Alternative sources of *Legionella* bacteria

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Alternative sources of *Legionella* bacteria

Alternatieve bronnen van legionella bacteriën
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

1.

General Introduction

1. *Legionella* and legionellosis

The family of Legionellaceae is composed of a single genus, *Legionella* (1). Together with the Coxiellaceae, Legionellaceae comprise the proposed order "*Legionellales*" within the class Gammaproteobacteria. There are currently 62 known species of *Legionella* of which approximately half have been associated with patients (2-6). Some species rarely cause disease and are only isolated from severely immunosuppressed patients (1). In contrast, the species *L. pneumophila* and *L. longbeachae* are frequent causative agents of disease (see text box 1 and 2).

Legionella are Gram-negative, rod shaped, flagellated bacteria with strict growth requirements (1, 7). They have a growth dependence for the amino acid L-cysteine (2). This characteristic is used to confirm that a suspected colony is *Legionella* by plating the strain on a media with and without L-cysteine. On plate, *Legionella* form colonies that are smooth, flat, and have a typical ground-glass appearance and an iridescent hue. Colors may vary between species and even in the same culture multiple colors can be observed (1).

L. pneumophila, the best-studied species of *Legionella*, has an optimal growth temperature of 35 to 37°C (8). However, this species can survive and possibly also grow at temperatures as high as 58°C (9). There are 16 known serogroups of *L. pneumophila* of which serogroup 1 (SG1) is the most frequently isolated from patients (1, 10). *L. pneumophila* strains can also be typed by monoclonal antibody (MAb) subgrouping, according to the expression of different epitopes by the bacteria (11). MAb 3/1 positivity of *L. pneumophila* SG1 strains is considered an indication of virulence since this monoclonal antibody recognizes a virulence-associated epitope (12).

1. A main causative agent of Legionnaires' disease: *Legionella pneumophila*

In 1976, a large outbreak of pneumonia took place in Philadelphia, affecting over 180 people, of which the majority were Legionnaires attending an American Legion convention in a hotel (151). The causative agent was a previously unrecognized bacterium which was named *Legionella pneumophila* (152). In retrospect, it was discovered that the newly found bacterial species was also linked to a large outbreak in 1968 of the so-called Pontiac fever (153).
Since the discovery of *L. pneumophila*, this species was linked to numerous aquatic sources, both man-made and natural, and caused many outbreaks. Several of these outbreaks, caused by cooling towers and whirlpools, affected over hundreds (27, 82, 154, 155).

2. A main causative agent of Legionnaires' disease: *Legionella longbeachae*

In 1980, *Legionella longbeachae* was first discovered in a patient with pneumonia in Long-beach, California (156). *L. longbeachae* is a main causative agent in Australia (85), New Zealand (12) and Thailand (157), but *L. longbeachae* infections have also been reported in the U.S. (158), Canada (159), Japan (160, 161), Taiwan (162) and Europe (62, 78, 80, 163).
In contrast to *L. pneumophila*, *L. longbeachae* is associated with exposure to potting soil and composted material (50-52). Outbreaks caused by this *Legionella* species are only little reported and have in general a smaller impact than outbreaks caused by *L. pneumophila*. The largest outbreak reported affected 9 people contracting Pontiac fever (164).

Legionella bacteria are subject to phagocytic predation by protozoa (13) but they are able to avoid the phagocyte-lysosome pathway and therefore degradation. *Legionella* multiplication inside protozoa was first described by Rowbotham in 1980 (14). The organisms have been shown to multiply in many species of amoebae such as *Acanthamoeba* and *Naegleria* (15, 16) but also in ciliated protozoa (17, 18). Furthermore, *Legionella* species have been detected in several fungi (19) and research by Rasch et al. (20) indicates that nematodes may also serve as natural hosts for *Legionella*.

Legionella are opportunistic pathogens. They avoid degradation by macrophages of the human immune system with a similar mechanism used to avoid degradation by protozoa (21). Infection occurs mainly through inhalation of *Legionella* contaminated aerosols, but aspiration or direct contact with wounds is also described (2). Person-to-person transmission of *Legionella* was assumed impossible but has recently been reported for the first time (22).

Legionellosis, the infection caused by *Legionella* bacteria, describes two distinct clinical syndromes, Legionnaires' disease (LD) and Pontiac fever. LD is an atypical pneumonia that clinically resembles other bacterial pneumonias (2). Symptoms can range from mild disease to severe pneumonia with sometimes fatal outcome (23). Recovery can be slow and patients may suffer sequelae such as fatigue (24-26). The incubation period is approximately 2-10 days but can extend up to 19 days (median 7 days) (27). Pontiac fever is a milder disease with flu-like symptoms (2, 28). The condition is associated with *Legionella* exposure but it is not clear if *Legionella* infection actually takes place (29). Several risk factors for LD have been identified, i.e., chronic lung disease, diabetes mellitus, tobacco smoking, and use of immunosuppressive medications or TNF_alpha inhibitors (1, 30-32). An important behavioral risk factor is travelling abroad (33). In the Netherlands, spending one or more nights away from home (not leaving the country) was also found to be a risk for contracting LD (30). Most cases of LD occur sporadically, i.e., not related to a cluster or

outbreak. From 2000 to 2009, outbreak associated LD accounted for 4% and 9% of all cases reported in the United States and Europe, respectively (29).

Methods available for diagnosis of LD are: culture, urine antigen tests, PCR, DFA (direct fluorescent-antibody) tests and IFA (indirect fluorescent-antibody) tests (29). The development and implementation of the urine antigen tests, in the Netherlands applied since 1999 (34), have greatly improved diagnosis rates and benefited LD patients by timely treatment of the condition (29). However, a negative urine antigen test does not rule out *Legionella* infection (35). The sensitivity of the test is dependent on disease severity (36), time since exposure (37), concentration of urine prior to analysis (38), and causative agent (35). Mercante and Winchell (29) recently reviewed *Legionella* diagnostics and they highlight the diagnostic "blind spot" for LD caused by non-SG1 strains. As urinary antigen testing is the most applied diagnostic test, the finding that *L. pneumophila* SG1 is the main infective agent is not surprising, as the urinary antigen tests currently available are developed to detect *L. pneumophila* SG1 primarily and have a low sensitivity for overall LD diagnosis (39).

Legionella bacteria are ubiquitous in the environment, in water and soil, and in man-made water systems. *L. pneumophila* infection is associated with aquatic sources, such as cooling towers (40), whirlpools (41, 42), water distribution systems (43, 44), thermal springs (45), indoor fountains (46, 47) and humidifiers (48, 49). Infection with *L. longbeachae* is linked to potting soil and composted materials (50-52). The transmission mode of *L. longbeachae* from potting soil and composted materials remains unclear. In two case-control studies, predictors of illness caused by *L. longbeachae* included: eating or drinking after gardening without washing hands (53), getting hands near face (smoking, eating or drinking, touching face) before washing hands (54), tipping or troweling compost (54) and being near dripping hanging flower pots (53). The standard method of *Legionella* detection in environmental samples is culture on plates (55).

2. Epidemiology

Legionellosis is believed to occur globally, however the rate of occurrence is unknown as many countries lack appropriate diagnostic methods or sufficient surveillance systems (56). Although legionellosis is a notifiable disease in many countries, the reported incidence varies widely due to differences in the level of surveillance and reporting. It is expected that notification rates do not reflect the true incidence because the disease is likely underdiagnosed.

2.1 Europe and the Netherlands

In Europe, LD is a relatively rare infectious disease with an overall notification rate of 1.4 per 100.000 population in 2015 (10). In 2015 the case-fatality ratio was 8.1% (range 2011-2015 is between 8 and 10%).

The European Centre for Disease Prevention and Control (ECDC) provides annual reports on LD in Europe. National surveillance data are collected retrospectively by the European Legionnaires' disease surveillance network (ELDSNet), which involves 28 EU member states and 2 EEA member states (Iceland and Norway), and via near-real-time reporting of travel-associated cases of LD (TALD). The 2012 EU/EEA case definition for LD cases states that a case is diagnosed with pneumonia and meets at least one of the laboratory criteria for confirmed or probable cases (see text box 3).

3. Case definition Legionnaires' disease (115)

Clinical criterion: Any person with pneumonia

Laboratory criteria for a confirmed case

At least one of the following three:

- Isolation of *Legionella* spp. from respiratory secretions or any normally sterile site;
- Detection of *Legionella pneumophila* antigen in urine;
- Significant rise in specific antibody level to *Legionella pneumophila* SG1 in paired serum samples.

Laboratory criteria for a probable case

At least one of the following four:

- Detection of *Legionella pneumophila* antigen in respiratory secretions or lung tissue e.g. by DFA staining using monoclonal antibody derived reagents;
- Detection of *Legionella* spp. nucleic acid in respiratory secretions, lung tissue or any normally sterile site;
- Significant rise in specific antibody level to *Legionella pneumophila* other than SG1 or other *Legionella* spp. in paired serum samples;
- Single high level of specific antibody to *Legionella pneumophila* SG1 in serum.

In 2015, 7034 cases of LD were reported in Europe, the highest number and notification rate since 1995 (10) (see Table 1 and Figure 1). The majority of the cases were classified as confirmed (6570, 93%). The notification rate in the Netherlands was 2.5/100.000 in 2015 (419 cases, of which 94% confirmed) (57). The overall European notification rate is influenced by several countries with very low notification rates. Notification rates in Europe vary from

less than 0.1 to 5.1 per 100.000 inhabitants (10). Nearly 75% of all notified cases in Europe in 2015 were reported by five countries: France, Italy, Spain, Germany and the Netherlands. These countries reported the highest numbers of cases, although their combined populations only represented approximately 53% of the EU/EEA population (58). Eastern and south-eastern European countries tend to report only few LD cases (59).

Table 1. Notified LD/legionellosis* cases and disease rates reported in Europe, the U.S., Canada, Australia, New Zealand and Japan

	n Cases/year	Notification rate per 100.000	Year of reporting	Increased incidence since	Reference
Europe	7034	1.4	2015	1995 (0.4-1.4)	(10, 60)
U.S.	6079	1.9	2015	2000 (0.4-1.9)	(61, 62)
Canada	328	0.9	2015	2010 (0.6-0.9)	(63)
Australia	365	1.5	2015	-	(64)
New Zealand	254	5.5	2015	2009 (1.7-5.5)	(12)
Japan	1248	1.0[†]	2014	2005 (0.2-1.0)[†]	(65)

– Not applicable. *The U.S., Canada, Australia, New Zealand and Japan record legionellosis (i.e., LD and Pontiac fever) instead of LD as is done in Europe. [†]Notification rate was not reported but calculated using population data (66).

As mentioned, LD is probably underdiagnosed and underreported. Thus, the disease burden of LD in Europe is probably higher than now recognized. For example, the outcome of a German study (CAPNETZ) suggests that 4% of all community acquired pneumonia (CAP) is due to *Legionella* infection (23). It was estimated that at least 15.000-30.000 sporadic LD cases occur in Germany per year, while in 2015 only 865 cases were notified (notification rate: 1.1/100.000) (10). Applying the Dutch incidence rate of 2015 (2.5/100.000) on the European population of the 30 EU/EAA countries in 2015 (~514 million), the expected number of notified LD cases would be 12.850. This means that almost half of the European LD cases are currently missed. It should be noted that the incidence of one country cannot be generalized to others because transmission will most probably be affected by local conditions (67).

An increasing burden of disease is experienced in Europe over the past years, as resembled by an increasing reported incidence from 2011 to 2015 (1.0/100.000 and 1.4/100.000 respectively) (see Figure 1) (10). Notifications in 2011 were relatively low compared to a peak year 2010. However, overall over the period of 1995 to 2015, an increase of reported LD was observed. Several explanations for the increasing trend in LD notification have been suggested, i.e., increased awareness, enhanced surveillance and an increasing proportion of older inhabitants that are at increased risk (68).

Figure 1. Notification rate of Legionnaires' disease in the EU/EEA*, by year of reporting, 1995–2015 (10)

*EWGLINET member countries not belonging to the EU/EEA were excluded for 1995–2008.

The majority of the notified European LD cases were sporadic and community-acquired (68.8% in 2015) (10). The other reported cases were associated with travel (21.7%, abroad and domestic travel), healthcare (7.7%) and other settings (1.9%). LD occurs throughout the year but most cases are reported in summer and early fall. In 2015, 59% of the cases had a date of onset between June and October. Older people are most susceptible to *Legionella* infection. People aged over 45 years accounted for 89% (6225) of 7027 cases with known age in 2015. Furthermore, men are more susceptible than women; the overall male-to-female ratio over the years 2011-2015 lies around 2.5:1. Urinary antigen testing is the most frequently used diagnostic method for confirmation of LD (77.4% of all laboratory tests in 2015) (10).

The most important infective agent in Europe is *L. pneumophila*. Of 890 culture-confirmed cases in 2015, 96.1% were due to *L. pneumophila* and 82.2% were due to *L. pneumophila* SG1 (see Table 2) (10). Other serogroups than SG1 comprised 14.4% of the isolated *L. pneumophila* strains.

Table 2. Main causative agent of LD/legionellosis* in Europe, the U.S., Canada, Australia, New Zealand and Japan

	Main causative agent(s)	Percentage of cases infected with main agent(s)	Year(s) of reporting	Reference
Europe	*L. pneumophila* SG1	82%[†]	2015	(10)
U.S.	*L. pneumophila* SG1	80%[†], 98%[‡]	2011-2013	(71)
Canada	*L. pneumophila* SG1	60%[†]; 66%[†]	1981-2009; 1978-2006	(72, 73)
Australia	*L. pneumophila*, *L. longbeachae*	53.9%, 45.3%[§]	2014	(74)
New Zealand	*L. longbeachae*, *L. pneumophila*	52.2%, 29.1%[†]	2015	(12)
Japan	*L. pneumophila* SG1	83%[†]	2008-2012	(75)

*The U.S., Canada, Australia, New Zealand and Japan record legionellosis (i.e., LD and Pontiac fever) instead of LD as is done in Europe. [†]Percentage based on culture results. [‡]Percentage mainly based on urine antigen testing. [§]Diagnostic method not specified.

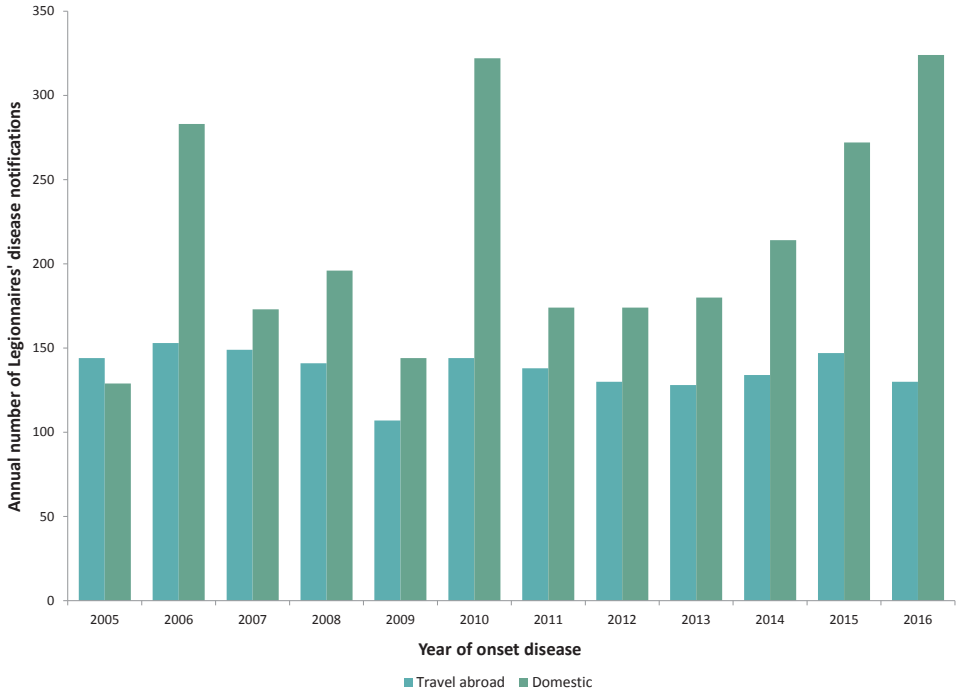
As in Europe, the reported LD incidence also increased in recent years in the Netherlands (see Figure 2) (69). The overall notification rate in 2016 was slightly higher than in 2015 (2.7/100.000, 454 cases vs. 2.5/100.000, 419 cases) and similar to the notification rate of the peak year 2010 (70) (see also paragraph 5). The number of travel associated cases has remained relatively stable over time and varied between 124 and 138 cases over the past 5 years. In 2016, of the 324 cases that had not travelled abroad (domestic cases), 300 cases were community acquired, 17 travelled in the Netherlands and seven were healthcare associated. The most important infective agent in the Netherlands is *L. pneumophila* (SG1).

The species *L. longbeachae* is more and more recognized as a causative agent in Europe. Since 2004, case reports from several European countries have described *L. longbeachae* infections (51, 62, 76-79). It is believed that *L. longbeachae* is an under-recognized causative agent of LD in Europe. In Scotland, since 2008, when the first infection with *L. longbeachae* was reported (52), an increase in reported *L. longbeachae* cases is experienced (80). This is in contrast to the rest of the U.K., which has not observed an increase in the incidence most likely reflecting underdiagnosing. The increase in Scotland is expected to be caused by improved detection.

2.2 LD in other parts of the world

To note: the U.S., Canada, Australia, New Zealand and Japan record legionellosis (i.e., LD and Pontiac fever) instead of LD as is done in Europe. However, the great majority of the legionellosis cases are LD (81).

Figure 2. Annual numbers of notified Legionnaires' disease cases, 2005 through 2016, by infection acquired abroad or domestic (i.e., within the Netherlands) (69)



The incidence rates of legionellosis in other parts of the world vary from less than the European incidence rate, i.e. Canada (0.9/100.000 in 2015) (63), to far more, i.e. New Zealand (5.5/100.000 in 2015) (12) (see Table 1). In New Zealand, after a long period of relatively stable notification rates (1997-2009), a strong increase of legionellosis was experienced in 2010 (12). In 2015, there was again a strong increase compared to the years before and the highest number of legionellosis cases ever was reported. The 2015 increase is likely due to the LegiNZ study, which began in May 2015 and includes 20 hospitals. In the LegiNZ study, hospitalized patients with suspected pneumonia are tested for *Legionella* spp. using PCR.

The disease rate in Canada is relatively low. In 2015 a total of 328 legionellosis cases were reported (0.9/100.000) (63). The notification rate of 2012, 1.4/100.000, was the highest ever reported and highly influenced by a large outbreak in Quebec concerning 182 cases (82). Overall, an increase in reported legionellosis is seen in Canada since 2010, although not as clear as the increase in New Zealand.

An increase in reported LD is also seen in the U.S. since 2000 (61, 83) and Japan since 2005 (65). Part of this increase in Japan probably reflects improved ascertainment due to an

increased use of urinary antigen testing (84). In Australia, no overall increase in notification rate is reported. In 2015, 365 cases of legionellosis were reported with a notification rate of 1.5/100.000 (64). This is the same as the average notification rate between 2005 and 2015.

As in Europe, *L. pneumophila* is also the main causative agent for legionellosis in the U.S., Canada and Japan (71-73, 75) (see Table 2). In contrast, legionellosis has two major causative agents in Australia and New Zealand, *L. pneumophila* and *L. longbeachae*. In 2015, *L. longbeachae* was identified in 52.2% of the cases and *L. pneumophila* in 29.1% of the cases in New Zealand, which is comparable to previous years (12). *In Australia*, the most common infective agent has alternated between *L. pneumophila* and *L. longbeachae* since 1998 (85). Of the cases where data on the causative agent were available in 2014 (the most recent data available), 53.9% was caused by *L. pneumophila* and 45.3% was caused by *L. longbeachae* (74).

3. Source investigations

Finding and eliminating the source of infection is of great importance in LD outbreak investigations. In the Netherlands, a National *Legionella* Outbreak Detection Program (NLODP) was implemented in 2002 (86). The goal of the NLODP is the early detection of small clusters of cases, identification of infection sources, and implementation of control measures to prevent additional LD cases. By means of an interview, potential sources to which a patient was possibly exposed to in the incubation period are identified. *Legionella* strains isolated from potential sources are compared genotypically to strains from patients associated with these sources. Mainly main-made water systems are investigated (86), such as whirlpools (private and public), cooling towers, potable water systems of health care facilities or hotels, and the water system of the patients home.

For sporadic cases, source finding investigations to identify possible sources of exposure are also performed, but environmental sampling is not always conducted. In the Netherlands, environmental samplings for solitary patients are pursued if the patient stayed in a healthcare center during the incubation period, or if a patient-derived *Legionella* isolate was available and the patient was exposed to possible sources of *Legionella* during the incubation period other than the patients home. A limiting factor in this is that only part of the LD patients produce sputum and therefore material for culture purposes is not easily available (87).

Finding the source of infection for sporadic LD cases has been proven difficult. A study that evaluated the findings of the NLODP over the period of 2002-2012 report that for only 2% (41 cases) of 1991 patients, who most probably contracted LD in the Netherlands, a probable infection source was identified (86). This low success rate is partly due to the fact

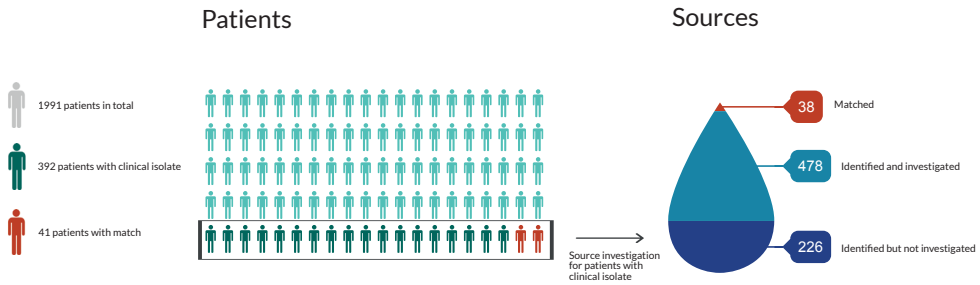
that only for 392 of the 1991 patients (20%) a clinical isolate could be retrieved (see Figure 3). So, for 41 of the 392 patients (10%) for whom a clinical strain was available, a genotype match could be made with a strain isolated from the environment. In total 704 possible sources, related to the 392 patients, were identified and 478 sources were sampled. Of these 120 sources were positive for *Legionella* species and 38 sources were matched to the 41 patients. In addition, the relative long incubation period makes it also difficult to identify sources, since people have to recall being exposed to possible sources and possibly *Legionella* concentrations in the source of infection have decreased in time below detection limits.

At the European scale, for 868 of 5359 LD cases in 2015 that had not travelled abroad, an environmental investigation was conducted and findings were reported to ECDC. Of these 868 LD cases, 42 clinical isolates were matched by genotyping to environmental isolates (4.8%).

For identifying the source of infection, multiple types of evidence are necessary. A match between the clinical and environmental strain is an important indication that the investigated source is the infection source. A match can be made by different molecular techniques, such as pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP), however, sequence based typing (SBT) has been the golden standard for *L. pneumophila* over the past years. The SBT method, developed by the European Working Group for *Legionella* Infections (EWGLI), uses seven genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*). These genes together form the SBT profile and translate to a sequence type (ST) number (88, 89). For other *Legionella* species SBT is not available.

At the time of writing, 2437 different sequence types are included in the SBT database (90). Despite the great genotype variation of *L. pneumophila*, some sequence types, such as ST1, are widely distributed (91, 92) and matching clinical and environmental isolates is not conclusive. Preferably, source investigations also provide epidemiological evidence to establish the source of infection or exclude alternative sources that were considered as a source of infection. This is more easily done for clusters and outbreaks, since the exposure to a common source evidently helps to exclude other considered sources.

Figure 3. Schematic presentation of the source investigation results from the National *Legionella* Outbreak Detection Program (2002–2012) (86) showing matches made between *L. pneumophila* strains isolated from LD patients and investigated sources



Furthermore, differentiation of species other than *L. pneumophila* (SG1) is even harder because of limited typing methods available. Whole genome sequencing (WGS)-based methods might make it easier to match sporadic cases, as this method is more discriminative for genotyping (93-95). Bacigalupe et al. (96) used WGS to investigate *L. longbeachae* isolates associated with five different LD cases and the compost samples they were previously linked to. For none of the patients, a genetic link to the corresponding compost sample could be established, as the clinical strains were not closely related to the environmental strains.

WGS is more and more used in *Legionella* typing. And although up until now it is mostly applied in outbreak investigations (82, 97-99), WGS can be very useful to investigate possible sources related to sporadic patients (100).

4. Sequence type distribution

To date, several European countries, Canada, the U.S., Japan and Israel, have used SBT to assess the genetic variability of local clinical *Legionella* types (see Table 3). From these reports it is clear that the majority of the LD cases are caused by only a few STs, while the variety of clinical STs is generally large. The contribution of the three main STs per country varies between 20.9% and 64.3% (average 38.8%). The index of discrimination (IOD) ranges from 0.775 to 0.979. The IOD is a measure to describe species diversity within an ecological habitat and ranges from 0 (no diversity) to 1 (high diversity).

L. pneumophila ST1, the oldest clone (91), is an important cause of disease in most of the reporting countries. ST1 is the main causative *Legionella* type in the U.S. (101), Canada (72), Germany (102), Belgium (103), Israel (104) and Japan (105). In Spain (region Catalonia) (106), Italy (107) and France (108), ST1 was the second or third most occurring. In the

Table 3. Clinical sequence type (ST) distribution data, showing the three or four most occuring STs per study

Country (region)	ST	Percentage of clinical strains	Percentage top 3 STs	Percentage of environmental strains	Only SG1 considered (Yes/ No)	Study period	No. of clinical strains, no. of unique STs	IOD	Reference
U.S.	**1**	25.2	35.4	49.0	Y	1982-2012	571, 153	0.924	(101)
	36	5.3		4.0					
	37	4.9		0					
	62	4.7		0					
Canada*	**1**	14.1	30.5	32.6	N	1981-2009	128, 64	0.964	(72)
	59	8.6		3.5					
	36	7.8		0					
	42	4.7		0					
Canada (Ontario)	**1**	14.2	34.1	-	Y	1978-2007	176, 62	-	(112)
	37	10.8		-					
	62	9.1		-					
	211	7.4		-					
Canada (Quebec)	62	12.8	27.0	0	N	2005-2015	141, 57	-	(113)
	213	7.8		0					
	1	6.4		26.5					
	37	6.4		0					
England & Wales	47	25.7	46.1	0.4	N	2000-2008	167, 42	0.901	(110)
	37	11.4		0.7					
	62	9.0		0					
	42	6.0		0					
Germany†	**1**	15.3	33.1	-	Y	until 2010	118, -	-	(102)
	182	9.3		-					

Table 3. Clinical sequence type (ST) distribution data, showing the three or four most occuring STs per study (*continued*)

Country (region)	ST	Percentage of clinical strains	Percentage top 3 STs	Percentage of environmental strains	Only SG1 considered (Yes/ No)	Study period	No. of clinical strains, no. of unique STs	IOD	Reference
	62	8.5		-					
	9	5.9		-					
Netherlands	47	41.3	60.9	0	Y	2007-2011	179, -	0.802	(109)
	62	12.8		0.5					
	46	6.7		0.5					
	45	5.6		0.5					
Spain	23	10.5	28.4	-	N	1989-2013	95, 44	0.964	(106)
(Catalonia)	37	9.5		-					
	1	8.4		-					
	42	7.4		-					
Belgium	**1**	19.8	53.5	-	Y	2000-2010	86, 31	0.879	(103)
	47	27.9		-					
	6	5.8		-					
	23	5.8		-					
Portugal*	44	9.1	23.6	-	N	1987-2012	55, 30	0.972	(111)
	62	7.3		-					
	99	7.3		-					
	1	3.6		-					
Italy*,‡	23	17.9	37.5	-	Y	1987-2012	56, 32	0.952	(107)
	1	14.3		-					
	42	5.4		-					

Table 3. Clinical sequence type (ST) distribution data, showing the three or four most occuring STs per study (*continued*)

Country (region)	ST	Percentage of clinical strains	Percentage top 3 STs	Percentage of environmental strains	Only SG1 considered (Yes/ No)	Study period	No. of clinical strains, no. of unique STs	IOD	Reference
France[†,§]	23	19.8	39.3	-	Y	2008-2012	1192, -	-	(108)
	47	10.3		-					
	1	9.1		-					
Israel	**1**	42.9	64.3	21.7	N	2006-2011	28, 12	0.775	(104)
	40	14.3		0.0					
	87	7.1		4.3					
	23	3.6		0.0					
Japan*	**1**	8.1	20.9	-	N	1980-2008	86, 53	0.979	(105)
	306	7.0		-					
	120	5.8		-					
	138	5.8		-					
Japan	505	23.5	47.1	-	Y	2002-2012	17, 12	0.934	(129)
(Toyama	384	17.6		-					
Prefecture)	644	5.9		-					

IOD: index of discrimination (clinical strains). - Not reported. *Outbreak strains were included. [†]Unclear if outbreak strains were included. [‡]Both unrelated and related strains were included (instead of only unrelated strains). [§]Unknown if strains were related.

Netherlands (109), England & Wales (110), and Portugal (111), ST1 is less important, and only responsible for 4 to 5% of the reported LD cases. Tijet et al. (112) report on a decrease in clinical ST1 strains in Ontario (Canada). A decreased incidence of cases caused by ST1 was also observed in Belgium and Japan (103, 105) but not in the U.S. (101).

L. pneumophila ST1 is also widely found in the environment. ST1 is found in the following countries reporting on environmental ST distributions: the U.S. (101), Canada (72, 113), England & Wales (110), Germany (102), the Netherlands (109), Spain (114), Israel (104), Japan (115), China (116-122), Gabon (123), Singapore (124), South-Korea (125) and Kuwait (126). In these studies, strains were mostly isolated from potable water systems and cooling towers. In Canada (72), Japan (115), China (116, 118, 119) and South-Korea (125), natural springs were also investigated. Some studies note that the ST distribution from natural reservoirs differs from strains from man-made environments (72, 115).

While ST1 is reported all over the world, other STs occur more locally. *L. pneumophila* ST23, is an important cause of disease in Europe, in Spain (region Catalonia) (106), Italy (107) and France (108), but is also reported in Israel (104) and Japan (105). In contrast, infection with ST23 is only once reported in the U.S. (127) and Canada (112). The distribution of a certain ST can also be limited to a region or city. For example, in Germany, the relatively rare ST182 causes disease mainly in Berlin (102).

In the Netherlands, *L. pneumophila* ST47 is the main cause of disease accountable for over 40% of the LD cases (109). This holds also for England & Wales (25.7%) (110), and ST47 is the second main cause of disease in Belgium (27.9%) (103) and France (10.3%) (108). This particular ST is not found in other reporting countries except for 3 cases in Canada (112).

Studies from the Netherlands (109), England & Wales (110), the U.S. (101), Canada (72, 113) and Israel (104), have compared the clinical ST distribution to the environmental ST distribution. In general, these studies show that common clinical strains are found in the environment very infrequently (see Table 3) and common environmental strains rarely cause disease. An exception is ST1. In the Netherlands, the three main clinical STs (ST47, ST62, ST46) account for more than half of the clinical isolates, but only 1.1% of environmental strains (mainly isolated from water systems and cooling towers) (109). There are various possible explanations for the observed discrepancy: i.e., important sources of *Legionella* are not targeted in source investigations, virulent STs are pathogenic in very low quantities and therefore not detected in environmental samples (disguised by more common STs or other bacteria) and current detection methods are not suitable for detecting the important STs in environmental samples, e.g. because bacteria are in a viable-but-not-culturable (VBNC) state (128).

5. Legionellosis and climate

The summers of 2006 and 2010 showed an unusual high incidence of LD in the Netherlands, with 283 and 317 domestic cases respectively (Figure 2) (70, 130). No sources were found that could explain the increase of cases in these two years and no changes in the notification system occurred. In 2006 there was one outbreak but after exclusion of the outbreak related strains, the LD incidence was still raised.

Other explanations were considered and it was observed that during the summer of 2006, a period of warm weather was followed by a period of intensive rainfall. A correlation between weather variables in the weeks before the probable date of infection, ambient temperature and precipitation, and LD incidence in 2006 (and later for 2010) was established (70, 130). A similar increase in cases was seen in England in 2006 (131) and this was found to be related to temperature and relative humidity (132).

Previously, meteorological variables had been related to increased legionellosis incidence in the U.S. and Canada (133-135). Fisman et al. (133) found that precipitation and increased relative humidity were predictors for the occurrence of legionellosis in the Greater Philadelphia Metropolitan Area from 1995-2003. In 2003 a significant rise in legionellosis cases was experienced in five states in the Mid-Atlantic region of the U.S. (also including the study area of the study by Fisman et al.) and Hicks et al. (134) found that increased rainfall was associated with increased risk of legionellosis. Ng et al. (135) studied legionellosis cases identified in the Greater Toronto Area of Ontario (GTA) from 1978 to 2006, and found that not rainfall but mainly changes in the local watershed (flow of water from rivers and creeks to Lake Ontario) were the strongest contributor to increased legionellosis risk. It was hypothesized that the environmental factors influence legionellosis risk through drinking water quality (Lake Ontario is the source of drinking water for most GTA residents).

Several other studies have established a link between LD incidence and relative humidity (136), vapour pressure (135, 137, 138), precipitation (136, 137, 139-141) and temperature (137, 138, 141). The most reported meteorological factor related to incidence is precipitation. It should be noted that relative humidity is influenced by precipitation so possibly the effect of this factor might have been captured partly in the precipitation effect.

It is not yet clear through which mechanism(s) meteorological variables influence LD incidence. Higher temperatures might support growth of *Legionella* bacteria in the environment and higher relative humidity might enhance *Legionella* survival in aerosols (142) and therefore spread of the bacteria and exposure risk. Possibly, heavy rainfall resulting in puddles and flooding of roads might contribute to increased dissemination of *Legionella* bacteria in the environment (143, 144).

France experienced a strong increase in LD in 2010 (145). Interestingly, a geographical west-east gradient was described with higher notification rates in eastern compared to western administrative regions. Regional disparities in notification were excluded as a possible cause. A relation with climatic factors was suggested, as several climates exist across the country, however this possible relationship was not further investigated (145).

6. Dutch legislation

After a large outbreak at a flower show in Bovenkarspel in 1999, affecting 188 people of whom 21 died (27, 146), elaborate control measures were introduced in a new drinking water law (147), with the aim to prevent growth of *Legionella* bacteria and thereby prevent infection. For certain water systems, to which third parties are exposed, it is mandatory to have a *Legionella* risk analysis and control plan which shows the risk of *Legionella* growth and measures taken to prevent growth. A log must be kept to document the control measures. Several risk factors for growth of *Legionella*, such as the presence of biofilm, the presence of dead ends, and water temperatures between 25°C and 50°C, are taken into account as well as protective factors, such as water temperatures that kill the bacterium (148). Furthermore, regular sampling should be performed. Sampling and analyzing the water samples should be done conform NEN-EN-ISO 11731:2017 (55) (replacement of NEN 6265:2007 since June 2017) by certified laboratories (148). The concentration *Legionella* bacteria in water should be less than 100 colony forming units (CFU) per Liter (147). When more than 1000 CFU/L are detected, the owner has to inform the supervisory body (the Dutch Human Environment and Transport Inspectorate (ILT)). Not only *L. pneumophila* is taken into account but also 20 other *Legionella* species, such as *Legionella anisa, Legionella feeleii* and *Legionella gormanii* (148). Examples of places that are obliged to prevent *Legionella* growth in the water systems are: hospitals and other health facilities, harbors, detention centers, pools, camp sites, and gas stations or truck stops with shower facilities (147).

Prevention of *Legionella* growth in wet cooling towers is also mandatory in the Netherlands (149) and under supervision of municipal or provincial authorities (150). As for drinking water, it is mandatory to have a *Legionella* risk analysis and control plan. However, there are fewer regulations, e.g. there is no maximum concentration norm.

Aim and outline of this thesis

The source of infection for sporadic LD cases is not often found (see paragraph 3) and common clinical *L. pneumophila* strains are almost never isolated from the environment (see paragraph 4). Therefore, the aim of this thesis is to explore alternative sources of *Legionella*

bacteria (i.e., that are not yet considered in source investigations) that might be the cause of sporadic *Legionella* infections.

First, **Chapter 2** presents a review that gives an overview of reservoirs and sources of *Legionella* bacteria described in literature. A level of evidence approach was applied to distinguish between unconfirmed and confirmed sources of infection by evaluating the evidence that was provided in studies that considered matrices or systems to be a source or reservoir of *Legionella*.

Then, in the following four chapters, results of several experimental studies on *Legionella* in two matrices, soil and rainwater, are described. **Chapter 3** describes the isolation of *L. pneumophila* ST47, the infective strain most associated with LD patients in the Netherlands, from garden soil. The isolated strain was linked to an outbreak of LD and Pontiac fever caused by an outdoor whirlpool. In **Chapter 4**, garden soil was further investigated as a reservoir of viable, pathogenic *Legionella* bacteria. Rainwater puddles and soil next to rainwater puddles were considered as reservoirs of *Legionella* in **Chapter 5**. In **Chapter 6**, the relationship of *Legionella* presence in natural soil and several soil characteristics, pH, soil humidity, granular composition and organic content, was investigated. In addition, *L. pneumophila* persistence in different soil types was studied. Two preliminary studies are presented on possible transmission routes of *Legionella* bacteria from soil to humans.

A general discussion of the research presented in this thesis is given in **Chapter 7**.

2.

Confirmed and potential sources of *Legionella* reviewed

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Abstract

Legionella bacteria are ubiquitous in natural matrices and man-made systems. However, it is not always clear if these reservoirs can act as source of infection resulting in cases of Legionnaires' disease. This review provides an overview of reservoirs of *Legionella* reported in the literature, other than drinking water distribution systems. Levels of evidence were developed to discriminate between potential and confirmed sources of *Legionella*. A total of 17 systems and matrices could be classified as confirmed sources of *Legionella.* Many other man-made systems or natural matrices were not classified as a confirmed source, since either no patients were linked to these reservoirs or the supporting evidence was weak. However, these systems or matrices could play an important role in the transmission of infectious *Legionella* bacteria; they might not yet be considered in source investigations, resulting in an underestimation of their importance. To optimize source investigations it is important to have knowledge about all the (potential) sources of *Legionella*. Further research is needed to unravel what the contribution is of each confirmed source, and possibly also potential sources, to the LD disease burden.

Introduction

Legionella are gram-negative bacteria that cause Legionnaires' disease (LD) and Pontiac fever in humans (58). *Legionella* bacteria are ubiquitous in the natural environment in both soil and water. *Legionella* infections are regularly traced to contaminated man-made water systems, such as water distribution systems (43, 44), cooling towers (40) and whirlpools (165). These systems often exhibit favorable growth conditions for *Legionella*. Transmission to humans occurs via contaminated water aerosolization (58). Potting soil is also an infection source (166), but the mode of transmission of *Legionella* originating from this source remains unclear. *Legionella pneumophila*, predominantly *L. pneumophila* serogroup 1 (SG1), constitutes over 90% of the clinical isolates in Europe (167) and the U.S. (168, 169). In Australia, New Zealand and Thailand, *L. longbeachae* is an important cause of LD (157, 170, 171).

Epidemiological studies are conducted to identify the infection source of sporadic LD cases or outbreaks, and microbiological methods are employed to isolate *Legionella* from suspected sources for confirmation. Molecular tracing is used to establish a link between clinical *Legionella* isolates and environmental isolates. However, the infection source remains unknown for most sporadic LD cases (172). Moreover, studies in the Netherlands, England and Wales showed that only a few sequence types of *L. pneumophila* cause the majority of sporadic *Legionella* infections, whereas these particular sequence types are only rarely detected in suspected sources (109, 110). The failure of standard culture methods to detect virulent strains in environmental samples could explain this discrepancy. *Legionella* bacteria are typically cultured on buffered charcoal yeast extract (BCYE, with or without antibiotics) medium plates for detection (173). False negative results can occur because these plates are easily overgrown by other bacteria in the sample or because *Legionella* bacteria in a viable-but-not-culturable (VBNC) state are not detected. Furthermore, virulent *Legionella* strains might only be present in low concentrations in the environment compared to other less virulent strains, thus masking their detection. Another explanation is that important *L. pneumophila* sources are not considered during source investigations (174).

To optimize LD source investigations it is important to have knowledge about all the reservoirs of *Legionella*, and whether exposure to these reservoirs can lead to infection. In the present study, a literature review was systematically conducted to obtain an overview of all reservoirs and sources of *Legionella*. The matrices and systems that are described in literature were classified according to the strength of evidence that implicates that the matrix or system could be a source of *Legionella* infection. For this purpose a level of evidence (LOE) approach was used. LOE approaches are used in evidence-based medicine (175) and other research areas such as waterborne infectious disease surveillance (176). These approaches did not fit our specific research question and therefore LOEs were developed and assigned

to every selected publication. Based on the assigned LOEs, systems and matrices were classified as potential source or confirmed source. A source was classified as confirmed if at least in one study sufficient evidence was provided that the described source was the cause of infection. Drinking water sources, such as showers and taps, are often targeted in outbreak investigations, and in many countries, legislation is already in place for preventive measures. Therefore, those sources are outside the scope of this review.

Methods

Literature search

A literature search was conducted using the MEDLINE database (publisher: U.S. National Library of Medicine), searched by OvidSP (Wolters Kluwer Health). The search was performed on June 25, 2013. The following terms were used to select publications about *Legionella*, LD or Pontiac fever: in the title and abstract: 'legionell*' OR 'legionnair*' OR 'Pontiac fever'; and in Medical Subject Headings (MeSH): 'exp legionellosis/' OR 'exp *Legionella*/'. These terms were combined with an extensive list of additional terms to identify publications that reported sources and reservoirs of *Legionella*. No restrictions for the publication date were imposed. PubMed (publisher: U.S. National Library of Medicine) was searched in addition to the MEDLINE database because the former also covers publications that are electronically published ahead of print. The same MeSH terms were applied, but only the publication titles, not the abstracts, were searched for the terms 'legionell*' OR 'legionnair*' OR 'Pontiac fever'. These terms were not combined with additional search terms. The publication date criterion was between June 1, 2012 and June 25, 2013.

Study inclusion criteria

A study was included if it was written in English, reported on primary research results and fulfilled at least one of the following criteria: 1. the study described the detection of *Legionella* spp. in environmental sources in surveillance or prevalence studies or in source investigations; 2. the study described risk factors that could be related to exposure to *Legionella* sources; 3. epidemiological data on LD or Pontiac fever cases were used to identify a possible infection source. Publications describing showers or taps as sources or reservoirs of *Legionella* were excluded (as discussed above). However, the indirect use of drinking water was included, e.g., the use of tap water for cleaning purposes, rinsing medical equipment or dental units. The use of tap water in baths was also included because baths involve the use of water for a prolonged period at a certain temperature, which might promote the *Legionella* growth.

Selection process

The selection process was conducted in two steps. First, the title and abstract of all publications were assessed independently by three researchers (SE, EH, and JS). Publications that were not relevant for the research objective were not selected for full-text assessment. In the second selection step, the full-text versions of the selected publications were assessed for eligibility on the basis of the selection criteria by one reviewer (EH). When multiple studies on the same outbreak were present, only the report providing the highest LOE was included, unless the other studies provided additional information resulting in a higher LOE.

Levels of evidence

LOEs were developed to discriminate between potential sources and confirmed sources of *Legionella*. Table 1 shows the different LOEs, from I, representing the highest LOE, to VI, representing the lowest LOE. For the highest LOE, cases must be epidemiologically linked to a suspected source and a match, either molecularly (LOE Ia) or by monoclonal antibody typing (LOE Ib), must be determined between the clinical and environmental isolates. Furthermore, there should be additional evidence demonstrating that these sources caused the infection: evidence that excludes other possible sources, evidence on the spread of *Legionella* from the suspected source, or evidence on the exposure of cases to the suspected source. The exclusion of other sources can be achieved by the environmental investigation of other sources, if there is only one common source in an outbreak situation, or if new cases ceased to occur after elimination of the suspected source. Evidence on the spread from the suspect source involves the isolation of *Legionella* from air samples. Evidence of exposure is achieved by case-control studies comparing the seroprevalence of *Legionella* or by case-control studies that imply exposure to the suspected source as a risk factor for contracting LD (in contrast to other considered sources). For LOE II, a match must be identified between clinical and environmental isolates, but additional evidence is not provided. LOE III was assigned when cases were epidemiologically linked to a suspected source, *Legionella* was isolated from this source, and additional evidence was provided, but environmental and clinical strains were not further typed or clinical strains were not available for comparison. LOE IV could be assigned in three situations: (1) cases were epidemiologically linked to a suspected source, and *Legionella* was isolated from this source; (2) cases were epidemiologically linked to a suspected source, no environmental isolates were obtained, but other possible sources were excluded or there was evidence of spread from or exposure to the source; and (3) no cases were linked to a suspected source, but environmental strains were isolated, and additional evidence was provided. When no LD cases were involved, additional evidence could include the isolation of *Legionella* from air samples or case-control studies on the seroprevalence of *Legionella*. LOE V was assigned when *Legionella* was isolated from a reservoir or potential source, or exposure was assessed, or risk factors for contracting LD were determined. LOE VI was assigned to

studies in which environmental *Legionella* was not isolated but was detected by molecular or antibody staining methods. A study may report the detection of *Legionella* in more than one type of system or reservoir; therefore, one study can be assigned multiple LOEs.

For the described sources and reservoirs of *Legionella,* the LOEs were assessed based on the selected literature. Subsequently, the sources of *Legionella* were subdivided into confirmed sources (at least one publication with LOE Ia or Ib) or potential sources (LOE II or lower).

Table 1: Levels of evidence for sources of *Legionella* bacteria

Level of evidence*	Cases[†]	Environmental *Legionella*	Evidence for spread/ evidence for exposure/ other sources excluded	Match by molecular method[‡, §]	Match by monoclonal antibody typing[‡]
Ia	X	X[‖]	X	X	
Ib	X	X[‖]	X		X
IIa	X	X[‖]		X	
IIb	X	X[‖]			X
III	X	X[‖]	X		
IV	X	X[‖]			
	X		X		
		X[‖]	X		
V		X[‖]			
			X		
VI		X[#]			

*From high to low. [†]One or more patients are epidemiologically linked to a source. [‡]Match is made between environmental and clinical isolate. [§]One of the following molecular methods: PFGE, AFLP, SBT. [‖]*Legionella* isolated from reservoirs or (potential) sources. [#]*Legionella* detected in reservoirs or (potential) sources by molecular methods or antibody staining methods only.

Results

A total of 2,189 publications were identified by searching the MEDLINE/PubMed databases, and 1,653 were excluded after screening the titles and abstracts. From the remaining publications, 138 were discarded after assessing the full-text, and 398 met the inclusion criteria. Figure 1 shows all of the reservoirs or (potential) sources of *Legionella* that were reported by more than two of the selected publications and the studies that described LD risk factors that could be related to exposure to sources of *Legionella*. Some (potential) sources were only reported in one or two studies ('other'). Table 2 shows the assigned LOEs per potential or confirmed source. In Table 3, all of the references per (potential) source are listed for studies that concerned outbreak investigations or case reports, with an assigned

LOE of IV or higher. Cooling towers and whirlpools are not presented in Table 2 or 3 because not all of the studies were assessed due to the large amount of studies reporting on these sources. Several studies provided the highest LOE for cooling towers and whirlpools, and a selection of these studies is described below. For wastewater, thermal springs, and surface water, all publications were assessed and are presented in Table 2, but only a portion of the publications are described in the text and incorporated in the reference list because of the large amount of studies (see also Appendix A: Tables S4, S5, and S6).

Figure 1. Number of publications per source type (green/solid gray=confirmed sources, blue/dashed=potential sources)

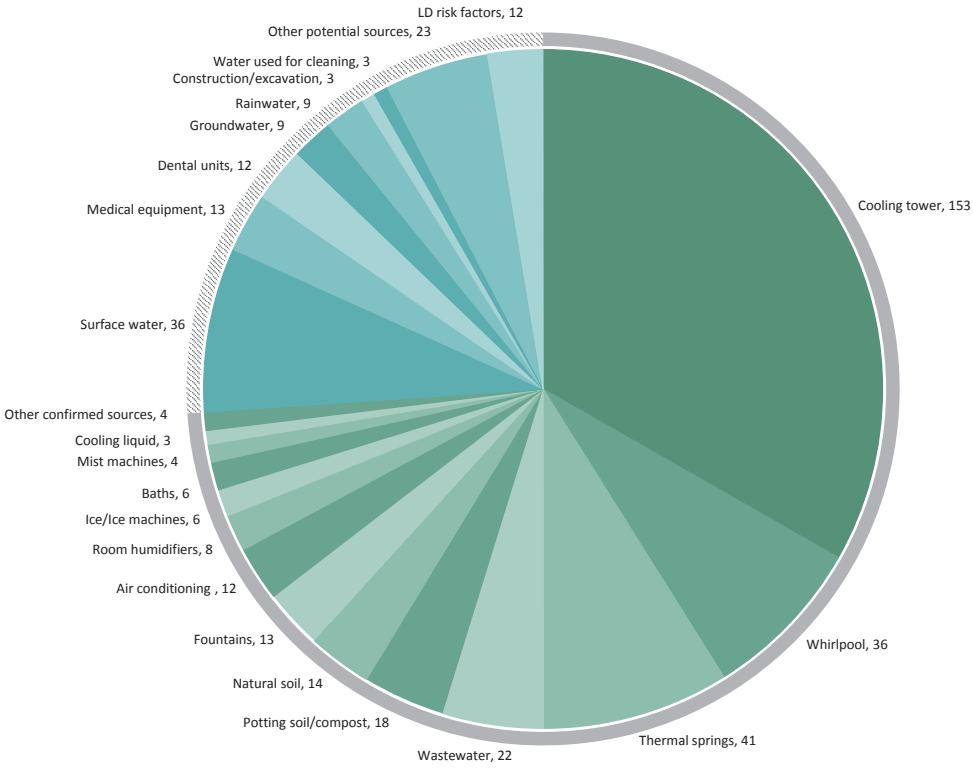

Table 2. Levels of evidence (LOEs) per source*

	Level of evidence						
(potential) *Legionella* source	**Ia**	**Ib**	**IIa†**	**III**	**IV**	**V**	**VI**
Potting soil/compost	4			2	3	9	1
Baths	3				2	1	
Fountains	3			3	1	6	
Wastewater/WWTPs	2			3	1	6	6
Room humidifiers	2			2	3	1	
Ice/Ice machines	1	1	1	1		2	
Mist machines	1	1				3	
Air conditioning systems		2		2		6	2
Natural water: thermal springs	1		5	1	3	27	4
Natural soil	1				2	9	2
Cooling liquid for machinery		1		2			
Milling Machine	1						
Ship water pump	1						
Foot bath	1						
Underwater chest drain		1					
Medical equipment: respiratory devices				3	2	2	
Medical equipment: other				2	1	3	
Water used for cleaning				1	1	1	
Sullage tanks collecting bilge				1			
Dental units					2	8	2
Roof-harvested rainwater					1	2	3
Construction and excavation					1	2	
Steam turbine condenser cleaning					1		
Inoperative bedpan flusher					1		
Natural water: surface water						22	14
Natural water: groundwater						7	2
Rainwater on the road						3	
Steam towel warmer						1	
Industrial air scrubber						1	
Garden hose						1	1

*The numbers in the table reflect the number of studies that were categorized as a certain LOE. Several studies provided the highest level of evidence for cooling towers and whirlpools; however these sources are not presented here because not all studies were assessed due to the great amount of studies reporting on these sources. †LOE IIb was never assigned.

Confirmed *Legionella* sources

Cooling towers

Cooling towers were described as a potential or confirmed source in 153 studies. Several of these studies provided the highest LOE (Ia) for cooling towers as an infection source (177-180). Many outbreaks of LD caused by the transmission of *Legionella* from contaminated cooling towers have been reported; hundreds of individuals have become ill at times (177, 179, 181-185). Cooling towers may also be responsible for some of the sporadic community-acquired LD cases. In three studies, an association was observed between the incidence of sporadic community-acquired LD and the proximity of place of residence to a cooling tower (186-188).

Whirlpools

A total of 36 articles were selected that described whirlpools as a potential or confirmed source. Several studies provided the highest LOE (Ia) in source investigations concerning whirlpools (41, 189-191). The use of private whirlpools (192, 193) and public whirlpools (191, 194, 195) were linked to patients with LD or Pontiac fever. Several outbreaks of LD were reported in which a whirlpool on display was considered the infection source (41, 42, 190, 196), and some outbreaks linked to whirlpools occurred on cruise ships (189, 197).

Potting soil and compost

In 18 studies, potting soil and/or compost were investigated. LD or Pontiac fever cases were related to exposure to potting soil in nine studies, and nine studies investigated the prevalence of *Legionella* in potting soil or compost. Koide et al. (198) investigated a case related to potting soil and also performed a prevalence study. One study identified risk factors associated with *L. longbeachae* infection (53).

The infectious agent in all described cases was *L. longbeachae*. The modes of possible exposure primarily included the use of potting soil at home or in the working place. Cramp et al. (164) described one outbreak of Pontiac fever in nine workers of a horticultural warehouse involved in the potting of plants that had the highest LOE (Ia). *L. longbeachae* SG2 isolates from potting soils were indistinguishable by pulsed-field gel electrophoresis (PFGE) from isolates obtained from cases. The water supply of the warehouse was eliminated as a potential source. Three studies reported on clusters or individual cases in which amplified fragment length polymorphism (AFLP) profiles of potting soil isolates and patient isolates were found to yield indistinguishable patterns (51, 52, 199). As other possible sources were excluded, these studies were categorized at the highest LOE (Ia). In eight studies reporting on potting soil as a possible source, no genotypic match was determined, or a source investigation was not conducted (158, 198, 200-205). The results from a case-control study

by O'Connor et al. (53) provided some insight into the possible transmission mode of *L. longbeachae.* The predictors of illness in a multivariate analysis included eating or drinking after gardening without washing hands (OR: 29.47, 95% CI: 1.96–412.14, p=0.014) and being near dripping hanging flower pots (OR: 8.97, 95% CI: 1.41–56.96, p=0.020). Surprisingly, the use of potting soil in the 4 weeks prior to hospitalization was not found to be a risk factor for illness.

Legionella was often detected in studies in which potting soil (198, 205-209) or compost (208, 210-212) were investigated. The maximum isolation rates were 91.7% for potting soil (207) and 84.8% for compost (211). Different species of *Legionella*, including *L. pneumophila*, are regularly isolated from potting soil and compost. Several studies even found *L. pneumophila* to be predominant (208, 210-212). The maximum reported concentrations for *L. pneumophila* were $2.8*10^6$ CFU/g in compost (210) and $2.8*10^4$ CFU/g in potting soil (206). Only one study reported a concentration for *L. longbeachae*, $2*10^4$ CFU/g compost (211).

Table 3. Levels of evidence (LOEs) per references per (potential) source*

(potential) *Legionella* source	Reference	LOE	LD/Pf[†]	No. of cases	Infective agent
Potting soil/compost	Cramp (164)	Ia	Pf	9	*L. longbeachae* SG2
	Den Boer (62)	Ia	LD	1	*L. longbeachae*
	Lindsay (51)	Ia	LD	4[‡]	*L. longbeachae* SG1
	Pravinkumar (52)	Ia	LD	3[‡]	*L. longbeachae* SG1
	Steele (50)	III	LD	4[‡]	*L. longbeachae* SG1
	Speers (200)	III	LD	1	*L. longbeachae*
	CDC (158)	IV	LD	3[‡]	*L. longbeachae*
	Dhillon (201)	IV	LD	1	*L. longbeachae*[§]
	Koide (198)	IV	LD	1	*L. longbeachae* SG1
Baths	Kura (213)	Ia	LD	3	*L. pneumophila* SG5
	Mineshita (214)	Ia	LD	1	*L. pneumophila* SG5
	Torii (215)	Ia	LD	1	*L. pneumophila* SG10
	Franzin (216)	IV	LD	1	*L. pneumophila*[§]
	Nagai (217)	IV	LD	1	*L. pneumophila*[§]
Fountains	Hlady (218)	Ia	LD	5	*L. pneumophila* SG1
	Palmore (47)	Ia	LD	2	*L. pneumophila* SG1
	O'Loughlin (46)	Ia	LD	18	*L. pneumophila* SG1
	Fenstersheib (219)	III	Pf	34	*L. anisa*[§]
	Jones (220)	III	Pf	117	*L. anisa*[§]
	Haupt (221)	III	LD	8	*L. pneumophila*[§]
	Correia (222)	IV	LD	11	*L. pneumophila*[§]

Table 3. Levels of evidence (LOEs) per references per (potential) source* (*continued*)

(potential) *Legionella* source	Reference	LOE	LD/Pf†	No. of cases	Infective agent
Wastewater/WWTPs	Borgen (223)	Ia	LD	5	*L. pneumophila* SG1
	Nygard (224)		LD	56	*L. pneumophila* SG1
	Blatny (225)		-	-	-
	Blatny (226)		-	-	-
	Olsen (227)		-	-	-
	Nguyen (228)	Ia	LD	86	*L. pneumophila* SG1
	Gregersen (229)	III	Pf	5	*L. pneumophila*§
	Castor (230)	III	Pf	15	*L. pneumophila*§
	Kusnetsov (231)	III	LD	2‡	*L. pneumophila*§
Room humidifiers	Yiallouros (49)	Ia	LD	9	*L. pneumophila* SG3
	Moran-Gilad (48)	Ia	LD	1	*L. pneumophila* SG1
	Arnow (232)	III	LD	5	*L. pneumophila*
	Joly (233)	III	LD	5	*L. dumoffii*
	Kaan (234)	IV	LD	1	*L. pneumophila*
Ice/Ice machines	Bencini (235)	Ia	LD	1	*L. pneumophila* SG1
	Bangsborg (236)	Ib	LD	2	*L. pneumophila* SG1
	Schuetz (237)	II	LD	1	*L. pneumophila* SG8
	Graman (238)	III	LD	1	*L. pneumophila* SG6
Mist machines	Barrabeig (239)	Ia	LD	12	*L. pneumophila* SG1
	Mahoney (240)	Ib	LD	33	*L. pneumophila* SG1
Air conditioning systems	Breiman (241)	Ib	LD	6	*L. pneumophila* SG1
	O'Mahony (242)	Ib	LD	68	*L. pneumophila* SG1
	Cordes (243)	III	LD	8	*L. pneumophila*§
	Kaufmann (153)	III	Pf	144	*L. pneumophila*§
Natural water: thermal springs	Miyamoto (45)	Ia	LD	1	*L. pneumophila* SG3
	Ito (244)	II	LD	1	*L. pneumophila* SG6
	Kurosawa (245)	II	LD	1	*L. pneumophila* SG1
	Matsui (246)	II	LD	1	*L. rubrilucens*
	Nozue (247)	II	LD	1	*L. pneumophila* SG3
	Tominaga (248)	II	LD	1	*L. pneumophila* SG1
	Gaia (249)	II	LD	1	*L. pneumophila* SG1
	Bornstein (250)	III	LD	5	*L. pneumophila*§
	Molmeret (251)	IV	LD	2‡	*L. pneumophila* SG1
Natural soil	Wallis (252)	Ia	LD	1	*L. pneumophila* SG1
	Parry (253)	IV	LD	5	*L. bozemanii*
	Haley (254)	IV	LD	49	LD bacterium
Cooling liquid for machinery	Allen (255)	Ib	LD	1	*L. pneumophila* SG1
	Herwaldt (256)	III	Pf	317	*L. feeleii*§

Table 3. Levels of evidence (LOEs) per references per (potential) source* (*continued*)

(potential) *Legionella* source	Reference	LOE	LD/Pf†	No. of cases	Infective agent
	O'Keefe (257)	III	LD, Pf	2	*L. pneumophila*§
Milling machine	Coscolla (258)	Ia	LD	11	*L. pneumophila* SG1
Ship water pump	Cayla (259)	Ia	LD	2	*L. pneumophila* SG1
Footh bath	Den Boer (260)	Ia	LD	3	*L. pneumophila* SG1
Under water chest drain	Moiraghi (261)	Ib	LD	12	*L. pneumophila* SG1
Respiratory devices	Arnow (232)	III	LD	5	*L. pneumophila*
	Joly (233)	III	LD	5	*L. dumoffii*
	Moiraghi (261)	III	LD	12	*L. pneumophila* SG1
	Aubert (262)	IV	LD	1	*L. pneumophila* SG1, SG8
	Pilon (263)	IV	LD	1	*L. pneumophila* SG1
TEE probes	Levy (264)	III	LD	3	*L. pneumophila* SG1
Hydrotherapy system	Leoni (265)	III	LD	1	*L. pneumophila*§
Medication nebulizer	Mastro (266)	IV	LD	13	*L. pneumophila* SG3
Water used for cleaning	Coetzee (267)	III	LD	2	*L. pneumophila*§
	Fry (63)	IV	LD	4	*L. pneumophila* SG1
Dental units	Atlas (268)	IV	LD	1	*L. dumoffii*
Roof-harvested rainwater	Simmons (269)	IV	LD	4	*L. pneumophila* SG1
Construction & excavation	Thacker (270)	IV	LD	81	LD bacterium§
Sullage tanks collecting bilge water	Hyland (271)	III	LD	7	*L. pneumophila*§
Steam turbine condenser cleaning	Fraser (272)	IV	Pf	10	*L. pneumophila*§
Inoperative bedpan flusher	Brown (55)	IV	LD	8	*L. pneumophila* SG1
Steam towel warmer	Higa (273)	III	LD	1	*L. pneumophila* SG1

*LOEs are listed for studies that concerned outbreak investigations or case reports. †Legionnaires' disease/Pontiac fever. ‡Study describes multiple sporadic cases. §No clinical isolate was obtained.

Baths

A total of six studies reported on *Legionella* contamination in baths; five of these studies originated from Japan. Most studies described LD cases that were exposed to contaminated baths, and one study described the prevalence of *Legionella* in bathing facilities.

In an outbreak study, *L. pneumophila* isolates from a spa bath on a cruise ship matched a clinical isolate by PFGE, representing the highest LOE (Ia) (213). The environmental isolates were obtained from the bath water and from porous natural stones in the filters of the spa. For two cases infected by *Legionella* after using all-day-running-baths (also referred to as 24-hour baths or ever-ready baths), a match between the patients and the environmental isolate was determined by PFGE, and other possible sources were excluded, resulting in

categorization at the highest LOE (214, 215). Two studies reported neonatal LD cases associated with water birth: one was nosocomial (216), and one involved a home water birth in an all-day-running-bath (217). The infection source could not be confirmed because no clinical isolates were available for comparison. Baba et al. (274) isolated *L. pneumophila* from 25 out of 91 bath water samples that originated from 30 bathing facilities.

Fountains

In 13 studies, fountains were described as potential or confirmed sources. Seven studies epidemiologically linked LD cases to the fountains, and six studies investigated the presence of *Legionella* in fountains.

Fountains inside buildings, a hotel (218), a restaurant (46) and a hospital (47) were identified as infection sources in three LD outbreak investigations. A molecular match between clinical and environmental isolates was determined in these studies by PFGE (47, 218) or sequence based typing (SBT) (46). Furthermore, other possible sources were excluded, and these studies were assigned the highest LOE (Ia). Two outbreaks of Pontiac fever have been described in which indoor fountains were considered to be the probable sources (219, 220). No clinical isolates were obtained but *Legionella anisa* was isolated from the suspected fountains in both outbreaks, and the majority of patients had elevated antibody titers to *L. anisa*. In both studies case-control studies revealed exposure to *L. anisa* between a control group and an exposed group. In two other outbreak studies, evidence for exposure to fountains was provided, but clinical and/or environmental isolates were not obtained (221, 222). In outbreaks in which fountains are the suspected source, many patients can be involved (up to 18 LD patients (46) and 117 Pontiac fever patients (220), see Table 3), suggesting that many people can be exposed to a *Legionella*-contaminated fountain. In environmental surveillance studies (275-280), the detection rates of *Legionella* in fountains varied from 2.2% (3/134) (277) to 80% (4/5) (276).

Wastewater/wastewater treatment plants

Wastewater or wastewater treatment plants (WWTPs) were reported as *Legionella* reservoirs or (potential) sources in 22 publications. Nine of these studies reported on WWTPs associated with outbreaks or sporadic LD or Pontiac fever cases. Thirteen of the 22 publications reported on the *Legionella* prevalence in WWTPs.

Borgen et al. (223) reported on a cluster five LD cases that were linked to a biological treatment plant at a company that produced wood-based chemicals in Norway in 2008. Clinical and environmental *L. pneumophila* isolates showed the same sequence type (ST462). Strain ST462 was isolated from an air scrubber that was the suspected infection source during a large outbreak of LD in 2005 (224). However, the outbreak strain was also found in high concentrations in the aeration ponds of the plant (10^{10} CFU/L), and Olsen et al.

(227) suggested that the aeration ponds, rather than the air scrubber, were the primary disseminators of *Legionella* during the outbreaks of 2005 and 2008. Blatny et al. (225, 226) studied the dissemination of *Legionella* originating from the aeration ponds. The studies surveying the biological treatment plant were categorized at the highest LOE (Ia). During an outbreak investigation concerning 86 LD cases in France, a petrochemical plant was considered the most likely infection source (228). *L. pneumophila*, which was indistinguishable by PFGE from clinical isolates, was isolated from cooling towers, from a waste basin at the plant and in air samples collected in the vicinity of the basin, representing the highest LOE (Ia). The authors argued that both the cooling towers and waste basin played a role in the transmission of *Legionella* in this outbreak. Three studies, describing two outbreaks of Pontiac fever and two individual cases, were assigned LOE III (229-231).

Many publications were identified that reported the detection of *Legionella* in WWTPs (see Table S4 for the complete set of references). Two studies investigated the prevalence of *Legionella* in a number of WWTPs and found that 27% (3/11) (281) and 59% (10/17) (282) of the plants were positive for *Legionella. Legionella* spp. and *L. pneumophila* were detected at different stages in the treatment process: in aeration ponds (144, 283), in WWTP influent and WWTP effluent (144, 282, 284-288), and in air sampled at WWTPs (286, 289, 290). One study detected *L. pneumophila* using qPCR in biosolids, which are made from sewage sludge and used for agricultural applications (291). *Legionella* in wastewater samples were primarily detected by direct fluorescent-antibody (DFA) tests and PCR. Three studies succeeded in isolating *Legionella* from WWTP influent, effluent, or aeration ponds (144, 283, 287).

Room humidifiers

Room humidifiers were the subject of investigation in eight publications. In five studies, contaminated room humidifiers were associated with LD cases (48, 49, 232-234). Three studies investigated *Legionella* contamination in humidifiers or aerosolization by humidifiers (292-294).

Two source investigations involving infants exposed to room humidifiers filled with contaminated tap water were assigned the highest LOE (Ia) (48, 49). Yiallouros et al. (49) reported a nosocomial outbreak, and Moran-Gilad et al. (48) described a community-acquired LD case. In both studies, a match was determined between the patient and environmental isolates by SBT. The detection of *Legionella* in humidifying systems was reported in two articles (292, 294), and two studies showed the aerosolization of *L. pneumophila* by humidifiers filled with contaminated water originating from hospital water systems (293, 294).

Ice/Ice machines

A total of six studies reported on ice or ice machines as reservoirs or potential or confirmed sources of LD. In four studies, *Legionella* infection occurred through contaminated ice, and in two studies, *Legionella* was detected in ice, but no transmission to patients was reported.

To date, five cases associated with contaminated ice have been reported, all in hospital settings (235-238). Four of the cases were suspected to have occurred through aspirated ice water, and *L. pneumophila* strains were isolated from patients and ice or ice machines (235, 236, 238). In one case, the environmental and patient isolates were found to be indistinguishable by AFLP, and other possible infection sources were excluded, representing the highest LOE (Ia) (235). LOE Ib was assigned to the publication of Bangsborg et al. (236), in which a match was determined based on monoclonal subtyping. The fifth patient developed LD after undergoing a bronchoscopy in which contaminated ice was used for cooling saline-filled syringes (237). In another study, ice used for cooling saline-filled syringes was contaminated, but no patients were reported (295). Stout et al. (296) detected *L. pneumophila* in 8 of 14 ice machines sampled in a single hospital.

Mist machines

Legionella contamination of mist machines was reported in four studies. Two studies considered mist machines as the infection source, and three studies investigated the prevalence of *Legionella*. Mahoney et al. (240) reported an outbreak and also performed a prevalence study.

Mist machines in supermarkets have been described as sources of *L. pneumophila* infection at the highest LOE (Ia, Ib) in two outbreak reports (239, 240). In both studies, *L. pneumophila* SG1 was isolated from the mist machines, and other possible sources were excluded. Clinical isolates and environmental isolates displayed the same PFGE pattern (239) or monoclonal subtype (240). In prevalence studies, 3 of 8 mist machines in grocery stores (240), 4 of 28 mist fans (124), and 2 of 20 greenhouse misting machines (297) were found positive for *Legionella*.

Air conditioning systems

Twelve studies considered air conditioning systems. Four of these studies reported on outbreaks and one case-control study found an association between LD and home air conditioning (153, 241-243, 298). Five studies reported the isolation of *Legionella* from air conditioning systems, but no patients were linked to these systems (299-303). Air conditioners in cars have also been suggested as a potential *Legionella* source (304, 305).

Two LD outbreak investigations were included that demonstrated the highest LOE (Ib) (241, 242). In these outbreaks, monoclonal antibody subtype patterns of clinical isolates

matched environmental isolates from an evaporative condenser (241) and a hospital air conditioning system (242). In the last study, the epidemic strain was also found in a cooling tower on the roof of the hospital, but the air conditioning system was considered the most likely transmission source because a fault in the design of the air conditioning system allowed for the generation of aerosols in a chiller unit of the system that served the floor in which most cases occurred (242). In a study by Cordes et al. (243), a contaminated evaporative condenser was the likely infection source, but no isolates were obtained from patients. One of the studies reported on the first (retrospectively recognized) Pontiac fever outbreak (1968), for which a defective air conditioning system was believed to be the infective source (153). Although the causative agent could not be identified at the time, *L. pneumophila* SG1 was isolated from the stored lung tissue of guinea pigs that had been exposed to evaporative condenser water in 1977. In a study by Broome et al. (298), significantly more cases were found to have home air conditioning compared to age-matched controls (p=0.03). However, compared to a second control group consisting of patients with pneumonia who were seronegative for *Legionella* (IFAT≤64), there was no significant difference.

L. pneumophila was detected by PCR in an LD patient who had condenser liquid leaking from the air conditioning system of his car (304). Because *Legionella* was not isolated from the patient or the environmental sample, the evidence for the car air conditioner as an infection source was limited. Sakamoto et al. (305) detected *Legionella* DNA in evaporator compartments of the air conditioning system of 11 of 22 cars.

Natural water: thermal springs

A total of 41 studies investigated hot or thermal springs. The detection of *Legionella* in thermal spring waters was described in 31 studies, and nine studies reported on cases linked to thermal spring waters. One study showed exposure to *Legionella* from thermal spring water. However, no cases were reported in this study.

For six cases, all related to Japanese spas, a genotypic match could be identified between the environmental and clinical isolates by PFGE (45, 244-248). In only one of the six studies, other possible infection sources were excluded; therefore, the highest LOE was assigned (Ia) (45). The other case reports were assigned the second highest LOE (II). One study reported on a case that was possibly related to a thermal spa in Switzerland (249). A French therapeutic thermal spa was investigated in a number of studies after several LD cases were found to be epidemiologically linked to this spa (250, 251). Researchers were not able to identify the infective strains, but an association was found between the exposure of visitors and employees to the hot spring water and antibody titers of these subjects against the majority of the *Legionella* species and serotypes isolated from the spa water (250). A similar study was conducted in a Portuguese spa in which visitors showed elevated antibody titers against the environmental *Legionella* strains (306).

Many studies described the detection of *Legionella* in thermal spring waters that were sampled from spring sources and spa facilities (i.e., hot tubs, spas, swimming pools, wastewater) (see Table S5 for the complete set of references). The detection rates of *Legionella* spp. by culture ranged from 4.5% (307) to 71.9% (308), and bacteria were detected at temperatures up to 66°C (309). The detection of *L. pneumophila* was reported in nearly all studies and the highest reported concentrations of *L. pneumophila* were over 10^4 CFU/L (310). Some spring waters that were contaminated with *Legionella* were for therapeutic use (310, 311).

Natural soil

A total of 14 publications concerned natural soil. The presence of several *Legionella* species in natural soil was described in seven publications, and seven studies considered natural soil as a potential *Legionella* source. One study provided evidence for natural soil as an infection source.

Wallis and Robinson (252) reported a single case infected with *L. pneumophila* SG1. A strain with an indistinguishable genotypic profile, determined by PFGE, was isolated from a field in which the patient had worked the week prior to illness. All other water and soil samples from the patient's home and workplace tested negative for *L. pneumophila* and LOE Ia was assigned. In 1985, Parry et al. (253) isolated *Legionella bozemanii* from four cases during an outbreak investigation of nosocomial LD. *L. bozemanii* was also cultured from soil in an area of excavation on hospital property and from tap water. The authors suggested that during the construction and installation of new plumbing, the plumbing system of the hospital became contaminated with *Legionella*. In another nosocomial outbreak study, soil was also considered a possible source (254). Significantly more groundskeepers had higher serum titers against *L. pneumophila* (titer≥1:128) compared to employees working indoors (p=0.018). However, no *Legionella* was isolated from the soil samples or other environmental samples. In four source investigations, *Legionella* was detected in the soil; however, there was no link to the cases (243, 312-314).

Seven publications described the detection of *Legionella* in natural soil (115, 205, 211, 315-318). In Japan and Thailand, *L. pneumophila* was isolated from the soil, and the allelic profiles of some strains were identified to have been previously associated with LD cases (115, 318). One study showed the presence of *Legionella* in garden soils mixed with composted materials (6 of 14 samples) (211). The 14 garden soil samples were obtained from gardeners shown to have *Legionella* in their compost. Concentration data of *L. pneumophila* in natural soil are not available.

Cooling liquid for machinery

Three studies described cases linked to contaminated liquid used for cooling industrial machinery. In a plastic factory in which an LD case occurred, a machine cooling system was the suspected infection source, and a match based on monoclonal subtyping was determined between clinical and environmental isolates (255). Other possible sources linked to the case were excluded; therefore, LOE Ib was assigned. In a large outbreak of Pontiac fever that affected 317 workers in an automobile plant, *Legionella feeleii* was found in a water-based coolant that was used to lubricate, cool, and clean the grinding and machining surfaces (256). Ill employees had significantly higher mean antibody titers against the environmental isolate than employees that were not ill and an unexposed control group, and attack rates decreased linearly with the distance from the system. One LD case and one Pontiac fever case were linked to *L. pneumophila* SG1 found in high concentrations ($1.3*10^5$ CFU/L) in an uncovered water tank that acted as a heat exchange for a welding cooling system (257). In the last two studies, no clinical isolates were obtained, but other possible sources were excluded, resulting in an LOE of III.

Other confirmed sources

Four studies reporting on other sources were assigned the highest LOE. In an outbreak investigation in Spain involving 11 patients, a milling machine used in street asphalt repaving was considered the infection source (LOE Ia) (258). *L. pneumophila* was isolated from the milling machine and the tank that supplied the machine with water. The water originated from a natural spring. Cayla et al. (259) reported on two patients who contracted LD after working on a cargo ship's cooling water circuit pump. *L. pneumophila* was isolated from the main valve of the pump (LOE Ia). An air-perfused footbath was the confirmed source in an outbreak report by Den Boer et al. (LOE Ia) (260). In a study by Moiraghi et al. (261), one of the 12 suspected LD cases could be explained by the use of a contaminated underwater chest drain postoperatively after cardiac surgery (LOE Ib).

Potential *Legionella* sources

Medical equipment

Respiratory devices were the subject of investigation in seven publications. Five studies reported on nosocomial cases that were potentially infected by exposure to contaminated aerosols from respiratory devices (232, 233, 261-263). In all investigations, *Legionella* bacteria were also detected in the hospital water system; for some studies, this could not be excluded as an infection source (262, 263). Furthermore, typing methods were not used in any of these studies to compare clinical and environmental isolates, resulting in LOEs of III or IV. The sixth publication reported the detection of *L. pneumophila* strains in air compressor systems that supply air for respirators (319). In the last publication, Woo et

al. (293) showed the aerosolization of *L. pneumophila* by respiratory equipment rinsed in contaminated tap water.

Additionally to respiratory devices, other types of medical equipment were investigated. In three studies, medical equipment was considered the most likely infection source, but the evidence was inconclusive (264-266). Results from a case-control study conducted after an LD outbreak revealed undergoing transesophageal echocardiography (TEE) as a risk factor (264). *L. pneumophila* SG1 strains from tap water used to rinse the TEE probes were nearly identical to clinical strains compared by PFGE. An LD case was linked to a contaminated respiratory hydrotherapy system with sulfurous spa water, but no clinical strain was available for comparison with environmental isolates (265). Nebulizers were investigated in two outbreak studies (55, 266). Mastro and co-workers (266) demonstrated the aerosolization of *Legionella* from a medication nebulizer in an experimental setting, but the infection source remained unclear because *Legionella* was also found in other suspected sources. In a study by Brown et al. (55), *L. micdadei* was isolated from nebulizers; however, the suspected infection source was an inoperative bedpan flusher. Medical appliances used for hydrotherapy (10) and incubator humidification trays at a neonatology ward (57) were found to be contaminated with *L. pneumophila,* but no cases were linked to these systems.

Water used for cleaning

Three of the selected publications reported on LD cases linked to cleaning with water in the work environment, but the evidence provided was extremely limited, resulting in low LOEs (III, IV, VI). In a report on a cluster of LD concerning two cases, an aqueous metal pre-treatment tunnel in a construction equipment manufacturing plant applied to degrease and rinse steel parts was suggested as a possible infection source (267). *L. pneumophila* was isolated from water samples from the aqueous pre-treatment system, but no clinical strains were available for comparison. A case-control study was conducted after an outbreak of LD at an automotive plant, and exposure to a cleaning line was associated with LD (63). However, no environmental isolates were obtained from the cleaning area, and strains isolated from other sites did not match with a clinical isolate. Castellani Pastoris et al. (320) reported on a patient who had been working on a drilling platform. A water gun supplied by a reservoir used for cleaning was the suspected infection source, although no environmental isolates were obtained.

Dental units

In total, 12 studies reported on dental units as a reservoir or potential source of *Legionella*. Two studies investigated *Legionella* seroprevalence, and nine studies described the contamination of dental units by *Legionella*. Only one of the 12 studies selected for in this review described an LD case linked to a dental unit involving a dentist.

A culture of *Legionella dumoffii* was obtained from the dentist, and *L. longbeachae* and *L. pneumophila* were detected in the lung tissue by monovalent fluorescent-antibody staining (268). All three species were also identified by monovalent fluorescent-antibody staining in samples from the dental operatory. Isolates of *Legionella* spp. were obtained from both the workplace and home but were not further typed (268).

Consistent with the frequent contamination of dental drill units with *Legionella*, Borella et al. (321) found that dental workers had a higher *Legionella* spp. seroprevalence (IFAT, cut-off for positivity 1:128) than office staff in Bari, Italy, suggesting that dental unit water is a *Legionella* source. The widespread *L. pneumophila* colonization of eight dental stations in the London Hospital Dental Institute has been reported (322). The *L. pneumophila* SG1 seroprevalence measured in the exposed dental staff was slightly increased compared to an unexposed group (RMAT≥8; IFAT≥16), but only significant with one of two used serological tests (322).

Legionella spp. and *L. pneumophila* are frequently detected in dental unit water samples (323-331). In one study, *Legionella* was detected in air samples in an experimental setting by qPCR (324). However, there was no significant difference in levels measured in the treatment room compared to background levels measured outside the treatment room.

Rainwater: roof-harvested rainwater

Six studies reported the detection of *Legionella* in roof-harvested rainwater. In one study, an outbreak in New Zealand was described, involving four LD cases linked to roof-harvested rainwater (269). *L. pneumophila* SG1 was isolated from a patient, and the same sequence type was found in roof-harvested rainwater and in a water blaster used on a nearby marina for cleaning boats. Patients were potentially infected through the use of the roof-harvested rainwater for showering, or they were infected outside by contaminated aerosols disseminated by the water blaster. Aerosols spread by the water blaster may also have seeded the roof-collected rainwater systems. In five studies, *Legionella* bacteria were detected in roof-harvested rainwater, either by culture (332, 333) or PCR (334-336).

Construction and excavation

Construction and excavation sites were occasionally considered as possible infection sources in older publications (270, 337, 338). However, evidence for construction and excavation sites as sources of *Legionella* was hardly provided. In 1978, a nosocomial outbreak of LD in 1965 was studied, and soil from an excavation site near the hospital was suggested as the potential reservoir of the infectious agent based on an epidemiological investigation (270). Sleeping by open windows and having free access to the grounds of the hospital were identified as risk factors. The results from a case-control study conducted by Storch et al. (337) implied that sporadic LD patients were more likely to have lived near excavation

sites than controls. Furthermore, there was a significant excess of construction workers among the patients. In a Spanish study, a significantly higher *L. pneumophila* antibody prevalence was measured (IFAT, titer≥1:64) in a group of 87 underground construction workers compared to a group of 150 healthy blood donors (16% and 1.2%, respectively) (338). It should be noted that the mean age of the blood donors was slightly lower than that of the construction workers. Furthermore, the group of workers consisted of all men, in contrast to the group of blood donors, which was comprised of 80 men and 70 women.

Natural water: surface water and groundwater

Surface water was the subject of investigation in 36 publications. In 34 publications, the presence of *Legionella* in surface water was described and two case-control studies found an association between LD incidence and surface water use. Nine publications concerned groundwater.

Den Boer et al. (339) investigated whether a geographical variation in LD incidence in the Netherlands coincided with geographical differences in the origin of drinking water (groundwater versus surface water). The price of water was used as a proxy because higher prices result from more intensive production processes using raw surface water. The results showed that a high water price was positively associated with a high LD incidence rate at the municipal level (OR: 5.1, 95% CI: 3.3 to 8.0). Ng et al. (135) discovered an association between the incidence of LD in the Greater Toronto Area, Canada, and lower river and creek levels and decreases in the temperature of Lake Ontario (the drinking water source for most residents). The authors hypothesized that low water levels might promote the growth and survival of *L. pneumophila* in treated or untreated water supplies, and cooling of the lake might influence LD risk via increased lake circulation.

Many selected publications described *Legionella* detection in surface water (see Table S6 for the complete set of references). In 1981, Fliermans et al. (340) demonstrated the ubiquity of *L. pneumophila* in surface waters by investigating 67 lakes and rivers in the U.S. The study showed that *L. pneumophila* was able to survive a wide range of psychical and chemical conditions (e.g., temperature, pH). Several other studies have found *Legionella* in natural waters with extremely diverse characteristics, e.g., a lake in Antarctica (341), rivers in a Brazilian rainforest (342), an acidic geothermal stream in the U.S. (343), hydrothermal lakes in Europe (344, 345), and marine and estuarine environments in the U.S., Puerto Rico and Brazil (287, 342, 346-348). Two outbreak strains involved in outbreaks related to a WWTP in Norway (see 'Wastewater/wastewater treatment plants') were isolated from a river downstream of the outlet of the plant, whereas they were not detected upstream of the outlet (227). The authors hypothesized that the river played a role in the dissemination of *L. pneumophila* during the outbreaks. The maximum reported concentration of *L. pneumophila* in surface water, $1.9*10^6$ CFU/L, was reported in this study.

Nine studies described *Legionella* prevalence in groundwater (10, 349-356). The maximum reported concentration of *L. pneumophila* in groundwater was $8*10^2$ CFU/L (349).

Rainwater: rainwater on the road

Legionella presence in rainwater on the road was shown in three studies. In a study investigating puddles at six locations on asphalt roads in Japan, 47.8% of 69 samples were positive for *Legionella* species (357). Strains of *L. pneumophila* SG1 were the most frequently isolated. Sakamoto et al. (143) found a relation between the prevalence of *Legionella* in rainwater puddles on roads in Japan and ambient temperature on the sampling date; the isolation rate of *L. pneumophila* increased from 15.8% at temperatures below 20°C to 58.3% at temperatures above 25°C. In the Netherlands, *L. pneumophila* bacteria were isolated by amoebal coculture from pluvial floods after intense rainfall (3 of 6 samples) (144). Two strains belonged to sequence types that had been previously identified in patients. Whether human exposure to rainwater on roads poses a risk for *Legionella* infection and possible LD remains unclear.

Other potential sources

In 23 publications, potential sources or reservoirs of *Legionella* were described that were only mentioned in one or two publications selected in this review. The following potential sources were considered in source investigations: discharge vessels of sullage tanks collecting bilge water from ships (271), a steam turbine condenser (272), an inoperative bedpan flusher (55), a steam towel warmer (273), an industrial air scrubber located at a WWTP (see 'Wastewater/wastewater treatment plants') (223, 224), and garden hoses (192, 358). Low LOEs (III-V) were assigned because the evidence was inconclusive or another suspected source was found to be the most likely source.

Furthermore, *Legionella* bacteria were isolated from the following potential sources/reservoirs: indoor pools (359), eyewash stations (360), bracts of rainforest epiphytic plants (347), telephone manholes (361), water and filters from a water-cooler (362), a material reclamation facility (69), machines used for street cleaning (258), car wash stations (228), and a steam discharge pipe extending from a boiler (363). *Legionella* bacteria were detected by molecular methods in the following potential sources/reservoirs: aquarium water (364), slow sand filters used in horticulture (365), coral (366), acid mine drainage (367) and bottled mineral water (368). Shahamat et al. (301) tested several potential sources of *Legionella* on a university campus and detected *Legionella* by DFA in a steam tunnel, a chiller, air handling units, sump pits and expansion tanks.

Legionnaires' disease risk factors

A total of 12 epidemiological studies identified risk factors for infection with *Legionella* and reported diving, exposure to industrial aerosols, factors related to driving, and meteorologi-

cal factors. Neubauer et al. (369) showed that divers had a significantly higher prevalence of positive antibody titers (titer≥1:64) than controls. The divers were significantly older than the controls, and no information other than age was provided regarding the controls. In a geographical ecological study by Che et al. (370), an association was found between exposure to industrial aerosols and the incidence of sporadic community-acquired LD cases in France. Two studies identified that professional drivers were at increased risk of acquiring LD (30, 150). In the study by Wallensten et al. (150), two risk exposures in relation to driving were identified: 'driving through industrial areas' (OR: 7.2, 95% CI: 1.5–33.7) and 'using no screenwash in the windscreen fluid' (OR: 47.2, 95% CI: 3.7–603.6). The publication also discussed isolating *Legionella* from windscreen fluid not containing screenwash of one car in a pilot study, but the data were not presented. Several studies have investigated the association between meteorological variables and LD incidence in Europe (the Netherlands, Spain, Switzerland, and the UK), the U.S., and Canada. Temperature (either average or short-term) (130, 132, 133, 138, 371), relative humidity (or vapor pressure) (130, 132, 133, 135, 138), and precipitation (130, 133, 134, 139) were identified as meteorological variables that are associated with LD incidence.

Discussion

This review shows that many different systems and matrices could be sources of *Legionella*. A total of 17 systems and matrices were classified as confirmed sources of *Legionella* (LOE I assigned to at least one study). For cooling towers, whirlpools, potting soil/compost, baths, fountains, wastewater/WWTPs, ice/ice machines, room humidifiers, mist machines, air conditioning systems, thermal springs, natural soil, a milling machine, a ship water pump, a foot bath, a chest drain, and cooling liquid for machinery, matches have been made between *Legionella* isolates from these systems and clinical isolates. Furthermore, for all of these sources, there was additional evidence provided that these sources were the infection source, such as evidence of the exposure of several cases to the same source or evidence of *Legionella* spread from the source. Several of these sources were confirmed sources in large outbreaks with five or more cases. This concerned not only well-known sources, such as cooling towers, whirlpools, air conditioning systems, potting soil/compost and fountains but also wastewater/WWTPs, room humidifiers, mist machines, and a milling machine. The evidence for some sources was derived from one or two studies, such as mist machines, whereas other sources, i.e., cooling towers, whirlpools, and potting soil/compost, were confirmed sources in at least four source investigations.

There were no systems or matrices where the highest LOE assigned was LOE II. LOE III was assigned to the following potential sources: TEE probes, a hydrotherapy system, water used for cleaning, and sullage tanks collecting bilge water. Besides the before mentioned, *Legionella* has been found in many other man-made systems or natural matrices, but either

no patients were linked to these reservoirs or the supporting evidence was weak, resulting in a low LOE (LOE IV-VI). However, these systems or matrices could play an important role in the transmission of infectious *Legionella* bacteria; they might not yet be considered in source investigations, resulting in an underestimation of their importance.

To compare environmental isolates and patient isolates, advanced methods currently available for typing *Legionella* should be used in addition to serotyping. In this review, molecular typing (AFLP, PFGE, SBT) was assumed to provide the best evidence that the *Legionella* bacteria isolated from a patient and from a suspected source belonged to the same strain (372). However, not all methods have the same discriminatory power. The best discriminatory method is whole genome sequencing (WGS) of *Legionella* isolates. This has only recently been conducted in a few LD outbreak investigations (82, 98, 99). Sánchez-Busó et al. (373) retrospectively performed WGS on clinical and environmental *L. pneumophila* strains linked to 13 outbreaks, and in some occasions the genome sequences of isolates of the same sequence type and outbreak did not cluster together and were more closely related to sequences from different outbreaks. This suggests that, for some sequence types, the current SBT approach provides insufficient resolution to establish the outbreak source. Definitive proof that a potential source was the infection source is difficult to obtain. The combination of evidence is necessary to confirm the source.

Potting soil and compost as a reservoir and source of *L. longbeachae* have been described in studies from many countries. A vast proportion of LD cases are reported to be caused by *L. longbeachae* in Australia and New Zealand (170, 171); however, in Europe and the U.S., *L. pneumophila* is dominant (167-169). Recently, it was also suggested that a certain proportion of LD cases in Europe are caused by *L. longbeachae* (77). Some studies identified *L. pneumophila* as the predominant species in potting soil and compost over *L. longbeachae* (206, 211). However, to our knowledge, no LD cases caused by *L. pneumophila* from potting soil or compost have been reported to date. Perhaps potting soil and compost are not yet considered potential sources of *L. pneumophila* in source investigations; therefore, it is important to assess whether *L. pneumophila* originating from potting soil or compost can cause disease.

Legionella spp. and *L. pneumophila* are also present in natural soil. However, only one study provided evidence for infection caused by *L. pneumophila* in natural soil (252). The patient worked at a nursery and had spent several days potting plants on a field. Possibly, the soil that harbored the infective strain, obtained from this field, was a mixture of natural soil and potting soil. In certain professions, exposure to natural soil might be substantial (254, 338); to assess the possible infection risk for *Legionella* originating from natural soil, concentration data should be determined and the virulence of soil-borne *Legionella* strains should be assessed.

Surface water, groundwater and rain are reservoirs of *Legionella* but evidence that these matrices are sources of infection is lacking. Data on aerosolization of *Legionella* from these matrices and subsequent exposure of humans, e.g. during heavy rain events or when surface water is used for cleaning purposes, could give insight in the possible risk that these reservoirs of *Legionella* pose to human health. River water has been described as a possible *L. pneumophila* source in two studies in which patients contracted LD after nearly drowning (193, 374). However, for both cases, *Legionella* was not detected in the river water. Based on the study inclusion criteria these studies were excluded from this review.

Only one case linked to dental treatment was identified in this review, and the evidence for the dental unit as the infection source was inconclusive (268). A recent study described a case of an 82-year-old woman who died of LD after dental treatment (375). This study was not identified in our literature search because the main search terms ['legionell*', 'legionnair*', 'Pontiac fever'] were not included in the title, and there was no abstract. This publication would have been assigned the highest LOE (Ia). It is surprising that not more cases are linked to dental units because dental units are often found to be colonized by *Legionella*, and risk groups are expected to visit dentists. Dental units may not always be targeted in outbreak investigations.

Several risk factors for acquiring LD are reported in the literature that might give insight into yet unknown sources. Further study is necessary to unravel the meaning of the risk factor 'being a driver as a profession' (30, 150). Furthermore, although the weather likely plays a role in the growth and transmission of *Legionella*, which sources or reservoirs of *Legionella* are under the influence of the weather and how weather conditions contribute to the growth and transmission of *Legionella* remains unclear.

After the literature search was conducted, some studies were published that reported on (potential) sources of *Legionella* that were not represented in this review or that supplied information that significantly changed the LOE assigned to a potential source. Euser et al. (376) identified a manually operated pressure test pump as the infection source for a sporadic LD case. Environmental and clinical isolates were matched by SBT. Wei et al. (377) found that *Legionella* strains from water dispensers used to make infant formula were indistinguishable by SBT from strains obtained from two nosocomial neonatal cases. Litwin et al. (378) linked an LD case to a contaminated recreational vehicle water reservoir. In a Greek study, *Legionella* was detected in car cabin air filters (379). Although *Legionella* was already detected in car wash stations (228), a recent publication provided essential evidence for these stations as a *Legionella* source (174). One study provided evidence for the possible transmission mode of *Legionella* originating from compost by demonstrating that compost could release bioaerosols containing *Legionella* (380).

This review has several methodical limitations. Relevant publications on potential sources of *Legionella* might have been missed because this review focused on publications published in biomedical journals because of the use of a single search engine: PubMed. However, in every publication selected for full-text assessment, the introduction and discussion sections were checked for relevant references that were missed in this review. It was concluded that literature regarding *Legionella* in dental units was missed in our search. Another search was conducted using two different search engines, but no other publications were found that supplied information that significantly changed the assigned LOE. Furthermore, the selection criteria may have excluded relevant publications on (potential) sources of *Legionella*. For example, studies on travel-related LD were excluded because it was assumed that most patients would be infected by the use of contaminated tap water through showering, possibly resulting in a loss of publications about other (potential) sources. And by excluding comments, a study describing the isolation of *L. pneumophila* from windscreen washer fluid was missed (381). Several publications on thermal springs as possible sources of *Legionella* were missed because these studies were published in Japanese (382-387). The inclusion of the missed publications would have likely resulted in more studies providing the highest LOE.

In designing the LOEs used in this review, *Legionella* concentration data were not taken into account. Concentration data could be used to assess the (relative) infection risk for a (potential) source. For example, Ahmed et al. (336) calculated that the concentration of *Legionella* in roof-harvested rainwater, as determined by PCR, was such that the infection risk associated with the use of rainwater for showering and garden hosing was to be well below the threshold value of one additional infection per 10,000 persons per year. However, data on concentrations are not always valuable depending on the type of detection method used. Molecular methods could overestimate the level of contamination and detection by culture could lead to false negative results (388).

This review demonstrates that many different water systems and non-water systems are reservoirs of *Legionella* and many different systems and matrices have been confirmed as sources of *Legionella*. LD can be acquired by exposure to relatively rare sources that may not yet be considered in source investigations. Therefore, when tracking a source of infection it is essential to consider all possible potential and confirmed sources. Further research is needed to unravel what the contribution is of each confirmed source, and possibly also potential sources, to the LD disease burden. The knowledge about sources of *Legionella* is imperative when developing policy for effective *Legionella* prevention and control.

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Supporting Information

The full text of the methods section and three tables (Tables S4-S6) are supplied in Appendix A.

3.

Soil as a source of *Legionella pneumophila* sequence type 47

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Abstract

Legionella pneumophila sequence type (ST) 47 was isolated from soil in a garden. We speculate that this strain was transmitted from soil to the whirlpool in the garden where it caused an outbreak of Legionnaires' disease and Pontiac fever. In the Netherlands, ST47 is frequently isolated from patients, but hardly ever from environmental sources. It is possible that human pathogenic *Legionella* strains, with ST47 as one of the predominant strains, are transmitted to humans from sources such as natural soil that are currently not targeted in outbreak investigations.

Introduction

Legionella pneumophila sequence type (ST) 47 is the sequence type that is most frequently isolated from patients in the Netherlands (41% of *L. pneumophila* serogroup 1 isolates (109)), Belgium (27.9% of *L. pneumophila* serogroup 1 isolates (103)), and the UK (25.7% of *L. pneumophila* isolates (110)). However, in source investigations, this sequence type is rarely found (109, 110). Therefore, it could be questioned whether the sources targeted in source investigations, such as tap water, whirlpools, cooling towers, and potting soil, are the most important sources for the transmission of *L. pneumophila* ST47 to humans.

Since the start of the National *Legionella* Outbreak Detection Program in the Netherlands in 2002, *L. pneumophila* ST47 has only been isolated from environmental sources on three occasions, all involving outdoor whirlpools (Table 1, (192)). Since all three whirlpools from which the *L. pneumophila* ST47 was isolated were outside in gardens, we speculate that *L. pneumophila* ST47 was introduced into the whirlpools from a reservoir in the open air, possibly soil in the gardens. To test this hypothesis, we sampled soil at two out of the three gardens in which the above mentioned outdoor whirlpools were situated. The soil samples were tested for the presence of *Legionella* spp. using an amoebal coculture procedure (144). Isolated *L. pneumophila* strains were typed by sequence-based typing (88, 89).

Table 1. Overview of source investigations in which *Legionella pneumophila* ST47 was isolated from environmental sources

Year of investigation	No. of cases	Environmental source	Clinical isolate
2013	5 (2 LD, 3 Pontiac fever)	Whirlpool in garden; 200 CFU/L	No
2009	4 (1 LD, 3 Pontiac fever)	Whirlpool in garden; swab sample (192)	No
2006	1 (LD)	Whirlpool in garden; 1 260 000 CFU/L	Yes (ST47)

LD: Legionnaires' disease. CFU: colony-forming units.

Methods

In the garden that was related to the cases in 2013 (garden A), five border soil samples, one soil sample from the lawn, one potting soil sample, and one sample from a compost bin were collected (Table 2). In the garden that was related to a case in 2006 (garden B), eight border soil samples were collected. The soil samples from these two gardens were all collected in September 2013. Samples were taken from the upper 2 cm of soil and stored in sterile bottles at 4°C until analysis.

For the analysis, approximately 5 g of soil was weighed and 5 ml of Page's amoeba saline (PAS) solution (144) was added. This was vortexed and then incubated at room temperature for 1 hour. *Legionella* spp. were detected by amoebal coculture (144). In brief, *Acanthamoeba castellanii* cells (American Type Culture Collection, Rockville, MD, USA)

were seeded in 12-well microplates (Corning Inc., New York, NY, USA) at a density of 5×10^5 cells per well in 1 ml of PAS. The incubated soil samples were vortexed vigorously again and for each sample, three wells with amoebae were inoculated with 100 ml sample. After incubation at 32°C for 3 days, the contents of each well were resuspended by pipetting and down. Subsequently, 100 ml of each well was transferred to a well with freshly seeded amoebae and these were incubated at 32°C for another 3 days. The contents of each well were 10-fold serially diluted in PAS and plated on buffered charcoal yeast extract (BCYE) plates (Oxoid Ltd, Hampshire, United Kingdom). *Legionella* strains were identified by latex agglutination test (Oxoid Ltd). *L. pneumophila* strains were further genotyped by sequence-based typing (88, 89).

Results

Three out of eight samples collected in garden A were positive for *Legionella* spp. (Table 2). One border soil sample contained *L. pneumophila* serogroup 1, ST47. The soil sample from the lawn and the potting soil sample contained non-*pneumophila Legionella* strains that were negative in the latex test. The eight soil samples from garden B were all negative for *Legionella* spp.

Table 2. Results of *Legionella* detection in samples from garden A

Sample no.	Sample type	Amoebal coculture*	Latex test†	Sequence-based typing
1	Border soil	3/3	*L. pneumophila* serogroup 1 (3/3)	ST47 (2/2)
2	Border soil	0/3		
3	Border soil	0/3		
4	Border soil	0/3		
5	Border soil	0/3		
6	Potting soil	3/3	Negative (0/3)	
7	Compost bin	0/3		
8	Lawn soil	3/3	Negative (0/3)	

*Number of *Legionella*-positive wells/number of inoculated wells. †Number of colonies with a positive test result/number of colonies tested.

Discussion

L. pneumophila serogroup 1, ST47 was isolated from natural soil in a garden. L *pneumophila* ST47 had previously been isolated from a whirlpool in that garden during source investigations following a combined Legionnaires' disease and Pontiac fever outbreak in 2013. It is possible that the ST47 strain was transmitted from soil to the whirlpool by wind or by people entering the whirlpool with soil on their feet.

We speculate that direct transmission of ST47 from soil to humans can also occur, for example when handling soil during gardening, causing solitary cases. For many solitary cases, the source of infection remains unknown.

ST47 is hardly ever isolated in source investigations. The isolation of ST47 from natural soil could be an indication that soil is an alternate, yet overlooked source of this human pathogenic strain.

Many studies have described the transmission of human pathogenic *Legionella longbeachae* from potting soil, even though the mode of transmission is not yet clear (171, 199). The transmission of *L. pneumophila* from soil to humans has also been described (252). Natural soil as a source of pathogenic *Legionella* bacteria causing human infections warrants further investigation.

4.

Presence and persistence of viable, clinically relevant *Legionella pneumophila* bacteria in garden soil in the Netherlands

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Abstract

Garden soils were investigated as reservoirs and potential sources of pathogenic *Legionella* bacteria. *Legionella* bacteria were detected in 22 of 177 garden soil samples (12%) by amoebal coculture. Of these 22 *Legionella*-positive soil samples, seven contained *Legionella pneumophila*. Several other species were found including the pathogenic *Legionella longbeachae* (4 gardens) and *Legionella sainthelensi* (9 gardens). The *L. pneumophila* isolates comprised 15 different sequence types (STs), and eight of these STs were previously isolated from patients according to the European Working Group for *Legionella* Infections (EWGLI) database. Six gardens that were found to be positive for *L. pneumophila* were resampled after several months, and in three gardens, *L. pneumophila* was again isolated. One of these gardens was resampled four times throughout the year and was found to be positive for *L. pneumophila* on all occasions.

Importance

Tracking the source of infection for sporadic cases of Legionnaires' disease (LD) has proven to be hard. *L. pneumophila* ST47, the sequence type that is most frequently isolated from LD patients in the Netherlands, is rarely found in potential environmental sources. As *L. pneumophila* ST47 was previously isolated from a garden soil sample during an outbreak investigation, garden soils were investigated as reservoirs and potential sources of pathogenic *Legionella* bacteria. The detection of viable, clinically relevant *Legionella* strains indicates that garden soil is a potential source of *Legionella* bacteria, and future research should assess the public health implication of the presence of *L. pneumophila* in garden soil.

Introduction

Legionella is an opportunistic pathogen that can cause legionellosis (16). Legionellosis refers to two distinct clinical syndromes: Legionnaires' disease (LD) and Pontiac fever. The vast majority of the clinical isolates in Europe (389-391) and the United States (168) constitute *Legionella pneumophila* serogroup 1 (SG1). In Australia, New Zealand, and Thailand, *Legionella longbeachae* is an important cause of disease (157, 171, 392). *Legionella* bacteria are ubiquitous in natural matrices and manmade water systems (393). Some of these systems and matrices can act as sources of *Legionella* infection, such as cooling towers (40), whirlpools (41), thermal springs (45) and wastewater treatment plants (223). Potting soil is a source of *L. longbeachae* infection (51, 52).

In source investigations, alongside epidemiological evidence, a genotypic match between environmental and clinical strains is needed to identify the source of infection. Tracking the source of infection for sporadic cases of LD has proven to be hard (393). Studies in the Netherlands, England, and Wales showed that common clinical *L. pneumophila* strains are only rarely found in the environment (109, 110). In these studies, *L. pneumophila* genotypes isolated from patients were compared with genotypes isolated from the environment. The environmental sources of *Legionella* in these studies comprised manmade water systems (domestic water distribution systems, cooling towers, spa pools).

L. pneumophila sequence type 47 (ST47) is the ST that is most frequently isolated from patients in the Netherlands (26.9% of clinical isolates available for typing) (393). Strikingly, since the start of the National *Legionella* Outbreak Detection Program in the Netherlands in 2002, this ST was only found three times in the environment during outbreak investigations, which concerned outdoor whirlpools that were involved in two combined outbreaks of LD and Pontiac fever and one solitary case of LD. As all three whirlpools were located outside, it was hypothesized that the outdoor environment was an influence, and after further investigation *L. pneumophila* ST47 was isolated from a soil sample from the garden of the most recent outbreak (394). It was suggested that the ST47 strain was transmitted from garden soil to the whirlpool by wind or by people entering the whirlpool with soil on their feet.

It is possible that garden soil plays a role in *Legionella* infection. Although potting soil is a well-studied reservoir and known source of *Legionella*, not much is known about *Legionella* in garden soil. Hughes and Steele (211) showed the presence of *Legionella* in six garden soils that were mixed with composted materials. Furthermore, there is evidence that natural soil is a reservoir and source of *Legionella*. Wallis and Robinson (252) reported an LD patient that had worked at a plant nursery in the week prior to illness. A *L. pneumophila* strain with an indistinguishable genotypic profile, determined by pulsed-field gel electrophoresis (PFGE), was isolated from a field in which the patient had spent time pot-

ting plants. In several other studies *L. pneumophila* strains have been isolated from natural soil (115, 243, 316, 318). In two of these studies, some of the obtained sequence types (STs) had previously been detected in cases of LD (115, 318).

The aim of the current study was to investigate garden soil as reservoir of viable, clinically relevant *Legionella* bacteria. Garden soils were sampled throughout the year. Furthermore, we studied whether *L. pneumophila* could persist in soil over time by resampling *Legionella*-positive gardens after several months. An amoebal coculture method was applied to detect *Legionella* bacteria in the soil. Amoebal coculture has been proven successful for the isolation of *Legionella* bacteria in samples, such as soil, with a lot of background flora (144, 395).

Materials and Methods

Sampling of garden soils

Garden soil samples were collected over a period of 1 year (February 2014 to January 2015). In order to obtain a large and diverse number of samples, colleagues from the National Institute for Public Health and the Environment and from the Regional Public Health Laboratory Kennemerland were asked to supply a soil sample from their own garden and/or the gardens of friends and family. Samples were to consist of soil from a soil bed at any place within their own garden, irrespective of type of garden, cultivation, or planting. Sterile spoons and jars were provided and were accompanied by an instruction form and short questionnaire. Participants were asked to sample the upper 2 cm of soil at one place in the garden. Upon arrival, the samples were stored at 4°C until analysis. If *L. pneumophila* was isolated from a garden, then the owners were asked to resample their garden after several months, at the same location as the first sampling.

Pre-treatment of the samples

Prior to analyses, 5 g of each soil sample was resuspended in 5 ml of sterile distilled water. These suspensions were vortexed for approximately 10 seconds and incubated at room temperature for 1 hour. The soil suspensions were vortexed again just before amoebal coculture.

Amoebal coculture method

The amoebal coculture method for detection of *Legionella* was performed as described previously (395). Briefly, *Acanthamoeba castellanii* ATCC #30234 (American Type Culture Collection, Manassas, VA, USA) was grown in 75-cm^2 culture flasks (Corning Inc., New York, NY, USA) with 15 ml of peptone-yeast extract-glucose (PYG) broth at 25°C. Prior to the infection, the PYG broth was removed, and the amoebae were resuspended in 15 ml

Page's amoeba saline (PAS) (396). The amoebae suspension was centrifuged at 850 X *g* for 10 minutes, and the pellet was subsequently resuspended in 15 ml PAS. This washing step was repeated 2 times. Cells were seeded in a 12-well microplate (Corning) at a density of 5×10^5 cells/ml of PAS. In each well, 1 ml of PAS with amoebae was inoculated with 100 µl of sample, and each sample was tested in triplicate (three wells). Thus, amoebae were inoculated with in total 300 µl of the suspension. By testing 300 µl of the soil suspension, 0.3 gram of the soil sample was tested. The theoretical detection limit was therefore 3.3 CFU/g soil.

The amoebal plates were incubated at 32°C. As a negative control, one well with amoebae was not inoculated with a sample. After 3 days of incubation, 100 µl of each suspension was subcultured on a new plate with freshly seeded amoebae. After another 3 days of incubation at 32°C, 100 µl of each well was serially diluted 10-fold in PAS. Of the 10^4-, 10^5-, 10^6-, 10^7- and 10^8-fold dilutions, 100 µl was plated on buffered charcoal yeast extract (BCYE) plates (Oxoid Ltd., Hampshire, United Kingdom). The 10^5- or 10^6-fold dilutions were also cultured on glycine-vancomycin-polymyxinB-cycloheximide (GVPC) plates (Oxoid Ltd., Hampshire, United Kingdom). After 4 and 7 days of incubation at 37°C, the BCYE and GVPC plates were inspected for *Legionella*-like colonies with a stereo microscope (magnification, 40 X; Olympus).

Confirmation and typing of *Legionella* isolates

Suspected *Legionella* colonies were tested for their inability to grow on BCYE medium without cysteine (Oxoid Ltd., Hampshire, United Kingdom). Strains that were unable to grow on the medium without cysteine were further subtyped by polyclonal antisera (*L. pneumophila* SG1, SG2 to SG14 and *Legionella* spp.) coupled to latex beads (*Legionella* latex test; Oxoid Limited, Hampshire, United Kingdom) and subsequently stored at -70°C. The strains were later identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Strains that could not be re-cultured prior to MALDI-TOF MS identification and that showed no clear agglutination response in the latex test were confirmed with a *Legionella* species-specific PCR targeting the 5S rRNA gene (397). *L. pneumophila* isolates were genotyped by the standard sequence-based typing (SBT) method of the European Working Group for *Legionella* Infections (EWGLI) using seven genes (*flaA, pilE, asd, mip, mompS, proA,* and *neuA*) (88, 89). The SBT profiles were generated using the high-throughput multilocus sequence typing (HiMLST) method that employs next-generation sequencing (398). *L. pneumophila* SG1 isolates were also subtyped by monoclonal antibody (MAb) subgrouping using the Dresden MAb Panel (12).

Statistical analysis

Univariate and multivariate logistic regressions were performed as described by Hosmer and Lemeshow (399). Univariate analysis was used to identify potential associations be-

tween positivity of gardens for *Legionella* and questionnaire and weather variables. The weather variables, i.e., precipitation and ambient temperature related to sampling days, were obtained from the Royal Netherlands Meteorological Institute (KNMI). For each garden, data were selected from the weather station closest to that garden. Continuous variables (garden age and weather characteristics) were categorized into classes of approximately equal size. Variables with a univariate *p*-value of ≤0.25 (two-tailed likelihood-ratio test) were selected for multivariate logistic regression. Prior to the multivariate analysis, the correlation coefficients of all variable combinations were determined, where a correlation coefficient of 0.25 or lower was considered non-interfering in the multivariate analysis. Subsequently, backward elimination was used until remaining variables exhibited a significant association with a *p*-value of <0.05 (two-tailed likelihood-ratio test). The statistical analyses were performed using SAS software v.9.3 (SAS institute, Cary, NC, USA).

Results

Isolation of *Legionella* spp. and *L. pneumophila* from garden soils

Over 12 months, a total of 177 unique gardens were sampled. Per month, between 10 and 20 samples were submitted by our colleagues, with an average of 15 (see Table 1). In total, 22 of 177 samples (12%) were positive for *Legionella* spp. Out of these 22 positive samples, seven (32%) contained *L. pneumophila* (garden samples 1, 4, 5, 8, 13, 18, and 22; see Table 2). Both *L. pneumophila* SG1 and non-SG1 isolates were obtained, and 15 different STs were identified. In five of these samples, other *Legionella* species were also detected besides *L. pneumophila,* namely *L. longbeachae* (4 samples), *Legionella sainthelensi* (2 samples), *Legionella bozemanii* (1 sample), and *Legionella feeleii* (1 sample). In the remaining 15 samples, several *Legionella* species were detected (Table 2), including *L. sainthelensi* (7 samples), *L. feeleii* (2 samples), *L. bozemanii* (1 sample), *Legionella cincinnatiensis* (1 sample), *Legionella anisa* (1 sample), and *Legionella wadsworthii* (1 sample). For 3 of the 15 samples, *Legionella* could not be further typed by MALDI-TOF MS because the bacteria could not be re-cultured.

Positive samples were found throughout the year (see Table 1). The ambient mean daily temperature on sampling days ranged from -1.9°C to 22.8°C (66). Positive garden soil samples were taken on days with ambient mean temperatures ranging between 0.7°C and 21.5°C. The precipitation sum of the 14 days preceding the sampling day varied between 0 and 113 mm for all sampling days and between 2 and 81 mm for the sampling days at which *Legionella* was detected.

Table 1. Number of *Legionella* spp.– and *L. pneumophila*–positive garden soil samples per month*

Sampling year	Sampling month	Mean temp (°C) (range)	Total precipitation (mm)	No. of samples analyzed	No. of *Legionella* spp.-positive samples (no. of *L. pneumophila*-positive samples)
2014	February	6.5 (3.9 – 10)	66.4	14	2 (1)
	March	8.4 (4.5 – 14.7)	25.7	16	1 (0)
	April	12.1 (7.2 – 17.1)	58.4	16	4 (2)
	May	13.2 (7.9 – 19.9)	102.0	15	0 (0)
	June	16.2 (13 – 21.6)	30.3	16	2 (1)
	July	19.8 (15 – 26.4)	137.1	20	3 (0)
	August	16.1 (12.4 – 20.5)	149.0	10	2 (1)
	September	15.9 (21.6 – 19.3)	20.5	16	3 (0)
	October	13.4 (9.9 – 17.3)	74.9	16	0 (0)
	November	8.2 (2.5 – 14.8)	46.8	15	2 (1)
	December	4.8 (-1.2 – 11.5)	99.5	11	2 (0)
2015	January	4.0 (-2.8 – 9.9)	115.7	12	1 (1)
Total				**177**	**22 (7)**

*The mean ambient temperature per sampling month and the total precipitation per month are indicated (66).

Resampling *L. pneumophila*–positive garden soils

Six of the seven *L. pneumophila*-positive gardens were resampled after several months, and four again tested positive for *Legionella* (gardens 1, 4, 13, and 18; see Table 3). One of the *L. pneumophila*-positive gardens was not resampled because this garden was found to be positive at the end of the project. For three of the four gardens, *L. pneumophila* was again isolated in the second sampling (gardens 1, 13, 18). Garden 1 was resampled four times throughout the year and was found to be positive for *L. pneumophila* on all occasions. Several *L. pneumophila* STs were detected, with ST477 present on all four occasions and ST710 and ST84 on three out of four occasions. *L. longbeachae* was also isolated on all 4 samplings of garden 1.

Clinical relevance of *L. pneumophila* isolates

A total of 70 *L. pneumophila* isolates were typed by SBT. For five isolates, a ST could not be retrieved due to failure of amplification of one or more gene targets. The remaining 65 isolates were classified into 15 different STs (see Table 4). According to the EWGLI SBT database ((400), accessed 17 February 2016), eight of these STs (ST84, ST115, ST462, ST465, ST477, ST710, ST863, and ST1856) were previously isolated from patients. Some STs were found regularly in garden soils, namely, ST84, ST115, ST477, and ST710. ST84 was detected most often; of the seven *L. pneumophila*-positive gardens, ST84 was found in all but one. Of the *L. pneumophila* SG1 isolates, 16 were MAb 3/1 positive and 10 were MAb 3/1 nega-

tive (see Table S5 in Appendix B, which shows all of the typing data of the *L. pneumophila* garden isolates).

Table 2. Detected *Legionella* species in garden soil samples

Sampling month, year	Garden soil sample	*Legionella* spp.*	*L. pneumophila* serogroup (no. of colonies analyzed)	Sequence type(s)
February 2014	*1*	*L. pneumophila*	1 (9)	84, 477, 863
		L. pneumophila	2-14 (2)	465, 710
		L. longbeachae	-	-
	2	*L. sainthelensi*	-	-
March 2014	*3*	*L. cincinnatiensis*	-	-
April 2014	*4*	*L. pneumophila*	1 (6)	84, 115, 477, 2028, 2032
		L. pneumophila	2-14 (12)	863, X[†]
		L. bozemanii	-	-
		L. feeleii	-	-
	5	*L. pneumophila*	2-14 (8)	2025, 2026, X
	6	*L. wadsworthii*	-	-
	7	*L. sainthelensi*	-	-
June 2014	*8*	*L. pneumophila*	1 (2)	84, 710
		L. pneumophila	2-14 (1)	462
		L. longbeachae	-	-
	9	*L. sainthelensi*	-	-
July 2014	*10*	*L. feeleii*	-	-
	11	*L. sainthelensi*	-	-
	12	*Legionella* spp.[‡]	-	-
August 2014	*13*	*L. pneumophila*	2-14 (4)	710
		L. longbeachae	-	-
		L. sainthelensi	-	-
	14	*L. sainthelensi*	-	-
September 2014	*15*	*L. sainthelensi*	-	-
	16	*Legionella* spp.[‡]	-	-
	17	*Legionella* spp.[‡]	-	-
November 2014	*18*	*L. pneumophila*	1 (15)	84, 477, 1856, 2022, 2029
		L. pneumophila	2-14 (3)	84, 115, 710
		L. longbeachae	-	-
		L. sainthelensi	-	-
	19	*L. feeleii*	-	-
		L. anisa	-	-
December 2014	*20*	*L. sainthelensi*	-	-
	21	*L. bozemanii*	-	-
January 2015	*22*	*L. pneumophila*	1 (2)	84

– Not applicable. *Typed by MALDI-TOF MS. [†]X: a sequence type could not be retrieved due to failure of amplification of one or more gene targets. [‡]*Legionella* spp. were not further typed because the bacteria could not be re-cultured.

Table 3. Persistence of *Legionella* species in garden 1, 4, 13 and 19, sampled on 2 or 4 occasions

Garden	Sampling parameters	1st sampling	2nd sampling	3rd sampling	4th sampling
1	**Sampling date**	February 2014	September 2014	November 2014	February 2015
	Detected species	*L. pneumophila* SG1	*L. pneumophila* SG1	*L. pneumophila* SG1	*L. pneumophila* SG1
		L. pneumophila SG2-14	*L. pneumophila* SG2-14	*L. longbeachae*	*L. pneumophila* SG2-14
		L. longbeachae	*L. longbeachae*		*L. longbeachae*
	Sequence types	84, 465, 477, 710, 863	84, 477, 710	84, 115, 477	477, 710
4	**Sampling date**	April 2014	September 2014		
	Detected species	*L. pneumophila* SG1	*L. feeleii*		
		L. pneumophila SG2-14	*L. sainthelensi*		
		L. bozemanii			
		L. feeleii			
	Sequence types	84, 115, 477, 863, 2028, 2032, X*	-		
13	**Sampling date**	August 2014	January 2015		
	Detected species	*L. pneumophila* SG2-14	*L. pneumophila* SG1		
		L. longbeachae	*L. pneumophila* SG2-14		
		L. sainthelensi	*L. bozemanii*		
			L. dumoffii		
	Sequence types	710	84, 115, 710, 2080, X		
18	**Sampling date**	November 2014	March 2015		
	Detected species	*L. pneumophila* SG1	*L. pneumophila* SG1		
		L. pneumophila SG2-14	*L. longbeachae*		
		L. longbeachae	*L. feeleii*		
		L. sainthelensi			
	Sequence types	84, 115, 477, 710, 1856, 2022, 2029	2022, X		

– Not applicable. *X: a sequence type could not be retrieved due to failure of amplification of one or more gene targets.

Table 4. Isolated sequence types from garden soils and clinical relevance of the sequence types according to the EWGLI SBT database*

Sequence type	No. of gardens†	No. of patients‡
84	6	13
115	4	8
710	4	2
477	3	4
863	2	1
462	1	1
465	1	2
1856	1	1
2022	1	0
2025	1	0
2026	1	0
2028	1	0
2029	1	0
2032	1	0
2080	1	0

*The database was accessed on 17 February 2016 (400). †Number of gardens from which the sequence type was isolated. ‡Number of patients from which the sequence type was isolated according to the EWGLI SBT database. A value of 0 indicates that no clinical strains were reported.

Univariate and multivariate analyses

The questionnaire covered the following variables: characteristics of the garden (type, size, location, age), use of potting soil/compost, origin of used potting soil/compost, use of other fertilizers (e.g., manure, artificial fertilizer), use of pesticides/herbicides, use of tap water for watering, presence of an outdoor whirlpool, season of sampling, and frequency of gardening in gardening season (see Table S6 in Appendix B). The owners of all 177 sampled gardens filled in the questionnaire. In addition, eight weather variables were analyzed, namely, precipitation sum on the sampling day, precipitation sum in the 14 days preceding the sampling day, ambient temperature (minimum, mean, maximum) on the sampling day, and ambient temperature (minimum, mean, maximum) in the 14 days preceding the sampling day.

Six of the examined variables reached a *p*-value of ≤0.25 in the univariate logistic regression and were analyzed in the multivariate analysis, i.e., garden size, garden age, use of potting soil/compost, use of other fertilizers, precipitation sum on the sampling day, and mean temperature in 14 days preceding the sampling day (see Table S6 in Appendix B). One variable (surrounding area) had a prevalence of zero in 2 out of 3 categories and could not be analyzed in the multivariate model. Correlations between all variables appeared to

be low (i.e., $r \leq 0.25$). Multivariate logistic regression analysis identified none of the variables to be statistically significantly associated with the presence of *Legionella* in the gardens.

Discussion

Viable *Legionella* strains, including *L. pneumophila*, were isolated by amoebal coculture from 12% of 177 investigated garden soils in the Netherlands, indicating that garden soil is a reservoir of *Legionella* bacteria. The majority of the isolated *L. pneumophila* SG1 strains were found to be MAb 3/1 positive. MAb 3/1 positivity is considered an indication of virulence, since this monoclonal antibody recognizes a virulence-associated epitope (12). Eight STs found in garden soils were clinically relevant according to the EWGLI SBT database (400). However, the STs that are most detected in patients in the Netherlands (ST1, ST47, and ST62) (393) were not detected in garden soils. Of the 15 detected STs in this study, three were also found in patients in the Netherlands (ST84, ST115, ST477). However, these STs are relatively uncommon; they were found in three patients, four patients, and one patient, respectively (since 2005). Strikingly however, these three clinically relevant STs belonged to the most frequently isolated STs in garden samples (see Table 4). It is possible that some conditions in garden soils are favorable for the growth of these clinically relevant STs. So, although garden soil is probably not the source of most infections with *L. pneumophila* in the Netherlands, it should be taken into account that it might play a role in *Legionella* transmission to humans, which warrants further investigation.

L. sainthelensi was found most often in garden soil; it was isolated from 10 of the 22 positive gardens (see Tables 2 and 3). *L. sainthelensi* can be infectious to humans (65, 401) and was first isolated in the United States from fresh water (402). *L. pneumophila* was the second most isolated species and *L. longbeachae* was the third. Strikingly, *L. longbeachae* was always detected in combination with *L. pneumophila*. *Legionella* bacteria were detected in garden soils throughout the year. No association was found between *Legionella*-positive gardens and temperature or precipitation. Evidence was found that *L. pneumophila* can persist in garden soil. For three gardens, *L. pneumophila* was detected again after four to seven months, and for one garden, *L. pneumophila* was found on four sampling occasions over a period of 1 year.

Interestingly, *Legionella* was absent from garden soils in rural areas, whereas 14.6% of garden samples in urban areas were positive for *Legionella* (see Table S6 in Appendix B, variable 'surrounding area'). However, since only 9 gardens in rural areas were investigated no firm conclusion can be drawn about the differences in *Legionella* presence in rural versus urban areas. *Legionella* was also absent from gardens in mixed areas (gardens located at the edge of a village, adjacent to a rural area).

It is possible that *Legionella* in garden soil originates from compost or potting soil. One Australian study, by Hughes and Steele (211), showed the presence of *Legionella* in six garden soils, of which five contained *L. pneumophila*. These soils were mixed with composted materials that were found to contain *Legionella*. Compost (210-212) and potting soil (50, 206, 207) are reservoirs of *Legionella,* and it is possible that *Legionella* is introduced into garden soil by the use of compost or potting soil in the garden. To investigate this possibility, genotypes isolated from garden soils could be compared to genotypes isolated from compost and potting soils. In this study, compost or potting soil samples were provided by the garden owners when available, but they were only analyzed when the compost/potting soil was ever applied to the area where the soil sample was taken and when the soil sample was found to be positive for *L. pneumophila*. For the seven *L. pneumophila*-positive gardens, only one potting soil sample was analyzed but not found to be positive. Furthermore, no association was found between *Legionella*-positive gardens and the reported use of potting soil or compost. We found only one publication that used SBT to type two *L. pneumophila* strains isolated from composted material (212). Interestingly, these were ST84, the most frequently isolated ST in this study. In contrast to the limited number of garden soil studies, several studies investigated natural soil as a reservoir of *L. pneumophila* (115, 316, 318, 395). No ST similarities were observed between the reported STs in these studies and the current study.

A limitation of the use of an amoebal coculture method for isolation of *Legionella* is that selectivity for certain *Legionella* strains might be introduced. It is possible that certain STs replicate better in *A. castellanii* than other STs, resulting in an outcome that is not representative for the natural situation. However, selectivity was not shown in a previous study in which a batch of 23 different *L. pneumophila* strains was tested (144). The batch was comprised of different monoclonal subtypes, both positive and negative for MAb 3/1, and 16 different STs. These strains, including the clinically relevant ST1 and ST47, were all shown to replicate similarly in amoebal coculture with *A. castellanii*. Another drawback of the amoebal coculture method is its limited sensitivity, since only a small amount of soil is investigated per sample. However, for soil samples, amoebal coculture seems the best method in order to obtain isolates. In previous studies we have shown that for samples containing many other bacteria, like soil, amoebal coculture has a higher positivity rate than culture techniques that are based solely on agar plates (144, 395). Another limitation of amoebal coculture is that only a restricted number of samples can be investigated since it is a rather laborious method. Therefore, only one sample per garden was analyzed, while multiple samples would probably have influenced the positivity rate. A more thorough investigation may have been done using molecular techniques, like PCR. However, molecular methods do not render isolates, which are necessary for typing purposes. Due to these limitations, low sensitivity and restricted number of samples, an accurate estimate

of the true prevalence of *Legionella* in garden soil cannot be made. In order to study the prevalence, another sampling strategy and other analysis methods should be chosen.

The significance of the presence of *Legionella* in garden soils to public health is not clear. No patients are known to have been infected by *Legionella* originating from garden soil. In several older publications, natural soil was considered to be a possible source of infection (243, 253, 254, 312-314) because excavation sites were believed to be associated with LD cases (270, 337). However, these studies provided no or little evidence for soil as a source of *Legionella* (393). One more recent study provided evidence for infection caused by *L. pneumophila* originating from natural soil (252). The infective strain was isolated from a field where the LD case had spent several days potting plants.

In conclusion, garden soil is a reservoir of *L. pneumophila* and *Legionella* spp. and may be an alternative source of *Legionella* that is not considered in source investigations, especially for some soil-specific strains like ST84, ST115 and ST477. Whether the presence of *Legionella* in garden soil has an impact on public health is not clear; no cases are linked to *Legionella* in garden soil, and none of the most prevalent Dutch clinical strains were identified in garden soil in this study. A case-control study may reveal whether gardening or working with garden soil is a risk factor for contracting LD, warranting targeted interventions. Prevalence should be studied in more detail, and *Legionella* concentrations in garden soil should be determined. Furthermore, it should be investigated how soil- and other environmental conditions, i.e., weather characteristics, influence viability, growth, and virulence of *Legionella* in garden soil. Furthermore, it is important to investigate if and how *Legionella* bacteria can aerosolize from soil and which gardening activities might pose a risk.

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Supporting Information

Two tables (Tables S5 and S6) are supplied in Appendix B.

5.

Viable *Legionella pneumophila* bacteria in natural soil and rainwater puddles

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Abstract

Aims: For the majority of sporadic Legionnaires' disease cases the source of infection remains unknown. Infection may possible result from exposure to *Legionella* bacteria in sources that are not yet considered in outbreak investigations. Therefore, potential sources of pathogenic *Legionella* bacteria - natural soil and rainwater puddles on roads - were studied in 2012.

Methods and Results: *Legionella* bacteria were detected in 30% (6/20) of soils and 3.9% (3/77) of rainwater puddles by amoebal coculture. *Legionella pneumophila* was isolated from two out of six *Legionella*-positive soil samples and two out of three *Legionella*-positive rainwater samples. Several other species were found including the pathogenic *Legionella gormanii* and *Legionella longbeachae.* Sequence types (ST) could be assigned to two *L. pneumophila* strains isolated from soil, ST710 and ST477, and one strain isolated from rainwater, ST1064. These sequence types were previously associated with Legionnaires' disease patients.

Conclusions: Rainwater and soil may be alternative sources for *Legionella.*

Significance and Impact of Study: The detection of clinically relevant strains indicates that rainwater and soil are potential sources of *Legionella* bacteria and future research should assess the public health implication of the presence of *L. pneumophila* in rainwater puddles and natural soil.

Introduction

Legionnaires' disease (LD) can be contracted after inhalation of aerosols containing *Legionella* (16). These bacteria are ubiquitous in the environment, in freshwater and in soil (16, 115, 318). *Legionella* bacteria have been shown to multiply intracellularly as parasites of free-living protozoa (58, 403). There are currently more than 50 known species of *Legionella* (404). *Legionella pneumophila* serogroup 1 is the cause of the majority of LD cases in Europe (167, 169) and the USA (168). *L. pneumophila* has been shown to replicate in water at temperatures between 25°C and 42°C, with an optimal growth temperature of around 35-37°C (405, 406). Known sources of *L. pneumophila*, often exhibiting these favourable growth temperatures, are, for example, cooling towers (228, 407), fountains (46, 47) and whirlpools (27, 41). In Australia and New Zealand, *Legionella longbeachae* is an important cause of LD (408). Potting soil has been described as a source of *L. longbeachae* (50, 52, 62, 164).

Infection with *Legionella* occurs both sporadically and in outbreaks, with most cases of LD being sporadic (16). For the majority of sporadic cases of LD, the source of infection remains unknown (409). One reason for this could be that in source investigations, pathogenic *Legionella* bacteria go undetected in potential sources because of the failure of the methods used for the detection of *Legionella* in environmental samples. The standard method of detection of *Legionella* in environmental matrices is culture on Buffered Charcoal Yeast Extract (BCYE) medium plates (173). These plates are easily overgrown by other bacteria in the sample, hence resulting in false negative results for *Legionella* bacteria. Furthermore, it is known that *Legionella* bacteria can enter a viable-but-not-culturable (VBNC) state and might therefore not be detected by culture methods using BCYE plates (128). A second explanation could be that infection has occurred through exposure to *Legionella* bacteria from sources that are not considered in source investigations. Viable *L. pneumophila* bacteria were recently detected in rainwater on asphalt roads (143, 357) and rainwater from pluvial floods (144). Rainwater may possible be an alternative source of infection, not yet targeted in routine source investigations. Several studies have reported an association between rainfall and LD. Hicks et al. (134) reported an association between increase in rainfall and increase in LD incidence. Fisman et al. (133) identified rainfall and increased humidity as predictors for the occurrence of LD. Karagiannis et al. (130) established that in the Netherlands, for the warmest period of the year (April-September), precipitation intensity, relative humidity and temperature were positively associated with LD incidence. Although the underlying mechanism that can explain this association has not yet been clarified, the results from these studies indicate that the natural environment may play an important role in exposure of humans to *Legionella*. Furthermore, natural soil has been described as a reservoir of *L. pneumophila* in Thailand and Japan (115, 316, 318, 357). Although potting soil is a known source of *L. longbeachae* (50, 52, 62, 164), it is yet unclear whether exposure to *Legionella* species in natural soil can lead to disease.

The aim of our study was to establish whether rainwater puddles on the road and natural soil near roads in the Netherlands could serve as reservoirs of *Legionella* and to investigate whether these potential sources harbour viable, pathogenic *Legionella* bacteria. We investigated the prevalence of *Legionella* spp. and *L. pneumophila* in rainwater puddles on roads in the spring and late summer of 2012. Based on more favourable growth conditions due to higher temperatures in summer, a higher prevalence was expected. Characteristics of the puddles and weather characteristics were also assessed in order to assess their relationship with *Legionella* prevalence. In late summer, we also investigated the prevalence of *Legionella* spp. and *L. pneumophila* in soil near roads. An amoebal coculture procedure was used for the detection of *Legionella* in the samples (144). Amoebal coculture has been successfully applied in previous studies to isolate *Legionella* bacteria that could not be recovered by plating on BCYE plates (144, 396, 410, 411).

Materials and Methods

Sampling

During two periods in 2012, water samples were taken from rainwater puddles on roads. In April and May, samples were collected at 43 different locations in the area around Utrecht, the Netherlands. From July to October, samples were taken at 40 locations that had also been sampled in the first period. GPS coordinates were used to document the locations of the rainwater puddles. An amount of approximately 125 ml water was scooped from puddles with sterile tubes or aspirated from the road surface with sterile syringes and then stored in sterile bottles. During the second period, soil samples were taken near roads at 20 locations where rainwater puddles had also been sampled. Within an area of 1 m^2, four subsamples from the upper 2 cm of soil were taken, mixed and stored in a sterile bottle. Water and soil samples were cooled during transportation and stored at 4°C until analysis. During sampling the following parameters were measured: ambient temperature, soil temperature, temperature of the rainwater in the puddle, size and depth of the puddle. In addition, pH, turbidity and electrical conductance of the sampled rainwater were measured.

Pre-treatment of the samples

Rainwater samples were concentrated by filtration of 100 ml of the sample through a 0.2 µm polycarbonate membrane filter (Merck Millipore, Darmstadt, Germany). After filtration, the membrane was placed in a sterile container with a layer of glass beads and 5 ml of the original sample. The container was placed in an ultrasound tank for 5 minutes in order to recover the organisms from the membrane. Not all samples could be filtrated due to the particulate content of the water. These samples were directly subjected to analysis. Of the soil samples, 5 g were resuspended in 5 ml of sterile distilled water. These suspensions were vortexed for approximately 10 seconds and allowed to stand at room temperature for

1 hour. Just before amoebal coculture (as described below), these soil suspensions were vortexed again.

Amoebal coculture method

The amoebal coculture method for detection of *Legionella* was performed as previously described by Schalk et al. (144): *Acanthamoeba castellanii* ATCC #30234 (American Type Culture Collection, Rockville, MD) was grown in 75-cm^2 culture flasks (Corning Inc., New York, NY) with 15 ml of peptone-yeast extract-glucose (PYG) broth (2% proteose peptone, 0.1% yeast extract, 0.1 M D-glucose, 4 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.1% sodium citrate dehydrate, 0.05 mM $Fe(NH_4)2(SO_4)_2.6H_2O$, 2.5 mM NaH_2PO_3, 2.5 mM K_2HPO_3) at 25°C. On the day of infection, the PYG broth was removed and the amoebae were resuspended in Page's amoeba saline (PAS) (396). The amoebae were washed three times with PAS followed by centrifugation at 850 X *g* for 10 minutes and subsequent resuspension of the pellet in 15 ml PAS. After the last resuspension in PAS, the number of cells was counted in a Bürker-Türk counting chamber. Cells were seeded in a 12-well microplate (Corning) at a density of 5×10^5 cells/ml of PAS. Per well, 1 ml of PAS with amoebae was added and this was inoculated with 100 µl of sample. Each sample was tested in triplicate (three wells). The amoebal plates were subsequently incubated at 32°C. As a negative control, one well with amoebae was not inoculated. After 3 days of infection, the amoebae were resuspended by pipetting the contents of each well up and down and 100 µl of each suspension was subcultured on a new plate with freshly seeded amoebae, prepared as described above. The plates were again incubated for another 3 days at 32°C. Subsequently, the content of each well was resuspended by pipetting up and down and was 10-fold serially diluted in PAS. Of the 10^3-, 10^4-, 10^5-, 10^6- and 10^7-fold dilutions, 100 µl was plated on BCYE plates (Oxoid Ltd., Hampshire, UK). Plates were incubated at 32°C. After 4 and 7 days of incubation, the BCYE plates were inspected for *Legionella*-like colonies, with a stereo microscope (magnification, 40 X; Olympus). Since 300 µl was analysed for each sample, the theoretical detection limit for unfiltrated water samples was $3.3*10^3$ CFU/L. The theoretical detection limit is the calculated detection limit, assuming that one bacterium in 300 µl can be detected. The filtrated water samples were 20 times concentrated, resulting in a theoretical detection limit of $1.7*10^2$ CFU/L. For the soil samples, 5 g was resuspended in 5 ml of sterile distilled water. Of this 5 ml, 300 µl was inoculated with amoebae and the theoretical detection limit was 3.3 CFU/g.

Confirmation and typing of *Legionella* colonies

Suspected *Legionella* colonies were tested for their inability to grow on BCYE medium without cysteine (Oxoid Ltd., Hampshire, UK). Strains unable to grow on media without cysteine were further identified by sequence analysis of part of the *mip* gene (144). To this end, colonies were frozen and subsequently diluted 1:10 in distilled water and heated in a heating block for 5 minutes at 95°C. A PCR was performed on 5 µl of this lysate with the

degenerated primers Mip-FP1 (5'-GAASARCAAATGAAAGAYGTTC-3') and Mip-RP8 (5'-CCAG-GRATAACTTGYGAWAC-3'). PCR was performed in a total volume of 50 µl PCR mixture consisting of 1×PCR buffer II (Roche, Almere, the Netherlands), 2.5 mM $MgCl_2$ (Roche), 0.2 mM dNTPs (Roche), 0.8 µM of each primer and 1.5 U Taq polymerase. The PCR program was as follows: 5' 95°C, 35 cycli of 30" 95°C, 30" 50°C, 30" 72°C, followed by 10' 72°C. The size of the PCR product (320 base pairs) was checked by DNA gelelectrophoresis on a 2% (w/v) agarose gel. PCR products were subsequently treated with ExoSAP (GE Healthcare, Diegem, Belgium). These PCR products were sequenced with a BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Applied Biosystems, Foster City, CA) with the Mip-FP1 and Mip-RP8 primers as forward and reverse primers. Sequences were analysed with BioNumerics software, version 6.6 (Applied Maths, Kortrijk, Belgium) and compared to *Legionella* sequences present in the GenBank database to subsequently type the isolated *Legionella* strains. Phylogenetic trees were constructed using the 320 nt sequence of the *mip* gene derived from the isolated strains and available sequences in the GenBank database, using the neighbour-joining (NJ) method, as implemented in the BioNumerics software. The accession numbers of the reference strains used in the phylogenetic tree are shown in Table 2. *L. pneumophila* strains were further genotyped by the standard sequence-based typing (SBT) method of the European Working Group for *Legionella* Infections (EWGLI) using seven genes (*flaA, pilE, asd, mip, mompS, proA,* and *neuA*) (88, 89, 412). The *L. pneumophila* strains were genotyped by our laboratory, as well as by the National *Legionella* Reference Laboratory. *Legionella* isolates were identified by polyclonal antisera, serogroup 1 and serogroups 2-14, coupled to latex beads (*Legionella* latex test, Oxoid Limited, Hampshire, UK). The identification of the separate serogroups of *L. pneumophila* was performed with monovalent antisera prepared by hyper-immunising rabbits with reference strains (Denka Seiken Co. Ltd., Tokyo, Japan).

Statistical analyses

The unpaired *t*-test was performed to evaluate statistically significant differences between the mean values of the following parameters in period 1 and period 2: temperature, pH, turbidity and electrical conductance of the puddles (Excel 2010, Microsoft, Schiphol, the Netherlands). Statistical significance was set at $p \leq 0.05$.

Results

Detection of *Legionella* in rainwater from puddles on the road

In this study, rainwater puddles on the road were sampled for *Legionella* during two periods. In the first sampling period, from 19 April 2012 to 23 May 2012, 46 puddles were sampled at 43 locations on five sampling days. Out of the 46 samples, nine were excluded from the results as cross-contamination occurred during analysis. Of the 37 rainwater

samples, 18 were filtrated. Filtration of the remaining samples was not possible due to particle content. *Legionella* was detected in 1 of 37 (2.7%) rainwater samples by the amoebal coculture method (Table 1). The isolated strains were typed as *Legionella gormanii* (Table 2, sample A).

In the second sampling period, which lasted from 30 July 2012 until 4 October 2012, 40 puddles were sampled on eight sampling days. These samples were taken at 40 of the 43 locations that were sampled in the first period. Road construction work at three of the 43 initially sampled locations prevented resampling at these sites. Of the 40 rainwater samples, 18 were filtrated. The remaining samples could not be filtrated due to particle content. *Legionella* was detected in 2 of 40 (5.0%) rainwater samples by amoebal coculture method (samples B and C). The strains from both samples were typed as *L. pneumophila* non-serogroup 1 (non-SG1). Three isolates originating from sample B could be further typed by SBT as sequence type (ST) 1064 (Table 2). The non-SG1 isolates from rainwater sample C did not yield a seven-allele profile, as the *neuA* gene could not be amplified (Table 2).

Table 1. Characteristics of the rainwater puddles and soil, sampled in periods 1 and 2

Period	1	2	2
Matrix	Rainwater	Rainwater	Soil
Total no. of samples	37	40	20
No. of *Legionella* spp.-positive samples (*L. pneumophila*-positive samples)	1 (0)	2 (2)	6 (2)
Puddle temperature (°C) Mean (range)	14.9 (8.3-24.4)	17.7 (12.3-24.4)	-
Soil temperature (°C) Mean (range)	-	-	17.7 (12.5-24.6)
pH Mean (range)	7.32 (6.29-8.02)	7.25 (6.69-8.75)	-
Turbidity (FTU) Mean (range)	228.1 (25.85-1468)	201.6 (9.98-1000)	-
Electrical conductance (µS) Mean (range)	143.5 (22.4-589)	105.6 (18.8-370)	-

– Not applicable.

Table 2. Serotyping and genotyping results of *Legionella* strains isolated from rainwater and soil samples

Rainwater sample	Soil sample	Date of sampling	*Legionella* spp.* (no. of colonies analysed)	Serotype† (no. of colonies analysed)	Sequence type or allelic profile‡
A		11 May	*L. gormanii* (2)	-	-
B		6 August	*L. pneumophila* (21)	7-14 (6)	1064
C		26 August	*L. pneumophila* (2)	7-14 (2)	5, 1, 22, 30, 6, 10, X
	D	23 August	*L. wadsworhtii* (3)	-	-
	D	23 August	*L. gratiana* (17)	-	-
	D	23 August	*Legionella* spp. (2)	-	-
	E	18 September	*L. gormanii* (43)	-	-
	F	3 October	*L. gormanii* (1)	-	-
	G	3 October	*Legionella* spp. (1)	-	-
	H	4 October	*L. pneumophila* (12)	1 (4)	6, 10, 23, 21, 33, 14, 15
	I	4 October	*L. feeleii* (13)	-	-
	I	4 October	*L. longbeachae* (4)	-	-
	I	4 October	*L. pneumophila* (16)	1 (2)	12, 10, 2, 5, 3, 17, 15
	I	4 October		1 (2)	12, 9, 2, 21, 3, 17, 39
	I	4 October		1 (1)	477
	I	4 October		1 (1)	nt
	I	4 October		3 (1)	710
	I	4 October		3 (4)	nt
	I	4 October		13 (1)	nt
	I	4 October		7-14 (1)	2, 10, 5, 10, 18, 14, 207
	I	4 October		7-14 (1)	2, 10, 14, 47, 18, 14, 207
	I	4 October		7-14 (1)	nt

– Not applicable. nt: Not further typed. **Legionella* reference strains: *L. gormanii* [GenBank: AF047748], *L. pneumophila* [GenBank: EU047264, EU047246], *L. wadsworthii* [GenBank: GQ265831], *L. gratiana* [GenBank: U92206], *L. feeleii* [GenBank: AF022340], *L. longbeachae* [GenBank: X83036]. *Legionella* spp. strains showed 87% or less similarity compared to *mip* sequences of known *Legionella* species present in the GenBank database. For the remaining strains, more than 92% similarity was observed with the reference strains, based on the 320 nt sequence of the *mip* gene. †7-14: One of the serogroups 7-14, exact serogroup not further determined. ‡If no ST could be assigned the allelic profile is provided, 'X' meaning that the gene target failed to amplify.

Characteristics of sampled puddles and weather characteristics

Table 1 shows the characteristics of the puddles in the two periods, such as temperature, pH and turbidity, as well as the temperature of the soil near the puddles. The water temperature was significantly higher in period 2 than in period 1 ($p<0.0024$). There was no significant difference in pH, turbidity or electrical conductance between the rainwaters sampled in period 1 and period 2. Table 3 shows the ambient temperature on the sampling

days as well as the mean temperature in the 14 days preceding the sampling days and the total precipitation in the 14 days before sampling. Of the 37 puddles in period 1 and 40 puddles in period 2, 64.9% (24/37) and 62.5% (25/40) respectively were completely located on the road, and 35.1% (13/37) and 37.5% (15/40) respectively were partly on the road and partly on the side of the road. The roads were constructed of asphalt (period 1: 24/37, period 2: 24/40), pavement (period 1: 11/37, period 2: 12/40), or a combination of asphalt and pavement (period 1: 2/37, period 2: 4/40).

The prevalence of viable *Legionella* spp. in rainwater puddles on roads appeared not to markedly differ between period 1 and period 2 (overall prevalence 3/77, 3.9%). The three positive samples had not been filtrated before analysis. The *Legionella* prevalence was too low to draw any conclusions about the association with puddle or weather characteristics. The three positive rainwater samples were all completely located on the road, but the roads were constructed of different materials. The puddles had different sizes and depths, and they were situated at different locations.

Detection of *Legionella* in soil

During period 2, soils near roads were sampled at 20 locations. *Legionella* spp. were detected in 6 out of 20 (30%) soil samples by amoebal coculture (Table 1). *L. pneumophila* serogroup 1 (SG1) was detected in two of the samples. For one of these samples (sample H), the four analysed strains had an identical but unknown allelic profile (Table 2). The other sample (sample I) contained a mixture of different *L. pneumophila* SG1 strains and non-SG1 strains. Typing of the *L. pneumophila* SG1 strains yielded two unknown allelic profiles and one known profile, namely ST477. Typing of the *L. pneumophila* non-SG1 strains also yielded two unknown profiles and one known profile for a SG3 strain, namely ST710. In addition, *Legionella feeleii* and *L. longbeachae* were detected in this sample. In another soil sample (sample D), a mixture was found of *Legionella wadsworthii, Legionella gratiana* and an undefined *Legionella* species, for which the sequence of the *mip* gene did not match *Legionella* sequences present in the NCBI database. In two samples (samples E and F) *L. gormanii* was detected, and the sixth sample (sample G) contained an undefined *Legionella* species.

Discussion

The use of amoebal coculture enables studies of complex environmental matrices that may represent yet unknown sources of *Legionella*. In this study, *Legionella* was isolated from rainwater puddles and soil using an amoebal coculture method with *A. castellanii* cells. For the rainwater samples, also a direct plating method on Glycine-Vancomycin-PolymyxinB-Cycloheximide (GVPC) medium plates was used. However, no *Legionella* bacteria could be isolated. Other bacteria from the samples probably inhibited the growth

of *Legionella*. Heat and acid treatments of the samples hardly reduced the presence of other microorganisms on the culture plates (data not shown).

Table 3. Ambient temperature and precipitation at and before sampling days of rainwater puddles*

Sampling date	No. of rainwater samples (positive/total)	No. of soil samples (positive/total)	Mean T (°C) sampling day	Mean T (°C) preceding days	Total precipitation (mm) preceding days
19 April	0/7		8.9	6.9	24.9
22 April	0/10		8.8	7.5	29.1
9 May	0/8		15.7	11.7	28.7
11 May	**1/7**		13.8	12.6	42.8
23 May	0/5		20.3	13.5	34.9
30 July	0/5		15.4	17.7	45.6
6 August	**1/6**		17.2	18.7	48.6
8 August	0/2		16.9	18.2	58.6
23 August		**1/6**	18.1	19.9	1.6
26 August	**1/7**	0/2	16.0	20.3	9.4
30 August	0/3	0/4	15.8	19.6	33.5
18 September	0/7	**1/2**	12.8	15.8	8.6
3 October	0/9	**2/3**	12.8	12.3	41.4
4 October	0/1	**2/3**	11.6	12.6	36.3

*The number of *Legionella*-positive samples per total rainwater or soil samples that were collected is indicated for each sampling day in columns 1 and 2 respectively. The mean ambient temperature (°C) on the sampling day, the mean T (°C) in the 14 days preceding the sampling day and the total precipitation (mm) in the 14 days preceding the sampling day are indicated (414). The bold values indicate the samplings that contain at least one positive sample.

Two *L. pneumophila* non-SG1 strains were detected in rainwater. One of these strains, ST1064, has previously been isolated from two patients in the Netherlands in 2011 (413). The prevalence of *Legionella* spp. in rainwater puddles during spring and late summer was low in comparison to other studies (3.9%). Schalk et al. (144) detected *Legionella* in 4 out of 13 (31%) pluvial floods by amoebal coculture, sampled in June and August 2011. Two out of 13 samples (15%) contained *L. pneumophila*. The pluvial flood samples were all taken after heavy rainfall. *Legionella* in rainwater puddles may possibly originate from surrounding soil, and the higher prevalence in pluvial floods could be explained by the fact that water enters pluvial floods by runoff from large surfaces of surrounding soil. In the current study, the number of positive samples was too small to be able to draw any conclusions about the effect of rainfall intensity on *Legionella* prevalence. Another explanation for the lower prevalence in rainwater samples could be that *Legionella* bacteria in shallow rainwater

puddles are subject to more UV light than in pluvial floods, inactivating the bacteria (415). Furthermore, prevalence of *Legionella* could be affected by temperature. In a study by Sakamoto et al. (143), *Legionella* prevalence was related to the mean ambient temperature on the sampling date. Three out of 19 samples (16%) were positive at temperatures below 20°C, 6 out of 14 samples (43%) were positive at temperatures between 20 and 25°C, and 7 out of 12 samples (58%) were positive at temperatures above 25°C. In our study, the mean measured ambient temperature on sampling days varied between 8.8 and 20.3°C. These relatively low temperatures could explain the low prevalence of *Legionella* in our study; however, similar ambient temperatures were measured in June and August 2011, when pluvial flood samples were studied and a higher *Legionella* prevalence was found by Schalk et al. (144). In contrast to the study by Sakamoto et al. (143), Kanatani et al. (357) found the prevalence of *Legionella* in puddles not to be related to ambient temperature on the date of sampling. Kanatani et al. (357) analysed puddles on roads for the presence of *Legionella* spp. by culture at six fixed locations in Japan. Throughout the year, samples were taken monthly and *Legionella* spp. were detected at temperatures ranging from -0.6°C to 32.2°C.

Sakamoto et al. (143) analysed rainwater that was collected directly from the air but did not detect any *Legionella* bacteria by culture. However, *Legionella* DNA was detected in the samples and possibly viable-but-not-culturable *Legionella* bacteria are present in raindrops (143). *Legionella* bacteria were previously isolated from rainwater collected from roofs (269, 416). However, it is not clear from these studies whether *Legionella* were already present in the raindrops or were present on the roof surfaces and were washed off by rain.

In soil samples, the prevalence of viable *Legionella* spp. and *L. pneumophila* (30% and 10% respectively) was higher than in rainwater puddles. There was also a greater diversity of *Legionella* strains in the soil samples. Two of the soil samples contained a mixture of *Legionella* spp. and from one of these samples at least six different *L. pneumophila* strains were isolated. Strain ST477 has been previously isolated from 5 patients in the Netherlands, Italy, Austria, the UK and Germany, according to the EWGLI SBT-database (413). Strain ST710 has been previously isolated from two patients in Canada and Germany. To our knowledge, our study is the first to detect *Legionella* bacteria in natural soil in Europe. Evstigneeva et al. (417) tested 11 soil samples by amoebal coculture from the city of Marseilles, France, but did not detect *Legionella*. Two recent studies described the isolation of clinically relevant *L. pneumophila* strains from natural soils in Thailand and Japan. Travis et al. (318) studied the prevalence of *Legionella* spp. in soil in Thailand and found 22 out of 39 (56.4%) samples, taken at eight rural sites, positive for *Legionella* spp. Of a total of 115 isolates, 17 typed as *L. pneumophila*. For eight strains allelic profiles were identified by SBT, and one identified ST had been previously associated with community-acquired cases. In a study by Amemura-Maekawa et al. (115), 35 *L. pneumophila* isolates from soils in Japan were typed by SBT. Eleven different STs were assigned, and nine of these STs had previously been detected in

clinical isolates. Our study and the studies by Travis et al. (318) and Amemura-Maekawa et al. (115) provide evidence that natural soil may serve as a reservoir for *L. pneumophila.* However, it is yet unknown whether humans are exposed to *Legionella* from natural soil.

Potting soil has been described as a source of *L. longbeachae* causing Legionnaires' disease (50, 52, 62). However, there is little evidence that exposure to *L. pneumophila* strains in natural soil may cause infection and possibly disease. Only Wallis and Robinson (252) reported on a case in Australia where the same *L. pneumophila* SG1 pulsovar was found in soil and in the patient. The soil was sampled from a field where the patient had spent several days potting plants.

Whether and how people are exposed to *Legionella* in natural soil and rainwater is unclear. During handling of soil, i.e., digging the ground, soil particles with *Legionella* bacteria may possibly be aerosolised and aerosols inhaled. Wind could also be a mechanism for aerosolization of soil particles (64), and it may be that people acquire LD by inhalation of aerosolised soil particles from the environment. *Legionella* in rainwater puddles could be aerosolised by splashing or by wind.

In conclusion, soil and rainwater may be alternative sources for *Legionella,* especially in periods when conditions for growth and aerosolization of *Legionella* are favourable, such as elevated temperature and relative humidity. The public health implication of the presence of *L. pneumophila* in natural soil and rainwater puddles is yet unclear and should not be overlooked. The *Legionella* concentration in rainwater puddles and soil and the rate of aerosolization should be quantified and new studies should focus on the effects of environmental conditions on the viability, growth and virulence of *Legionella* in rainwater and diverse soil types. Risk of infection from exposure to *Legionella* in the natural environment can be estimated by means of quantitative microbial risk assessment (QMRA), highlighting the need for estimations of exposure levels and dose-response relations (418).

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6.

Effect of soil characteristics on the presence and persistence of *Legionella* bacteria

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Abstract

The relationship between soil characteristics of natural soils and the presence and persistence of *Legionella* bacteria was investigated. Furthermore, two possible transmission routes of *Legionella* from soil to humans, i.e., aerosolization of dry soil and of water from rainwater puddles followed by inhalation, were hypothesized upon. *Legionella* spp. were isolated from natural soils by amoebal coculture and a relationship was found between *Legionella* presence and soil type. Compared to *Legionella* negative soils, the *Legionella*-positive ones contained an enhanced level of clay and silt, in relation to sand. *Legionella pneumophila* persisted in spiked clayey and sandy soils over a period of up to 78 days, but did not survive after 7 days in soil under low soil moisture conditions. The clayey soil was more favorable for *Legionella* survival than the sandy soil. It was found that *L. pneumophila* was transferred from sandy soil to a water layer on the soil, mimicking a rainwater puddle, showing that *Legionella* can potentially move to a compartment relevant for aerosolization.

Importance

Soil is a reservoir of *Legionella* bacteria, and therefore natural soil may constitute an infection source of Legionnaires' disease, a fact that is not yet considered in source investigations. We showed that *Legionella* presence and persistence are related to soil type and these findings help to focus further studies on soil as an infection source. Studies are needed to establish if aerosolization of *Legionella* from rainwater puddles or directly from (dried) soil is possible. Future research should assess the public health implication of the presence of clinical relevant *Legionella* species in natural soil.

Introduction

Legionella is an opportunistic pathogen that causes Legionnaires' disease (LD) and Pontiac fever in humans (16). LD is a severe disease involving pneumonia. Pontiac fever is a far milder infection with flu-like symptoms. *Legionella pneumophila*, predominantly *L. pneumophila* serogroup 1 (SG1), is the most common pathogenic species seen in patients, in Europe (419, 420) and the United States (168). In Australia, New Zealand and Thailand, *L. longbeachae* is an important cause of LD, associated with exposure to potting soil (157, 166, 171, 421). *Legionella* bacteria are ubiquitous in the natural environment, in soil and water, where they can thrive intracellularly within protozoa (14, 16). Natural matrices and man-made water systems colonized with *Legionella* bacteria, such as cooling towers and whirlpools, can act as sources of infection (40, 41, 45, 209). After inhalation of *Legionella* -infested aerosols that are spread from a source, *Legionella* can infect macrophages in the lungs of humans and cause disease (16).

Over the past five years (2012-2016), between 173 and 324 LD cases (non-travel related) were annually identified in the Netherlands (422). Only a small part of these *Legionella* infections occurred in clusters or outbreaks; most cases of LD were sporadic. The source of infection of such sporadic LD cases is almost never identified (393). *L. pneumophila* strains that are clinically common in the Netherlands are only rarely detected in frequently investigated environmental sources, such as cooling towers, spa pools and domestic water distribution systems. The same phenomenon is experienced in England and Wales and Canada (110, 113). One explanation for the discrepancy between the distribution of clinical and environmental *Legionella* strains is that in source investigations, pathogenic *Legionella* bacteria go undetected because of the failure of detection of *Legionella* from environmental sources, via culture on buffered charcoal yeast extract (BCYE) medium plates (the current standard method of detection). Another explanation for the discrepancy is that infection possibly occurs by *Legionella* bacteria from sources that have not yet been considered in source investigations.

Soil is a reservoir of *Legionella* bacteria and, possibly, natural soil constitutes an infection source of LD (393). *L. pneumophila*, among other *Legionella* species, has been detected in natural soil in several studies (115, 316, 318, 395 , 423). Among the soil-borne *L. pneumophila* strains, clinically relevant sequence types (STs) were identified (115, 318, 395). Furthermore, in one study, a sporadic infection with soil-borne *L. pneumophila* was described (252). The case had been working on a field in the week prior to illness, and so a soil origin of the infectious agent was suspected. In a study by Schalk et al. (394) it was hypothesized that a garden soil-borne *L. pneumophila* strain had contaminated a whirlpool in a garden, which was involved in an outbreak of LD and Pontiac fever.

At present, the extent to which a transmission route of *Legionella* from soil exists and poses a threat to human health is unknown. As mentioned, possibly humans could be indirectly exposed to soil-borne *Legionella* through contamination of water systems like a whirlpool. We here hypothesize that there are two other possible routes of transmission. The bacterium *Coxiella burnetii*, the causative agent of Q fever in humans, is very likely spread via aerosolised dust particles (424, 425). Likewise, soil-borne *Legionella* might be transferred to humans via aerosolization of dried soil. Aerosolization of contaminated soil particles could be caused by wind or another disturbance of the soil, like digging. Furthermore, when soil-borne *Legionella* bacteria are transferred, by water flow, from soil to a rainwater puddle, aerosolization of *Legionella* bacteria could take place when the puddle is disturbed, for example by intense rainfall or a car driving through. Rainwater puddles as possible infection source have been studied before (143, 357, 395), and *Legionella* bacteria have been found to be present in rainwater puddles. However, there is no evidence for their transmission from puddles to humans and the origin of the bacteria in the puddles is unclear.

In a previous study, we established that natural soil near roads, at locations where rainwater puddles had formed, and garden soil constitute reservoirs of viable *L. pneumophila* and *Legionella* spp. (395, 426). In the current study, we investigated if there is a relationship between soil characteristics, pH, soil humidity, granular composition and organic content and the presence of *Legionella* in natural soils. Furthermore, we studied if *L. pneumophila* can persist in different soil types over time. Also, in order to investigate possible transmission routes of *Legionella* bacteria from soil to humans, we tested if *L. pneumophila* is transferred from soil to a water layer on the soil, and if it is able to survive in soil under low moisture conditions.

Materials and Methods

Sampling of natural soils

Soils were sampled on six days in July and August 2013. In total eighty sampling locations were selected by random choice of geographical coordinates in the province of Utrecht, the Netherlands. The coordinates were chosen via a digital map, excluding urban areas. On every sampling day, an equal number of samples were taken. Within an area of 1 m^2, four subsamples were taken with a sterile spoon from the upper 2 cm of soil (including the surface layer), mixed and stored in a sterile jar. Samples were taken from both shadowed and bare areas. The soil samples were cooled during transportation and stored at 4°C until analysis. During sampling the ambient temperature and soil surface temperature were measured. In addition, pH, soil humidity and organic content (weight loss on ignition) were determined for all samples. The granular composition (percentage sand, silt and clay

content) of the soils was determined (conform NEN 5753 (192)) for 54 of the 80 samples. The volume of the remaining soil samples was insufficient for the analyses. The soils were classified according to the USDA soil texture classification system (37). An amoebal coculture method was applied to isolate *Legionella* bacteria.

Amoebal coculture method

Five gram of each natural soil sample was resuspended in 5 ml of Page's amoeba saline (PAS) (396) and vortexed for approximately 10 seconds. The suspensions were incubated at room temperature for 1 hour and subsequently vortexed shortly before amoebal coculture analyses. The amoebal coculture method for detection of *Legionella* was performed as described previously (395). Briefly, *Acanthamoeba castellanii* ATCC #30234 (American Type Culture Collection, Manassas, VA, USA) was cultured in 75-cm^2 culture flasks (Corning Inc., New York, NY, USA) with peptone-yeast extract-glucose (PYG) broth at 25°C. Prior to the infection, the PYG broth was removed and the amoebae were resuspended in 15 ml PAS (396). The amoebae were washed three times by centrifugation for 10 minutes at 850 X *g* and resuspension of the pellet in PAS. After the third washing step the amoebal suspension was diluted with PAS to a density of 5×10^5 cells/ml. The cells were seeded in a 12-well microplate (Corning) at a volume of 1 ml per well. Per well, 100 µl of soil sample was added. Each sample was tested in triplicate (three wells). As a negative control, one well with amoebae was not inoculated with a sample. After 3 days of incubation at 32°C, 100 µl of each well was subcultured on new microplates with freshly seeded amoebae. After another 3 days of incubation at 32°C, 100 µl of each well was 10-fold serially diluted in PAS. Of the 10^4- to 10^8-fold dilutions, 100 µl was plated on BCYE medium plates. The BCYE plates were incubated at 37°C and after 4 and 7 days inspected for *Legionella*-like colonies with a stereo microscope (magnification, 40 X; Olympus). The theoretical detection limit of this analysis was 3.3 CFU/g soil, as 300 µl of the suspension, containing 0.3 g soil, was inoculated with amoebae.

***L. pneumophila* persistence study in moist and dried soil**

To study persistence of *Legionella* in soil, two different soils, a clayey and a sandy soil, were spiked with a *Legionella* suspension and incubated at 25°C. The sandy soil came from the organic experimental farm Droevendaal (Wageningen University and Research, the Netherlands, sampling site 'Wildekamp'), from a grass field that had not been cultivated for several years (sandy loam, pH 4.7, organic content 3.9% (427)). The clayey soil was sampled from a dike and characteristics of the soil were not further assessed. All roots, stones or other debris were removed from the soils prior to the experiment. For each soil type, six samples were spiked and incubated in parallel (see Figure 1). Of the six samples, three samples were kept at constant soil moisture content and three samples were left uncontrolled. Furthermore, for each soil type, a seventh sample was not spiked with *Legionella* and served as a negative control.

Prior to the experiment, the moisture content of the two soils was determined by the oven-drying method (24h drying at approximately 105°C). Using the moisture content data, seven 300 g dry weight portions were prepared per soil. A previously isolated soil-borne *L. pneumophila* strain (ST477) (395) was grown overnight in Yeast Extract Medium (YEM) with BCYE supplement (Oxoid Ltd., Hampshire, United Kingdom). The bacterial suspension was serially diluted (10-fold) in PAS and plated on BCYE medium plates (Oxoid Ltd., Hampshire, United Kingdom) in order to calculate the *Legionella* concentration of the suspension afterwards. For each of the two soils, six portions were spiked with 3 ml of the 10^{-2} diluted *Legionella* suspension and thoroughly mixed. The target end concentration of *Legionella* in soil was 10^5 CFU/g (dry weight). To facilitate the mixing, also 10.5 ml PAS was added. Mixing was performed as follows; the soil, the bacterial suspension and the PAS were combined in a sterile plastic bag. The bag was sealed and the soil in the bag was manually kneaded for exact 5 minutes. Three ml of sterile water was added to the seventh soil portion that served as a negative control. The (calculated) soil humidity of the sandy soil, based on dry weight, was 11.6% before spiking and 16.2% after spiking. For the clayey soil, the humidity was 27.2% before and 31.8% after spiking.

The fourteen prepared soil portions were placed in sterile plastic jars with a volume of 1 L. All filled jars were weighed before the start of the experiment. All soils were incubated at 25°C for 78 days total. Samples that were kept at stable soil moisture content over the experiment were sprayed with sterile water every three days. The added water compensated the weight loss of the jars over three days. The samples for which the moisture content was left uncontrolled were also weighed at every sampling and the measured weight loss was used to calculate the decline in soil humidity. All jars were stored without lid in bigger containers with lid. The humidity level in the containers that held the jars that were kept at a constant moisture content was kept high by adding paper towels that were drenched with sterile water.

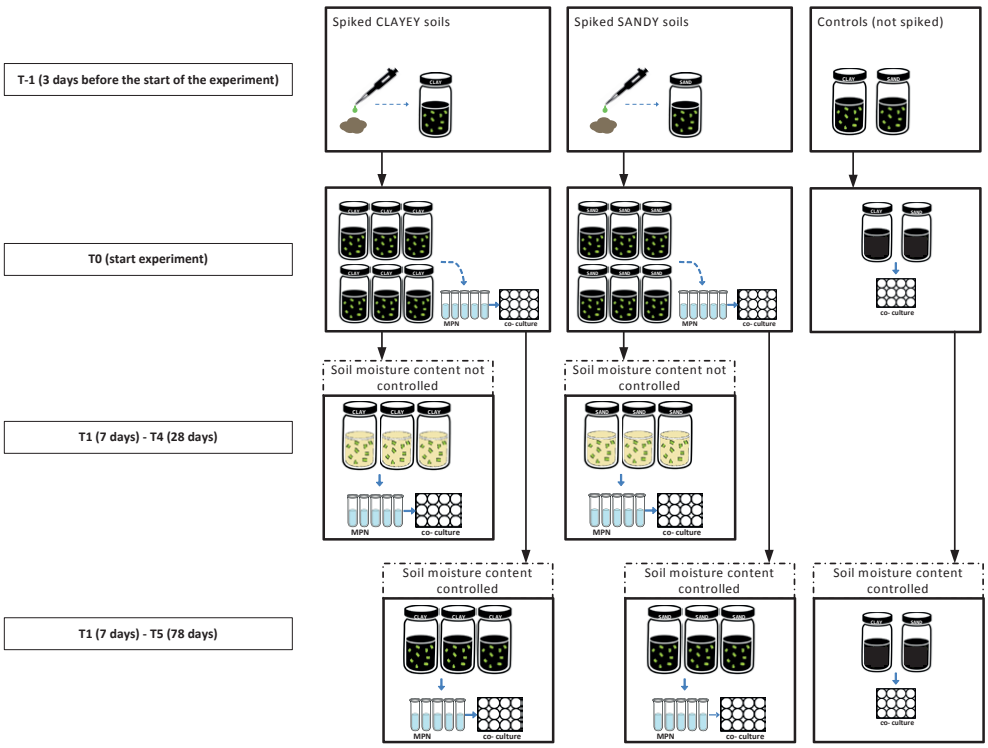
Sampling of the soils started three days after spiking (t=0, see Figure 1). All of the spiked soils and the negative control soils were sampled. A sample of approximately 5 g was collected by scraping or scoping soil from different places of the top layer of soil. These samples were used to determine the starting quantity of *Legionella* bacteria per g clayey or sandy soil. Subsequently, soil samples were taken on t=7, t=14, t=21, t=28 and t=78 days. *Legionella* was quantified by amoebal coculture PCR MPN method.

Amoebal coculture PCR MPN method for quantitation of *L. pneumophila* in the persistence study

Each soil sample (5 g) from the persistence study was resuspended in 5 ml of PAS and vortexed for approximately 10 seconds. The suspensions were incubated at room temperature for 1 hour and subsequently vortexed shortly before amoebal coculture analyses.

The method for detection by amoebal coculture was mostly similar as for the detection of *Legionella* in natural soil (as described above), except for that a most probable number (MPN) method was used to estimate the concentration *Legionella* bacteria in the samples. The suspended soil samples were serially diluted (10-fold) and of four dilutions, three times 100 µl was inoculated with the amoebae suspension. Instead of culturing on BCYE plates, qPCR was used for confirmation of *Legionella* presence. Samples were analyzed before and after the coculture incubation period to establish if growth of *Legionella* occurred during the inoculation with amoebae. Growth indicates that viable *Legionella* were present in the soil sample. Of each well, a 100 µl sample for qPCR was taken immediately after inoculating the soil samples with the amoebae (t=0) and after 6 days of incubation (t=6). Each qPCR sample was kept at -20°C until qPCR analysis. As a positive control in the amoebal coculture PCR MPN method, a *L. pneumophila* strain was used that was previously isolated from soil. This strain was cultured and diluted portions of the culture were frozen until used in the amoebal coculture PCR MPN method. One well with amoebae suspension was inoculated with this *L. pneumophila* strain.

Figure 1. Experimental setup *L. pneumophila* persistence study



qPCR detection of *Legionella*

The qPCR samples, taken before and after the incubation period on amoebae (t=0 and t=6), were heated to 100°C for 10 minutes to release DNA. The following primers and probe were used, LPneuF (5' CCGATGCCACATCATTAGC 3'), LPneuR (5' CCAATTGAGCGCCACTCATAG 3'), LPneuP (5' TGCCTTTAGCCATTGCTTCCG 3'), targeting part of the *Legionella mip*-gen (NEN 6254 (189)). PCR amplification was performed with a LightCycler® 480 (Roche Molecular Systems, Inc., Pleasanton, California). The reaction mixture contained 1,1 µl 20 µM of each primer, 0,7µl 20 µM of the probe, 10µl LightCycler® 480 Probes Master mix (Roche Molecular Systems, Inc., Pleasanton, California), and 5 µl of sample in a total volume of 20 µl. Amplification began with denaturation and polymerase activation at 95°C for 10 minutes, followed by 45 cycles of 15 seconds at 94°C, and 50 seconds at the annealing temperature (60°C).

Release of *Legionella* from soil to water

To investigate release of *Legionella* from soil to water, a *Legionella* suspension was prepared as described above. A sandy soil and a clay soil were spiked with 1 ml of the 10^{-1} diluted suspension per 100 g (dry weight). The target end concentration of *Legionella* in the soils was 10^6 CFU/g (dry weight). The spiked soils were incubated over 3 hours and then divided in portions of 100 g (dry weight) over eight sterile jars (150 ml) for each soil type. Also four jars were filled with unspiked sandy and clayey soil. The soils were pressed to achieve a bulk density of 1.2 g/cm^3 for the sandy soil and of 1.25 g/cm^3 for the clayey soil. To mimic a water puddle, rainwater was carefully added to each jar, until the soil was completely saturated and a water layer of approximately 13 mm formed on the soils. The rainwater had been collected in sterile bins, during several heavy rain events. The rainwater was tested for the presence of *Legionella* bacteria by culture on BCYE-plates, but no *Legionella* were detected. The pH of the rainwater was 6.2. At the start of the experiment (t=0), a 100 µl water sample was taken from the water layer on the soil, without disturbing the soil and with minimal disturbance of the water layer. Samples were also taken after t=30, 60, 120 and 180 minutes. The samples were frozen and analyzed by qPCR as described above. The whole experiment was conducted at room temperature.

Confirmation and typing of *Legionella* colonies isolated from natural soil

As *Legionella* bacteria have growth dependence for the amino acid L-cysteine, suspected *Legionella* colonies, detected after 4/7 days incubation on BCYE-plates, were tested for their inability to grow on BCYE medium without cysteine (Oxoid Ltd., Hampshire, United Kingdom). Strains unable to grow on media without cysteine were further identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Statistical analyses

The unpaired *t*-test was performed to evaluate statistically significant differences between the mean values of the following sampling and soil parameters for *Legionella*-positive and *Legionella*-negative samples: ambient temperature, soil surface temperature, pH, humidity, organic content and granular composition (Excel 2010, Microsoft). Statistical significance was set at $p \leq 0.05$. To classify soil samples into soil textural classes, based on granular composition, the R-package 'soiltexture' (428) was used in R (version 3.3.0) (429).

For the persistence study, presence of *L. pneumophila* was scored as either present or absent in a titration series per sample. Die-off rates, and differences in die-off rates between soil types with and without drying, were examined following an MPN-approach in a Bayesian setting with the presence or absence of growth as the response variable using JAGS through the 'rjags'-package in R (429, 430). *Legionella* concentrations at time t (in days) were assumed to be homogenously (i.e., Poisson) distributed in the samples and assumed to follow a monophasic exponential decay with decay rate λ (431). This decay rate was modelled to be a linear function of soil type, drying and experimental jar. Variables significantly influencing die-off were selected from this model through backward selection. Variables that were 95% probable to have a parameter value of zero excluded from the credible interval (i.e., the variable is highly likely associated with die-off) were retained in the final model. Vague priors were used as a priori information in the Bayesian analysis: N (0, 10000) for the decay rate parameters and Gamma (0.001, 0.001) for the *Legionella* concentration at time t=0 and for the standard deviation of the concentration. The simulated Markov chains (n=3) consisted of 100,000 iterations, of which the first 1,000 were discarded for burn-in.

Results

In this study, the relationship between soil characteristics and the presence of *Legionella* in natural soils was investigated. Furthermore, the persistence of *L. pneumophila* in two different soils, a clayey and a sandy soil, over time was experimentally tested. Finally, in order to investigate possible transmission routes of *Legionella* bacteria from soil to humans, it was tested if *L. pneumophila* was able to survive in soil with low moisture content and if *L. pneumophila* was transferred from soil to a water layer on the soil.

Relationship between soil characteristics and presence of *Legionella* in natural soils

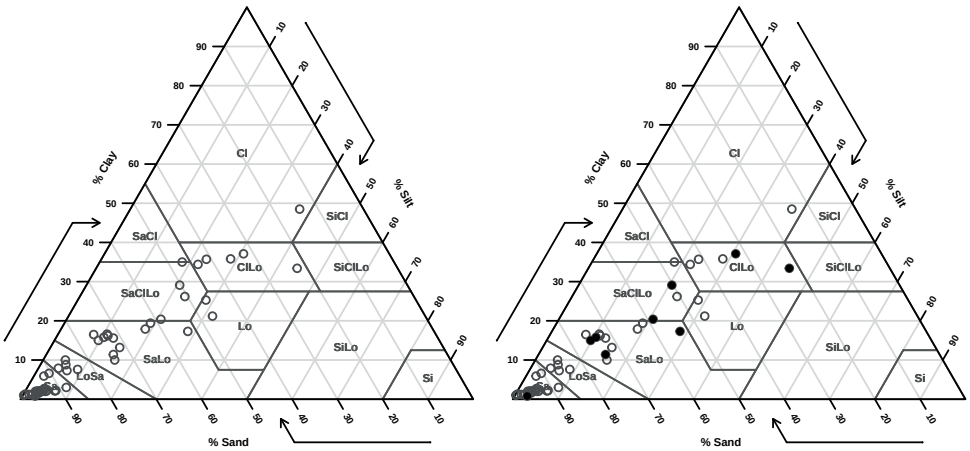
In the summer of 2013, a total of 80 randomly selected natural soil samples were collected from the area of the province of Utrecht, the Netherlands. Table 1 shows the characteristics of several sampling and sample parameters. Figure 2a shows a triangle plot with soil textural classes based on the USDA classification system (432). The soil samples were classified according to their granular composition (fractions sand, silt and clay particles)

and represented in the plot. Granular composition data were available for 54 of the 80 soil samples. The samples were classified as sand (25 samples), loamy sand (5 samples), sandy loam (12 samples), sandy clay loam (3 samples), loam (2 samples), clay loam (5 samples) and clay (1 sample). For one sample, the classification was ambiguous; it could be classified as sandy clay or as sandy clay loam.

Table 1. Soil sampling and sample parameters for all samples, for *Legionella*–negative samples and for *Legionella*–positive samples

	Total samples (n=80) mean (range)	*Legionella* spp. negative samples (n=68) mean (range)	*Legionella* spp. positive samples (n=12) mean (range)	*p*=
Ambient temperature (°C)	23.3 (16.2-29.9)	23.4 (16.2-29.9)	23.2 (17.4-27.9)	0.84
Soil surface temperature (°C)	22.0 (15.6-28.1)	22.3 (15.6-31.4)	22.9 (19.4-27.6)	0.47
pH	6.7 (3.5-8.6)	6.6 (3.5-8.6)	7.2 (5.4-8.6)	0.13
Soil humidity (%)	19.2 (0.8-60.2)	18.9 (0.8-60.2)	20.7 (1.2-48.6)	0.65
Organic content (%)	8.9 (1.3-28.9)	8.7 (1.3-28.9)	10.2 (3.3-23.4)	0.42

Figure 2. Soil texture triangle plot



A) All soil samples (circles), B) Soil samples positive for *Legionella* in black. Cl: clay, SiCl: silty clay, SaCl: sandy clay, ClLo: clay loam, SiClLo: silty clay loam, SaClLo: sandy clay loam, Lo: loam, SiLo: silty loam, SaLo: sandy loam, Si: silt, LoSa: loamy sand, Sa: sand.

From twelve of the 80 samples (15%) *Legionella* spp. could be cultured. For six of the 12 *Legionella*-positive samples, *Legionella* could not be typed further by MALDI-TOF MS because the bacteria could not be re-cultured. The following *Legionella* species were detected: *Legionella bozemanii* (3 samples), *Legionella dumoffii* (1 sample), *Legionella feeleii* (1 sample), *Legionella gormanii* (1 sample), *Legionella wadsworthii* (3 samples), *L. non-pneumophila* (4 samples). In three samples, more than one species was found. None of the samples contained *L. pneumophila.* The soil samples positive for *Legionella* had a higher pH value (mean 7.2 [5.4-8.6] vs. 6.6 [3.5-8.6]), and were on average more humid (mean 20.7% [1.2-48.6] vs. 18.9% [0.8-60.2]) and higher in organic content (mean 10.2% [3.3-23.4] vs. 8.7% [1.3-28.9]) compared to the negative samples, but differences were not statistically significant (see Table 1).

Figure 2b shows the *Legionella*-negative and *Legionella*-positive samples in the soil textural triangle. The positive samples were classified as sandy loam (4 samples), sandy clay loam (2 samples), clay loam (2 samples) and sand (1 sample). Looking at the granular composition data, negative and positive soil samples were significantly different (see Table 2). Soils in which *Legionella* spp. were detected contained more soil particles within the lower size range (<50 µm, i.e., silt and clay particles) in relation to particles from the higher size range (>50 µm, i.e., sand particles). On average 40.9% of the weight of *Legionella*-positive soils consisted of particles smaller than 50 µm. Of the negative samples, on average 20.3% of the volume contained these small particles.

Table 2. Comparison of the granular composition of *Legionella*–negative versus *Legionella*–positive samples*

Soil particle size classes	*Legionella* spp.-negative samples (n=45)†	*Legionella* spp.-positive samples (n=9)†	*p*=
<2 µm	10.7	20.2	0.03
2 - 50 µm	9.6	20.7	0.003
>50 µm	79.7	59.1	0.01

*The data in the table reflect the mean volume of soil consisting of soil particles from a certain particle size class (%). †The volume of part of the soil samples was not sufficient for determination of the granular composition.

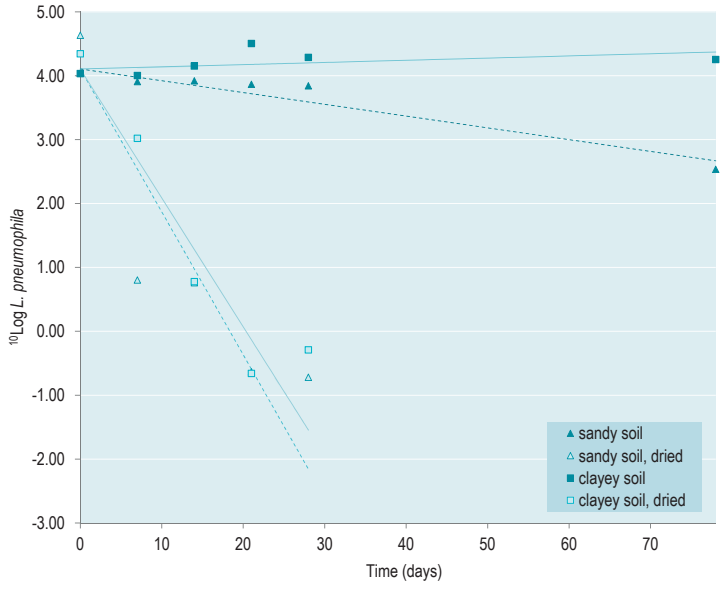
L. pneumophila persistence in moist and dried soil

L. pneumophila suspensions with $1.6*10^7$ CFU/ml and $1.3*10^7$ CFU/ml respectively, estimated from plate counts, were used to spike the sandy and clayey soil samples. As 300 g dry weight of the sandy and clayey soil samples were spiked with 3 ml of these suspensions, the theoretical *Legionella* concentration in sandy soil was $1.6*10^5$ CFU/g dry weight (=5.2 log10 units) and in clayey soil $1.3*10^5$ CFU/g dry weight (=5.1 log10 units). On t=0, the concentration of *Legionella* in the spiked soils was determined experimentally with the amoebal coculture PCR MPN method (see Figure 1). The mean concentration of *Legionella* in the soils was $1.3*10^4$

CFU/g (95% CI is $1.3*10^3$-$1.6*10^4$ CFU/g). The concentration of *Legionella* in clayey soil ranged from 3.96 to 4.63 log10 units CFU/g soil. *L. pneumophila* was not detected in the unspiked clayey and sandy soils that served as negative controls, at any time point.

The statistical analysis showed no differences in decay rates between the three experimental jars of both soil types and this variable was removed from the model. Both the soil type and drying influenced the die-off rate (see Figure 3), with $p<0.001$ that 0 is included in the credible interval. In the spiked soils that were kept at stable soil moisture content, *L. pneumophila* was detected over a period of 78 days. Prior to incubation (t=0), the threshold cycle values were >38 (the highest threshold cycle value measured for a serially diluted *L. pneumophila* suspension), and after incubation (t=6) these were 25-29. For clayey soils, the *Legionella* population was stable over time. In the sandy soils, the *Legionella* concentration decreased with approximately 1.4 orders of magnitude over 78 days. In the spiked soils without moisture control, *L. pneumophila* was not detected in the sandy soils from t=7 days and on. The (calculated) soil humidity declined from 16.2% to 10.3% over 7 days. The humidity declined further, in an exponential way, to 5.4% from day 28 until day 78. For clayey soils the *Legionella* concentration declined from t=0 to t=7 and the organism was not detected at later measurements. The soil humidity declined from 31.8% to 14.9% over 7 days and to 7.6% after 28 days.

Figure 3. Concentration of *L. pneumophila* in spiked sandy and clayey soils over a study period of 78 days, at 25°C, with and without moisture control (dried)*



*Plot markers indicate the estimated concentration (considering non-detects as censored observations).

Release of *Legionella* from soil to water

A *Legionella* suspension with $4.0*10^7$ CFU/ml, estimated from plate counts, was used to spike the sandy and clayey soil samples. As 100 g dry weight of the sandy and clayey soil samples were spiked with 1 ml of the suspension, the theoretical *Legionella* concentration in the soils was $4.0*10^5$ CFU/g dry weight (=5.6 log10 units). In the water layer on top of the unspiked clayey and sandy soils, which served as negative controls, *L. pneumophila* was not detected by PCR. After saturation of the spiked soils with rainwater, *L. pneumophila* was detected in the water layer on sandy soil by PCR, but not in the water layer on the clayey soil. The threshold cycle values decreased slightly over time (average 37.5 on t=0 and 36.2 on t=180 minutes).

Discussion

Fifteen percent of the natural soil samples were *Legionella*-positive, and *L. bozemanii* and *L. wadsworthii* were the most common species isolated. These, and also the other three identified species, *L. dumoffii, L. feeleii* and *L. gormanii,* were previously associated with soil (316, 395, 426, 433). All these *Legionella* spp. are pathogenic (2). In contrast to our earlier soil studies (395, 426), *L. pneumophila* was not detected in this study. In the current study, soil was collected from randomly chosen locations on a digital soil map, excluding urban areas. In practice, the main sampled locations were fields, forests and road sides (not close to the road). Possibly, these locations are less favorable for *L. pneumophila,* because of climatological or soil characteristic differences, compared to soils near roads (395) and garden soils (426). Only on 5 out of 80 locations, samples were taken directly (within approximately 1 m) next to the road (asphalt), and 2 of these were found to be positive. The characteristics of soils next to (asphalt) roads should be investigated in relation to *Legionella* presence.

Legionella presence is related to soil type. Soils harboring *Legionella* spp. had a different granular composition than soils without *Legionella*. Compared to *Legionella*-negative soils, the *Legionella*-positive soils contained more clay and silt particles, in proportion to sand particles (size >50 µm).

Soil texture is related to soil moisture content, where particle size is inversely related to the ability to retain water (434). Soils with a high clay/silt content have a high number of habitable, moisture-containing pores (with neck diameter <3 µm) that are protective for bacteria (435, 436). The ability of a soil to retain water is possibly a favourable factor for *Legionella* bacteria.

Based on the USDA soil classification system (432), the soil textural classes of *Legionella*-positive soils in this study were sand, sandy loam, sandy clay loam and clay loam. Although

the main sampled soil texture was sand (25 samples), only one of these was positive for *Legionella* (sample taken directly next to a road).

Comparing the group of sand samples to the other soil types sampled in this study, the sand samples had on average a statistically significant lower pH (6.4 v. 7.4), soil moisture content (10.1% v. 25.8%) and organic content (4.2% v. 9.1%). Like soil moisture, possibly also soil pH and organic content influence *Legionella* presence (8, 353, 437).

We showed that *L. pneumophila* could persist in soil over time (78 days). The clayey soil was more favorable for *Legionella* persistence than the sandy soil. That *L. pneumophila* reveals the capacity to remain viable, at quite raised densities, in soil is in line with a previous study, where we showed persistence of indigenous *L. pneumophila* in a garden soil (426). In a recent study by Graham and Harte (438), persistence of *L. bozemanii* in liquefaction affected natural soils could not be shown. Besides the soil type and species, also the culture method and incubation parameters (i.e., temperature and moisture content) differed from our study. Survival and growth of *Legionella* spp. in potting soil has been established before (205, 438, 439).

In the experimental set up of the current study, an incubation temperature of 25°C was chosen, so as to mimick summer temperatures. Increased levels of LD incidence were previously related to exceptionally high temperatures followed by a period of intense rainfall and it was hypothesized that *Legionella* bacteria in natural matrices, such as soil, may thrive under warm conditions (70, 130). However, as it is not very common to have a temperature of 25°C over a long period in the Netherlands, the persistence study was repeated for *L. pneumophila* in clayey soil at 15°C. The spiked strain persisted at a constant concentration over 105 days (data not shown). To study the influence of temperature on *Legionella* bacteria in soil, studies should test *Legionella* persistence at other temperatures than 15°C and 25°C, and under circumstances with varying temperatures (to resemble day and night variations). Ideally, a natural soil known to harbor *Legionella* should be used for these experiments, in contrast to an artificially contaminated soil.

Several publications report natural soil as a reservoir of pathogenic *Legionella* species (115, 312, 316, 318, 423). However, the significance of the presence of *Legionella* in natural soil for public health is still unclear. One study by Wallis and Robinson (252) provided evidence for infection caused by *L. pneumophila* originating from a field where an LD patient had spent several days potting plants prior to illness. The genotypic profile of the strains isolated from the patient and soil were indistinguishable by PFGE. PFGE is considered highly discriminatory, however this depends on the method and type of restriction enzyme used, which was not elaborated in the publication by Wallis and Robinson (252). Different studies have identified clinically relevant *L. pneumophila* sequence types in soil (115, 318, 395,

426). Amemura-Maekawa et al. (115) analyzed the distribution of *L. pneumophila* SG1 STs from three environments: cooling tower water, bath water and soil, and compared these to clinical isolates. Almost all STs of soil isolates (9 out of 11 STs) were also detected in clinical isolates, in contrast to bath water (11 out of 34 STs) and cooling tower water (3 out of 8 STs). Furthermore, except for two STs, the soil STs identified in this study were unique to soil, suggesting that *Legionella* types are specifically associated with a certain environment.

The hypothesized transmission route of soil-borne *Legionella*, via aerosolization of dry soil by wind, as showed for e.g. *C. burnetii*. (424, 425), seems unlikely based on our results. After seven days without moisture control, *L. pneumophila* did not survive in sandy soil (humidity level 10.3%) and strongly declined in concentration in the clayey soils (humidity level 14.9%). However, it cannot be excluded that *Legionella* bacteria are present in a viable but uncultivable state. Furthermore, survival of *Legionella* could be influenced by the rate of moisture loss or duration of dry conditions and the survival in dry soil conditions over a shorter time span, i.e., less than 7 days, or at more moderate humidity levels should be investigated. To note, the humidity levels in the soil samples were probably lower than the measured humidity levels, as the samples were taken from the surface layer in which humidity loss is faster compared to the deeper soil layers. The measured humidity levels are based on weight loss of the entire soil portions. Although *L. pneumophila* survived longer in the clayey soils, aerosolization of sandy soil as compared to clayey soil seems more likely due to the loose structure of sandy soil.

It is commonly accepted that moisture is essential for survival of *Legionella* bacteria. However, for soil-borne *Legionella* bacteria this has not been investigated before. Katz and Hammel (8) concluded that *L. pneumophila* is susceptible to drying after a bacterial dilution was dried on a glass slide. Possible, soil provides conditions for *Legionella* bacteria to survive periods of drying stress, e.g. by living intracellular in protozoa. Moreover, microorganisms live in the thin film of water bound to the soil matrix (434), and even under dry conditions, this water might still be available for the bacteria. The effect of amoebae presence in soil in relation to *Legionella* survival under dry conditions should be investigated. Furthermore, it is essential to know if *Legionella* bacteria keep their virulence traits under dry conditions.

We were able to show release from *L. pneumophila* from spiked sandy soils to rainwater, in contrast to clayey soils. *L. pneumophila* was detected by qPCR in a water layer on top of the spiked sandy soils, mimicking a rainwater puddle. The threshold cycle values for sandy soil were quite high and possibly *Legionella* bacteria were also released from clayey soil but in quantities below the detection limit. The difference in release of the bacteria from sandy soil and clayey soil could be explained by a greater adhesion of bacteria to clayey soil particles, compared to sandy soil particles (440). Furthermore, during the process of

adding the rainwater, the sandy soil was visibly disturbed which may have enhanced the release of bacteria from the sandy soil, or alternatively, bacteria attached to soil particles were sampled. Possibly, when the water would have been added more roughly, in a way mimicking heavy rainfall, the clayey soils would also have been disturbed. For the sandy soils, the threshold cycle values decreased slightly over time, which indicates some passive release of bacteria to water. To further study the exposure route through aerosolization of *Legionella* from rainwater puddles, new studies should focus on determining the *Legionella* concentration in rainwater puddles and quantifying the rate of aerosolization.

In conclusion, natural soil is a reservoir of *Legionella* spp. and *Legionella* presence is related to soil type. *Legionella* can persist in different types of soil; however, certain types of soil might be more favorable for *Legionella*, especially under environmental stress such as drought. These favorable soil types might act as alternative sources of *Legionella* bacteria. *Legionella* concentrations in natural soil should be determined and it should be investigated further how soil conditions influence viability, growth and virulence of *Legionella*. More studies are needed to establish if aerosolization of *Legionella* from rainwater puddles or directly from (dried) soil is possible and under which circumstances people are exposed. Exposure to natural soil might be substantial in certain professions (254, 338) or activities such as gardening, and should be investigated by case-control studies or risk factor analyses.

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7.

General Discussion

1. Soil and rainwater puddles as alternative sources of *Legionella* bacteria

1.1 *Legionella* in natural soil and garden soil

Presence and persistence

Viable *Legionella pneumophila* SG1 bacteria, the main causative agent of LD in the Netherlands, are present in garden soils (**Chapters 3 and 4**) and natural soils (**Chapters 5 and 6**) (see Table 1). Of the ten non-*pneumophila* species that were detected in soil, nine have been associated with disease (2). The presence of *Legionella* species in soil was described before (115, 243, 312-318) but is not as extensively investigated as aquatic sources and reservoirs (**Chapter 2**). Soil as a source of *Legionella* has been mostly considered in the late seventies and eighties, but later on the focus shifted to aquatic sources.

We have shown persistence of *L. pneumophila* in garden soils (**Chapter 4**) and natural soils (**Chapter 6**).The persistence of *Legionella* in soil was not described before. In **Chapter 4**, persistence was observed in the natural situation, by resampling *Legionella*-positive gardens, and in **Chapter 6** it was shown in an experiment with *L. pneumophila* spiked soils. Persistence in soil is an important finding; the bacteria can survive and probably even grow in this matrix. Especially detecting *Legionella* bacteria over a period of a year in garden soil suggests that that garden soil was inhabited by *Legionella* bacteria. It seems unlikely that the bacteria were introduced to the garden soil on multiple occasions. As soils are rich in amoebae and other protozoa that can act as hosts for *Legionella* (434), it is plausible that soil actually constitutes a natural habitat for *Legionella*.

Table 1. *Legionella* species isolated from soil and rainwater

	Garden soil (ch. 3 & ch. 4)	Natural soil (ch. 5 & ch. 6)	Rainwater puddles (ch. 5)	Related to disease (Yes, No)
L. pneumophila (STs*)	+ (84, 115, 477, 710, 863, 462, 465, 1856)	+ (477, 710)	+ (1064)	Y
L. longbeachae	+	+		Y
L. bozemanii	+	+		Y
L. feeleii	+	+		Y
L. wadsworthii	+	+		Y
L. dumoffii	+	+		Y
L. gormanii		+	+	Y
L. anisa	+			Y
L. sainthelensi	+			Y
L. cincinnatiensis	+			Y
L. gratiana		+		N

*Only sequence types (STs) that were ever related to patients are shown.

Soil as a source

Although until now no cases were linked to *Legionella* in soil in the Netherlands, the lack of source investigation for this matrix means that it cannot be excluded as a source of *Legionella*. In a source investigation related to a contaminated private outdoor whirlpool that caused disease in five people (2 LD and 3 Pontiac fever) (**Chapter 3**), *L. pneumophila* ST47 was isolated from garden soil. This sequence type (ST) is responsible for most disease cases in the Netherlands. However, the absence of the most prevalent Dutch clinical strains in the soil studies from **Chapters 4**, **5** and **6**, could lead to the conclusion that soil is not a reservoir of *L. pneumophila* ST47 and other important clinical strains. On the other hand, ST47 is almost never found in environmental investigations and detecting it in garden soil might therefore be an important lead on the possible reservoir of *L. pneumophila* ST47. In the Netherlands, ST47 was only isolated from environmental samples, i.e., water and swab samples, related to private outdoor whirlpools. Indoor whirlpools are also investigated in source investigation of wellness centers (recreational facility offering spas, saunas etc.) and *L. pneumophila* SG1 is regularly found (86) (personal communication dr. S.M. Euser, clinical epidemiologist at the Regional Public Health Laboratory Kennemerland). The fact that strain ST47 was related to outdoor whirlpools in particular, makes a strong case for the influence of the natural environment. However, possibly, ST47 is related to outdoor whirlpools because certain circumstances of outdoor whirlpools are favorable for this particular ST compared to indoor whirlpools, e.g. differences in water temperature or maintenance of the pools.

To asses if soil is a source and a potential cause of disease in the Netherlands, soil should be targeted in source investigations more often, in particular in relation to cases that are infected by a ST found regularly in soil. In garden soil, ST84, ST115, ST710 and ST477 are regularly found and ST710 and ST477 were also detected in natural soil (see Table 1).

To get a better understanding of the presence of *L. pneumophila* in natural soils and the sequence type distribution in this matrix, a larger sampling campaign is needed, like was done for garden soil in **Chapter 4**. For future research on *Legionella* presence in natural soils, we here give argument for the tenet that the focus should be on soils next to roads. In the first study describing *Legionella* detection in natural soil (**Chapter 5**), soils near roads, at locations where rainwater puddles on the road had formed, were sampled. The prevalence of *Legionella* spp. and *L. pneumophila* was higher than in the second study, where soil samples were not related to roads (**Chapter 6**) (30% and 10% vs. 12% and none respectively). It should be noted that in the first sampling campaign only 20 samples were analyzed. Possibly, this number is too low to give a robust representation of the prevalence of *Legionella* in soil. However, it is striking that, despite sampling 80 locations in the second sampling campaign, *L. pneumophila* was not once isolated. Possibly, soils next to roads

have certain characteristics that are favorable for *Legionella (pneumophila)* presence and growth, such as higher temperatures and influence from run-off water from the road.

Exposure to Legionella in soils

The possible attribution of soil as a source of LD could also be explored in a risk factor analysis, i.e., it could be investigated if gardening or working with soil pose a risk for contracting LD. This could also shed light on the possible mode of transmission, by studying what activities in particular enhance the risk, as was done by O'Connor et al. (53) and Kenagy et al. (54) for *L. longbeachae* infection.

Exposure to garden soil might be more regular and more extensive than exposure to natural soil. Especially in summertime, when people work in the garden or sit outside, exposure to garden soil seems inevitable. *L. pneumophila* was detected in 4% of the sampled garden soils (**Chapter 4**). This percentage might seem low; however, the chance of detection was presumably also low, as one sample was taken from a garden, and from one spot (not a combined sample). If multiple samples would have been analyzed for every garden, or a mixed sample was made by combining several samples of one garden, the detection rate of *Legionella* in garden soils might have been higher. Furthermore, only a limited amount of soil was analyzed (0.3 gram per sample), restricting the sampling scope. Exposure to natural soil from e.g. fields might only be relevant for people who work with soil in certain professions, e.g. construction or agricultural workers (64, 338).

L. pneumophila bacteria were isolated from natural soils close to roads (**Chapter 5**) and possibly they can wash out to puddles that form during rainfall. This is supported by the experiment that showed that *Legionella* bacteria can be released from sandy soil to a water layer after saturation of the soil, mimicking a rainwater puddle (**Chapter 6**). We hypothesize, as done by Sakamoto et al. (143), that *Legionella* bacteria can be aerosolized by vehicles disturbing puddles on roads (see also paragraph 1.2). In an ongoing case-control study, we investigate if there is a relationship between the risk of contracting LD and living near roads.

In **Chapter 6**, we speculated on another possible mode of transmission, namely via aerosolization of dry soil through wind, like is the case for the zoonotic pathogen *Coxiella burnetii*. Recently, the possible link between aerosolized soil (by liquefaction, i.e., soil disturbance) exposure and the elevated LD incidence after two major earthquakes in New Zealand was investigated (438). It was tested if *Legionella bozemanii* spiked into liquefaction-affected soil could persist over a 60-day period. *L. bozemanii* only survived in a control sample (compost). An important difference between the control sample and the liquefaction samples was that the control sample retained moisture. In our study, *L. pneumophila* did not survive in soil samples without moisture control (**Chapter 6**). As *Legionella* bacteria are ubiquitous

in aquatic matrices, their lack of harshness upon soil drying seems logical. However, we have isolated *Legionella* from several soil samples with a moisture content of only 6% (dry weight based), and once from a sample with only 1.2% moisture (*L. bozemanii*). Some *Legionella* species might have adapted to low moisture conditions and possibly, amoebae, which are drought resistant (441), play a role in the protection of *Legionella* under such dry conditions. Moreover, the soil moisture content should be considered in the context of soil textural type, as soil texture determines the extent of the moisture-containing habitable pore space. In the persistence experiment described in **Chapter 6** we did not investigate the presence of possible amoebal hosts for *Legionella*, although it seems plausible that they were present, as amoebae are regularly found in soil (434). For future research, the effect of soil amoebae and survival and virulence of *Legionella* bacteria under dry conditions should be investigated.

1.2 *Legionella* in rainwater puddles

Too little is known about *Legionella* in rainwater puddles in the Netherlands to exclude (or confirm) these matrices as infective sources. We have detected *Legionella* bacteria in rainwater puddles (see Table 1) and the prevalence was rather low compared to soil (**Chapter 5**). *L. pneumophila* strains were isolated but these were non-SG1 strains, which are considered less virulent than SG1 strains. In one sample, ST1064 was detected which was also found in patients although only rarely (413). Because detection rates were low, and relevant STs were not detected, it could be argued that the risk of *Legionella* contamination from rainwater puddles is low. However, it should be noted that too few samples were analyzed to assess the true prevalence of *L. pneumophila* in rainwater puddles in the Netherlands. Furthermore, the detection limit of the amoebal coculture method was quite high ($3.3*10^3$ CFU/L). In a Japanese study, the prevalence of *L. pneumophila* in rainwater puddles was much higher than in our study, which could have been influenced by the lower detection limit of 200 CFU/L (143). However, other conditions, like climate and sampling location, possibly also explain the difference in detection rates.

It will be difficult to assess if rainwater puddles are a source of *Legionella*, as targeting puddles in source investigations is impossible for the simple reason that rainwater puddles will most probably not exist anymore at the moment of investigation. It would be useful to explore if *Legionella* prevalence in rainwater puddles is affected by the intensity of the rainfall event creating the puddles, which was not taken into account in our study. We hypothesized that *Legionella* in puddles originate from surrounding or underlying soil, and heavy rainfall might influence the transfer from soil to water. As mentioned before, *Legionella* bacteria were transferred from sandy soil to a water layer (**Chapter 6**) in contrast to clayey soil. The sandy soils were visibly disturbed. In pluvial flood samples, after heavy rainfall, detection rates were higher than for the rainwater puddles (31% for *Legionella* spp. and 15% for *L. pneumophila*) (144). The influence of heavy rainfall could explain the higher

prevalence in pluvial floods but it could also be explained by the fact that water enters pluvial floods by runoff from large surfaces of surrounding soil.

In two Japanese studies, *Legionella* bacteria were found on recurrent events in puddles on roads on fixed locations (143, 357). It would be interesting to investigate if contaminated rainwater puddles in the Netherlands are also found positive on more than one occasion, indicating that possible exposure to *Legionella* from puddles is prolonged. If this is the case than targeting rainwater puddles in source investigations might be possible. Recurrent contamination of rainwater puddles (that disappear and form again by rainfall events) could also be studied in an experimental situation.

2. Other alternative sources for sporadic Legionnaires' disease

Legionella bacteria are ubiquitous in the environment, both in the natural as well as in the man-made environment. In **Chapter 2**, a wide range of reservoirs of *Legionella* bacteria, reported on in scientific literature, are described. For part of these reservoirs, there is evidence that they acted as an infective source at least once (i.e., confirmed sources). However, some confirmed sources of LD are not routinely targeted in source investigations and might therefore not be recognized as a source of sporadic LD. Moreover, the lack of evidence for other described reservoirs (i.e., potential sources) does not rule out that the reservoir can also be an infective source.

2.1 Possible under–recognized sources of sporadic LD

Wastewater treatment plants

Wastewater treatment plants (WWTPs) are confirmed sources of LD, with two outbreaks providing the highest Level of Evidence (LOE) (see **Chapter 2**). WWTPS are not routinely targeted in source investigations and could therefore be an overlooked source. In the Netherlands, viable *Legionella* bacteria were detected at WWTPs (144). There are approximately 360 WWTPs (113) that treat the urban wastewater in the Netherlands and the median distance of place of residence to the nearest WWTP is 3428 m. This distance could be even lower when industrial WWTPs are taken into account.

Depending on the distance of possible spread of *Legionella* bacteria, exposure to *Legionella* originating from WWTPs could be substantial in the Netherlands. In a recurrent outbreak in Norway, an increased risk was demonstrated for zones up to a distance of 3 km from the suspected source (224). However, eight of the 56 LD cases involved, neither lived nor reported visiting places within 10 km of the probable source, and so it was speculated that the spread of aerosols might be as far as over 10 km. To note, in later investigations of this outbreak, the river Glomma, onto which the treated wastewater was discharged, was

suspected to have played a role in the dissemination of the bacteria (227). *L. pneumophila* SG1 (including the outbreak strain) was detected in the river at high concentrations (10^4-10^5 CFU/L). The eight cases indeed lived near the river downstream of the plant (seven cases <1 km, one case ~2 km), at locations where *L. pneumophila* SG1 was also detected (224). Blatny et al. (225) studied the WWTP involved in the Norway outbreak and have proven the spread of *Legionella* bacteria from the aeration ponds up to 200 m downwind by air sampling.

In conclusion, WWTPs could be a source of sporadic LD in the Netherlands but the risk of being in the proximity of WWTPs is unknown. The distance, size and treatment type of the WWTPs might influence the risk. As well as the influence of weather conditions, such as prevailing wind direction and humidity levels, as these influence the spread and survival of *Legionella* contaminated aerosols (142, 442, 443). In an ongoing study we investigate if proximity of residence to the nearest WWTP is a risk factor of contracting LD.

By considering WWTPs as possible sources of infection in source investigations, the importance of this alternative source of *Legionella* can be assessed. Multiple sources at the WWTPs should be considered as *Legionella* bacteria are widely present at WWTPs (**Chapter 2**), which makes it difficult to establish the (main) disseminator of the bacteria. In the two reported WWTPs causing outbreaks, the causative agents were detected at multiple locations, namely cooling towers (228), an air scrubber (227), the aeration ponds (227, 228) and, as mentioned, a river (227).

Cooling towers

Cooling towers are a recognized source of *Legionella* bacteria and it is very well possible that these are an important cause of sporadic LD, which is at the moment undervalued. From 2002 to 2012, 1991 domestic LD cases were reported in the Netherlands, and 1418 unique potential sources related to these patients were sampled (86). Cooling towers were not investigated regularly; only 43 out of the 1418 potential sources were cooling towers. However, these source types were frequently found to be contaminated with *Legionella*. *L. pneumophila* was found in 39.5% of the cooling towers and *L. pneumophila* SG1 in 20.9%. Only in one other source type (i.e., wellness centers) *L. pneumophila* was detected more often (51.3%).

Rickets et al. (187) studied the geographical association between sporadic cases of LD in England and Wales, and wet cooling systems (WCS, i.e., cooling towers and evaporative condensers). They found that cases lived closer to WCS than their matched controls (mean distance 2.11 km and 2.58 km respectively; mean difference 0.47 km). The risk attributable to proximity to the nearest WCS was calculated, under the assumption that the risk of Legionnaires' disease above 6 km from a WCS represents the background ('unexposed')

rate of disease occurrence. The researchers estimated that residential proximity to a WCS may account for 20% of sporadic community-acquired cases. In ecological studies, often the place of residence of cases and controls are used, as these data are easily accessible. However, the exposure to cooling towers is probably much more extensive, when other locations e.g. workplace, regular travelling routes and leisure locations are considered.

The study by Ricketts at al. (187) was possible since a geographically complete inventory of WCS could be assembled, because of the legal requirement in England and Wales for businesses to register all such devices. In the Netherlands, since 2010, owners of newly installed cooling towers are obliged to report these to the authorities. The municipal authorities are obliged to make an inventory of all active cooling towers installed before 2010 (150). However, to date, the registration of cooling towers in the Netherlands is incomplete (339).

In conclusion, cooling towers might be responsible for a substantial part of the sporadic LD cases. However, as long as it is unclear where the cooling towers are located in the Netherlands, and thus where possible exposure exists, it is impossible to say to what extent cooling towers are responsible for sporadic cases of LD in the Netherlands, or to put its importance in perspective with other possible sources of LD.

2.2 Many sources responsible for sporadic LD

The general presence of *Legionella* bacteria in our surroundings makes it plausible that sporadic LD cases are caused by various source types, instead of one or a few main sources. This in contrast to outbreaks, which are generally caused by two sources, i.e., cooling towers (40) and whirlpools (41, 42).

The diversity of all possible sources for sporadic LD cases could hamper source investigations, as it would be costly to investigate all sources a case was possible exposed to. A solution for this would be to conduct an extensive source investigation campaign for a set period, e.g. for 1 or 2 years. During this time, an extensive sampling of potential infective sources should be pursued for LD patients in the Netherlands. Sources and reservoirs (i.e., confirmed sources and potential sources, see **Chapter 2**) that are normally not or irregularly targeted in source investigations should be the focus of this campaign, such as WWTPs, cooling towers, potting soil and compost, (garden) soil and possible other systems that contain and aerosolize water. For this sampling campaign, it would be essential to actively obtain human respiratory samples to make sure that for a considerable number of cases a clinical strain is available that can be compared to environmental strains and thus supply a substantial evidence base.

Another possibility is an extensive sampling campaign focused on the before mentioned reservoirs and sources, unrelated to patients. This would render a database of environ-

mental *Legionella* strains that could be compared, after typing, to the distribution of STs in the clinical database, as was done before in the study by Euser et al. (109).

Legionella is perceived as a preventable disease, which is in part true. *Legionella* growth can be prevented or controlled in man-made water systems by maintenance, disinfection or proper design of the systems (444-446). Therefore, many disease cases and deaths could have been prevented if e.g. whirlpools (on display) (41, 42, 447) or cooling towers (82, 154, 155) would have been better designed or maintained. However, if multiple source types are responsible for the sporadic LD cases in the Netherlands, then it will be very difficult to control *Legionella* growth in all these sources. Moreover, controlling *Legionella* growth in natural reservoirs, such as soil, might not even be possible or desirable.

3. What we can learn from known environmental risk factors for LD

Several risk factors for contracting LD have been described that might shed light on under-recognized sources of *Legionella*.

Over the past ten years, it has become clear that weather characteristics are linked to LD. Especially precipitation has been related to an increased risk of disease (70, 130, 133, 134, 136, 137, 139-141). It is unknown how precipitation influences LD incidence but the overwhelming evidence for this relationship warrants further investigation. Moreover, as more intensive rain events are predicted to occur more regularly due to climate change (448), the mechanisms by which precipitation influence LD incidence should be further investigated. Depending on the outcome, preventive measures can be initiated.

In this thesis, we hypothesized that rainfall facilitates the transport of *Legionella* bacteria from soil to rainwater puddles from which the bacteria are subsequently aerosolized by disturbance of the rainwater by e.g. cars. Car or truck drivers, cyclists and, depending on how far the contaminated aerosols can spread, people living close to roads are at increased risk. If precipitation influences LD incidence through this mechanism, preventive measures could be the prevention of rainwater puddles forming on busy roads, i.e., facilitating water runoff. Furthermore, awareness of clinicians after wet periods could be increased.

Two studies have identified that professional drivers are at increased risk of acquiring LD (30, 150). Possibly, this relates to increased exposure to aerosols from rainwater puddles. However, in the study by Wallensten (150), the risk factors 'driving through industrial areas' and 'using no screenwash in the windscreen fluid' were identified, which point to other possible sources of *Legionella*. *L. pneumophila* has been isolated from windscreen fluid (381, 449) and especially the study by Schwake et al. (449) supports the idea that windscreen washer fluids are a possible source of *Legionella*. The bacteria were isolated

from ten out of 12 school buses up to concentrations of $8.1*10^4$ CFU/ml. In six buses *L. pneumophila* was detected and *Legionella* bacteria were also detected in three air samples from the windshield washer spray of two buses. Furthermore, the researchers showed that *L. pneumophila* can survive to a certain extent in windscreen washer fluid. The high detection rate in this study suggests that contamination of washer fluid with *Legionella* could be a common situation and the presence of pathogenic *Legionella* bacteria in washer fluid should also be assessed for vehicles in the Netherlands. To assess if windscreen washer fluid is an actual source of *Legionella*, this potential source should be targeted in source investigations, especially when a case is a frequent driver.

Returning to the weather-related risk factors, besides rainfall, relative humidity has also been indicated as an LD risk factor (132, 133, 135, 136). Possibly, precipitation is not directly influencing the dissemination of *Legionella* but enhances *Legionella* survival in aerosols because it influences the relative humidity. Enhanced aerosol survival influences the spread of the bacteria which could increase the exposure risk to e.g. *Legionella* bacteria disseminated from cooling towers. In the Netherlands, the daily mean relative humidity was on average 79% for the months July, August and September (calculated over the past 5 years) (450). Hambleton et al. (443) have shown that aerosolized *L. pneumophila* is relatively stable at a humidity level of 65% but less stable at 90%. The daily mean relative humidity levels can vary significantly per day (between 59% and 94% for the months July, August and September) (450). Moreover, there can be strong variances within a day. For example, in the months July, August and September of 2016, the mean difference between the measured minimum relative humidity and maximum relative humidity was 41% and the difference could go up to as high as 64%. Hourly data on the effects of heavy rain events on relative humidity, and knowledge of the stability of aerosolized *L. pneumophila* at humidity levels between 65% and 90%, could help identify if and how relative humidity/precipitation influence survival of contaminated aerosols, and possible identify a time window of high risk.

4. Detection methods of environmental *Legionella*

4.1 Detection of *Legionella* by amoebal coculture

For the research presented in this thesis, we used an amoeba coculture method with *Acanthamoeba castellanii* to detect *Legionella* (**Chapters 3-6**). Soil and rainwater from rainwater puddles contain a large number of non-*Legionella* micro-organisms. The standard method of detection, culture on plates (55), is not suitable for heavily contaminated samples as they are easily overgrown by other microorganism in the samples, resulting in false negatives.

In **Chapter 5**, rainwater samples were analyzed with both the amoebal coculture method and with direct culture on glycine-vancomycin-polymyxinB-cycloheximide (GVPC) plates. *Legionella* bacteria were only detected with the coculture method. The successful use of the amoebal coculture methods has been proven before in several studies, detecting *Legionella* bacteria in WWTP samples (144), whirlpool filter samples (411), bath water (451) and potting soil (unpublished results). Moreover, it might also be useful for diagnostic purposes, when conventional culture methods fail (410).

4.2 Amoebal coculture use in source investigations

In this thesis we followed the hypothesis that important clinically related *L. pneumophila* types are not found in investigated environmental sources because the focus was not on the right source types. However, a second explanation for the discrepancy between the ST distribution of clinical *L. pneumophila* strains and environmentally detected *L. pneumophila* is that the currently used detection method, i.e., culture on plates, is not suitable for detection of the clinically relevant STs in the regularly investigated sources. For example, growth of *Legionella* from cooling tower samples might be hampered by the presence of other microorganisms. For this, the amoebal coculture method could pose a solution.

Different pre-treatment steps can be applied to reduce the load of other microorganisms in samples before standard culture, such as heat and acid treatment, as recommended for samples with many other microorganisms in the NEN-EN-ISO 11731:2017 norm (55). However, in our experience acid treatment was only partly successful for rainwater and soil samples (data not shown) and in the study by Schwake et al. (449) it was experienced that heat-treatment only partly reduced the level and occurrence of other bacteria in heavily contaminated windscreen washer fluids. Furthermore, media plates with antibiotic supplements can be used to reduce growth of other microorganisms. However, this was not sufficient for rainwater (**Chapter 5**) or potting soil sample analysis (unpublished results), for which the culture method did not gain results in contrast to amoebal coculture.

A third explanation for not finding clinically relevant STs in the environment, is that the virulent STs are present at very low concentrations and are therefore missed with the currently used detection methods. Conza et al. (452) established that the detection limit of the amoebal coculture method, using *Acanthamoeba polyphaga,* for spiked compost and air samples was 10^2-10^3 CFU in 1 g compost/1 m^3 air and found that the coculture method was more sensitive than direct culture (10^5-10^6 cells in 1 g compost/1 m^3 air). Therefore, the amoebal coculture method could have an advantage over standard culture for detection of low numbers of *Legionella*.

4.3 Disadvantages of the amoebal coculture method

The coculture method has some disadvantages that makes it a less suitable method for source identification purposes. First, amoebal coculture is, as compared to culture, a rather laborious method. Second, the method has a long turnover time (9-13 days). The method could be shortened to 7 days by detection of *Legionella* after coculture with PCR instead of culture on plates, as we have used for the soil persistence study (**Chapter 6**). However, as *Legionella* would not be isolated from the sample, the opportunity to match clinical isolates to environmental isolates is impossible. Third, there possibly exists a difference in sensitivity between *Legionella* strains for cocultivation with *A. castellanii*. Therefore, the strain diversity of a sample might not reflect the actual situation. In the study by Conza et al. (452), the isolates obtained from unspiked compost samples yielded with coculture showed less diversity than the isolates obtained by the culture method. To exclude that certain *Legionella* strains are not able, or are less successful, to replicate in amoebae, a batch of 23 different *L. pneumophila* strains was tested (144). All of the strains replicated similarly in amoebal coculture. However, it was not tested how different *Legionella* strains react in a mixed sample. It may very well be possible that certain types of *Legionella* are more capable of growth in amoebae, giving them an advantage when competing with other (*Legionella*) bacteria. Moreover, different *Legionella* strains could be present in unequal concentrations and possibly bacteria in low concentrations are not significantly phagocytized by the amoebae compared to bacteria at high concentrations, and therefore have less chance to replicate. However, strains present in very low concentrations would probably be missed by standard culture too.

4.4 Culture and ST diversity within samples

Even if the currently used methods are suitable for isolation of clinically relevant STs in environmental samples, i.e., important *Legionella* strains are present in concentrations above the detection limit and growth on plates is not hampered by non-*Legionella* organisms or *Legionella* strains present in much higher concentrations, the relevant STs could still go undetected. On 11 occasions we have isolated *L. pneumophila* from rainwater and soil samples (**Chapters 4 and 5**) and in 7 of these samples more than one ST was detected (up to seven different STs per sample). The diversity of these samples would have gone unrecognized when a 'first colony pick' sampling strategy was followed. In the new norm for *Legionella* detection, NEN-EN-ISO 11731:2017 (55) , it is stated that three presumptive colonies should be picked when there is only one colony type on the plate. If more morphological different types of *Legionella* are present, at least one colony from each type should be picked. However, *L. pneumophila* colonies look very much alike and it seems unlikely that different STs of *Legionella* have different appearances. It is therefore essential to pick multiple colonies from a plate, even if the colonies are phenotypically alike. This might be especially important if the clinically relevant strains are present in low numbers compared to other *Legionella* bacteria in the sample. Furthermore, the morphological

characteristics of colonies is best inspected with a stereo microscope (magnification, 40 X), especially when other microorganisms are present on the plates. To limit costs and workload, a subset of all picked *L. pneumophila* strains from a single plate could be used for sequence typing to investigate if more than one ST is present in the sample and if further typing of more strains is needed.

4.5 Alternative methods for source investigations

The amoebal coculture method might not be the ideal method for detecting *Legionella* in source investigations because of the disadvantages discussed above. However, it could be used to assess the possibility that environmental *Legionella* species are missed by conventional culture methods. By analyzing e.g. cooling tower samples with both methods, it can be investigated if conventional culture fails to detect (certain) *L. pneumophila* strains and to what extent. Such a comparison can be relevant in two ways. First, it can shed light on the important sources of sporadic LD, when clinically relevant strains, such as ST47, are detected by coculture. Second, even if the clinically most relevant strains are not detected, but there is a difference in detection rate or a higher strain diversity, it can put the usefulness of detecting *Legionella* in certain sample types with the current standard method under discussion.

An ideal method for source investigations is relatively easy to perform, has a short time to get a result, detects relevant *Legionella* species, is not hindered by high background flora and has a low detection limit. Quantitative PCR would be suitable although the performance can be influenced by PCR-inhibiting substances in certain samples (453). Another limitation is that no differentiation can be made between live and dead cells or free DNA. However, the main limitation is that because no isolates are gained, it is impossible to match environmental strains to clinical strains. In 2012, Public Health England adopted a real-time PCR specific for *L. pneumophila* SG1 combined with direct molecular typing (nSBT, a nested modification of the SBT protocol), in order to reduce reporting time of preliminary typing results (454). This approach proved to be effective, even on heavily contaminated environmental samples where culture methods failed to isolate any strains. However, this typing method is not suitable for specimens containing more than one *L. pneumophila* ST. As the majority of Dutch LD patients are caused by only a few STs, a *L. pneumophila* sequence type specific PCR could also be applied, such as the recently developed ST47 qPCR assay (455). This assay should be further evaluated for usability of detecting *L. pneumophila* ST47 in the environment as it was only tested on three environmental samples. A disadvantage of the assay is that ST109 strains were also detected.

5. A view on the primary and secondary *Legionella* prevention strategies and recommendations for future research

In the Netherlands, drinking water legislation is in place as the primary strategy for LD prevention (109). This strategy focuses on prevention of *Legionella* spp. growth in water systems to which the general public is exposed (such as water systems of hospitals and hotels). The so-called secondary prevention of LD uses an outbreak detection program that identifies and eliminates potential infective sources that LD patients have been exposed to during their incubation period. Euser et al. (109) compared the clinical and environmental *L. pneumophila* SG1 genotype distribution to evaluate both strategies and their results suggest that primary prevention is not aiming at the correct reservoir, whereas the secondary prevention strategy is only partially focused.

5.1 Primary prevention

As stated by Euser et al. (109), in an ideal situation, preventive efforts focus on the niches that harbour the *Legionella* genotypes that cause human disease. Despite the drinking water legislation, sporadic LD incidence is still increasing in the Netherlands. Apparently the primary prevention efforts do not target all sources responsible for sporadic LD. However, this does not mean that water systems targeted under de current drinking water legislation are not partly responsible for sporadic LD. It is very difficult to investigate if current legislation is reducing the LD burden. ST1 is widely represented among the *L. pneumophila* strains isolated during primary prevention (68%) (109), while ST1 is not found to be a frequent cause of disease in the Netherlands (4%) compared to other countries. Possibly, the primary prevention strategy reduces infection with ST1. This hypothesis could be investigated by comparing *Legionella* prevention strategies in countries with a high ST1 disease burden (the United States) (101), to countries with a low ST1 disease burden (England & Wales, Portugal) (110, 111) and countries with a decreasing ST1 disease burden (Canada, Belgium, Japan) (103, 105, 112).

Strikingly, to date, despite the fact that the probable infective source of the largest outbreak in the Netherlands was a whirlpool on display (27), there is no regulation to prevent a similar situation from happening.

5.2 Secondary prevention

Den Boer et al. (86) have evaluated the results from the outbreak detection program from 2002-2012 and concluded that, despite great efforts by collaborators and a systematic method for source identification, most sources of LD infections remained undiscovered. A standardized questionnaire covering over 20 different source types is used for source identification, which primarily covers sources identified from the literature (86). Some of these source types are possibly less useful to further investigate in the source investigations.

The main sampled source type from 2002-2012 was the place of residence (51.3%, 762 out of 1484 sampling results); however, only in 7% of the samples *L. pneumophila* was detected (86). Over a ten year period, patients' residences were 7 times found to be the probable source of infection by matching clinical and environmental isolates. It could be argued that, because of the low detection rate and low risk of exposure (only people living in the house or sleeping over), the place of residence should not be further investigated. However, although the residence is probably not an important cause of disease, including the residence in source investigations might still be relevant. Besides isolating the relevant ST from a suspected source, source investigations preferably also gain additional evidence demonstrating that a suspected source caused the infection (**Chapter 2**). Excluding the patients' residence as source of infection is in that way important.

Decorative fountains (mainly outdoor) were sometimes investigated and *L. pneumophila* was never isolated from this source type (86) (personal communication dr. S.M. Euser, clinical epidemiologist at the Regional Public Health Laboratory Kennemerland). More importantly, three outbreak studies that provided the highest LOE (see **Chapter 2**) that the suspected fountain was the cause of disease, all concerned indoor fountains (46, 47, 218). Therefore, in the future, only indoor fountains should be considered in source investigations.

Garden centres were the most frequently identified cluster site (26%, 27 out of 105 clusters) and the third most investigated location (86 times, after place of residence and hospitals/ health care centres) (86). However, in the possible sources investigated at garden centres, *L. pneumophila* was only found in 2.4% of the samples and a match was never made with clinical isolates. Visiting a garden centre seems to be a risk factor for contracting LD as many patients report visiting garden centres in the incubation period. However, this hypothesis should be confirmed with a risk factor analyses. Furthermore, it should be investigated if gardening and visiting a garden centre are related, so possibly gardening is the actual risk factor for contracting LD. Until further research confirms that people visiting garden centres are at risk, these places should not be targeted in source investigations anymore.

As mentioned before, some possible under-recognized or overlooked sources of LD should be included regularly in source investigations, i.e., cooling towers, WWTPs, potting soil and compost, (garden) soil and windscreen washer fluid.

5.3 Recommendations

Knowledge on which sources of *Legionella* are responsible for sporadic LD cases is essential to better focus primary and secondary prevention strategies. In the schematic representation on the next page, the recommendations regarding discovering alternative *Legionella* sources of sporadic LD in the Netherlands as discussed are summarized.

The presence of clinically relevant STs in alternative sources of LD should be investigated, either related to patients in source investigations (I) or in environmental sampling campaigns (II). In addition to, or instead of the standard culture method, other detection techniques should be applied, such as amoebal coculture and ST specific PCRs (IV). In this way the possible limitations of traditional culture can be ascertained. Cooling towers are difficult to target in source investigations because of the incomplete registration. So, generating a ST database for this source type by environmental sampling of (registered) cooling towers is probably more successful. Besides targeting garden or natural soil in source investigations (I), possible risk factors related to exposure to soil should be assessed (III). Furthermore, the virulence of soil-borne *Legionella* bacteria and the relationship between the bacteria and soil protozoa should be studied (VI). Investigating the role of relative humidity/precipitation on aerosol survival of *Legionella* bacteria could shed light on the relation between wet weather and increased *Legionella* incidence (V). Determining the possible role of contaminated rainwater puddles in this relation is difficult. A larger sampling campaign is needed to assess the prevalence of *Legionella* bacteria in puddles in the Netherlands, but planning of sampling is difficult because of the dependence on the weather. Therefore, it might be more useful to study other aspects further, such as *Legionella* survival and virulence in rainwater puddles. This could be done in an experimental setting or, if possible, at a location that has been recurrently found to be positive for *Legionella* in puddles (VI).

Source investigations (I)

Include alternative *Legionella* sources in source investigations, such as:

- WWTPs
- Cooling towers
- Garden soil or natural soil
- Compost or potting soil
- Windscreen washer fluid

! Apply multiple detection techniques

Research

Environmental sampling (II)

Generate a database of STs that are common in the following alternative sources:

- WWTPs
- Cooling towers
- Soils near roads
- Windscreen washer fluid
- Compost or potting soil
- Garden soil or natural soil (optional)

Risk factor analysis (III)

Investigate if the following activities are risk factors for *L. pneumophila* infection:

- Visiting a garden center
- Gardening and certain gardening activities
- Working with (natural/garden) soil in occupational settings

Detection methods (IV)

- Compare amoebal coculture results with standard culture (as stated in NEN-EN-ISO 11731:2017) for samples with high background flora
- Investigate how multiple *L. pneumophila* strains in a sample, and strains present in low quantities in a sample are detected by amoebal coculture
- Evaluate the use of the ST47 PCR on environmental samples

Aerosol survival (V)

- Assess if precipitation and/or relative humidity influence LD incidence by increased survival of *Legionella* contaminated aerosols

Soil as a source of L. pneumophila (VI)

- Investigate relationship with soil protozoa
- Further experimentally investigate possible exposure routes
- Assess virulence of *Legionella* strains in soil

Rainwater as a source of L. pneumophila (VII)

- Investigate more rainwater puddles for *Legionella* presence and assess *Legionella* concentration in puddles
- Study puddles at a location (road), where a *Legionella*-positive puddle has been found before, over a longer period of time
- Study *Legionella* behavior in rainwater puddles in an experimental setting

Appendix A

Supporting information Chapter 2

Complete description of the Methods section

Literature search

A literature search was conducted using the MEDLINE database (publisher: U.S. National Library of Medicine), searched by OvidSP (Wolters Kluwer Health). The search was performed on June 25, 2013. The following terms were used to select publications about *Legionella*, LD or Pontiac fever: in the title and abstract: 'legionell*' OR 'legionnair*' OR 'Pontiac fever'; and in Medical Subject Headings (MeSH): 'exp legionellosis/' OR 'exp *Legionella*/'. These terms were combined with an extensive list of additional terms to identify publications that reported sources and reservoirs of *Legionella*. No restrictions for the publication date were imposed. PubMed (publisher: U.S. National Library of Medicine) was searched in addition to the MEDLINE database because the former also covers publications that are electronically published ahead of print. The same MeSH terms were applied, but only the publication titles, not the abstracts, were searched for the terms 'legionell*' OR 'legionnair*' OR 'Pontiac fever'. These terms were not combined with additional search terms. The publication date criterion was between June 1, 2012 and June 25, 2013.

Study inclusion criteria

A study was included if it was written in English, reported on primary research results and fulfilled at least one of the following criteria: 1. the study described the detection of *Legionella* spp. in environmental sources in surveillance or prevalence studies or in source investigations; 2. the study described risk factors that could be related to exposure to *Legionella* sources; 3. epidemiological data on LD or Pontiac fever cases were used to identify a possible infection source.

Publications describing showers or taps as sources or reservoirs of *Legionella* were excluded (as discussed above). However, the indirect use of drinking water was included, e.g., the use of tap water for cleaning purposes, rinsing medical equipment or dental units. The use of tap water in baths was also included because baths involve the use of water for a prolonged period at a certain temperature, which might promote the *Legionella* growth. The following study topics were also excluded from this review: disinfection methods of *Legionella*-contaminated water systems, the effect of antibiotics against environmental *Legionella* strains, the geographical distribution of clinical and environmental types of *Legionella*, the distribution of virulence genes among *Legionella* isolates, *Legionella* detected in animals, and travel-related LD. Studies on travel-related LD were excluded because it was assumed that most patients would be infected by the use of contaminated tap water through showering. A study was also excluded if more than one source was investigated but the results on *Legionella* presence were not presented separately for the different sources. Editorials, comments and conference abstracts were excluded. Letters to the editor were only included if they reported on a case study.

Selection process

The selection process was conducted in two steps. First, the title and abstract of all publications were assessed independently by three researchers (SE, EH, and JS). Publications that were not relevant for the research objective were not selected for full-text assessment. Case study publications were selected if a (potential) infection source was stated in the title or abstract or if a source investigation was performed. The exact origin of the tested environmental samples remained unclear in some publications. In these cases, a publication was selected for further assessment if the study focused on the prevalence or isolation of *Legionella*. Publications without available abstracts were considered possibly relevant in the following cases: the title implied that a source investigation was conducted, some type of source was mentioned in the title, or the study concerned a case report or outbreak description. If it was doubtful whether the publication should be included or excluded, the three reviewers discussed the publication until a consensus was reached. In the second selection step, the full-text versions of the selected publications were assessed for eligibility on the basis of the selection criteria by one reviewer (EH). When multiple studies on the same outbreak were present, only the report providing the highest LOE was included, unless the other studies provided additional information resulting in a higher LOE.

Levels of evidence

LOEs were developed to discriminate between potential sources and confirmed sources of *Legionella*. Table 1 shows the different LOEs, from I, representing the highest LOE, to VI, representing the lowest LOE. For the highest LOE, cases must be epidemiologically linked to a suspected source and a match, either molecularly (LOE Ia) or by monoclonal antibody typing (LOE Ib), must be determined between the clinical and environmental isolates. Furthermore, there should be additional evidence demonstrating that these sources caused the infection: evidence that excludes other possible sources, evidence on the spread of *Legionella* from the suspected source, or evidence on the exposure of cases to the suspected source. The exclusion of other sources can be achieved by the environmental investigation of other sources, if there is only one common source in an outbreak situation, or if new cases ceased to occur after elimination of the suspected source. Evidence on the spread from the suspect source involves the isolation of *Legionella* from air samples. Evidence of exposure is achieved by case-control studies comparing the seroprevalence of *Legionella* or by case-control studies that imply exposure to the suspected source as a risk factor for contracting LD (in contrast to other considered sources). For LOE II, a match must be identified between clinical and environmental isolates, but additional evidence is not provided. LOE III was assigned when cases were epidemiologically linked to a suspected source, *Legionella* was isolated from this source, and additional evidence was provided, but environmental and clinical strains were not further typed or clinical strains were not available for comparison. LOE IV could be assigned in three situations: (1) cases were epidemiologically linked to a suspected source, and *Legionella* was isolated from this

source; (2) cases were epidemiologically linked to a suspected source, no environmental isolates were obtained, but other possible sources were excluded or there was evidence of spread from or exposure to the source; and (3) no cases were linked to a suspected source, but environmental strains were isolated, and additional evidence was provided. When no LD cases were involved, additional evidence could include the isolation of *Legionella* from air samples or case-control studies on the seroprevalence of *Legionella*. LOE V was assigned when *Legionella* was isolated from a reservoir or potential source, or exposure was assessed, or risk factors for contracting LD were determined. LOE VI was assigned to studies in which environmental *Legionella* was not isolated but was detected by molecular or antibody staining methods. A study may report the detection of *Legionella* in more than one type of system or reservoir; therefore, one study can be assigned multiple LOEs.

For the described sources and reservoirs of *Legionella,* the LOEs were assessed based on the selected literature. Subsequently, the sources of *Legionella* were subdivided into confirmed sources (at least one publication with LOE Ia or Ib) or potential sources (LOE II or lower).

Table S4. Selected publications on wastewater/wastewater treatment plants (WWTPs)

Reference	Country	No. of tested WWTPs	Samples analyzed	Detection method	Detected *Legionella* species
Alonso et al. (2006)(288)	Spain/Portugal	2	Effluent	PCR	*L. pneumophila*
Bercovier et al. (1986)(283)	Israel	unknown	Aeration ponds	Culture	*Legionella* spp. (4 out of 60 oxidation ponds)
Brissaud et al. (2008)(284)	France	1	Effluent	qPCR	*Legionella* spp. *L. pneumophila* either not detected or at a content to low for quantification
Calvo et al. (2013)(281)	Spain	11	Unknown	PCR	*L. pneumophila* (3 out of 11 WWTPs)
Catalan et al. (1997)(285)	Spain	1	Primary effluent	Culture PCR	*L. pneumophila* detected by PCR only (9 out of 12 samples)
Cherry et al. (1982)(456)	USA	1	Trickling filter bed	Guinea pig inoculation	*L. jordanis*
Huang et al. (2009)(282)	Taiwan	17	Influent Final effluent	PCR	*Legionella* spp. (10 of the 17 WWTPs and 25 out of 41 samples) *L. pneumophila* (2 out of 41 samples)
Palmer et al. (1995)(286)	USA	3	Chlorinated tertiary effluent Air	Culture DFA PCR	*Legionella* spp. detected with DFA and PCR (3 out of 3 WWTPs) *L. pneumophila* detected only with DFA, in very low numbers (2 out of 3 WWTPs) *Legionella* spp. detected with all three methods in air
Palmer et al. (1993)(287)	USA	1	Primary influent Primary effluent Secondary effluent	Culture DFA PCR	*Legionella* spp. detected with all methods in all phases of sewage treatment *L. pneumophila* detected with PCR and/or DFA in all phases of sewage treatment
Pascual et al. (2001)(289)	Spain	1	Air	Culture PCR	*L. pneumophila* detected by PCR only (2 out of 6 sampling sites)
Schalk et al. (2012)(144)	Netherlands	5	Influent Aeration ponds	Amoebal coculture	*Legionella* spp. (9 out of 24 samples, all WWTPs) *L. pneumophila* (5 out of 24 samples, 2 WWTPs)
Stampi et al. (2000)(290)	Italy	1	Air	Culture	*Legionella* spp. (6 out of 201 samples)
Viau & Peccia (2009)(291)	USA	29	Biosolids	qPCR	*L. pneumophila* (12 out of 36 samples)

Table S5. Selected publications on natural water: thermal springs

Reference	Country	Source type	Samples analyzed	Detection method	Detected *Legionella* species
Amemura-Maekawa et al. (2005)(457)	Japan	Hot spring	10 public spas and/or hot spring baths	Culture	*L. pneumophila*
Bornstein et al. (1989)(458)	France	Hot spring		Culture	*L. gratiana*
Costa et al. (2005)(459)	Portugal	Thermal spring	6 boreholes were sampled, at two different sites (water supplied two therapeutic spas)	Culture	*Legionella* spp. (both sites) *L. pneumophila* (both sites)
Costa et al. (2010)(460)	Portugal	Thermal spring	Geothermally heated therapeutic spa	Culture	*Legionella* spp.
Dutka & Evans (1986)(461)	Canada	Hot spring	3 hot springs	Culture DFA	*L. pneumophila*
Furuhata et al. (2011)(462)	Japan	Hot spring		Culture	*L. nagasakiensis*
Furuhata et al. (2012)(463)	Japan	Hot spring	Spa facility	Culture	*L. rubrilucens*
Furuhata et al. (2010)(464)	Japan	Hot spring	Spa facility	Culture	*L. londiniensis*
Furuhata et al. (2009)(465)	Japan	Hot spring	Spa facility	Culture	*L. pneumophila* (25.5%, 14 out of 55 water samples) *L. micdadei* (5.5%, 3 out of 55 water samples)
Ghrairi et al. (2013)(309)	Tunisia	Hot spring	Hot spring therapeutic spas, sampled at various locations: springs appearance, reservoirs, cooling water tank, swimming pool and bathroom outlets	Culture qPCR	*Legionella* spp. (culture; qPCR, 70.1% 54 out of 77 samples) *L. pneumophila* (culture, 22%, 17 out of 77 samples) Not in spring groundwater
Hsu et al. (2006)(466)	Taiwan	Hot spring	Spa facilities in 7 recreation areas (samples taken from hot tubs, spas and swimming pools)	PCR	*Legionella* spp. (27.5%, 25 out of 91 sampled sites) *L. pneumophila* (8.8%, 8 out of 91 sampled sites)
Hsu et al. (2011)(467)	Taiwan	Hot spring	Floating and fixed biofilm, sampled at six locations	Culture PCR PCR detection on indigenous amoebae	*Legionella* spp. (28.1%, 45 out of 160 sites; 26.9%, 43 out of 160 floating biofilm samples; 3.1%, 5 out of 160 fixed biofilm samples) Positives found by 1 or 2 of all 3 methods *L. pneumophila* (12 out of 160 sites)

Table S5. Selected publications on natural water: thermal springs (*continued*)

Reference	Country	Source type	Samples analyzed	Detection method	Detected *Legionella* species
Hsu et al. (2009)(468)	Taiwan	Hot spring	13 spring resorts, 34 sampling sites, throughout a mud spring recreation area. Source water, facility water (hot tubs, spas, swimming pools) and wastewater from spring facilities was sampled, mud samples	Culture PCR PCR detection on indigenous amoebae	*Legionella* spp. (47.1%, 16 out of 34 sites, both water and mud samples) Positives found by at least 1 of 3 methods *L. pneumophila* (8 out of 34 sites)
Huang & Hsu (2010)(469)	Taiwan	Hot spring	Spring recreation areas. Source water, facility water (hot tubs, spas, swimming pools) and wastewater from spring facilities was sampled	Culture	*Legionella* spp. (wastewater 40.0%, facility water 10.4%, source water 5.7%) *L. pneumophila*
Huang et al. (2011)(470)	Taiwan	Hot spring	Spring recreation areas. Source water, facility water (hot tubs, spas, swimming pools) and wastewater from spring facilities was sampled	Culture PCR PCR detection on indigenous amoebae	*Legionella* spp. (29.4%, 15 out of 51 samples, 30.8% (8/26) in source water including hot spring water and cold stream water, 29.2% (7/24) facility water) Positives found by at least 1 of 3 methods *L. pneumophila*
Huang et al. (2011)(471)	Taiwan	Hot spring	Hot spring water, cold stream water, and wastewater from spring resorts (carbonate spring water, sodium bicarbonate spring water, mud spring water)	Culture PCR	*Legionella* spp. (26,5%, 18 out of 68 samples, respectively 25.8% (8/31), 17.9% (5/28), and 55.6% (5/9) in hot spring water, cold stream water and wastewater) *L. pneumophila*: most frequently identified (n=12)
Huang et al. (2010)(472)	Taiwan	Hot spring	Spring recreation areas. Facility water (hot tubs, spas, swimming pools)	Culture PCR	*Legionella* spp. (27.8%, 12 out of 72 samples, hot tubs and spas) *L. pneumophila* (2.8%)
Jeminez et al. (2012)(473)	Columbia	Acidic hot spring		PCR	*Legionellae*
Kao et al. (2013)(474)	Taiwan	Thermal spring	Spring water recreation areas	qPCR	*Legionella* spp. (16.6%, 8 out of 48 samples)

Table S5. Selected publications on natural water: thermal springs (*continued*)

Reference	Country	Source type	Samples analyzed	Detection method	Detected *Legionella* species
Kao et al. (2013)(475)	Taiwan	Thermal spring	Three spring water recreation areas (sodium bicarbonate and sulfur spring)	Culture PCR PCR detection on indigenous amoebae	*Legionella* spp. (38%, 19 out of 50 samples)
Lee et al. (2010)(125)	South-Korea	Spring		Culture	*L. pneumophila* *L. spiritensis* *Legionella* spp.
Lin et al. (2007)(476)	Taiwan	Hot spring	19 hot spring resorts (sulfur-based springs and carbonate-based springs)	Culture	*L. pneumophila* (11%, 6 out of 55 samples; 21%, 4 out of 19 resorts)
Martinelli et al. (2001)(310)	Italy	Hot spring	Three therapeutic thermal spas	Culture	*L. pneumophila* (34.8%, 40 out of 115 samples)
Parthuisot et al. (2011) (477)	France	Thermal spring		Culture Immunofluorescence-Based Assays (IF) + Solid-Phase Cytometry	*L. pneumophila* (culture, IF)
Paveenkittiporn et al. (2012)(478)	Thailand	Hot spring		Culture	*L. longbeachae* *L. gormanii*
Qin et al. (2012)(479)	China	Hot spring		Culture PCR qPCR	*Legionella* spp. (culture, 54.4%, 49/90; PCR, 71.1%, 64/90; qPCR, 93.3%, 84/90)
Qin et al. (2013)(480)	China	Hot spring	Three hot spring recreation areas (spa pools)	Culture	*Legionella* spp. (51.9%, 160 out of 308 samples) *L. pneumophila:* most frequently isolated (98.9%)
Rocha et al. (1995)(306)	Portugal	Thermal spring	Therapeutic spa	Culture	*Legionella* spp. (10 out of 10 sampled sites) *L. pneumophila*: most frequently identified
Sommese et al. (1996)(307)	Italy	Thermal spring		Culture	*Legionella* spp. (3 out of 66 sampled sources) *L. pneumophila* *L. dumoffii*

Table S5. Selected publications on natural water: thermal springs (*continued*)

Reference	Country	Source type	Samples analyzed	Detection method	Detected *Legionella* species
Sukthana et al. (2005)(308)	Thailand	Hot spring		Culture	*L. pneumophila* (63.4%, 45 out of 71 samples, 71.9%, 41 out of 57 springs)
Verissimo et al. (1991)(345)	Portugal	Hot Spring	19 aquatic environments in 4 hydrothermal areas	Culture	*Legionella* spp. *L. pneumophila* (74% of a total of 288 isolates)
Zbikowska et al. (2013) (311)	Poland	Thermal saline spring	Three thermal baths	FISH	*Legionella* spp. *L. pneumophila* (3 out of 3 sampled baths)

Table S6. Selected publications on natural water: surface water

Reference	Country	Source/reservoir	Samples analyzed	Detection method	Detected *Legionella* species
Bercovier et al. (1986)(283)	Israel	Ponds, surface water	Fish ponds with and without sewage, irrigation water without sewage	Culture	*Legionella* spp. (2 out of 30 fish ponds, 10 out of 22 irrigation water samples)
Brissaud et al. (2008)(284)	France	River	Water samples	qPCR	*Legionella* spp. *L. pneumophila* low concentrations or not detected
Calvo et al. (2013)(281)	Spain	Water reservoirs (swamps)	Water samples	qPCR	*L. pneumophila* (2 out of 3 reservoirs)
Campbell et al. (1984)(402)	USA	Spring		Culture	*L. sainthelensi*
Campocasso et al. (2012) (481)	Tunisia	Hypersaline lake		Coculture*	*L. tunisiensis*
Carvalho et al. (2008)(341)	Antarctica	Polar lakes		Culture PCR	*L. pneumophila* (by culture and PCR) *L. lytica* (PCR) *L. jeonii* (PCR) *L. erythra* (PCR)
Carvalho et al. (2007)(342)	Brazil	Rivers	One sample from an upstream pristine region, one sample from a downstream estuarine region moderately affected by untreated domestic sewage	Culture PCR	pristine water: *L. pneumophila* (PCR) estuarine water: *L. pneumophila* and non-*pneumophila* (PCR) *L. dumoffii* *L. lytica* *L. birminghamensis* *L. bozemanii*
Corsaro et al. (2010)(351)	Spain	Surface water, groundwater	From treatment plants producing drinking water	Coculture† PCR detection on indigenous amoebae	*Legionella* spp.
Cherry et al. (1982)(456)	USA	River		Guinea pig inoculation	*L. jordanis*

Table S6. Selected publications on natural water: surface water (*continued*)

Reference	Country	Source/reservoir	Samples analyzed	Detection method	Detected *Legionella* species
Declerck et al. (2007)(482)	Belgium	Lakes, ponds, brooks, creeks	Water, substrate-associated and floating biofilm samples Surface waters were not related to the anthropogenic aquatic systems	qPCR	*Legionella* spp. (81% of all sampling points, i.e., water and/or substrate-associated biofilm and/or floating biofilm, floating biofilm positive in all 30 samples, 30 out of 37 samples) *L. pneumophila* (70%, 21 out of 30 samples)
Devos et al. (2005)(483)	Belgium	Pond, canal, diving place, percolating water		Culture PCR	*L. pneumophila* (PCR, 3 out of 4 samples; culture, 2 out of 4 samples)
Fliermans et al. (1981)(340)	USA	River, lake		DFA Guinea pig inoculation	*L. pneumophila* (DFA, 66 out of 67 sampling locations)
Fliermans et al. (1979)(484)	USA	Lake		DFA Guinea pig inoculation	*L. pneumophila* (DFA)
Gast et al. (2011)(346)	USA	Marine water	Eustuarine system and salt lake	PCR detection on indigenous amoebae	*Legionella* spp. (48%, 185 out of 388 isolated amoebae) *L. pneumophila* (9.2%, 17 out of 185 *Legionella* spp.-positive amoebae)
Joly et al. (1984)(485)	Canada	Streams, lakes	Some samples came from heavily polluted areas	Culture DFA Guinea pig inoculation	*L. pneumophila* (culture) *L. micdadei* (culture) *Legionella* spp. (DFA, 43 out of 100 samples) *L. pneumophila* (55% of the positive samples)
Kao et al. (2013)(474)	China	River		PCR qPCR	*Legionella* spp. (10 out of 100 samples) *L. pneumophila* (4 out of 100 samples)
Luck et al. (2010)(486)	Germany	Stream		Culture	*L. dresdenensis*
Morris et al. (1979)(312)	USA	Stream		Guinea pig inoculation	LD bacterium

Table S6. Selected publications on natural water: surface water (*continued*)

Reference	Country	Source/reservoir	Samples analyzed	Detection method	Detected *Legionella* species
Okpara et al. (1996)(487)	Germany	Surface water	Obtained at different levels of purification of surface water (in a waterworks)	Culture PCR	*Legionella* spp. (PCR, 4 out of 5 samples)
Olsen et al. (2010)(227)	Norway	River	River downstream of the outlet of a wastewater treatment plant	Culture	*L. pneumphila*
Ortiz-Roque & Hazen (1987)(347)	Puerto Rico	Marine water, fresh water	Most of the sampling sites were under anthropogenic impact (sewage discharge)	DFA Guinea pig inoculation	*Legionella* spp. (culture, DFA, at all 5 sampling sites) *L. pneumophila* (DFA, at all 5 sampling sites)
Palmer et al. (1993)(287)	USA	Marine water	Part of the sampling sites were under anthropogenic impact (treated sewage discharge)	Culture DFA PCR	*Legionella* spp. (5 out of 6 sampling sites: DFA) *L. pneumophila* (DFA and/or PCR, 5 out of 6 sampling sites)
Palmer et al. (1995)(286)	USA	Lake	One lake was filled with groundwater, one lake was under anthropogenic impact (treated sewage discharge)	Culture DFA PCR	*Legionella* spp. (both lakes: DFA)
Parthuisot et al. (2011)(477)	France	Fresh water		Culture Immunofluorescence-Based Assays (IF) + Solid-Phase Cytometry	*L. pneumophila* (culture, IF)
Parthuisot et al. (2010)(488)	France	River	Sampling sites were partly under anthropogenic impact; samples were taken up- and downstream of discharge sites (i.e., treated sewage discharge or discharge from thermal baths)	Culture qPCR	*Legionella* spp. (qPCR, at all three sampling sites, upstream and downstream) *L. pneumophila* (culture, 2 out of three sampling sites, both downstream of the discharge sites)
Paveenkittiporn et al. (2012)(478)	Thailand	Pond		Culture PCR	*L. dumoffi, L. micdadei* (culture)

Table S6. Selected publications on natural water: surface water (*continued*)

Reference	Country	Source/reservoir	Samples analyzed	Detection method	Detected *Legionella* species
Sheehan et al. (2005)(343)	USA	Stream	Acid-sulfate-chloride stream, originates from multiple hot springs and contains an extensive algal mat, algal mat was sampled	Culture PCR	*Legionella* spp. (PCR, 3 out of 4 sites)
Sinigalliano et al. (2007) (348)	USA	Estuary lake, canals	Sampling after flooding of the city of New Orleans, Louisiana, caused by Hurricane Katrina and Hurricane Rita	PCR	*Legionella* spp. (all sampled sites lake, both sampled canal sites) *L. pneumophila* (1 site)
States et al. (1987)(489)	USA	River		Culture	*L. pneumohila*
Thomas et al. (2008)(490)	France	River		Coculture‡ PCR detection on indigenous amoebae	*L. anisa* (coculture)
Tomov et al. (1981)(344)	Bulgaria	Thermal lake	Samples obtained from small lakes formed around warm mineral springs	Guinea pig inoculation	*L. pneumophila*
Verissimo et al. (1991)(345)	Portugal	Thermal lake		Culture	*Legionella* spp. (1 isolate)
Wullings & van der Kooij (2006)(352)	Netherlands	Groundwater, rivers	Aerobic groundwater and anaerobic groundwater used for drinking water production	Culture qPCR	*Legionella* spp. (qPCR, 8 out of 9 aerobic groundwater samples, 3 out of 7 anaerobic groundwater samples, all 10 river water samples) *L. pneumophila* (qPCR, 3 out of 10 river water samples)
Yamamoto et al. (1993)(491)	Japan	Stream, marine water	Tide pools	Culture DFA PCR	*Legionella* spp. (PCR, all samples) *L. pneumophila* (DFA, all samples)

**Acanthamoeba polyphaga.* †*Acanthamoeba* spp. ‡*Acanthamoeba castellanii.*

Appendix B

Supporting information Chapter 4

Table S5. Typing results of the *L. pneumophila* strains isolated from garden soil samples

Garden	Sampling	Sampling year	Sampling month	Serogroup	MAb 3/1 positive/ negative	MAb subtype	ST	No. of typed strains
1	1st	2014	February	SG1	pos	Benidorm	84	2
1	1st	2014	February	SG1	pos	Benidorm	477	4
1	1st	2014	February	SG1	pos	France/Allentown	863	1
1	1st	2014	February	SG2–14*	-	-	465	1
1	1st	2014	February	SG2–14	-	-	710	1
1	2nd	2014	September	SG1	pos	Benidorm	84	2
1	2nd	2014	September	SG1	pos	Benidorm	477	1
1	2nd	2014	September	SG2–14	-	-	710	3
1	3rd	2014	November	SG1	pos	Benidorm	84	1
1	3rd	2014	November	SG1	pos	Benidorm	115	2
1	3rd	2014	November	SG1	pos	Benidorm	477	3
1	4th	2015	February	SG1	neg	Camperdown	477	4
1	4th	2015	February	SG2–14	-	-	710	2
4	1st	2014	April	SG1	pos	Benidorm	84	1
4	1st	2014	April	SG1	pos	Benidorm	115	2
4	1st	2014	April	SG1	pos	Benidorm	477	1
4	1st	2014	April	SG1	pos	Philadelphia	2032	1
4	1st	2014	April	SG1	neg	Camperdown	2028	1
4	1st	2014	April	SG2–14	-	-	863	1
4	1st	2014	April	SG2–14	-	-	X†	2
5	1st	2014	April	SG2–14	-	-	2025	2
5	1st	2014	April	SG2–14	-	-	2026	1
5	1st	2014	April	SG2–14	-	-	X	1

Table S5. Typing results of the *L. pneumophila* strains isolated from garden soil samples (*continued*)

Garden	Sampling	Sampling year	Sampling month	Serogroup	MAb 3/1 positive/ negative	MAb subtype	ST	No. of typed strains
8	1st	2014	June	SG1	pos	Benidorm	84	1
8	1st	2014	June	SG1	pos	Benidorm	710	1
8	1st	2014	June	SG2–14	-	-	462	1
13	1st	2014	August	SG2–14	-	-	710	2
13	2nd	2015	January	SG1	neg	Camperdown	84	5
13	2nd	2015	January	SG1	neg	Camperdown	115	1
13	2nd	2015	January	SG2–14	-	-	84	1
13	2nd	2015	January	SG2–14	-	-	710	1
13	2nd	2015	January	SG2–14	-	-	2080	1
13	2nd	2015	January	SG2–14	-	-	X	1
18	1st	2014	November	SG1	pos	Benidorm	84	1
18	1st	2014	November	SG1	pos	Benidorm	477	3
18	1st	2014	November	SG1	neg	Camperdown	1856	1
18	1st	2014	November	SG1	neg	Olda	1856	1
18	1st	2014	November	SG1	neg	Olda	2022	1
18	1st	2014	November	SG1	neg	Olda	2029	1
18	1st	2014	November	SG2–14	-	-	84	1
18	1st	2014	November	SG2–14	-	-	115	1
18	1st	2014	November	SG2–14	-	-	710	1
18	2nd	2015	March	SG1	neg	Camperdown	X	1
18	2nd	2015	March	SG1	neg	Olda	2022	1
22	1st	2015	January	SG2–14	-	-	84	2

– Not applicable. ST: Sequence type. *SG2-14: one of the serogroups 2-14, exact serogroup not further determined. [†]X: a sequence type could not be retrieved due to failure of amplification of one or more gene targets.

Table S6. Questionnaire and weather variables univariately analyzed for association with the presence of *Legionella* in garden soils*

Variable		Frequency (no.)	Frequency (%)	Positive for *Legionella* (%)	*p*–value
Garden type	normal garden	164	92.7	12.2	0.74
	vegetable garden	7	4.0	28.6	
	allotment garden	6	3.4	0	
Size garden	≤30 m2	82	46.3	15.9	0.17
	>30 m2	80	45.2	8.8	
Surrounding area	rural	9	5.1	0	0.02
	urban	144	81.4	14.6	
	mixed	17	9.6	0	
Age garden	<1 year	10	6.4	30	0.10
	>1 year	146	93.6	10.3	
Use potting soil/compost	sometimes/regularly (at least 1/year)	103	59.9	14.6	0.24
	never	69	40.1	8.7	
Use of potting soil and origin potting soil/compost	yes, homemade	12	6.8	0	0.33
	yes, bought	77	43.5	16.9	
	yes, homemade and bought	8	4.5	12.5	
	yes, unknown	1	0.6	0	
	no, no use of potting soil/compost	69	39.0	8.7	
	yes, other	5	2.8	20.0	

Table S6. Questionnaire and weather variables univariately analyzed for association with the presence of *Legionella* in garden soils* (*continued*)

Variable		Frequency (no.)	Frequency (%)	Positive for *Legionella* (%)	*p*–value
Use of other fertilizers	sometimes/regularly (at least 1/year)	77	44.8	14.3	0.23
	never	95	55.2	8.4	
Frequency of gardening in gardening season	1/week or more	99	57.2	11.1	0.82
	1/month	40	23.1	15.0	
	<1/month	34	19.7	11.8	
Watering garden with tap water	yes	133	75.1	12.8	0.67
	no	39	22.0	10.3	
Whirlpool in garden	yes	3	1.7	0	0.37
	no	174	98.3	12.6	
Use of pesticide/herbicide	yes	36	20.8	13.9	0.72
	no	137	79.2	11.7	
Season of sampling	winter	37	20.9	13.5	0.89
	spring	47	26.6	10.6	
	summer	46	26.0	15.2	
	fall	47	26.6	10.6	
Precipitation on the day of sampling (mm)	0	71	40.1	16.9	0.22
	>0 – 1	19	10.7	10.5	
	>1 – 2	42	23.7	7.1	
	>2	45	25.4	11.1	

Table S6. Questionnaire and weather variables univariately analyzed for association with the presence of *Legionella* in garden soils* (*continued*)

Variable		Frequency (no.)	Frequency (%)	Positive for *Legionella* (%)	*p*–value
Precipitation sum in the 14 days preceding sampling (mm)	≤15	38	21.5	10.5	0.37
	>15 – 30	59	33.3	18.6	
	>30 – 45	31	17.5	9.7	
	>45	49	27.7	8.2	
Mean temperature on sampling day (°C)	≤6	41	23.2	12.2	0.56
	>6 – 12	27	15.3	11.1	
	>12 – 18	51	28.8	17.7	
	>18	58	32.8	8.6	
Minimum temperature on sampling day (°C)	≤4	44	24.9	13.6	0.93
	>4 – 9	39	22.0	10.3	
	>9 – 14	57	32.2	14.0	
	>14	37	20.9	10.8	
Maximum temperature on sampling day (°C)	≤11	50	28.2	12.0	0.70
	>11 – 16	25	14.1	8.0	
	>16 – 21	40	22.6	17.5	
	>21	62	35.0	11.3	
Mean temperature in 14 days preceding sampling day (°C)	≤8	39	22.0	15.4	0.16
	>8 – 12	46	26.0	13.0	
	>12 – 16	47	26.6	4.3	
	>16	45	25.4	17.8	

Table S6. Questionnaire and weather variables univariately analyzed for association with the presence of *Legionella* in garden soils* (*continued*)

Variable		Frequency (no.)	Frequency (%)	Positive for *Legionella* (%)	*p*–value
Minimum temperature in 14 days preceding sampling day (°C)	≤–1	37	20.9	10.8	0.58
	>–1 – 3	49	27.7	16.3	
	>3 – 7	41	23.2	7.3	
	>7	50	28.2	14.0	
Maximum temperature in 14 days preceding sampling day (°C)	≤17	49	27.7	16.3	0.68
	>17 – 22	37	20.9	10.8	
	>22 – 27	47	26.6	8.5	
	>27	44	24.9	13.6	

*A total of 177 gardens were sampled, if the total per variable does not tally to 177 then the variable contained missing data. Variables in bold were analyzed multivariately ($p \leq 0.25$).

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Summary

Legionella are Gram-negative, rod shaped, flagellated bacteria. There are currently 62 known species of which approximately half have been associated with patients. Legionellosis, the infection caused by *Legionella* bacteria, describes two distinct clinical syndromes, Legionnaires' disease (LD) and Pontiac fever. Infection occurs mainly through inhalation of *Legionella* contaminated aerosols. Symptoms of LD can range from mild disease to severe pneumonia with sometimes fatal outcome. Pontiac fever is a far milder disease with flu-like symptoms. In the Netherlands and Europe, the most important infective agent is *Legionella pneumophila*. In other parts of the world, such as Australia and New Zealand, the species *Legionella longbeachae* is a frequent cause of disease.

Legionella bacteria are ubiquitous in the environment, in water and soil, and in man-made water systems. They are subject to phagocytic predation by protozoa but are able to avoid degradation and they can multiply inside the protozoa. *L. pneumophila* infection is associated with aquatic sources, such as tap water systems, cooling towers and whirlpools. Infection with *L. longbeachae* has been linked to potting soil and composted materials.

LD is a notifiable disease and in 2016, 324 LD cases were reported that most probably contracted the disease in the Netherlands. The majority of these LD cases occur sporadically, i.e., not related to a cluster or outbreak. Interestingly, the source of infection of such sporadic LD cases is almost never identified. Infection may possibly result from exposure to *Legionella* bacteria in sources that are not yet considered in source investigations. Therefore, the aim of this thesis was to explore such alternative sources of *Legionella* bacteria (i.e., that are not yet considered in source investigations). Knowledge about the sources that cause the LD patients in the Netherlands can help focus preventive measures.

To optimize LD source investigations, it is important to have knowledge about all the reservoirs of *Legionella*, and whether exposure to these reservoirs can lead to infection. In the literature review described in **Chapter 2**, we provide an overview of reservoirs of *Legionella* reported in the literature, other than drinking water distribution systems. The matrices and systems that are described in the literature were classified according to the strength of evidence that implicates that the matrix or system could be a source of *Legionella* infection. For this purpose, a level of evidence (LOE) approach was used. Every selected study was assigned an LOE and based on this, the systems and matrices were classified as a potential source or confirmed source. A confirmed source was assigned at least once the highest LOE (LOE I). For LOE I, cases must be epidemiologically linked to a suspected source and a match must be determined between the clinical and environmental isolates. Furthermore, there should be additional evidence demonstrating that the source caused the infection(s), e.g., evidence that excludes other possible sources.

This review demonstrates that many different water systems and non-water systems are reservoirs of *Legionella* and 17 different systems and matrices have been confirmed as sources of *Legionella,* such as, cooling towers, whirlpools, potting soil/compost, wastewater/wastewater treatment plants (WWTPs), fountains, room humidifiers, mist machines and baths. Many other man-made systems or natural matrices were classified as a potential source. Examples of these are respiratory devices, water used for cleaning, surface water, groundwater and rainwater. Although the strength of the available evidence for these potential sources is low, these systems or matrices could play a role in the transmission of infectious *Legionella* bacteria. They might not yet be considered in source investigations, resulting in an underestimation of their importance. In addition, some of the confirmed sources are also not yet considered regularly in source investigations.

In the four studies described in the next chapters, we considered natural soil (**Chapters 3-6**) and rainwater puddles (**Chapters 5 and 6**) as alternative sources of *Legionella* bacteria. An amoebal coculture method was used to isolate *Legionella* bacteria from these matrices.

In **Chapter 3**, we describe the isolation of *L. pneumophila* sequence type (ST) 47 from garden soil. In the Netherlands, *L. pneumophila* ST47 is an important cause of sporadic LD, but this ST is hardly ever isolated from environmental sources. Since the start of the National *Legionella* Outbreak Detection Program in the Netherlands in 2002, ST47 was only found three times in the environment during outbreak investigations, which concerned outdoor whirlpools that were involved in two outbreaks of legionellosis and one solitary case. As all three whirlpools were located outside, it was hypothesized that the outdoor environment was an influence. Two gardens were investigated and strain ST47 was detected in the garden soil of the most recent outbreak. We speculate that this strain was transmitted from soil to the whirlpool in the garden where it caused the outbreak.

In the study described in **Chapter 4**, we further investigated garden soil as a reservoir of viable *Legionella* bacteria. *Legionella* bacteria were detected in 22 of the 177 sampled gardens (12%). Of these 22 soil samples, seven contained *L. pneumophila*. The *Legionella*-positive samples also regularly contained other species, including *L. longbeachae*, *Legionella sainthelensi* and *Legionella bozemanii*. The *L. pneumophila* isolates comprised 15 different STs. Three of these STs were also found in patients in the Netherlands (ST84, ST115, ST477) but they are relatively uncommon. Strikingly, these three clinically relevant STs belonged to the most frequently isolated STs in garden samples. We speculate that it is possible that some conditions in garden soils are favorable for the growth of these clinically relevant STs. Furthermore, evidence was found that *L. pneumophila* can persist in garden soil over time. For three gardens, *L. pneumophila* was detected again after four

to seven months. One garden was sampled four times over a period of one year and *L. pneumophila* was found on all occasions.

Several studies have reported an association between rainfall and LD incidence. Furthermore, viable *Legionella* bacteria were detected in rainwater from pluvial floods in the Netherlands and in rainwater on roads in Japan. Therefore, in the study presented in **Chapter 5**, we considered rainwater puddles on roads in the Netherlands and soil next to roads as reservoirs and alternative sources of *Legionella*. *Legionella* bacteria were isolated from 4% (3/77) of the rainwater puddles and 30% (6/20) of the soils. *L. pneumophila* was isolated from 2 rainwater samples and 2 soil samples. Several other species were found including *L. longbeachae* and *Legionella gormanii.* STs could be assigned to two *L. pneumophila* strains isolated from soil, ST710 and ST477, and one strain isolated from rainwater, ST1064. These STs were previously associated with LD patients.

Due to the relatively high detection rate of *Legionella* bacteria in soil next to roads, we further investigated natural soil as a reservoir of *Legionella*. In **Chapter 6**, we present data on the relationship between soil characteristics of natural soils and the presence and persistence of *Legionella* bacteria. *Legionella* spp. were isolated from natural soils and a relationship was found between *Legionella* presence and soil type. Compared to *Legionella*-negative soils, the *Legionella*-positive ones contained an enhanced level of clay and silt, in relation to sand. Furthermore, we found that, in an experimental situation, clayey soil was more favorable for *L. pneumophila* persistence over time than sandy soil. In order to investigate a possible transmission route of *Legionella* bacteria from soil to humans, we tested if *L. pneumophila* is transferred from soil to a water layer on the soil, mimicking a rainwater puddle. It was found that *L. pneumophila* was transferred from sandy soil to the water layer, showing that *Legionella* in soil can potentially move to a compartment relevant for aerosolization.

In conclusion, soil and rainwater puddles are reservoirs of *Legionella* bacteria and therefore potential alternative sources of LD. If people are exposed to *Legionella* from soil and rainwater is unknown. We recommend to target soil in source investigations, in particular in relation to cases that are infected by a soil specific ST. WWTPs and cooling towers are known sources of *Legionella* but they are possibly under-recognized as sources of sporadic LD because they are not regularly targeted in source investigations. Further research is needed to unravel what the contribution of these sources is to the disease burden in the Netherlands.

Samenvatting in het Nederlands

Legionellabacteriën zijn Gram-negatieve, staafvormige bacteriën met flagella. Legionellabacteriën komen overal in het milieu voor, in zowel water als grond, maar ook in kunstmatige watersystemen. De bacteriën kunnen in het milieu opgenomen worden door bepaalde micro-organismen (zoals amoeben). Legionellabacteriën hebben echter de eigenschap dat ze kunnen voorkomen dat ze afgebroken worden door deze micro-organismen. De bacteriën gebruiken de micro-organismen zelfs om te kunnen vermeerderen.

Momenteel zijn er 62 legionellasoorten bekend en ongeveer de helft hiervan kan ziekte veroorzaken bij de mens. *Legionella pneumophila* is de soort die in Nederland en Europa de meeste ziektegevallen veroorzaakt. In andere delen van de wereld, zoals Australië en Nieuw-Zeeland, is *Legionella longbeachae* een belangrijke oorzaak van ziekte. Infectie met *L. pneumophila* wordt voornamelijk geassocieerd met kunstmatige aquatische bronnen, zoals leidingwatersystemen (bijv. douches), koeltorens en whirlpools. Deze watersystemen kunnen water vernevelen dat legionellabacteriën bevat en dit kan door mensen worden ingeademd. Infectie met *L. longbeachae* wordt veroorzaakt door blootstelling aan potgrond of compost waar deze legionellasoort zich in bevindt.

De infectie die veroorzaakt wordt door legionellabacteriën zoals *L. pneumophila* en *L. longbeachae* wordt legionellose genoemd. Dit is een verzamelnaam voor twee ziektebeelden: Pontiac fever en veteranenziekte. Pontiac fever is een griepachtige aandoening die vanzelf over gaat. Bij veteranenziekte is het belangrijkste symptoom longontsteking en doorgaans is ziekenhuisopname hierbij noodzakelijk. In ernstige gevallen kunnen patiënten komen te overlijden.

Veteranenziekte is een meldingsplichtige ziekte en in 2016 werden 324 geïnfecteerde patiënten gerapporteerd die de ziekte opliepen in Nederland. Het overgrote deel van deze patiënten zijn solitair, wat wil zeggen dat ze niet gerelateerd zijn aan andere patiënten in clusters of uitbraken. Het is opvallend dat voor deze patiënten bijna nooit een oorzaak van infectie wordt gevonden, ondanks bronopsporingsactiviteiten. Mogelijk raken patiënten geïnfecteerd door blootstelling aan bronnen van legionella die tijdens bronopsporingen niet worden onderzocht. Het doel van het onderzoek beschreven in dit proefschrift was daarom, het onderzoeken van alternatieve bronnen van legionellabacteriën die de oorzaak zouden kunnen zijn van solitaire patiënten met veteranenziekte. Met alternatieve bronnen worden bronnen bedoeld die niet standaard meegenomen worden bij bronopsporingen. Meer inzicht in infectiebronnen in Nederland kan handvatten bieden voor gerichte preventiemaatregelen.

Om brononderzoeken te optimaliseren is het belangrijk om kennis te hebben van de reservoirs waar legionellabacteriën zich in kunnen bevinden en of blootstelling aan deze reservoirs kan leiden tot infectie. Als blootstelling aan legionellabacteriën uit een reservoir

leidt tot infectie, noemen we het reservoir een bron. In **Hoofdstuk 2** wordt een overzicht gegeven van reservoirs van legionella beschreven in wetenschappelijke studies. Alle beschreven reservoirs zijn ingedeeld als bevestigde bron of als potentiële bron op basis van de onderzoeksresultaten van de geselecteerde studies. Als een reservoir als bevestigde bron is geclassificeerd, dan zijn er voldoende bewijzen die aantonen dat mensen door legionella uit het reservoir zijn geïnfecteerd. De volgende bewijzen moeten daarvoor zijn geleverd: patiënten waren epidemiologisch gelinkt aan de mogelijke bron en de geïsoleerde klinische stam en de stam uit het milieu waren niet van elkaar te onderscheiden. Daarnaast moet er aanvullend bewijs worden geleverd dat de legionellabacteriën uit de mogelijke bron de infectie(s) hadden veroorzaakt. Het uitsluiten van andere potentiële bronnen waaraan de patiënt(en) werd(en) blootgesteld is daar een voorbeeld van. Als er onvoldoende bewijzen waren dan werd het reservoir geclassificeerd als potentiële bron.

Dit literatuuronderzoek laat zien dat legionellabacteriën in een groot scala aan watersystemen en niet-watersystemen aanwezig zijn. Er zijn 17 verschillende systemen en matrices geclassificeerd als bevestigde bron van legionella, zoals koeltorens, whirlpools, potgrond en compost, rioolwater en rioolwaterzuiveringsinstallaties, fonteinen, luchtbevochtigers, mistmachines en baden. Voor veel andere systemen of matrices die legionellabacteriën bevatten werd niet voldoende bewijs geleverd. Voorbeelden hiervan zijn: beademingsapparatuur, water gebruikt voor schoonmaken, oppervlakte- en grondwater, of regenwater. Het kan echter niet worden uitgesloten dat deze systemen en matrices een rol spelen in het veroorzaken van solitaire legionellose-patiënten; mogelijk worden ze niet overwogen en onderzocht tijdens bronopsporingsactiviteiten. Hierdoor kan de bijdrage van sommige reservoirs van legionella aan de ziektelast worden onderschat. Daarnaast zijn er bevestigde bronnen die niet routinematig worden onderzocht bij het zoeken naar de oorzaak van infectie.

In de vier studies die in de volgende hoofdstukken worden beschreven, hebben we onderzoek gedaan naar grond (**Hoofdstuk 3-6**) en regenwaterplassen (**Hoofdstuk 5 en 6**) als alternatieve bronnen van legionellabacteriën. Een kweekmethode met amoeben werd toegepast om legionellabacteriën te isoleren uit grond en regenwater.

In **Hoofdstuk 3** beschrijven we de isolatie van een *L. pneumophila* stam, sequentietype (ST) 47, uit tuingrond. Binnen de soort *L. pneumophila* bestaan verschillende sequentietypes. Met sequentie wordt de volgorde bedoeld van bepaalde stukken van het DNA van de bacterie. Hoewel er meer dan 2000 sequentietypes bekend zijn, is slechts een klein aantal *L. pneumophila*-sequentietypes de oorzaak van infectie van patiënten. In Nederland is *L. pneumophila*-ST47 een belangrijke ziekteverwekker. Dit type legionella wordt dus vaak bij patiënten aangetroffen, maar het wordt bijna nooit geïsoleerd uit mogelijke bronnen. In Nederland is ST47 sinds 2002 slechts drie keer gevonden tijdens brononderzoek, waarbij

in alle gevallen de bacterie werd aangetroffen in tuinwhirlpools. Deze whirlpools waren de oorzaak van 2 uitbraken en 1 solitaire patiënt. Omdat alle drie de whirlpools buiten stonden, was onze hypothese dat de natuurlijke omgeving van invloed moet zijn geweest. Twee van de drie tuinen werden onderzocht en uit de tuin van de meest recente uitbraak werd stam ST47 geïsoleerd. Mogelijk is deze stam vanuit de tuingrond in de whirlpool terecht gekomen.

Na deze studie hebben we verder onderzoek gedaan naar tuingrond, beschreven in **Hoofdstuk 4**. Van de 177 onderzochte tuinen, vonden we in 22 tuinen legionellabacteriën in de grond (12%). Zeven van deze 22 grondmonsters bevatten de soort *L. pneumophila*. In de legionella-positieve monsters werden ook regelmatig andere legionellasoorten aangetroffen, zoals *L. longbeachae*, *Legionella sainthelensi* en *Legionella bozemanii.* De geïsoleerde *L. pneumophila* stammen bestonden uit 15 verschillende sequentietypes. Drie van deze types werden eerder ook bij patiënten gevonden (ST84, ST115, ST477), hoewel ze niet veelvoorkomend zijn. Opvallend is dat deze drie klinisch-relevante sequentietypes tot de meestvoorkomende sequentietypes in de tuingrondmonsters behoorden. Mogelijk zijn bepaalde condities in tuingrond gunstig voor deze specifieke *L. pneumophila*-types om te overleven en groeien. Verder hebben we bewijs gevonden dat legionellabacteriën kunnen persisteren in tuingrond. Een zestal *L. pneumophila*-positieve tuinen werd na een aantal maanden opnieuw onderzocht en uit drie tuinen werden dezelfde types geïsoleerd. Eén tuin werd in totaal viermaal bemonsterd over een periode van een jaar en op alle momenten positief bevonden. Dit betekent dat legionellabacteriën naar alle waarschijnlijkheid kunnen persisteren in tuingrond.

Verschillende studies hebben afgelopen jaren aangetoond dat neerslag gerelateerd is aan het voorkomen van legionellose-patiënten. Dat wil zeggen dat er bij veel neerslag een stijging is in het aantal ziektegevallen. Daarnaast werden legionellabacteriën in Nederland geïsoleerd uit overstromingswater na hevige regenval ('water op straat') en uit regenwaterplassen in Japan. Naar aanleiding van deze studies hebben we regenwaterplassen op de weg en grond naast de weg onderzocht. In **Hoofdstuk 5** rapporteren we dat er legionellabacteriën werden geïsoleerd uit drie van de 77 (4%) onderzochte regenwaterplassen en zes van de 20 (30%) grondmonsters. *L. pneumophila* werd gevonden in twee regenwaterplassen en in twee grondmonsters. Verschillende andere legionellasoorten werden aangetroffen, zoals *L. longbeachae* en *Legionella gormanii*. Van de geïsoleerde *L. pneumophila* stammen hadden er drie een klinisch-relevant sequentietype (ST710, ST477 en ST1064).

In bovengenoemde studie werden in relatief veel grondmonsters legionellabacteriën gevonden. We hebben daarom verder onderzoek verricht naar natuurlijke grond (d.w.z. geen potgrond/tuingrond) als reservoir en potentiële bron van legionella. In het onderzoek beschreven in **Hoofdstuk 6** is een relatie aangetoond tussen de aanwezigheid van

legionellabacteriën in natuurlijke grond en grondtype. In vergelijking tot de negatieve grondmonsters, hadden de grondmonsters waaruit legionella werd geïsoleerd een hogere proportie zilt- en kleideeltjes in verhouding tot zanddeeltjes. Daarnaast vonden we dat *L. pneumophila* bacteriën beter overleefden in kleigrond dan in zandgrond. Dat legionellabacteriën aanwezig zijn in grond wil nog niet zeggen dat grond ook een bron is. Mensen moeten namelijk blootgesteld worden aan de bacteriën uit grond om ziek te worden. In een experimentele opstelling hebben we het ontstaan van een regenplas op grond nagebootst en vonden dat de bacteriën vanuit zandgrond naar een waterlaag op de grond verplaatsten. De waterlaag stelde in dit experiment een regenplas voor. Legionellabacteriën kunnen dus mogelijk verplaatsen van grond naar een matrix waaruit de bacterie kan worden verneveld. Verneveling zou plaats kunnen vinden wanneer er bijvoorbeeld een auto door de regenplas rijdt.

Samengevat zijn (tuin)grond en regenwaterplassen reservoirs van legionellabacteriën en mogelijk alternatieve bronnen van legionellose. Of mensen blootgesteld kunnen worden aan legionellabacteriën afkomstig uit grond (via regenwaterplassen) is nog onduidelijk. Het is aan te bevelen om in de toekomst (tuin)grond mee te nemen in brononderzoeken, in het bijzonder voor sequentietypes die veel voorkomen in grond. Rioolwaterzuiveringsinstallaties en koeltorens zijn bekende bronnen van legionellabacteriën, maar ze worden niet routinematig meegenomen bij bronopsporing. Daarom worden ze mogelijk onderschat als oorzaak van solitaire legionellose-patiënten. Verder onderzoek is nodig om vast te stellen wat de bijdrage is van deze bronnen aan de legionellose-ziektelast in Nederland.

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About the author

I, Eri van Heijnsbergen, was born on April 16, 1982 in 's-Hertogenbosch (the Netherlands). I spent my youth in Berlicum, a small town in the south of the Netherlands. I attended high school at the Fioretti College in Veghel and graduated in 2000.

After working for one year after high school graduation, I started the study Veterinary Medicine in 2001 at Utrecht University. After one year I switched to the bachelor education Biology from which I graduated *Cum laude* in 2005. Between my bachelor and master study I worked for one year at IKEA in Utrecht to have some time to figure out which direction I wanted to pursue. In 2006, I started the master Science Communication & Education. In 2008, I started an internship at the Institute for Risk Assessment Sciences (IRAS) and only then did I discover my true interest in environmental microbiology. I studied the influence of an antibiotic on the presence of bacterial resistance genes in soil amended with manure. In 2009, I received my master degree *Cum laude*.

After graduation I first worked as an editorial coordinator at a medical science publisher in Baarn. In October 2011, I got the opportunity to start as a PhD candidate at the National Institute for Public Health and the Environment (RIVM) on the subject of alternative sources of *Legionella* bacteria, as described in this thesis.

Since April 2016, I work as a researcher at IRAS on a project about antibiotic resistance in the environment. After living for 12 years in Utrecht and Noord-Holland, I currently live in Brabant, in Vught.

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