undersökning av samband mellan Legionellaförekomst och teknisk utformning av bioreningsanläggningar – En utvärdering utförd på uppdrag av Skogsindustrierna. Extract Information AB (in Swedish).

Mathieu, L., Robine, E., Deloge-Abarkan, M., Ritoux, R., Pauly, D., Hartemann, P. & Zmirou-Navier, D. 2006 *Legionella* bacteria in aerosols: Sampling and analytical approaches used during the Legionnaires' disease outbreak in Pas-de-Calais. *J. Infect. Dis.* **193** (9), 1333–1335.

Medema, G., Wullings, B., Roeleveld, P. & van der Kooij, D. 2004 Risk assessment of *Legionella* and enteric pathogens in sewage treatment works. *Water Supply* **4** (2), 125–132.

Nederbragt, A. J., Balasingham, A., Sirevåg, R., Utkilen, H., Jakobsen, K. S. & Anderson-Glenna, M. 2008 Multiple-locus variable-number tandem repeat analysis of *Legionella pneumophila* using multi-colored capillary electrophoresis. *J. Microbial. Methods* **73** (2), 111–117.

Nygård, K., Werner-Johansen, O., Rønsen, S., Caugant, D. A., Simonsen, O., Kanestrøm, A., Ask, E., Ringstad, J., Ødegård, R., Jensen, T., Krogh, T., Høiby, E. A., Ragnhildstveit, E., Aaberge, I. S. & Aavitsland, P. 2008 An outbreak of Legionnaires' disease caused by long-distance spread from an industrial air scrubber in Sarpsborg, Norway. *Clin. Infect. Dis.* **46** (1), 61–69.

Ratzow, S., Gaia, V., Helbig, J. H., Fry, N. K. & Luck, P. C. 2007 Addition of neuA, the gene encoding N-acylneuraminate cytidylyl transferase, increases the discriminatory ability of the consensus sequence-based scheme for typing *Legionella pneumophila* serogroup 1 strains. *J. Clin. Microbiol.* **45**, 1965–1968.

Ruotsalainen, E., Kusnetsov, J., Seppälä, I., Kanervo-Nordström, A., Virolainen-Julkunen, A. & Lyytikäinen, O. 2008 A cluster of Pontiac fever among employees in the biological waste-water treatment plants of a chemical mill. Abstract book, European Working Group of *Legionella* Infections. 23rd Meeting, Madrid, May 11–13th 2008.

Tran Minh, N. N., Ilef, D., Jarraud, S., Rouil, L., Campese, C. & Che, D. 2006 A community-wide outbreak of Legionnaires' disease linked to industrial cooling towers – How far can contaminated aerosols spread? *J. Infect. Dis.* **193**, 102–111.

Wedege, E., Bergdal, T., Bolstad, K., Caugant, D. A., Efskind, J., Heier, H. E., Kanestrøm, A., Strand, B. H. & Aaberge, I. S. 2009 Seroepidemiological study after a long-distance industrial outbreak of Legionnaires' disease. *Clin. Vaccine Immunol.* **16** (4), 528–534.

Wedege, E., Bolstad, K., Borgen, K., Fritzønn, E. & Caugant, D. A. 2013 Molecular characterization of clinical and environmental isolates of *Legionella pneumophila* in Norway. *Scand. J. Infect. Dis.* **45**, 59–64.

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