Occurrence of *Legionella pneumophila* and *Hartmannella vermiformis* in fresh water environments and their interactions in biofilms

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Occurrence of *Legionella pneumophila* and *Hartmannella vermiformis* in fresh water environments and their interactions in biofilms

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Abstract

Legionella pneumophila, the causative agent of Legionnaires' disease, is widespread in natural fresh water environments and is also frequently found in man-made water systems. Microbial biofilms and protozoa are known to play a major role in the proliferation of *L. pneumophila*. Biofilms provide shelter and a gradient of nutrients, and protozoa may act as host for *L. pneumophila*, since intracellular multiplication of *L. pneumophila* was shown in a variety of protozoan species. The need for protozoa for the proliferation of *L. pneumophila* in aquatic habitats is still not fully understood and is even questioned by some investigators. This thesis shows the *in vivo* growth of *L. pneumophila* in protozoa in aquatic biofilms developing at high population densities on plasticized polyvinylchloride in a biofilm-batch model system with autoclaved tap water. The protozoan species was identified as *Hartmannella vermiformis*, a free-living amoebae which is frequently found co-occurring with *L. pneumophila*. *H. vermiformis* grazed on several specific bacteria in a multi-species biofilm, and it was suggested that several bacterial species and not just one specific organism might serve as food source for *H. vermiformis* in natural environments. Biofilms in tap water distributing systems show a large microbial diversity and are therefore excellent feeding grounds for protozoa. To enable investigations on the contribution of *H. vermiformis* to the occurrence, persistence and proliferation of *L. pneumophila* in fresh water environments, an 18S rRNA-gene targeted real-time PCR method was developed for the specific detection and quantification of *H. vermiformis*. Applying this method to water samples originating from cooling towers, in combination with the detection and quantification of *L. pneumophila*, a significant positive correlation between the log concentrations of both organisms was shown, indicating the importance of *H. vermiformis* as host for *L. pneumophila*. However, despite the common presence of *H. vermiformis* in surface waters, cooling towers, warm water systems and waste water treatment plants, results suggested that other protozoa also contribute to the multiplication of *L. pneumophila*. *L. pneumophila* in natural environments can most probably only multiply in the presence of proliferating protozoa. Modeling of data from a biofilm-batch model indicated that protozoa can only multiply if a threshold concentration of prey bacteria is reached. In tap water installations such a threshold concentration is only reached in the biofilm. Hence, limiting biofilm formation to a level below this threshold concentration and prevention of a rapid biofilm development may be effective in limiting *L. pneumophila* proliferation. A multiple barrier approach, including the use of a suitable control method, the supply of biologically stable low nutrient water, and the application of construction materials with a low biofilm formation potential, is proposed as an effective way for limiting *L. pneumophila* proliferation and survival in man-made water systems.

Contents

CHAPTER 1

Introduction

Introduction

In February 1999, 188 visitors of a flower show near Bovenkarspel in the Netherlands became ill and twenty-nine of them died. The illness, a serious pneumonia, was caused by *Legionella pneumophila*. A whirlpool on display, operated with water without a disinfectant, was identified as the source of the infection (Den Boer *et al*., 2001 and 2002). The first recognized outbreak of pneumonia due to *L. pneumophila* occurred in Philadelphia (USA) in July 1976 among persons attending an American Legion Convention. Here, a total of 180 attendees became ill, of which twenty-nine died. The disease became known as the Legionnaires' disease (Fraser *et al*., 1977) and the etiologic agent was isolated and identified early 1977 (McDade *et al*., 1977). The source of the infection during the Legionnaires' convention was later found to be the air-conditioning system in the lobby of the hotel. Despite all scientific knowledge collected since the first reported outbreak, technical guidelines and regulations, aiming at prevention and control of the growth of *L. pneumophila* in man-made water systems (Joseph *et al*., 2005, Waterleidingbesluit, 2001 and 2004), large outbreaks due to *L. pneumophila* continue to occur, e.g. in Spain, France, Norway and Canada (Anonymous, 2005a and 2005b, Garcia-Fulgueiras *et al*., 2003). Also the number of incidental community-acquired cases does not decrease. Recent reports show that the reported incidence in Europe was more than 4500 cases in 2003 and 2004 (Ricketts and Joseph, 2005).

The bacterium causing the Legionnaires' disease in Philadelphia was isolated via guinea-pigs by using procedures previously developed for the isolation of *Rickettsia* (McDade *et al*., 1977). This was however not the first isolate of *Legionella*. In 1943 Tatlock (1944) obtained an isolate that was then termed a "*Rickettsia*-like organism". In 1954, Drozanski (1956) isolated a bacterium that infected free-living amoebae from soil in Poland. This organism was later (in 1996) classified as a species of *Legionella* (Hookey *et al*., 1996).

Nowadays it is known that *L. pneumophila* is present in many natural fresh water environments as well as in man-made water systems (Fliermans *et al*., 1981, Leoni *et al*., 2001, Tobin *et al*., 1980, Yamamoto *et al*., 1992). Within these environments *L. pneumophila* proliferates mainly in biofilms (Colbourne *et al*., 1984, Rogers *et al*., 1994a). Furthermore legionellae have been shown to multiply intracellularly in eukaryotic host cells (Rowbotham, 1980a). A total of 14 species of amoebae, two species of ciliated protozoa, and one species of slime mold, have been identified as potential hosts for *L. pneumophila* (Fields *et al*., 2002, Shadrach *et al*., 2005). However, a few investigators still question the need for protozoa for the proliferation of *L. pneumophila* in biofilms (Rogers and Keevil, 1992, Surman *et al*., 2002).

This introduction provides an overview of the current knowledge on characteristics of the genus *Legionella*, with specific attention to *L. pneumophila*, as well as protozoa and biofilms, which are shown to be important factors for the proliferation of *L. pneumophila* in aquatic environments, both natural and man-made.

Figure 1.1. Electron microscopy photograph of *L. pneumophila* (http://www.kcl.ac.uk/kis/schools/life_sciences/life_sci/msc/msc_pub_health.html, accession date: 6 January 2006).

Legionella pneumophila

Introduction and characteristics

L. pneumophila is a facultative intracellular pathogen, which belongs to the genus *Legionella*. Bacteria of this genus are Gram-negative, non-spore forming, rod-shaped, aerobic bacteria within the γ-Proteobacteria (Fig. 1.1). They contain branched-chain fatty acids as components of their lipopolysaccharides, have a non-fermentative metabolism, and require L-cysteine and iron salts for growth. The genus *Legionella* was established in 1979, three years after the first recognized outbreak of legionellosis (Brenner *et al.*, 1979, Fraser *et al.*, 1977). The family Legionellaceae consists of the single genus *Legionella*. This genus currently includes 50 identified species, comprising 71 distinct serogroups, and the number of species and serogroups continues to increase (Table 1.1).

The genome of *L. pneumophila* subsp. *L. pneumophila* (serogroup 1), strain Philadelphia 1, consists of a single circular chromosome of 3,397,754 base pairs (Chien *et al.*, 2004). This strain also possesses a plasmid-like element of 45 kbp that exists in a circular episomal form, but can also be integrated in the chromosome. The genome includes selective expansions of important gene families involved in intracellular growth, such as *icm/dot* genes, the *lvh/lvr* cluster, and the *ralF* gene that actually is of eukaryotic origin. Also genes for unexpected metabolic pathways, including an intact glycolytic chain, pyruvate dehydrogenase complex, tricarboxylic acid cycle and respiratory chain, and a glucose-6-phosphate transporter have been identified. Moreover, sequence analysis revealed the presence of previously unknown candidate virulence determinants. Knowledge of the genome sequence of *L. pneumophila* offers the opportunity to examine its broad host range and its ability to multiply in nutrient poor aquatic environments, including water supplies. Having lists of genes unique to *Legionella* or shared with unrelated bacteria with similar life-styles, it should now be possible to determine experimentally, which of these genes distinguish *Legionella* species displaying different host preferences or pathogenicity (Chien *et al.*, 2004).

Legionnaires' disease and Pontiac fever

Inhalation of airborne droplets containing cells of *Legionella* is generally thought to be the most common mode of transmission. The aerosols can be generated by mechanical devices (e.g. cooling towers, air-conditioning systems) or by the use of potable water, especially from domestic warm water installations (e.g. showers)

Table 1.1. *Legionella* species and serogroups
(Fields *et al.*, 2002, La Scola *et al.*, 2004, Park *et al.*, 2003, Park *et al.*, 2004)

Species	No. of serogroups	No. of serogroups associated with disease	Species	No. of serogroups	No. of serogroups associated with disease
L. adelaidensis	1	0	*L. lansingensis*	1	1
L. anisa	1	1	*L. londiniensis*	1	0
L. beliardensis	1	0	*L. longbeachae*	2	2
L. birminghamensis	1	1	*L. lytica*	1	0
L. bozemanae	2	2	*L. maceachernii*	1	1
L. brunensis	1	0	*L. micdadei*	1	1
L. busanensis	1	0	*L. moravica*	1	0
L. cherrii	1	0	*L. nautarum*	1	0
L. cincinnatiensis	1	1	*L. oakridgensis*	1	1
L. drancourtii	1	0	*L. parisiensis*	1	1
L. drozanskii	1	0	*L. pneumophila*	15	15
L. dumoffii	1	1	*L. quateirensis*	1	0
L. erythra	2	1[a]	*L. quinlivanii*	2	0
L. fairfieldensis	1	0	*L. rowbothamii*	1	0
L. fallonii	1	0	*L. rubrilucens*	1	0
L. feeleii	2	2	*L. sainthelensi*	2	2
L. geestiana	1	0	*L. santicrucis*	1	0
L. gormanii	1	1	*L. shakespearei*	1	0
L. gratiana	1	0	*L. spiritensis*	1	0
L. gresilensis	1	0	*L. steigerwaltii*	1	0
L. hackeliae	2	2	*L. taurinensis*	1	0
L. israelensis	1	0	*L. tucsonensis*	1	1
L. jamestowniensis	1	0	*L. wadsworthii*	1	1
L. jeonii	1	0	*L. waltersii*	1	0
L. jordanis	1	1	*L. worsleiensis*	1	0

[a]Serogroup 2 of *L. erythra* has been associated with human disease.

(Breiman *et al.*, 1990, Muder *et al.*, 1986). When inhaled into the deepest parts of the lung, *L. pneumophila* enters mammalian phagocytes and proliferates within these cells (Fig. 1.2).

Legionella spp. can be responsible for two types of illnesses: Legionnaires' disease (Fraser *et al.*, 1977) and Pontiac fever (Glick *et al.*, 1978). During the early phase of Legionnaires' disease constitutional symptoms predominate. Fever, malaise, myalgia, rigors, confusion, headache, and diarrhea are usually followed by nonproductive cough and dyspnea (Tsai *et al.*, 1979). Although no chest X-ray pattern can separate this infection from other types of pneumonia, alveolar infiltrates are common to Legionnaires' disease (Macfarlane *et al.*, 1984). The key to diagnosis is performing appropriate microbiological testing when a patient belongs to a high-risk category. Typically, high-risk categories include subjects of advanced age and people with sustained damage to the host defenses that normally protect lungs from infection (Marston *et al.*, 1994, Winn and Myerowitz, 1981). Some of the most common risk factors for legionellosis are cigarette smoking, emphysema or chronic lung diseases, lung and hematologic malignancies, and clinical immunosuppression or cytotoxic

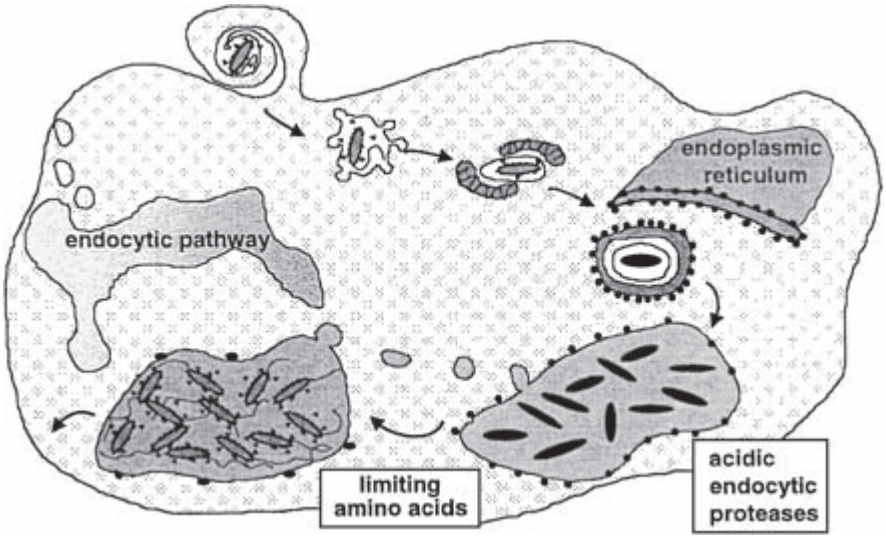

Figure 1.2. Model for the pathway of *Legionella pneumophila* proliferation in macrophages. After being engulfed within coiled phagosomes, post-exponential phase bacteria establish a vacuole that does not acidify or interact with the endosomal pathway, but is surrounded by endoplasmic reticulum. Within this protected vacuole, bacteria convert to an acid-tolerant, replicative form and no longer express virulence factors. Consequently, vacuoles merge with the lysosomal compartment, an acidic, nutrient-rich niche allowing for replication. Once the local amino acid supply is depleted, the progeny convert to the virulent form, expressing factors to escape the spent host, survive and disperse in the environment, and establish a protected replication niche in another phagocyte (Swanson and Hammer, 2000). Reprinted with permission.

chemotherapy (Carratala *et al.*, 1994, Marston *et al.*, 1994).

While not all *Legionella* species have been shown to cause clinical symptoms (Table 1.1), *L. pneumophila* is by far the most pathogenic species. It has been identified as the causative agent of approximately 90% of all reported cases of legionellosis in the United States (Fields *et al.*, 2002). In Europe *L. pneumophila* accounted for 93.5% of the 10,322 cases reported in the period from 2000 – 2002 and for 93.1% of the 9,166 cases in the period from 2003-2004. This is in contrast to the situation in Australia and New Zealand, where 30% of the cases of Legionnaires' disease are caused by *L. longbeachae* (Yu *et al.*, 2002). Of the 15 serogroups presently known for *L. pneumophila*, serogroup 1 was found to be most infective, since 76.5 and 76.4% of all infections were caused by this type in the 2000-2002 and the 2003-2004 period, respectively (Joseph, 2004, Ricketts and Joseph, 2005).

In the United States an average of 356 cases were reported each year between 1980 and 1988 to the center for disease control each year, with no trend (Fields *et al.*, 2002). This is a fraction of the 8,000 to 18,000 cases estimated to occur each year in the United States, based on the incidence of legionellosis in cases of hospitalized pneumonia (Marston *et al.*, 1997). A major increase in reported cases of Legionnaires' disease

occurred in many European countries. The rate per million inhabitants in Europe increased from 4.1 in 1993 to 10.1 in 2002, but decreased afterwards to 9.8 in 2003 and 8.2 in 2004. The mortality rate seemed to decline, with observed rates of 11% in 2000 and 6% in 2002 (Joseph, 2004). In 2003 and 2004, however, higher fatality rates of 7.7 and 8.6%, respectively, were observed (Ricketts and Joseph, 2005). Since the first recognized outbreak of Legionnaires' disease in Philadelphia, incidents have occurred frequently. In the periods 2000-2002 and 2003-2004, 189 and 218 outbreaks were reported in Europe, respectively (Joseph, 2004, Ricketts and Joseph, 2005). Examples of these outbreaks are given in Table 1.2.

The second disease caused by some *Legionella* species is Pontiac fever, which is an acute, self-limited form of legionellosis characterized by fever, chills, myalgia, and headaches (Kaufmann *et al.*, 1981). Like Legionnaires' disease, Pontiac fever is also thought to be transmitted by inhalation of aerosolized water colonized with legionellae. The disease has been associated with four species of *Legionella*, namely *L. pneumophila*, *L. micdadei*, *L. anisa* and *L. feeleii* (Fields *et al.*, 1990a, Goldberg *et al.*, 1989, Herwaldt *et al.*, 1984, Kaufmann *et al.*, 1981). Epidemiologically, Pontiac fever differs from Legionnaires' disease in the characteristic short incubation period (6-48 h), high attack rate, and absence of mortality or long-term complications. The pathogenesis of Pontiac fever is poorly understood, and it remains unclear why exposure to legionellae can result in two clinically and epidemiologically distinct syndromes. One theory is that Pontiac fever is caused by hypersensitivity to a cellular component of either legionellae or the protozoan hosts of the bacteria (Rowbotham, 1980b). Another hypothesis is that aerosols containing dead cells of *Legionella* can cause the disease (Miller *et al.*, 1993). According to a third theory, the disease is caused by an endotoxin produced by legionellae or other bacteria (Fields *et al.*, 2001).

Table 1.2. Examples of Legionnaires' disease outbreaks caused by *L. pneumophila*

Country	Year	No. of cases (casualties)	Source of infection	Reference
USA	1976	180 (29)	Air conditioning system	(Fraser *et al.*, 1977)
Netherlands	1978-1982	14 (3)	Hot water system	(Meenhorst *et al.*, 1983)
UK	1985	68 (22)	Chiller unit air conditioning plant	(O'Mahony et al., 1990)
UK	1989	33 (5)	Cooling tower	(Watson *et al.*, 1994)
USA	1992	5 (0)	Decorative fountain	(Hlady *et al.*, 1993)
USA	1996	23 (2)	Whirlpool	(Benkel *et al.*, 2000)
Netherlands	1999	188 (29)	Whirlpool	(Den Boer *et al.*, 2002)
Australia	2000	125 (4)	Cooling tower	(Greig *et al.*, 2004)
Japan	2000	34 (3)	Bath water	(Nakamura *et al.*, 2003)
Spain	2001	449 (6)	Cooling towers	(Garcia-Fulgueiras *et al.*, 2003)
France	2004	85 (13)	Cooling towers	(Anonymous, 2005b)
Norway	2005	53 (10)	Air scrubber	(Anonymous, 2005a)
Canada	2005	127 (21)	Cooling tower	(Anonymous, 2005b)

Ecology

Water is the major reservoir for legionellae, and the bacteria are found widespread in natural fresh water environments, despite their fastidious nature (Fliermans *et al.*, 1981). The bacterium has also frequently been observed in engineered water systems such as warm water distributing systems, cooling towers, humidifiers and fountains (Leoni *et al.*, 2001, Tobin *et al.*, 1980, Yamamoto *et al.*, 1992). Multiplication of the organism in water systems poses a potential human health risk wherever aerosolisation can occur (Fields, 1996). An exception to this observation is *L. longbeachae*, a frequent isolate from potting soil (Steele *et al.*, 1990). This species is the leading cause of legionellosis in Australia and occurs in gardeners and those exposed to commercial potting soil (Ruehlemann and Crawford, 1996).

L. pneumophila multiplies at temperatures between 20 and 43°C, with an optimal growth temperature of 35°C (Katz and Hammel, 1987, Rogers *et al.*, 1994a, Wadowsky *et al.*, 1985). *In vitro*, legionellae grow only in complex media with supplements of cysteine and iron salts (Edelstein, 1981). However, *L. pneumophila* was observed to satellite around colonies of *Flavobacterium breve* on an L-cysteine-deficient medium which did not support growth of legionellae. The pathogen also grows in association with certain cyanobacteria (Tison *et al.*, 1980, Wadowsky and Yee, 1983). For their multiplication in natural and man-made aquatic environments other organisms are required, but different species of heterotrophic bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Flavobacterium* sp.) alone did not support the growth of *L. pneumophila*, although prolonged survival was observed in the presence of these bacteria (Murga *et al.*, 2001, Wadowsky *et al.*, 1988). *In vitro* studies using cocultures have repeatedly demonstrated intracellular multiplication of *L. pneumophila* in amoebae (*Acanthamoeba*, *Echinamoeba*, *Hartmannella*, *Naegleria*, *Vahlkampfia*, *Dictyostelium*) and in ciliated protozoa (*Tetrahymena*) (Table 1.3) (Fields, 1996, Fields *et al.*, 1984, Hagele *et al.*, 2000, Rowbotham, 1980a). Amoebae have been observed in water systems associated with Legionnaires' disease (Barbaree *et al.*, 1986, Breiman *et al.*, 1990) and *L. pneumophila* was shown to recolonize water-distributing systems within a few weeks after disinfection (Levin *et al.*, 1995, Lin *et al.*, 1998). Batch experiments in tap water revealed that *L. pneumophila* did not multiply in the absence of protozoa, while growth with protozoa (*Hartmannella*) could be confirmed using cocultures (Fields *et al.*, 1989, Wadowsky *et al.*, 1988).

In 1984 it was shown that *L. pneumophila* is present in the biofilm attached to rubber components in water fittings (Colbourne *et al.*, 1984). Later it was demonstrated that biofilms actually are the preferred extracellular habitat for *L. pneumophila* (Armon *et al.*, 1997, Rogers and Keevil, 1992). Rogers and coworkers (Rogers *et al.*, 1994a and 1994b) found that growth of *L. pneumophila* on a variety of materials in contact with tap water is linked to biofilm development. However, they concluded that (i) the number of *L. pneumophila* in the biofilms was unrelated to the total number as determined with plate counts and that (ii), growth of *L. pneumophila* coincided with undetectable numbers of potential hosts on some materials. From several studies it has been concluded that intracellular growth of *L. pneumophila* in protozoa is not always

Table 1.3. Protozoa for which intracellular multiplication of legionellae has been reported

Category	Species	Reference
Amoebae	*Acanthamoeba castellanii*	(Rowbotham, 1980a)
	Acanthamoeba culbertsoni	(Miyamoto *et al.*, 2003)
	Acanthamoeba palestinensis	(Anand *et al.*, 1983)
	Acanthamoeba polyphaga	(Rowbotham, 1980a)
	Acanthamoeba royreba	(Tyndall and Domingue, 1982)
	Balamuthia mandrillaris	(Shadrach *et al.*, 2005)
	Echinamoeba exundans	(Fields *et al.*, 1989)
	Hartmannella cantabrigiensis	(Rowbotham, 1986)
	Hartmannella vermiformis	(Rowbotham, 1986)
	Naegleria fowleri	(Newsome *et al.*, 1985)
	Naegleria gruberi	(Rowbotham, 1980a)
	Naegleria jadini	(Rowbotham, 1980a)
	Naegleria lovaniensis	(Tyndall and Domingue, 1982)
	Vahlkampfia jugosa	(Rowbotham, 1986)
Ciliates	*Tetrahymena pyriformis*	(Fields *et al.*, 1984)
	Tetrahymena thermophila	(Kikuhara *et al.*, 1994)
	Tetrahymena vorax	(Smith-Somerville *et al.*, 1991)
Slime mold	*Dictyostelium discoideum*	(Hagele *et al.*, 2000, Solomon *et al.*, 2000)

essential for the multiplication of *L. pneumophila* within a mixed bacterial consortium in water (Rogers and Keevil, 1992, Surman *et al.*, 2002).

Detection

To identify the bacterium responsible for the first recognized outbreak of pneumonia due to *L. pneumophila*, guinea-pigs were infected with post-mortem lung tissue from the patients with fatal Legionnaires' disease, and embryonated yolk-sacks were inoculated with spleen homogenates from infected guinea-pigs. In January 1977, a Gram-negative bacterium was isolated and designated *L. pneumophila* (McDade *et al.*, 1977). *L. pneumophila* was first isolated by using Mueller-Hilton agar, supplemented with hemoglobin and IsoVitaleX (MH-IH) (Feeley *et al.*, 1978). The essential component in hemoglobin was found to be a soluble form of iron, and L-cysteine was the essential amino acid provided by the IsoVitaleX (MH-IH). These refinements led to the development of Feeley-Gorman agar, which provides better recovery of the organism from tissue (Feeley *et al.*, 1978). Later, starch was replaced with charcoal to detoxify the medium and the amino acid source was changed to yeast extract, resulting in charcoal yeast extract agar (Feeley *et al.*, 1979). This is the base form for most media used to grow legionellae. The medium has been improved several times, eventually resulting in the medium currently used, namely buffered charcoal yeast extract (BCYE) agar enriched with α-keto-glutarate with and without selective agents added (Edelstein, 1981 and 1982, Pasculle *et al.*, 1980). Nevertheless, although cultivation of viable *Legionella* species remains the standard method, various factors, e.g. other bacteria can

interfere with its growth on selective media.
Because of the difficulty in culturing *Legionella* from various environments, other methods have been developed for its detection. Direct fluorescent antibody (DFA) staining has proved to be very useful for detecting *L. pneumophila* in natural aquatic systems (Alary and Joly, 1992, Fliermans *et al.*, 1981). DFA staining has also been used for serological identification of *Legionella* isolates. A gas chromatographic mass spectrometric method, based on unique 3-hydroxy- and 2,3 dihydroxy fatty acids of the *L. pneumophila* lipopolysaccharides, has been used to detect *L. pneumophila* in biofilms in potable water containing a complex microbial consortium (Walker *et al.*, 1993). For diagnostic purposes also several serological tests, for detecting antibodies to *Legionella* spp., and urine antigen detection tests, are available (Fields *et al.*, 2002).
Various molecular methods have also been developed for detecting *Legionella*. The first assay designed to detect the DNA of *L. pneumophila* used a radio labeled ribosomal probe specific for all strains of *Legionella* (Gen-Probe, San Diego, Calif.). However, after a pseudo-outbreak due to false-positive cases, the assay was removed from the market (Laussucq *et al.*, 1988). Amplification of diagnostic marker sequences by Polymerase Chain Reaction (PCR) represents one of the few tests with the potential to detect infections caused by all of the known species of *Legionella*. Various PCR assays that have been developed for detection of *Legionella* target either DNA sequences specific for *L. pneumophila* (Starnbach *et al.*, 1989), the 5S rRNA gene (MacDonell and Colwell, 1987, Mahbubani *et al.*, 1990), the 16S rRNA gene (Jonas *et al.*, 1995, Lisby and Dessau, 1994, Miyamoto *et al.*, 1997), or the *mip* gene (Engleberg *et al.*, 1989, Mahbubani *et al.*, 1990, Ramirez *et al.*, 1996). Fluorescent in situ hybridization (FISH) of whole cells with a 16S rRNA-targeted oligonucleotide probe has also proven to be a valuable tool for the specific detection of *L. pneumophila* (Grimm *et al.*, 1998).
Several researchers have reported on the use of real-time PCR combined with a hybridization probe to confirm the product identity for rapid detection of legionellae in clinical and environmental samples (Ballard *et al.*, 2000, Hayden *et al.*, 2001, Herpers *et al.*, 2003, Yanez *et al.*, 2005). The quantitative nature and speed with which real-time PCR procedures can be performed offers significant advantages over both culture-based and previous biomolecular techniques. However, cultivation was surprisingly the best for detecting multiple *Legionella* species in lung tissue (Hayden *et al.*, 2001). On the other hand, real-time PCR combined with an immunomagnetic separation technique provided a sensitive, specific and accurate method for the rapid quantification of *L. pneumophila* in water samples (Yanez *et al.*, 2005). Real-time PCR might be a better method than cultivation for detection and quantification of *L. pneumophila* in environmental samples and in samples from man-made systems, since other bacteria do not interfere with *L. pneumophila* in the PCR based approach.

Protozoa

Introduction

Protozoa, comprising thousands of species, are a heterogeneous group of unicellular eukaryotic microorganisms that range in size from 2 μm to 3 mm. Free-living protozoa can be taxonomically divided on the basis of locomotory organelles into flagellates, amoebae, and ciliates (Fig. 1.3 A-C). In addition, they can obtain nutrition through a variety of processes that include autotrophy, mixotrophy, and heterotrophy. Some flagellates, for example, are commonly autotrophic and fix carbon using photosynthesis. Others, including ciliates and testate amoebae are however mixotrophic and obtain carbon through a combination of heterotrophic and autotrophic processes mediated through the presence of functional chloroplasts or symbiotic algae. Most species are heterotrophic and feed predominantly on other free-living life forms such as bacteria, fungi, algae and smaller protozoa. Prey capture by heterotrophic forms varies between the three groups, with naked amoebae and flagellates commonly being raptorial, and ciliates being mainly filter feeders (Symmons *et al.*, 2003).

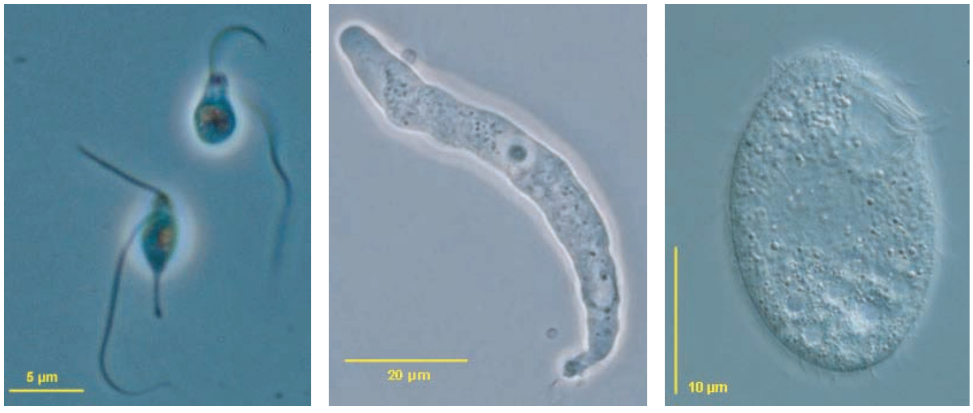

Figure 1.3. Pictures of a flagellate (*Bodo* sp.), amoeba (*Hartmannella* sp.), and ciliate (*Tetrahymena pyriformis*) (http://microscope.mbl.edu/baypaul/microscope/images/t_imgAZ/, accession date: 6 January 2006)

The primary characteristic of amoebae is the presence of some forms of pseudopodia, which show considerable diversity of structures and types, reflected in the variety of shapes among the free-living naked amoebae. The smaller amoebae, including *H. vermiformis*, which occur in water and soils, lack clear morphological features (Page, 1988). The cell body organization of amoebae has a subcellular nature and the diversity of anatomical features as seen in the "higher eukaryotes" is absent. Two zones can be distinguished in the cytoplasm of amoebae: a thin transparent outer layer or ectoplasm and a more granular endoplasm which forms the bulk of the cytoplasm and which flows forward through the body into the cylindrical

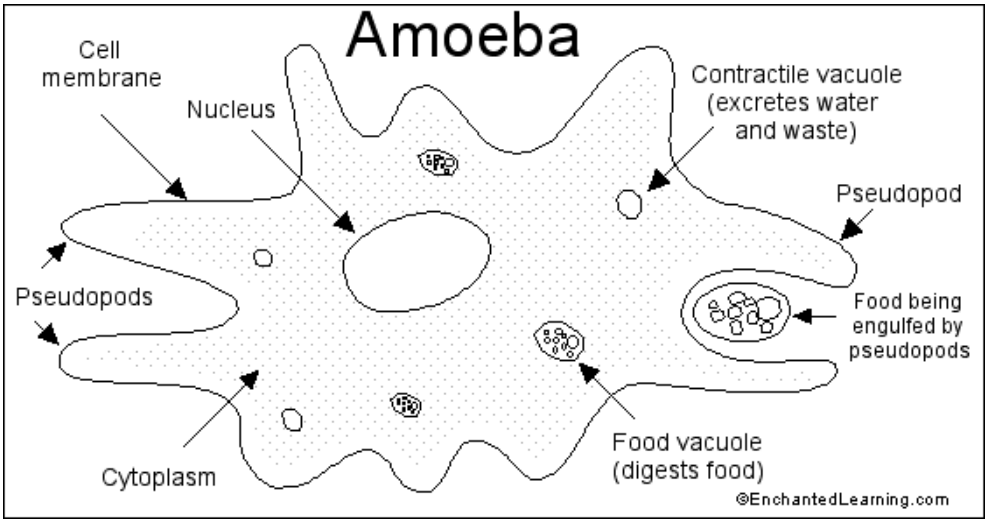

Figure 1.4. Schematic drawing of an amoeba cell (http://www.enchantedlearning.com/agifs/Amoeba_bw.GIF, accession date: 6 January 2006).

pseudopodia (Fig. 1.4). The ectoplasm is most clearly visible at the tip of a pseudopodium where it forms a hyaline cup. The endoplasm contains one or many nuclei, a variety of vacuoles, food storage granules, mitochondria and other membranous or crystalline inclusions (Andresen, 1973). The phagotrophic lobose amoebae engulf microorganisms, which they encounter, with their pseudopodia as they creep around, and which they use as food source.

Once food capture is completed, the food vacuole separates from the surface plasma membrane. Nevertheless, the food does not pass directly into the cytoplasm, and the food vacuole keeps it separate from intracellular cell constituents. The food passes through a series of changes, starting with death of prey due to dehydration and followed by its gradual digestion over several hours (Bovee and Jahn, 1973, Chapman-Andresen, 1973, Dewey, 1967, Muller, 1967). Whether food uptake occurs by means of phagocytosis of particles or other organisms, or pinocytosis of molecules, the process is essentially the same and accidental uptake of substances occurs during both processes (Nisbet, 1984).

Protozoa are the most important predators of bacteria in soil and aquatic systems, with free-living amoebae being responsible for 60% of the total decrease of the bacterial microbiota (Bamforth, 1985, Barcina *et al.*, 1991, Clarholm, 1981 and 1984, Elliott and Coleman, 1977, González *et al.*, 1990 and 1992, Stout and Heal, 1967). So, amoebae play a major role in controlling the bacterial community (Alexander, 1981, Casida, 1989, Fenchel, 1987). However, not all bacteria seem to be an equally suitable food source for protozoa and amoebae (Singh, 1941, 1942 and 1946, Stout and Heal, 1967). Gram-negative bacteria were able to survive the presence of many protozoa (Alexander, 1981), and biologically-formed toxins in bacteria may prevent attack by

protozoa (Habte and Alexander, 1978a). Protozoan grazing stimulates microbial activity and enhances the turnover of phosphorus and nitrogen, which is, especially in the rhizosphere, an important function in soil ecosystems (Rodriguez-Zaragoza, 1994). Free-living amoebae are ubiquitous and have been isolated from various natural environments such as soil, fresh water, salt water, dust, and air. Free-living amoebae are also frequently isolated from man-made ecosystems such as tap water, air-conditioning units, and cooling towers, where they feed on the biofilms present in these systems. Several bacteria, including human pathogens, have developed mechanisms to survive phagocytosis by free-living amoebae and are able to exploit them as hosts (Greub and Raoult, 2004). Examples include the pathogenic bacteria *L. pneumophila* (Rowbotham, 1980a), *Listeria monocytogenes* (Ly and Muller, 1990), *Vibrio cholerae* (Thom *et al.*, 1992), various *Mycobacterium* spp. (Greub and Raoult, 2004), *Salmonella typhimurium* (Gaze *et al.*, 2003), *Salmonella enterica* (Tezcan-Merdol *et al.*, 2004), *Escherichia coli* O157 (Barker *et al.*, 1999), *Francisella tularenis* (Abd *et al.*, 2003), *Chlamydia pneumoniae* (Essig *et al.*, 1997), *Helicobacter pylori* (Winiecka-Krusnell *et al.*, 2002) and *Campylobacter jejuni* (Axelsson-Olsson *et al.*, 2005), which all have been observed in association with amoebae. Therefore, it has been suggested that amoebae represent the environmental reservoirs for *Legionella*, acting as "Trojan Horses" of the microbial world (Barker and Brown, 1994). In addition to those facultative intracellular bacteria, various obligate endosymbionts belonging to five different evolutionary lineages within the *Proteobacteria*, the *Bacteroidetes*, and the *Chlamydiae*, have been observed in *Acanthamoeba* spp. and *Hartmannella* sp., respectively (Horn and Wagner, 2004).

L. pneumophila and protozoa

L. pneumophila requires a unique combination of nutrients in order to proliferate in pure culture in the laboratory. These unusual nutritional requirements contradict with the widespread distribution of legionellae in fresh water environments. The levels of nutrients that legionellae require are rarely found in fresh water and, if present, would serve only to amplify faster growing bacteria that would compete with the legionellae. However, this apparent contradiction can be explained by assuming that these nutrients are present in an intracellular environment, rather than dissolved in fresh water.

Indeed, legionellae survive and proliferate in aquatic and moist soil environments as intracellular parasites of free-living protozoa (Fields, 1996, Rowbotham, 1980a). These bacteria have been shown to multiply in 14 species of amoebae, three species of ciliated protozoa, and one species of slime mold (Table 1.3).

The intracellular life cycle of *L. pneumophila* in protozoa is very similar to the life cycle of *L. pneumophila* in macrophages (Fig. 1.2). Microscopically, the processes are virtually identical, although notable differences in the mechanism of entering and exiting the host cell do exist. An overview of the life cycle of *L. pneumophila* in protozoa and human macrophages is given in Figure 1.5 (Fields *et al.*, 2002).

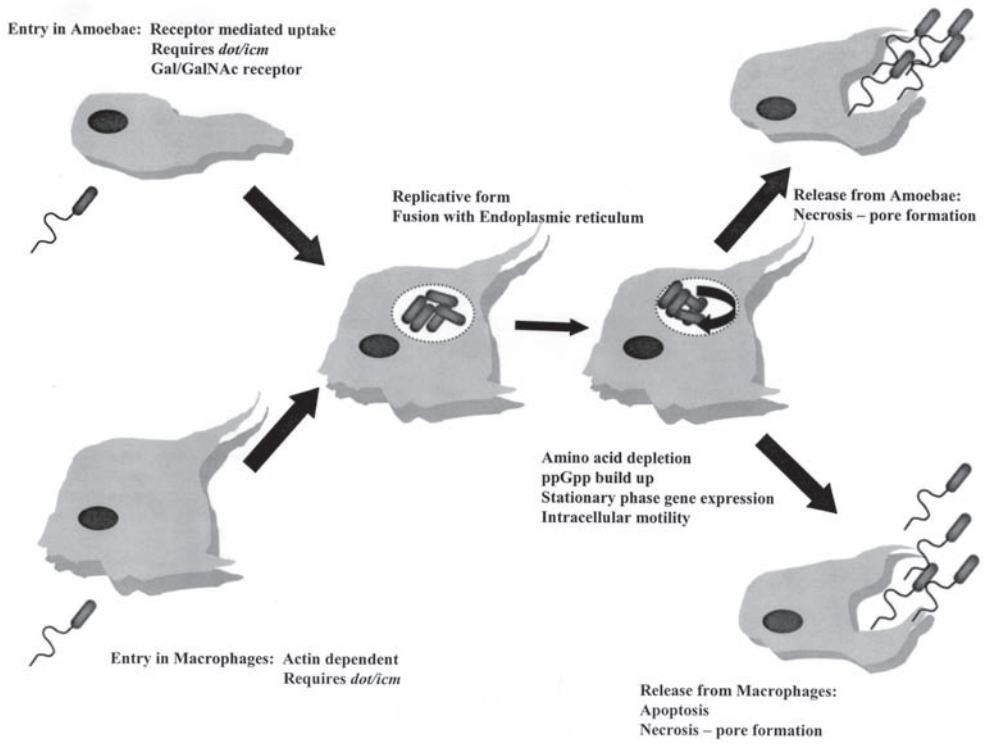

Figure 1.5. Life cycle of *L. pneumophila* in protozoa and human macrophages (Fields *et al.*, 2002). Reprinted with permission.

Hartmannella vermiformis

H. vermiformis is a free-living amoeba, belonging to the genus *Hartmannella*. Page first described this amoeba in 1967 (Page, 1967). *H. vermiformis* are limax amoebae that move by steady state advance of the usually single pseudopod. *Hartmannella* are elongated thin amoebae with a length in active locomotion between 12-33 μm, and a length:width ratio averaging 6.3, but ranging up to 8.4. Other species of the genus *Hartmannella* are the marine amoebae *H. abertawensis*, *H. lobifera* and *H. vacuolata*, and the fresh water amoebae *H. cantabrigiensis* and *H. limax* (Anderson *et al.*, 1997, Page, 1969 and 1988, Smirnov, 1996). Other genera of the family Hartmannellidae are *Glaeseria*, *Saccamoeba* and *Cashia* (Page, 1988).

The life cycle of *H. vermiformis* is simple. The active cell or feeding stage in which multiplication takes place is called amoeba or trophozoite and reproduces by equal binary fission. The inactive or dormant cell, called the cyst, with a smooth spherical or slight oval appearance with a single wall, is formed to survive unfavorable situations (Page, 1967, Taylor and Sanders, 1991).

Hartmannella feed mainly on bacteria, and it has been repeatedly shown that *H. vermiformis* prefers some bacterial species above others (Fenchel, 1987, Singh, 1941 and

1942, Weekers *et al.*, 1993). For example the non-pigmented Enterobacteriaceae *E. coli* K-12 and *Klebsiella aerogenes* appeared to be excellent feed for *H. vermiformis*, while *H. vermiformis* could hardly use the pigmented bacteria *Micrococcus luteus*, *Chromatium vinosum* and *Serratia marcescens* for growth (Weekers *et al.*, 1993).
H. vermiformis is widespread in nature, and has been isolated from soil, fresh water, air samples and different engineered water systems (Breiman *et al.*, 1990, Page, 1974, Page, 1967, Rohr *et al.*, 1998, Walker *et al.*, 1986). *H. vermiformis*, as other aquatic protozoa, plays an important role in nutrient cycling in ecosystems, since free-living amoebae take part in nitrogen mineralization processes (Clarholm, 1985, Elliott *et al.*, 1979). The organism has direct and indirect public health significance. *H. vermiformis* has been isolated from the cerebrospinal fluid of a patient with meningoencephalitis and bronchopneumonia (Centeno *et al.*, 1996). Furthermore it has been suggested that *H. vermiformis* can cause keratitis, although this is questioned by other investigators (Aitken *et al.*, 1996, De Jonckheere and Brown, 1998, Inoue *et al.*, 1998, Kennedy *et al.*, 1995). The indirect public health significance of the organisms is related to its role as host for *L. pneumophila* (Fields *et al.*, 1990b, Fields *et al.*, 1989, Kuchta *et al.*, 1998, Wadowsky *et al.*, 1988, Wadowsky *et al.*, 1991).

Detection

Protozoa are important hosts for the multiplication of *Legionella* (Rowbotham, 1980a) and play a role in the pathogenicity of *Legionella* including its transmission (Fields *et al.*, 2002). However, appropriate detection methods are still lacking. Traditionally, cultivation methods based on enrichments are applied for the detection of protozoa in aquatic habitats (Page, 1988). Cultivated protozoa can subsequently be classified based upon their morphological characteristics and by using biochemical and immunological methods (Szénasi *et al.*, 1998). To circumvent the need for cultivation, molecular tools for protozoa detection have been developed during the past decade, mainly using rRNA-targeted FISH with DNA oligonucleotide probes. Probes have been designed for the specific detection of *Legionella*-growth promoting free-living amoebae *Acanthamoeba*, *Naegleria* and *Hartmannella*, allowing simultaneous detection and classification of amoebae in situ (Grimm *et al.*, 2001, Stothard *et al.*, 1999). Nevertheless, unless operated in an automated fashion, these techniques are time consuming, and therefore PCR-based detection methods for the free-living amoebae *Acanthamoebae* and *Naegleria* were developed (Kilvington and Beeching, 1995, Schroeder *et al.*, 2001, Vodkin *et al.*, 1992). For the detection of *H. vermiformis*, however, such a method is lacking.

Biofilms

Introduction

Already more then 75 years ago it was mentioned that bacteria and protozoa in drinking water distribution systems mainly exist attached to the inner walls of the pipes

(Heymann, 1928). Later it was established that under oligotrophic circumstances as prevailing in marine and fresh water environments, bacteria were found to attach to solid surfaces (Zobell, 1943). Also under nutrient rich circumstances, often encountered in installations used in the food industry, bacteria were found attached to the solid surface (Carpentier and Cerf, 1993). These attached bacteria form biofilms: microbially derived sessile communities characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype, with respect to growth rate and gene expression (Donlan and Costerton, 2002).

Biofilms are found on almost each moist surface and surfaces in direct contact with water or other fluids. Next to the above-mentioned environments, biofilms are also found on rocks immersed in a stream, on implants in the human body, on teeth, in wastewater treatment plants and in the human gut (Carpentier and Cerf, 1993, Characklis, 1973, Savage, 1977). In some places biofilm formation is desirable, e.g. in oxidation beds in wastewater treatment plants, and in slow sand filters in water treatment plants, where biofilms are used to remove organic and inorganic compounds from water (Characklis, 1973). In other places biofilm formation can cause problems, such as in prosthetic implants and as dental plaque (Addy *et al.*, 1992, Dougherty, 1988). Biofilms in water distributing systems may be a source of undesired microorganisms, bad taste and odor or biofouling. In addition to these problems biofilms can also cause energy losses and blockages in condenser tubes, cooling fill materials, water and wastewater circuits, and heat exchange tubes (Characklis, 1981).

The development of a biofilm can be divided in five stages (Fig. 1.6). The first step is the initial reversible attachment of cells to the surface. In the second step extracellular polymeric substances (EPS) are produced, by which the cells more firmly adhere to the surface (irreversible attachment). The third step comprises the early development of biofilm architecture, which is then further matured in the fourth step. In the final step, single cells, but sometimes whole micro-colonies are dispersed from the biofilm (Stoodley *et al.*, 2002). EPS is the construction material for biofilms. The major components of EPS are not only polysaccharides, but also proteins and in some cases lipids, with minor contents of nucleic acids and other biopolymers. Furthermore, EPS matrix sequesters nutrients from the planktonic phase (Flemming and Wingerder, 2001).

In 1978 it was stated that the majority of bacteria in aquatic ecosystems, both nutrient-sufficient and oligotrophic, grow in matrix-enclosed biofilms adherent to surfaces and that these sessile bacterial cells differ profoundly from their planktonic counterparts (Costerton *et al.*, 1978). This predominance of biofilms was established in all natural ecosystems except deep groundwater and abyssal oceans, and we now realize that these sessile populations account for most physiological processes in these ecosystems (Costerton *et al.*, 1995). Biofilms are thus perceived as being hot spots for biotic interactions, genetic exchange, and the biogeochemical cycling of elements. Furthermore, in drinking water distribution systems, biofilms may well be the main

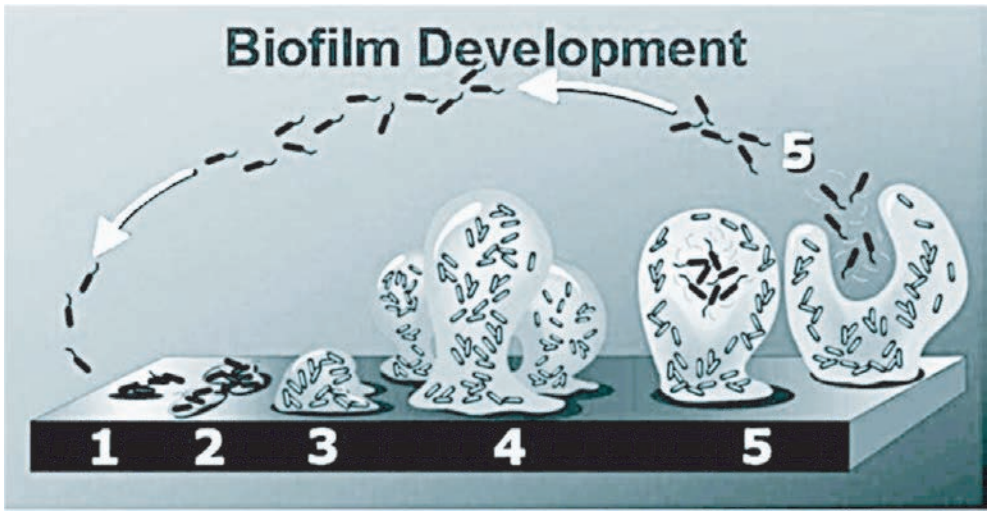

Figure 1.6. Diagram showing the development of a biofilm as a five-stage process. Stage 1: initial attachment of cells to the surface. Stage 2: production of EPS resulting in more firmly adhered "irreversible" attachment. Stage 3: early development of biofilm architecture. Stage 4: maturation of biofilm architecture. Stage 5: dispersion of single cells from the biofilm (Stoodley *et al.*, 2002). Reprinted with permission.

source of planktonic bacteria since up to 1,000 sessile microorganisms can be present for each planktonic cell which is detected (Momba *et al.*, 2000). Biofilm concentrations on materials including cast iron, galvanized steel, cement and PVC, originating from a drinking water distribution system, ranged from 4×10^5 to 2×10^8 cells/cm^2 (Wingender and Flemming, 2004).

Within the biofilm, bacteria are phenotypically different from their planktonic counterparts and are required to sense their surroundings, scavenge nutrients and survive to cytotoxic compounds (Costerton *et al.*, 1995, Stickler, 1999). Additionally, bacteria may co-operate in these environments through multicellular co-ordination and communication (quorum sensing) (Shapiro, 1998). Interestingly, bacteria within biofilms undergo high rates of conjugation, which indicates that biofilms are genetically dynamic and may play an essential role in the exchange of genetic material among their members (Dahlberg *et al.*, 1998, Hausner and Wuertz, 1999). Furthermore, it is generally recognized that cells in biofilms are more resistant to antimicrobial agents compared to their planktonic counterparts, whether these antimicrobial agents are antibiotics, disinfectants, or germicides in a clinical setting, biocides in an industrial setting or protozoa in nature (Costerton *et al.*, 1999, Donlan and Costerton, 2002). Mechanisms responsible for resistance may be one or more of the following: (i) delayed penetration of the antimicrobial agent through the biofilm matrix, (ii) altered growth rate of biofilm organisms, and (iii) other physiological changes due to the biofilm mode of growth (Donlan and Costerton, 2002).

Biofilms and *L. pneumophila*

Colbourne and coworkers (1984) showed that *L. pneumophila* is present in the biofilm attached to rubber components in water fittings. Later it was demonstrated that biofilms actually are the preferred extracellular habitat for *L. pneumophila* (Armon *et al.*, 1997, Rogers and Keevil, 1992). Biofilm matrices are known to provide shelter and a gradient of nutrients. The complex nutrients available within biofilms have led some researchers to propose that the biofilms support the survival and multiplication of legionellae outside a host cell (Rogers and Keevil, 1992, Surman *et al.*, 2002). Investigators have attempted to detect extracellular growth of *L. pneumophila* by using a biofilm reactor and a defined bacterial biofilm grown on non-supplemented potable water. The base biofilm was composed of *P. aeruginosa*, *K. pneumoniae*, and a *Flavobacterium*-like organism. However, *L. pneumophila* did not multiply in the absence of amoebae (Murga *et al.*, 2001).

Biofilms and protozoa

The presence of protozoa and metazoa in close association with and within environmental bacterial biofilms is now well accepted. Keevil *et al.* (1995) demonstrated motile bacteria, protozoa and nematodes, passing through the water channels of oligotrophic environmental biofilms, whilst Hunt and Parry (1998) found that protozoan numbers associated with biofilm communities exceeded those in the surrounding water column.

However, despite the close proximity of microfauna and biofilm communities, it has been proposed that sessile bacteria are protected from predation and that therefore protozoa will have little impact upon biofilm population dynamics (Costerton *et al.*, 1987, Decho and Castenholz, 1986). In contrast to these surveys, Pedersen (1990) stated that the effects of protozoan grazing on the biofilm community is probably the most important factor controlling biofilm dynamics. Biofilms in dental unit waterlines support proliferation of a variety of free-living amoebae, namely *Hartmannella*, *Vanella*, *Vahlkampfia*, *Naegleria* and *Acanthamoebae* spp. (Barbeau and Buhler, 2001), suggesting that they obtain nutrients through digestion of biofilm bacteria. Wolfaardt *et al.* (1994) found that protozoa living in association with herbicide-rich biofilms possessed high levels of the same herbicide within their food vacuole. This indicated that the protozoa had fed preferentially upon surrounding biofilm bacteria.

Although many different protozoan species are found in association with biofilms, their level of association and ultimate grazing impact on the biofilm-prey will differ. Therefore biofilm-associated protozoa were separated into four groups: (i) transient protozoa, which are predominantly planktonic but can swim close to the biofilm and feed on suspended prey, (ii) sessile protozoa, using the surface attachment but feed on suspended prey, (iii) browser protozoa, species that are able to feed on suspended prey when free-swimming and feed on attached prey when browsing over surfaces, and (iv) amoebae, protozoa that browse over surfaces and feed directly on biofilm-associated prey (Parry, 2004).

Although legionellae can multiply under laboratory conditions in cocultures in 14 species of amoebae, 3 species of ciliates and one species of slime mold (Fields *et al.*, 2002, Miyamoto *et al.*, 2003, Shadrach *et al.*, 2005), the intracellular proliferation of *Legionella* in natural aquatic environment has so far only been shown for *Acanthamoeba* spp., *Naegleria* spp. and *Hartmannella* sp. (Harf and Monteil, 1988). These three amoebal species and species belonging to the genera *Echinamoeba*, *Saccamoeba*, and *Vahlkampfia* respectively, have also been isolated from *Legionella*-contaminated plumbing systems (Breiman *et al.*, 1990, Henke and Seidel, 1986, Michel and Borneff, 1989, Rohr *et al.*, 1998). Therefore the elucidation of the behaviour of amoebae in biofilms is important to obtain information about the proliferation of legionellae.
In a study by Heaton and colleagues (2001), it was shown that the naked amoebae *Vexillifera bacillipedes*, *Vahlkampfia avara*, *Hartmannella cantabrigiensis*, and *Platyamoeba placida* had the capability of ingesting and digesting *E. coli* that had been embedded within alginate, whilst, surprisingly, the larger naked amoeba *Saccamoeba limax* was unable to do so. Furthermore, mathematical models have suggested that even at high prey density, chemoreception would confer an advantage to protozoa, while at low prey concentration it would be essential (Blackburn and Fenchel, 1999). It was indeed shown that flagellates prefer to ingest microspheres coated with bovine serum albumin as compared with other protein and starch coatings (Matz *et al.*, 2002). It was also shown that cell-to-cell communication by diffusible signal molecules occurred across the prokaryote-eukaryote boundary, between a bacterial biofilm and zoospores of a eukaryote alga (Joint *et al.*, 2002). However, a biofilm consisting of *P. aeruginosa* may resist protozoan grazing and therefore persist in the environment by both the formation of microcolonies and the production of toxins (Matz *et al.*, 2004).

Research objectives and thesis outline

L. pneumophila is widespread in the environment, and is also frequently found in man-made water systems. Exposure to aerosols containing *L. pneumophila* can lead to Legionnaires' disease. One of the worst outbreaks of this disease occurred in the Netherlands in 1999, due to the proliferation of *L. pneumophila* in a whirlpool spa at a flower show in Bovenkarspel (Den Boer *et al.*, 2002). This outbreak caused much concern and dispute. An advice of the Health Council of the Netherlands was published (Health Council, 2003) and new regulations aiming at prevention and control of the growth of *Legionella* in man-made water systems were introduced in the Netherlands (Tijdelijke regeling *Legionella* preventie, 2000, Waterleidingbesluit, 2004). Risk assessment for *Legionella* growth in warm water systems is the main approach, followed by corrective measures where needed. Initially, the approach was required for water systems in all public buildings (Tijdelijke regeling *Legionella* preventie, 2000), but in the latest regulation only the 'high-risk' buildings were included (Waterleidingbesluit, 2004). Additional measures proposed by the Health Council included: application of rapid diagnosis of the disease, adequate treatment of patients with Legionnaires'

disease, more scientific research and implementation of rapid detection methods for *Legionella*, and implementation of modern regulations regarding *L. pneumophila* prevention in all European member states (Health Council, 2003).
Biofilms and protozoa play a major role in the proliferation of *L. pneumophila* (Colbourne *et al.*, 1984, Rogers *et al.*, 1994a, Rowbotham, 1980a). In the Netherlands drinking water is distributed without a disinfectant residual, because disinfectants have a detrimental effect on the taste and odor and result in the presence of compounds with toxic properties (Van der Kooij *et al.*, 1999). To limit biofilm formation in the water distributing systems, biologically stable drinking water is prepared by the water supply companies and biologically stable materials are applied in the water distributing systems (Van der Kooij *et al.*, 1999). This approach is in contrast with the general practice in the USA, the UK and other European countries, where a disinfectant residual is maintained in the distribution system. Research on biological stability was mainly aiming at preventing the increase of heterotrophic plate counts and multiplication of undesirable bacteria such as aeromads and coliforms (Van der Kooij *et al.*, 1999). The outbreak of legionellosis in Bovenkarspel initiated research on the conditions favouring the growth of *L. pneumophila* in warm water systems connected to public water supplies.
The objectives of the present study were (i) to determine whether specific bacteria and/or protozoa are needed for growth of *L. pneumophila* in biofilms present in tap water installations, (ii) to collect quantitative information about the relationships between *L. pneumophila* and required organism(s) in fresh water environments used for drinking water production, and (iii) to obtain quantitative information on the proliferation of *L. pneumophila* in aquatic biofilms under conditions resembling those in installations for warm tap water. With this information control measures for limiting the multiplication of *L. pneumophila* in water installations might be proposed.
Chapter 2 of this thesis reports the *in vivo* growth of *L. pneumophila* in *H. vermiformis* in aquatic biofilms developing at high cell densities on plasticized polyvinyl chloride (PVCp) in a biofilm-batch system with autoclaved tap water. In an aquatic biofilm with comparable cell densities, but without the presence of protozoa, no growth of *L. pneumophila* was observed. Calculations confirmed that intracellular growth was most likely the only way for *L. pneumophila* to proliferate within the biofilm.
Chapter 3 focuses on the identification and comparison of the dominant bacterial populations in the two different aquatic biofilms developed on PVCp, namely the aquatic biofilm with the amoeba *H. vermiformis* and the biofilm without protozoa, using a culture independent approach. Furthermore cultured bacteria, obtained from the biofilm containing *H. vermiformis*, were identified and compared with the dominant bacterial groups detected with the culture independent method.
Chapter 4 describes the development of an 18S ribosomal RNA gene-targeted real-time PCR method for the specific detection and quantification of *H. vermiformis*. This amoeba is an important host for *L. pneumophila* and is frequently found in the same source as *L. pneumophila* during outbreaks. Therefore, cooling towers, surface waters and warm water systems were investigated for the presence and abundance of *L.*

pneumophila and *H. vermiformis* **(chapter 5)**. The results were compared with other parameters (ATP (adenosine triphosphate, temperature, HPC (heterotrophic plate count), DOC (dissolved organic carbon) and thermotolerant coliform bacteria and *E. coli*). Furthermore, 18S rRNA gene sequences of *H. vermiformis* amplified from the different sources by the real-time PCR were analyzed and compared to assess sub-species heterogeneity.

Chapter 6 describes the development of a biofilm-model for the proliferation of *L. pneumophila*. This model is used to study the quantification of *L. pneumophila* in autoclaved tap water with two essential microbial components: a host protozoan (*H. vermiformis*) and a bacterial strain (*Acidovorax* sp.) serving as food source. **Chapter 7** includes a general discussion, the conclusions and future perspectives of the research described in this thesis.

CHAPTER 2

Intracellular proliferation of *Legionella pneumophila* in *Hartmannella vermiformis* in aquatic biofilms grown on plasticized polyvinyl chloride

Co-authored by: Bart A. Wullings, Antoon D. L. Akkermans, Rijkelt R. Beumer, and Dick van der Kooij
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Abstract

The need for protozoa for the proliferation of *Legionella pneumophila* in aquatic habitats is still not fully understood and is even questioned by some investigators. This study showed the *in vivo* growth of *L. pneumophila* in protozoa in aquatic biofilms developing at high concentrations on plasticized polyvinyl chloride in a batch system with autoclaved tap water. The inoculum, a mixed microbial community including indigenous *L. pneumophila* originating from a tap water system, was added in an unfiltered as well as a filtered (cellulose nitrate, 3.0-μm pore size) state. Both the attached and suspended biomasses were examined for their total amounts of ATP, for cultivable *L. pneumophila*, and for their concentrations of protozoa. *L. pneumophila* grew to high numbers (6.3 log CFU/cm^2) only in flasks with an unfiltered inoculum. Filtration obviously removed the growth-supporting factor, but it did not affect biofilm formation, as determined by measuring ATP. Cultivation, direct counting, and 18S ribosomal DNA-targeted PCR with subsequent sequencing revealed the presence of *Hartmannella vermiformis* in all flasks in which *L. pneumophila* multiplied and also when cycloheximide had been added. Fluorescent in situ hybridization clearly demonstrated the intracellular growth of *L. pneumophila* in trophozoites of *H. vermiformis*, with 25.9% ± 10.5% of the trophozoites containing *L. pneumophila* on day 10 and >90% containing *L. pneumophila* on day 14. Calculations confirmed that intracellular growth was most likely the only way for *L. pneumophila* to proliferate within the biofilm. Higher biofilm concentrations, measured as amounts of ATP, gave higher *L. pneumophila* concentrations, and therefore the growth of *L. pneumophila* within engineered water systems can be limited by controlling biofilm formation.

Introduction

Legionella pneumophila is widespread in natural fresh water environments, despite its fastidious nature (Fliermans *et al.*, 1981). The bacterium has also frequently been observed in engineered water systems such as warm water distributing systems, cooling towers, humidifiers, and fountains (Leoni *et al.*, 2001, Tobin *et al.*, 1980, Yamamoto *et al.*, 1992). The multiplication of the organism in water systems poses a potential human health risk when aerosolization can occur (Fields, 1996).

Legionellae grow *in vitro*, only in complex media with supplements of cysteine and iron salts (Edelstein, 1981). For their multiplication *in vivo*, other microorganisms are required, but different species of heterotrophic bacteria (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Flavobacterium* sp.) alone do not support the growth of *L. pneumophila* (Murga *et al.*, 2001, Wadowsky *et al.*, 1988). *In vitro* studies using cocultures have repeatedly demonstrated the intracellular multiplication of *L. pneumophila* in amoebae (*Acanthamoeba*, *Echinamoeba*, *Hartmannella*, *Naegleria*, *Vahlkampfia*, and *Dictyostelium*) and in a ciliated protozoon (*Tetrahymena pyriformis*) (Fields, 1996, Fields *et al.*, 1984, Hagele *et al.*, 2000, Rowbotham, 1980a). Amoebae have been observed in water systems associated

with Legionnaires' disease (Barbaree *et al.*, 1986, Breiman *et al.*, 1990), and *L. pneumophila* can recolonize water distributing systems within a few weeks after disinfection (Levin *et al.*, 1995, Lin *et al.*, 1998). Batch experiments with tap water showed that *L. pneumophila* did not multiply in the absence of protozoa, and growth in the presence of a protozoon (*Hartmannella*) was confirmed by the use of cocultures (Fields *et al.*, 1989, Wadowsky *et al.*, 1988).

Rogers and coworkers (1994a and 1994b) found that the growth of *L. pneumophila* on a variety of materials in contact with tap water is coupled with biofilm development. However, they concluded that (i) the numbers of *L. pneumophila* in the biofilms were unrelated to the total numbers of microorganisms present and they concluded that (ii), the growth of *L. pneumophila* coincided with undetectable numbers of potential hosts on some materials. Furthermore, it had been concluded from several studies that the intracellular growth of *L. pneumophila* in protozoa is not always essential for the multiplication of *L. pneumophila* within a mixed bacterial consortium in water (Rogers and Keevil, 1992, Surman *et al.*, 2002).

This chapter describes the use of a batch test system for the development of different concentrations of aquatic biofilms by using polyvinyl chloride, in both unplasticized (PVCu) and plasticized (PVCp) versions, as a support and as a source of energy and carbon. *In vivo* observations revealed that the intracellular multiplication of *L. pneumophila* in *Hartmannella vermiformis* was the mechanism of proliferation for *L. pneumophila* in biofilms on PVCp, which contained a high concentration of heterotrophic bacteria.

Materials and methods

Batch test system

Biofilms were developed on pieces of PVCu (about 9.5 cm^2 of surface area for each) and pieces of PVCp tubes (diameter 0.80 cm; about 6.2 cm^2 of surface area for each) in a static test using heat-cleaned (550°C) Erlenmeyer flasks with a volume of 1 liter. All material pieces were pasteurized at 70°C for 30 minutes and then incubated in 600 ml of sterilized tap water (pH 7.8) at surface-to-volume ratios of 0.24 and 0.16 cm^{-1} for PVCu and PVCp, respectively. These surface-to-volume ratios were kept constant during the test; after the collection of a piece of PVCu or PVCp, a proportional volume of water was also removed from the flask. Nitrate and phosphate from autoclaved stock solutions were added to the flasks at final concentrations of 72.5 μM and 13.5 μM, respectively, to prevent growth limitation by these nutrients. A mixed microbial community including indigenous *L. pneumophila* serotype 1, originating from a plumbing system in the Netherlands, was used as the inoculum. Before use as an inoculum, this mixed microbial community had been maintained in tap water containing pieces of silicone tubing at 37°C, followed by storage at -80°C. The inoculum was added in an unfiltered as well as a filtered state (3.0-μm pore size cellulose nitrate filter, Sartorius, Goettingen, Germany). Filtration reduced the

indigenous *L. pneumophila* concentration in the inoculum from 4.1 to 2.4 log CFU/ml and the concentration of the heterotrophic bacteria from 5.1 to 4.2 log CFU/ml. Compared to that of the unfiltered inoculum, a 10-fold larger volume of the filtered inoculum was added to the test flasks to partly correct for these differences. The protozoon concentration in the unfiltered inoculum, as determined by a cultivation method, was 1.4 log protozoa/ml; no protozoa were detected in the filtered inoculum. A pure culture of the same indigenous *L. pneumophila* serotype 1 strain was also used as an inoculum, after two passages on buffered charcoal yeast extract agar (Edelstein, 1981) and subsequent suspension in sterile tap water. Filter-sterilized (0.2-μm pore size, Schleicher & Schuell, Dassel, Germany) cycloheximide was added at a final concentration of 100 μM to two flasks with the unfiltered inoculum to prevent growth of protozoa. All tests were conducted in duplicate, and incubation took place at 37°C without shaking for several weeks.

Biomass measurements in biofilm and planktonic phase

The attached biomass (biofilm) and the suspended biomass (planktonic phase) were analyzed for their total ATP concentrations and for the numbers of *Legionella*, heterotrophic bacteria, and protozoa at different time points during the experiments. The microorganisms in the biofilm were removed from the material pieces by six 2-min sonication steps in 10 ml of sterilized tap water (U950D, Ultrawave Limited, Cardiff, United Kingdom) at a frequency of 30 kHz and an average power input of 0.11 W/ml. The total ATP concentrations, representing the active biomasses, in the biofilm and planktonic phase were determined by ATP analysis with a commercially available ATP kit (Celsis International B.V., Landgraaf, the Netherlands). *Legionella* concentrations were determined by plating on buffered charcoal yeast extract agar and

Table 2.1. Primer sequences used in this study

Primer	Sequence	Reference
5' → 3' directed primers:		
Euk 516	ACC AGA CTT GCC CTC C	(Amann *et al.*, 1990)
LEGPNE1	ATC TGA CCG TCC CAG GTT	(Grimm *et al.*, 1998)
EUKf	ACC TGG TTG ATC CTG CCA G	(Moon-Van der Staay *et al.*, 2000)
373C	GAT TCC GGA GAG GGA GCC TGA	(Weekers *et al.*, 1994)
892C[a]	GTC AGA GGT GAA ATT CTA GG	(Schroeder *et al.*, 2001)
1262C	GTG GTG CAT GGC CGT TCT TA	(Weekers *et al.*, 1994)
3' → 5' directed primers:		
EUKr	TGA TCC TTC YGC AGG TTC AC	(Moon-Van der Staay *et al.*, 2000)
373	TCA GGC TCC CTC TCC GGA ATC	(Weekers *et al.*, 1994)
892[a]	CCT AGA ATT TCA CCT CTG AC	(Schroeder *et al.*, 2001)
1262	GAA CGG CCA TGC ACC AC	(Weekers *et al.*, 1994)

[a] The primer sequence was slightly modified, being adjusted to *H. vermiformis* Nijmegen (Weekers et al., 1994).

incubation at 37°C for 7 days (Edelstein, 1981). The heterotrophic bacterial concentration was determined by the heterotrophic plate count (HPC) method with R2A agar (Reasoner and Geldreich, 1985). Protozoon concentrations were determined by cultivation, direct counting with the fluorochrome primulin, and/or fluorescent in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes. Cultivation was done according to the method described by Darbyshire *et al.* (1974), adjusted for water samples by the addition of 50 µl of the biofilm or planktonic phase to the first wells. *Pseudomonas fluorescens* (about 7 log CFU/ml) was used as a food source for the protozoa. The protozoon concentration was calculated with GenStat, 6th ed. (VSN International Ltd., Oxford, United Kingdom). The cultivated protozoa were identified by the use of "*An illustrated key to freshwater and soil amoebae*" (Page, 1976). The fluorochrome primulin (Aldrich Chemical Company, Inc., Milwaukee, Wis.) was applied to stain the protozoa, followed by direct counting as described elsewhere (Bloem *et al.*, 1986). Direct counting with FISH is described below.

FISH

At each time point, 30 ml of the planktonic phase and 50 ml of the biofilm phase were filtered over a 0.4-µm pore size HTTP Isopore Membrane (Millipore, Bedford, Mass.) in a vacuum not exceeding 3 kPa. The samples were fixed on the filter with a 4% paraformaldehyde-phosphate-buffered saline solution at room temperature for 30 min, washed with distilled water, and dehydrated at 46°C for 5 min. The filters were placed on glass slides for hybridization by the application of 40 µl hybridization buffer (20% [vol/vol] formamide, 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 15 mM Tris-HCl, [pH 7.4]), 200 ng of the fluorescein-labeled probe EUK516 (Table 2.1, Amann *et al.*, 1990), 225 ng of the Cy3-labeled probe LEGPNE1 (Table 2.1, Grimm *et al.*, 1998), 38 ng of Poly(U), and 76 ng of bovine serum albumine and then were covered with a coverslip. Incubation was performed at 46°C for 3 h in an isotonically humid chamber. The labeled oligonucleotides were removed by incubating the filters upside down in preheated washing buffer (40 mM NaCl, 15 mM Tris-HCl [pH 7.4], 0.01% sodium dodecyl sulfate). After incubation for 30 min at 46°C, the filters were rinsed with distilled water (room temperature) and dried at 46°C for 5 min. The filters were mounted in Vectorshield (Vector Laboratories, Inc., Burlingame, Calif.) on a glass slide. Fluorescence was detected by use of a Leica DMRXA fluorescence microscope, supplied with a COHU high-performance charge-coupled device digital camera. Leica Q Fluoro, v. V1.0b, software was used to record the pictures.

PCR for detection of eukaryotes

All Erlenmeyer flasks with PVCp test pieces were checked for the presence of eukaryotes by use of a PCR assay. At each time point, 30 ml of the planktonic phase and 50 ml of the biofilm phase were filtered over a 0.2-µm pore size GTTP Isopore Membrane (Millipore). DNA extraction of the filter retained cells was done by use of a FastDNA spin kit for soil (BIO 101, Carlsbad, Calif.) according to the instructions

supplied by the manufacturer. The 18S rRNA gene was amplified by a PCR with the oligonucleotide primers EUKf and EUKr (Table 2.1), which were complementary to regions of conserved sequences located proximal to the 5' and 3' termini of known 18S rRNA genes (Moon-Van der Staay *et al.*, 2000). The PCR mixtures (50 µl) contained about 100 ng of template DNA, each primer at a concentration of 0.5 µM, a 0.2 mM concentration of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 2.5 Units *Taq* DNA polymerase (Invitrogen, Life technologies, Breda, the Netherlands), and the PCR buffer supplied with the enzyme. PCR cycling was performed in an UNO II thermocycler Biometra (Westburg, Leusden, the Netherlands) with the following program: predenaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 60 s, and extension at 72°C for 90 s; and a final extension step at 72°C for 10 min.

Cloning of PCR-amplified products

PCR amplicons were purified by the use of a QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). PCR products were cloned into *Escherichia coli* JM109 by use of the Promega pGEM-T easy vector system (Promega, Madison, Wis.). PCRs were performed on cell lysates of ampicillin-resistant transformants, using pGEM-T specific primers T7 and Sp6 to confirm the sizes of the inserts. To establish the eukaryotic diversity between the clones obtained from the aquatic biofilm with *L. pneumophila*, we subjected the amplicons to restriction fragment length polymorphism analysis with the restriction enzymes MspI, AluI and CfoI. The amplicons derived from plasmids containing unique inserts were purified by use of a QIAquick PCR purification kit and were subjected to DNA sequence analysis.

Sequence analysis

Sequence analysis was done by MWG-Biotech (Ebersberg, Germany). The primers used to sequence the total 18S rRNA gene are given in Table 2.1 (Schroeder *et al.*, 2001, Weekers *et al.*, 1994).

Phylogenetic analysis

The determined 18S rRNA gene sequences were aligned by use of the ARB software package (Ludwig *et al.*, 2004). Different phylogenetic trees were constructed by different methods and by using different filters as implemented in the ARB package.

Nucleotide sequence accession numbers

The 18S ribosomal DNA (rDNA) sequences from *H. vermiformis* KWR-1, KWR-2 and KWR-3 have been deposited in GenBank under accession numbers AY502959, AY502960 and AY502961, respectively.

Results

Batch test system

A maximum ATP concentration of $9.1 \times 10^2 \pm 2.8 \times 10^1$ pg/cm² was reached within 3 days in the batch test system with PVCu. The HPC values showed that exponential growth occurred up to day 3. With PVCp a maximum ATP concentration of $3.8 \times 10^4 \pm 2.7 \times 10^3$ pg/cm² was reached within 6 days. The total ATP concentrations declined to about $4.6 \times 10^2 \pm 0.5 \times 10^1$ pg/cm² on day 10 for PVCu and to about $1.6 \times 10^4 \pm 9.8 \times 10^2$ pg/cm² on day 14 for PVCp (Fig. 2.1A to C) and remained stable when we continued the experiment until day 40 (data not shown). Also, the HPC values observed with PVCu remained at a constant level during prolonged incubations.

The *L. pneumophila* concentration increased exponentially in flasks with the unfiltered inoculum when the ATP concentrations and HPC values had reached maximum levels. Prolonged incubations showed that the *Legionella* concentration remained stable until the end of the experiment (Fig. 2.1A and B). The maximum *L. pneumophila* concentration for the experiments with PVCp was 6.3 ± 0.1 log CFU/cm² and was lower for experiments with PVCu (4.9 ± 0.1 log CFU/cm²).

The percentage of biomass present in the biofilm relative to that in the planktonic phase depended on the material used for biofilm development. For PVCu, 57% ± 6% of the total ATP, 77% ± 6% of *L. pneumophila* cells, and 38% ± 11% of the HPC value were present in the biofilm. For PVCp, these values were 37% ± 9% for total ATP and 52% ± 15% for *L. pneumophila*. The concentrations of total ATP and *L. pneumophila* were highest with PVCp, and this material was used in further experiments for the identification of growth-supporting factor(s) for *L. pneumophila*.

Identification of growth-supporting factor(s) for *L. pneumophila*

Growth of *L. pneumophila* was detected in the flasks with the unfiltered inoculum (with or without cycloheximide). No proliferation of *L. pneumophila* was observed in the flasks with the filtered inoculum, although the total ATP concentrations under both conditions were similar (Fig 2.1B and C). Also, no growth of *L. pneumophila* was observed when a pure culture of *L. pneumophila* serotype 1 was used as the inoculum. The addition of an untreated filter with its retentate to the flasks gave similar results to those for flasks with the unfiltered inoculum, but the addition of a pasteurized (30 min, 70°C) filter with its retentate to the flasks with the filtered inoculum did not induce the growth of *L. pneumophila* (data not shown).

Protozoa were detected by cultivation and direct counting in all of the flasks in which the growth of *L. pneumophila* was observed. No protozoa were detected ($n < 1.6$ log protozoa/cm² for cultivation and $n < 2.1$ log protozoa/cm² for direct counting) in the flasks in which no proliferation of *L. pneumophila* occurred. Based on their morphology, all of the protozoa cultivated in the 96-well plates were identified as *H. vermiformis*. The direct counting methods also revealed that only one type of protozoa with the typical features of *H. vermiformis* was present in the Erlenmeyer flasks.

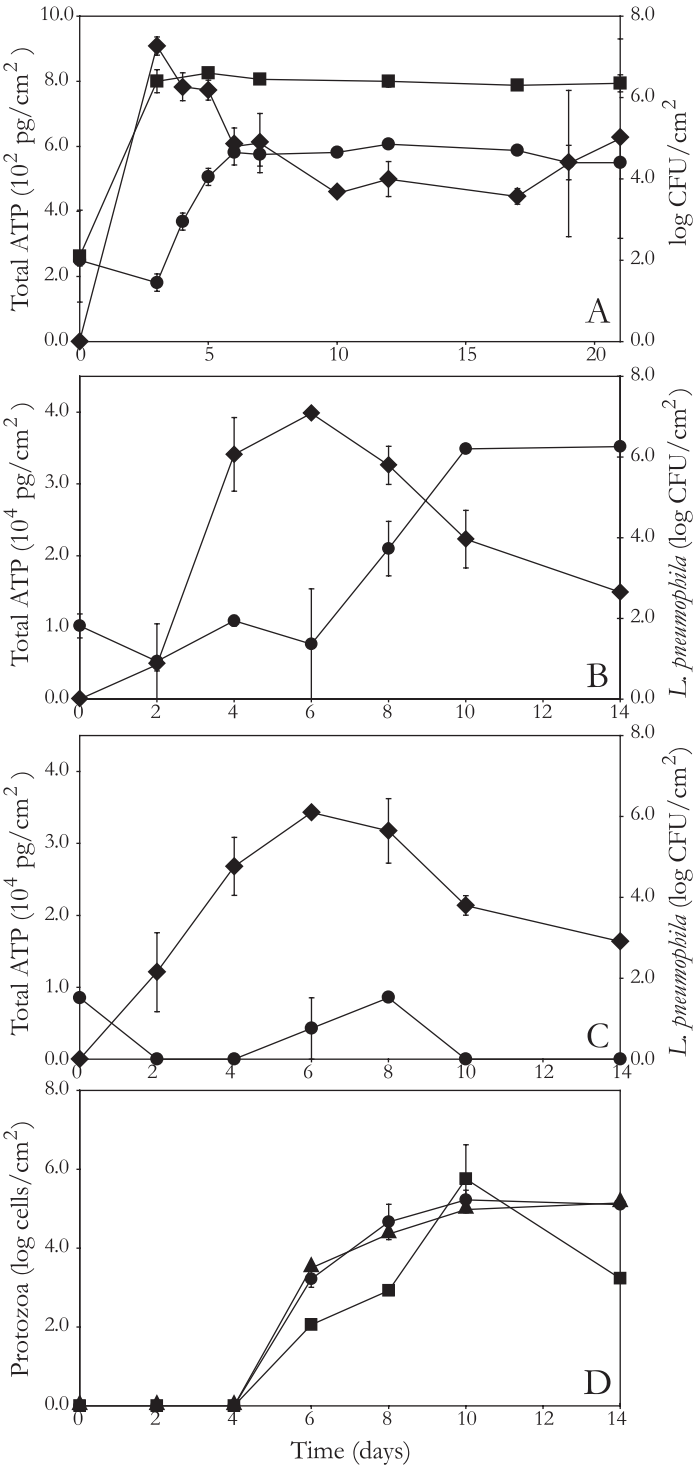
Total ATP (10^2 pg/cm^2)
log CFU/cm^2
A
Total ATP (10^4 pg/cm^2)
L. pneumophila (log CFU/cm^2)
B
C
Protozoa (log cells/cm^2)
D
Time (days)

Figure 2.1 (left page). A. Concentrations of total ATP (◆), *L. pneumophila* (●) and heterotrophic bacteria (■) in flasks with the unfiltered inoculum and PVCu as the biofilm carrier. Each point represents the mean concentration from two experiments, and bars indicate standard deviations. **B.** Concentrations of total ATP (◆) and *L. pneumophila* (●) in flasks with the unfiltered inoculum and PVCp as the biofilm carrier. Each point represents the mean concentration from two experiments, and bars indicate standard deviations. **C.** Concentrations of total ATP (◆) and *L. pneumophila* (●) in flasks with the filtered inoculum and PVCp as the biofilm carrier. Each point represents the mean concentration from two experiments, and bars indicate standard deviations. **D.** Protozoon concentrations in flasks with the unfiltered inoculum with PVCp. Squares (■) represent the concentrations determined by the cultivation method; circles (●) and triangles (▲) represent the concentrations determined by direct counting methods using primulin and FISH, respectively. Bars indicate standard deviations. No protozoa were detected in flasks with the filtered inoculum.

From day 6 on, eukaryotic DNAs were detected with 18S rDNA-targeted PCRs in the biofilm and the planktonic phase in flasks that showed growth of *L. pneumophila* (with and without cycloheximide). Although protozoa were present in the flasks with the unfiltered inoculum before day 6, their DNAs were not detectable by PCR, indicating that the initial protozoon concentration was below the detection threshold. Cloning and subsequent sequencing confirmed that *H. vermiformis* was the only protozoon species present. No eukaryotic DNAs were detected in the flasks with the filtered inoculum (Fig. 2.2).
The FISH technique revealed the different stages of the intracellular proliferation of *L. pneumophila* within protozoa (Fig. 2.3).

Quantitative relationship between *L. pneumophila* and *H. vermiformis*
Protozoa were first detected on day 6 (Fig. 2.1D). The cultivation method revealed that the concentration of protozoa increased to 5.8 ± 0.9 log protozoa/cm^2 by day 10 and decreased afterwards. The results of the direct counting methods using primulin and FISH were similar to the results of the cultivation method (Fig. 2.1D), but with these methods no decline in protozoon concentration was detected after day 10. Furthermore, it was found that 93% ± 4% of the protozoa were present in the biofilm rather than in the planktonic phase. During the experiments, the amoebae changed from the trophozoite form on day 6 (Fig. 2.3A) to the cyst form on day 14 (Fig. 2.3E).

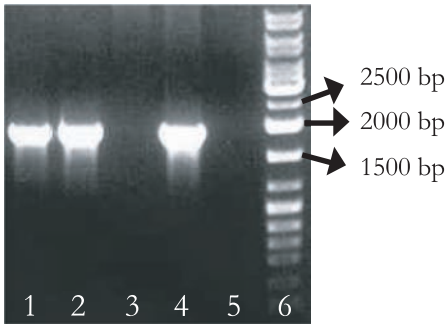

Figure 2.2. Detection of eukaryotic DNA. Lane 1, DNA from biofilm from flasks with unfiltered inoculum in the presence of 100 µM cycloheximide; lane 2 and 4, DNAs from biofilms from flasks with unfiltered inoculum; lane 3 and 5, DNAs from biofilms from flasks with filtered inoculum; lane 6: marker (sm 0333, Gene ruler DNA Ladder Mix, Fermentas, St. Leon-Rot, Germany)

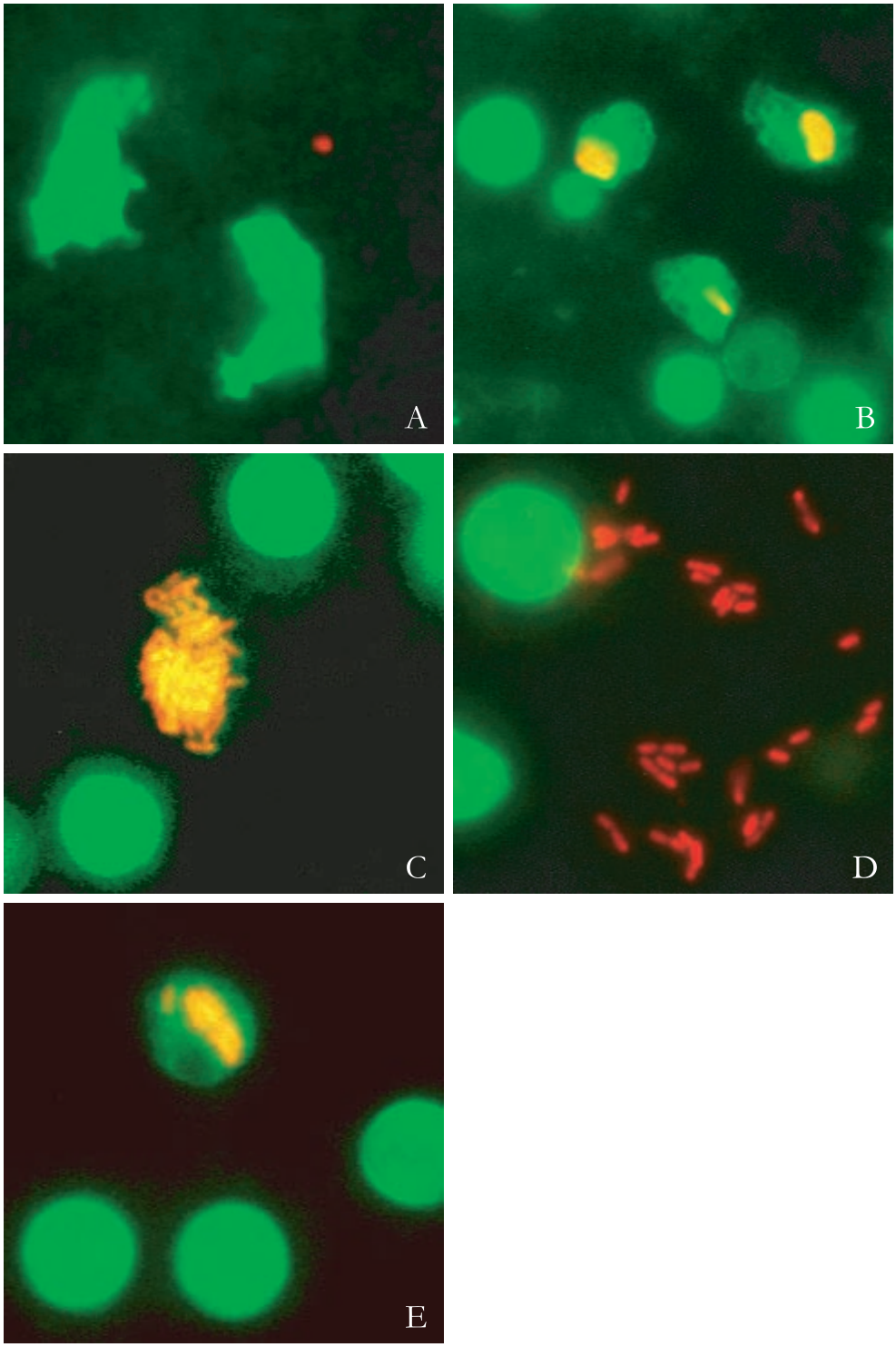
A
B
C
D
E

Figure 2.3 (left page). Different stages of intracellular proliferation of *L. pneumophila* within amoebae. All images were made by using material from the biofilm phase, since amoebae were mainly present in this phase. **A.** Trophozoite amoebae on day 6 of the experiment. Magnification, ×1,000. **B.** Trophozoite amoebae that were just infected with *L. pneumophila* (day 10). Magnification, ×1,000. **C.** Heavily infected amoeba (day 14). Magnification, ×1,600. **D.** Free *L. pneumophila* bacteria, just escaped from a lysed amoeba (day 14). Magnification, ×1,600. **E.** Cysts of amoebae, with one infected by *L. pneumophila* (day 14). Magnification, ×1,600.

Table 2.2 gives the different states of the protozoa as percentages of the total concentrations of protozoa observed by direct cell counting using primulin and FISH. For a time interval of T (hours), the specific growth rate m (per hour) of the protozoa was calculated as follows:

$$\mu = \frac{\ln y_T - \ln y_0}{T}$$

where y_0 and y_T are the protozoon numbers at the beginning and end of the interval. During exponential growth these numbers are related by the equation

$$y_T = y_0 \cdot e^{(\mu T)}$$

The maximum growth rate of the *H. vermiformis* strain, as observed with the cultivation method, was 0.14 h^{-1}. With both direct counting methods a maximum growth rate of 0.15 h^{-1} was calculated.

The *L. pneumophila* concentrations observed in amoebae ranged from 1 cell per amoeba, when they were just infected (Fig. 2.3B) to a maximum of about 100 cells per amoeba just before lysis (Fig. 2.3C). All but one infected amoebae were present as trophozoites (Fig. 2.3B and C), and only one infected cyst was detected (0.02%) (Fig. 2.3E). Table 2.2 gives the numbers of protozoa that were infected with *L. pneumophila* as percentages of all protozoa and as percentages of the trophozoites.

With the different methods for determining the protozoon concentration (cultivation and direct counting using primulin and FISH), a maximal protozoon concentration of 5.3 ± 0.4 log protozoa/cm^2 was detected on day 10 (Fig. 2.1D). On that day 6.2 ± 0.1 log CFU of *L. pneumophila*/cm^2 were present (Fig. 2.1B). Lysis of the protozoa occurred after about 100 *L. pneumophila* cells per infected amoeba were observed (Fig. 2.3C). Hence, 4.2 ± 0.1 log protozoa/cm^2 should account for the total *L. pneumophila* concentration. These lysed trophozoites were not included in the number of protozoa detected on day 10. From this estimated number of lysed protozoa, we calculated that an average of 10.5% ± 6.1% (n = 5) of all protozoa had been infected between days 6 and 10. With FISH analysis, the intracellular proliferation of *L. pneumophila* within trophozoites was observed in 25.9% ± 10.5% (n = 2) of the trophozoites that were present on day 10 (Table 2.2). This percentage was significantly different from the

Table 2.2. Numbers of protozoa, as determined by direct cell counting using primulin and FISH, in a batch test system with PVCp as the biofilm carrier in duplicate flasks

Time point (day)	Total concentration of protozoa (log cells/cm^2)[a]	% of protozoa in indicated state[a]		Trophozoites (%) containing *L. pneumophila*[a]
		Trophozoite	Cyst	
0	ND	ND	ND	ND
2	ND	ND	ND	ND
4	ND	ND	ND	ND
6	3.4 ± 0.2	100.0 ± 0.0	0 ± 0.0	ND
8	4.6 ± 0.2	93.9 ± 6.1	6.1 ± 6.1	ND
10	5.1 ± 0.1	5.7 ± 3.0	94.3 ± 3.0	25.9 ± 10.5
14	5.1 ± 0.1	0.2 ± 0.1	99.8 ± 0.1	92.3[b]

[a]ND, not detected: the concentration was below the detection limit (n<2.1 log protozoa/cm^2)
[b]A total of 13 trophozoites were observed in three different tests, and 12 of them contained *Legionella*

estimated percentage for days 6 to 10 ($p = 0.06$ by a *t* test). On day 14, the percentage of infected trophozoites had increased further (>90%), and only 0.2% of the protozoa were present as trophozoites (Table 2.2).

Eukaryotic identification

The 18S rDNA-targeted PCR amplicons were cloned into *E. coli* JM109 and subsequently subjected to restriction fragment length polymorphism analysis. With this analysis, 19 different restriction patterns were obtained for 50 clones. After partly sequencing their 18S rRNA genes, we identified all 19 sequences as being from *H. vermiformis*, with ≥98% similarities. These 19 clones could be divided into three groups (KWR-1, KWR-2 and KWR-3). The complete (1,838 bp) 18S rRNA genes for these three different operating taxonomic units were determined and showed G+C contents of 49.62, 49.51 and 49.67% for KWR-1, KWR-2, and KWR-3, respectively.

The sequences (KWR-1, KWR-2 and KWR-3) were compared by phylogenetic analysis with those of other protozoa in which the intracellular growth of *L. pneumophila* had been shown (Fig. 2.4). The obtained sequences fell into the group of *H. vermiformis*, consistently forming a subgroup. A total of two nucleotide variations were present in all sequence types and were different from seven other *H. vermiformis* sequences at positions 1550 and 1628 (nucleotide numbering according to the *H. vermiformis* strain Nijmegen sequence [Weekers *et al.*, 1994]). A single base pair insertion (G) was identified between positions 1818 and 1819. In addition to the previously mentioned two mutations, only a few additional single nucleotide polymorphisms were observed among the different *H. vermiformis* strains. These observations indicate that there is a high degree of sequence similarity between *H. vermiformis* strains originating from different habitats and different parts of the world.

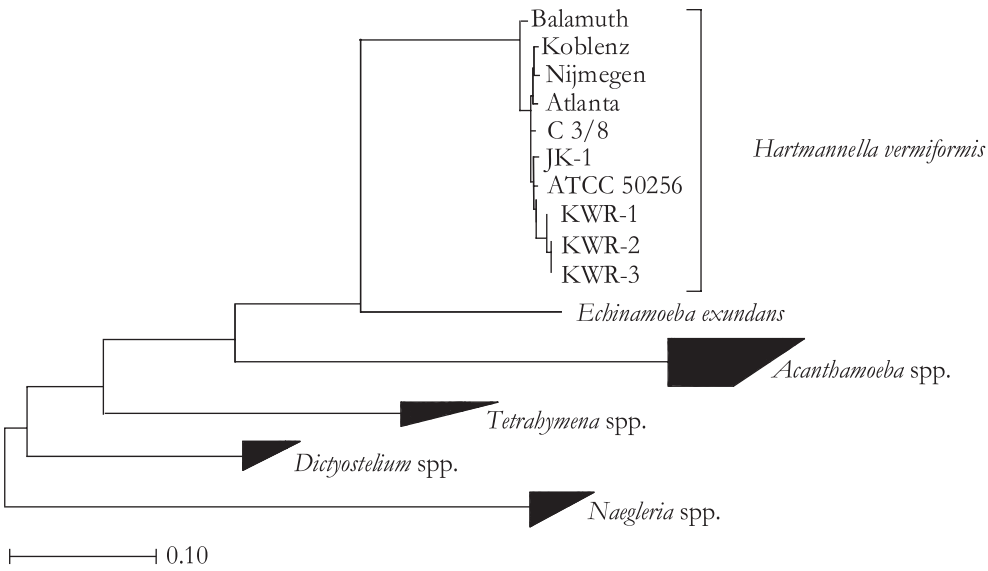

Figure 2.4. Phylogenetic tree from 18S rRNA gene sequences showing the relationships of the examined sequences to those of other protozoa in which intracellular growth of *L. pneumophila* was shown (Fields, 1996, Fields *et al.*, 1984, Hagele *et al.*, 2000, Rowbotham, 1980a). Different trees were calculated, and all trees gave the same consensus, as shown in the figure. Bar, 10% sequence divergence. Strains Koblenz (X75514), Nijmegen (X75515), and Atlanta (X75513) were previously sequenced by Weekers *et al.* (1994), strain Balamuth (ATCC 30966, M95168) was sequenced by Gunderson *et al.* (1994), strain C3/8 (AF426157) was sequenced by Walochnik *et al.* (2002), and strains JK-1 and ATCC 50256 were sequenced by Kuchta *et al.* (1998).

Discussion

Experimental design

For the present study, we used a batch test system for the simulation of biofilm development in tap water. This batch test system has previously been compared with a dynamic model of biofilm development on different materials that are in contact with tap water (Van der Kooij *et al.*, 2002). That previous study showed that the batch test system is well suited for the study of biofilm formation on material surfaces that are in contact with tap water. With this model, it is easy to obtain different biofilm concentrations by using different materials. ATP was used to assess the concentration of active biomasses. The similarity between the total biomass concentrations in tests with a filtered inoculum and with an unfiltered inoculum indicates that ATP represented bacterial biomasses. The concentrations of total ATP and *L. pneumophila* were clearly higher in the presence of PVCp than in the presence of PVCu. Obviously, the concentration of growth-supporting factors for *L. pneumophila* was higher with PVCp, thus facilitating their identification. The high biomass concentrations observed with PVCp were caused by the leaching of biodegradable plastizicers from this

material (Colbourne, 1985). From the obtained percentages of attached and suspended biomasses and the applied surface-to-volume ratios (0.24 to 0.16 cm^{-1}), and assuming a biofilm thickness of 100 µm or less, it can be estimated that the biofilm concentrations were about 100 times higher than the concentrations of suspended biomass. The high proportion (>90%) of protozoa observed in the biofilm on PVCp further indicates that the biofilm was the place where the multiplication of protozoa and *L. pneumophila* occurred.

The following relationship between *L. pneumophila* and total ATP concentration has been reported for the batch test system with incubation at 25°C: log (*Legionella* growth potential) = 0.81 log (biomass production potential) + 1.1 (Van der Kooij *et al.*, 2002). The concentrations of *L. pneumophila* and total ATP observed in the present study after 14 days for PVCp and after 21 days for PVCu did not fit this equation. The log (*Legionella* growth potential) values in the present study were 1.4 and 1.3 times higher for PVCp and PVCu, respectively, than that calculated with the given equation. This difference was attributed to the different temperatures used for incubation of the test flasks because a temperature of 37°C is more favorable for the growth of *L. pneumophila* than is 25°C (Van der Kooij *et al.*, 2002, Wadowsky *et al.*, 1985).

Growth of *Legionella* in aquatic biofilm only in the presence of *H. vermiformis*

Inoculum filtration (3.0-µm pore size) did not influence the total biomass yield (total ATP) but removed a factor that enabled *L. pneumophila* to grow, despite the presence of this bacterium in the inoculum (Fig. 2.1C). Also, no growth of *L. pneumophila* was observed when it was added to the flasks as a pure culture. The inactivation of the growth-supporting factor by pasteurization indicated that a live organism served as the growth-supporting factor. These findings are in complete agreement with the observations of Wadowsky *et al.* (1988), who tested the effect of inoculum filtration (1.0-µm pore size) on the growth of *L. pneumophila* in tap water and observed inactivation of the growth-supporting factor at 60°C.

The cultivation method for the detection of protozoa showed only *H. vermiformis*, and this organism was only present in the flasks with the unfiltered inoculum. FISH analysis revealed the *in vivo* intracellular multiplication of *L. pneumophila* in amoebae, which were identified as *H. vermiformis* by PCR, cloning, and sequence analysis. *H. vermiformis* has also repeatedly been identified as a growth-supporting factor for *L. pneumophila* by researchers in the United States (Fields *et al.*, 1989 and 1990b, Kuchta *et al.*, 1998, Murga *et al.*, 2001, Wadowsky *et al.*, 1988 and 1991). Several authors have questioned the fact that protozoa are required as a host for *L. pneumophila*, and the extracellular growth of *L. pneumophila* has been reported in two studies. In one study, cycloheximide inhibited the growth of protozoa, but *L. pneumophila* proliferated (Surman *et al.*, 2002). The other study suggested that the bacterial consortium within a biofilm supplies sufficient nutrients to enable legionellae to grow extracellularly (Rogers and Keevil, 1992). In the present study, however, growth of *L. pneumophila* was observed at a similar concentration of cycloheximide, but *H. vermiformis* was still detected by 18S rDNA-

targeted PCR (Fig. 2.2). Figure 2.3C shows a microcolony of *L. pneumophila* inside an amoeba just before lysis. The amoeba itself is hardly visible. After the lysis of amoebae, the bacteria are free in the environment, where they may occasionally cluster together (Fig. 2.3D).

Intracellular growth of *L. pneumophila* has been observed *in vitro* in *T. pyriformis*, *Acanthamoeba castellanii* and *H. vermiformis* by the use of FISH (Grimm *et al.*, 2001, Grimm *et al.*, 1998, Manz *et al.*, 1995). The protozoon cultures in these studies were inoculated with a high concentration of *L. pneumophila* (6 to 8 log cells/ml). In the present study, the development of biofilms in tap water was simulated by the use of relatively small inoculum sizes for both *L. pneumophila* and *H. vermiformis* (4.1 and 1.4 log cells/ml, respectively), thus more closely resembling the actual situation in water systems.

The present study also demonstrated the presence of *L. pneumophila* inside a cyst of *H. vermiformis* (Fig. 2.3E) *in vivo*, but the percentage of infected cysts was very low (0.02%). Kuchta *et al.* (1993 and 1998) did not detect *L. pneumophila* within cysts of *H. vermiformis*, and Greub and Raoult (2003) observed the bacterium within the cyst wall, but not within vacuoles or the cytoplasm, of a mature cyst of *H. vermiformis*. *L. pneumophila*-infected cysts of *Acanthamoebae palestinensis* have been observed in another study (Skinner *et al.*, 1983), and 2 to 5% of *Acanthamoeba polyphaga* cysts were found to be infected with *L. pneumophila* (Rowbotham, 1986). Our observations support earlier suggestions that trophozoites and cysts may both provide a protective environment for *L. pneumophila* in engineered water systems (Harf and Monteil, 1988, Skinner *et al.*, 1983).

Quantitative relationship between total ATP, *L. pneumophila*, and *H. vermiformis*

Heterotrophic bacteria that contributed to the peak of the total ATP concentration (Fig. 2.1A) most likely served as a food source for *H. vermiformis*, since protozoa were first detected on day 6, when the maximum total ATP concentration was observed (Fig. 2.1D). The concentration of *L. pneumophila* increased exponentially after the first *H. vermiformis* protozoa were detected. The growth rates of *H. vermiformis* obtained in this study were 2.9-fold higher than the maximum growth rates of naked amoebae in their natural environment (Arndt, 1993) and were similar to the highest growth rates of naked amoebae obtained under laboratory conditions (Baldock *et al.*, 1980, Butler and Rogerson, 1996). Hence, the aquatic biofilm developing on the pieces of PVCp provided a favorable environment for the growth of *H. vermiformis*.

Our observations and calculations further showed that under the test conditions used, a significant percentage of the *H. vermiformis* population was infected with *L. pneumophila*. No infected protozoa were detected on day 8 of the experiment, when 93.9% ± 6.1% of the protozoa were present as trophozoites. On day 10, only 5.7% ± 3.0% of the protozoa were present as trophozoites, 25.9% ± 10.5% of which contained *L. pneumophila*. These percentages were even more extreme on day 14, when 0.2% ± 0.1% of the protozoa were present as trophozoites and >90 % of the

trophozoites were infected (Table 2.2). After day 10, no further growth of either *L. pneumophila* or *H. vermiformis* was observed (Fig 2.1B and D). This observation supports the conclusion that the growth of *L. pneumophila* depended on intracellular proliferation. The high percentage of infected trophozoites on day 14 suggests that *Legionella* can inhibit the growth of *H. vermiformis* under batch test conditions. On the other hand, the growth of protozoa had already ceased on day 10, possibly by the depletion of preferred prey bacteria. A clarification of these processes requires further research.

Phylogenetic analysis of *H. vermiformis*

Among the protozoa in which intracellular growth of *L. pneumophila* occurs, the cluster of *H. vermiformis* showed the highest similarity with *Echinamoeba exundans*. This was also found by Amaral Zettler *et al.* (2000). In fact, *E. exundans* was first described as a species of *Hartmannella* (Page, 1967). The clones KWR-1, KWR-2 and KWR-3 clearly formed a subgroup within the *H. vermiformis* group, although there are very few sequence variations within the *H. vermiformis* cluster (Weekers *et al.*, 1994). This subgroup showed the highest similarity with *H. vermiformis* strain ATCC 50256, which was originally isolated from a warm water tank in relation with *L. pneumophila* (Kuchta *et al.*, 1998). The obtained phylogenetic tree and the results of Walochnik *et al.* (2002) do not support the previously suggested separation between European and North American strains of *H. vermiformis* (Kuchta *et al.*, 1998, Weekers *et al.*, 1994).

In conclusion, this study demonstrated that *L. pneumophila* multiplies in trophozoites of *H. vermiformis* that are present in aquatic biofilms developing on plastic materials in contact with tap water in a batch test system. Different materials (PVCu and PVCp) gave different biofilm concentrations (total ATP concentrations), and higher total ATP concentration gave higher concentrations of *L. pneumophila*. The proliferation of *L. pneumophila* was due to its intracellular multiplication in *H. vermiformis* in the presence of a high concentration of heterotrophic bacteria in the biofilm. Prevention of the growth of *L. pneumophila* within engineered water systems thus requires the prevention of growth of protozoa in biofilms. This objective can be achieved by the removal of biodegradable compounds from the water, the selection of materials that do not support biofilm formation, and/or the maintenance of a disinfectant residual.

Acknowledgement

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CHAPTER 3

Composition of the bacterial community in a biofilm-batch model system with *Legionella pneumophila* and *Hartmannella vermiformis*

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Abstract

Legionella pneumophila can only proliferate in aquatic habitats in the presence of a eukaryotic host. It is unknown, whether specific bacterial species are needed for the proliferation of such a host. Erlenmeyer flasks, containing tap water and pieces of plasticized PVC were inoculated either with an unfiltered inoculum originating from a tap water system with *L. pneumophila* and *Hartmannella vermiformis* as indigenous populations, or with the same, but filtered (cellulose nitrate, 3.0-μm pore size) inoculum, to remove *H. vermiformis*, but not *L. pneumophila*.
16S rRNA gene-targeted denaturing gradient gel electrophoresis (DGGE), complemented by cloning and sequencing analysis, showed that the bacterial composition in the biofilm and in the planktonic phase changed in time, in both environments. The bacterial composition of the planktonic phase in the flasks inoculated with *L. pneumophila* and *H. vermiformis* was more related to the biofilm and planktonic phase in the flasks without *H. vermiformis* than to its biofilm counterpart. This was due to (i) the presence of *L. pneumophila* as dominant group in the biofilm of the environment with *H. vermiformis* and (ii) the decrease in intensity of two bands representing a *Phenylobacterium lituiforme*-related and a *Limnobacter thiooxidans*-related population in the DGGE profile of the biofilm in the flasks with the unfiltered inoculum. The decrease in the abundance of these bacteria was attributed to selective grazing of *H. vermiformis* on the biofilm.
The results obtained in this study showed that *H. vermiformis*, a potential host for *L. pneumophila*, grazes on several specific bacteria in a multi-species biofilm. It is therefore suggested that several bacterial species, and not just one specific organism, may serve as food source for *H. vermiformis* in natural environments.

Introduction

Legionella pneumophila is ubiquitous in natural fresh water environments at elevated temperatures and is also found in man-made water systems (Fliermans *et al.*, 1981, Tobin *et al.*, 1980, Yamamoto *et al.*, 1992). The multiplication of the organism in man-made systems, e.g. warm water installations and cooling towers, poses a potential human health risk when aerosolization can occur (Fields, 1996).
L. pneumophila is a fastidious organism, and under laboratory conditions it only grows in complex media containing amino acids with supplements of cysteine and iron salts (Edelstein, 1981). In the natural environment its high nutritional demands are satisfied in the intracellular environment of protozoan hosts, rather than with nutrients dissolved in fresh water. Intracellular proliferation of *L. pneumophila* has repeatedly been demonstrated (Rowbotham, 1980a), but the need for protozoa in a nutrient rich biofilm is still questioned by some investigators (Rogers and Keevil, 1992, Surman *et al.*, 2002). In an aquatic biofilm grown on plasticized polyvinyl chloride (PVCp), it was observed that (i) *L. pneumophila* did not multiply in a biofilm in the absence of protozoa

and (ii) the free-living amoeba *Hartmannella vermiformis* served as host for the intracellular proliferation of *L. pneumophila* under the conditions of the test (Kuiper *et al.*, 2004).

Free-living amoebae are the dominant bacterial consumers in soil (Clarholm, 1981, Elliott and Coleman, 1977) and are responsible for 60% of the total decrease of the bacterial biomass (Clarholm, 1981). Protozoa are also important predators in aquatic systems (Barcina *et al.*, 1991, González, 1990 and 1992), and it has been shown that naked amoebae are able to ingest and digest bacteria embedded within alginate, an environment similar to bacterial biofilms (Heaton *et al.*, 2001). Differences in the ability for amoebae to feed on various food bacteria have been observed (Singh, 1941, 1942, and 1946, Stout and Heal, 1967). Non-pigmented Enterobacteriaceae *Escherichia coli* K-12 and *Klebsiella aerogenes* are excellent food for *H. vermiformis*, but the pigmented *Chromatium vinosum* and *Serratia marcescens* were not consumed (Weekers *et al.*, 1993). In compliance with legislation, coliform bacteria, including *E. coli* and *K. aerogenes*, are absent in 100 ml tap water (Waterleidingbesluit, 2001). Consequently, these bacteria do not act as food for amoebae in tap water installations and other bacteria multiplying in this environment serve as prey for *H. vermiformis*.

In this study, the bacterial composition was compared of two batch type aquatic mesocosms (biofilm and planktonic phase) also used in chapter 2 (Kuiper *et al.*, 2004), one containing *L. pneumophila* and *H. vermiformis* and a control environment without protozoa. This comparison was performed to assess whether specific bacterial populations served as prey for *H. vermiformis*, thus indirectly promoting the proliferation of *L. pneumophila*. The dominant bacterial groups were identified by cloning and sequence analysis of PCR amplicons, and compared to the bacterial isolates that were cultured from the biofilm containing *L. pneumophila* and *H. vermiformis*.

Materials and methods

Experimental approach

Two 600-ml Erlenmeyer flasks (biofilm-batch model [BBM] flasks), inoculated in duplicate with filtered and unfiltered water, originating from a warm tap water system with *L. pneumophila*, respectively, as described previously (chapter 2 of this thesis [Kuiper *et al.*, 2004]), were used in this study. The composition of the attached (biofilm) and the suspended biomass (planktonic phase) of samples taken at different time points during the experiments were analyzed with denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal RNA gene-targeted PCR amplicons, and further investigated with cloning and sequencing. Also cultured isolates were retrieved from the flasks that received the unfiltered inoculum. Biofilm material was removed from the PVCp pieces by six 2-min sonication steps in 10 ml of sterilized tap water (U950D, Ultrawave Limited, Cardiff, United Kingdom) at a frequency of 30 kHz and an average power input of 0.11 W/ml. At each time point (0, 2, 4, 6, 8, 10 and 14 days after inoculation), 30 ml of the planktonic phase and 50 ml of the biofilm phase were

filtered over a 0.2-µm pore size GTTP Isopore Membrane (Millipore, Bedford, Mass.). DNA extraction of the filter-retained cells was done by use of the FastDNA spin kit for soil (BIO 101, Carlsbad, Calif.) according to the instructions supplied by the manufacturer and DNA was eluted in a final volume of 50 µl.

In order to retrieve readily cultivable isolates that could potentially be used as food source for *H. vermiformis* in subsequent model studies, additional BBM flasks were prepared following the standard experimental set-up as described in chapter 6 of this thesis. Briefly, biofilms were developed on pieces of PVCp (diameter, 0.80 cm; about 6.2 cm^2 of surface area for each) using thoroughly cleaned Erlenmeyer flasks with a volume of 1 liter. Each Erlenmeyer flask (closed with a cotton-wool plug), containing 600 ml tap water (pH 7.8), pieces of PVCp (surface-to-volume ratio of 0.16 cm^{-1}), and nitrate and phosphate at final concentrations of 72.5 and 13.5 µM respectively, was autoclaved (15 min, 121°C). The BBM flasks were inoculated with a mixed microbial community including indigenous *L. pneumophila* serotype 1 and *H. vermiformis*, originating from a plumbing system in the Netherlands. After 7 days of incubation at 37°C under static conditions the attached microbial community (biofilm) was removed by sonication and used as a new inoculum (1 ml) in freshly prepared BBM flasks. Inoculation and incubation of these flasks was repeated four times and subsequently an appropriate dilution of the biofilm microbial community was plated on R2A medium (Reasoner and Geldreich, 1985)(Difco, BD, Sparks, Maryland). Cultivable bacteria, apparently different strains as judged by colony morphology, were isolated with the streak plate method. Cell lysis of a single colony of each strain was performed in DNA free water at 95°C for 10 min. This suspension was subsequently used for PCR amplification.

PCR amplification

For determining the bacterial diversity in the biofilm and in the planktonic phase, primers GC-0968f and 1401r (Table 3.1) were used to amplify V6-V8 regions of bacterial 16S rRNA genes, yielding amplicons of 470 bp in length. PCR was performed using the *Taq* DNA polymerase kit from Invitrogen (Life Technologies, the Netherlands). PCR mixtures (50 µl) contained 0.25 µl of *Taq* polymerase (1.25 U), 1× PCR buffer supplied with the enzyme, 3.0 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM

Table 3.1. Sequences of PCR primers used in this study

Primer	Sequence	Source or reference
27f	5'-GTT TGA TCC TGG CTC AG-3'	(Lane, 1991)
1492r	5'-CGG CTA CCT TGT TAC GAC-3'	(Lane, 1991)
T7	5'-AAT ACG ACT CAC TAT AGG-3'	Promega
Sp6	5'-ATT TAG GTG ACA CTA TAG-3'	Promega
GC-0968f	5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA CCT TAC-3'	(Nübel *et al.*, 1996)
1401r	5'-CGG TGT GTA CAA GAC CC-3'	(Nübel *et al.*, 1996)

of both primers, 1 µl of template DNA and UV sterile water. PCR cycling was performed in an UNO II thermocycler Biometra (Westburg, Leusden, the Netherlands) with the following program: predenaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 20 s, and extension at 72°C for 40 s; and a final extension step at 72°C for 10 min.
The identification of the cultured bacterial isolates and of the indigenous *L. pneumophila*, which was cultivated on buffered charcoal yeast extract agar medium (Edelstein, 1981), was based on the 16S rRNA gene sequence. Primers 27f and 1492r (Table 3.1) were used to amplify an almost complete fragment of 1465bp of the 16S rRNA gene. The PCR mixtures (50 µl) contained 1 µl of template DNA, each primer at a concentration of 0.2 µM, 200 µM of each dNTP, 3 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Invitrogen), and the PCR buffer supplied with the enzyme. PCR cycling was performed with the equipment mentioned before, with the following program: predenaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 20 s, and extension at 72°C for 40 s; and a final extension step at 72°C for 7 min. Aliquots of 5 µl of each PCR product were analyzed by electrophoresis on a 1.2% agarose gel (w/v) containing ethidium bromide to check size and amount of the amplicons.

DGGE analysis
The amplicons obtained by V6-V8 PCR were separated by DGGE according to the specifications by Muyzer *et al.* (1993), using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was performed in an 8% polyacrylamide gel 37.5:1 acrylamide-bisacrylamide (dimensions 200×200×1 mm) using a 30-60% denaturing gradient (Muyzer *et al.*, 1998). The gels were electrophoresed for 16 h at 85 V in 0.5×TAE buffer (Maniatis *et al.*, 1989) at a constant temperature of 60°C and stained with $AgNO_3$ (Sanguinetti *et al.*, 1994). All gels were scanned at 600 dpi and analyzed using the Bionumerics software package version 4.0 (Applied Maths, Kortrijk, Belgium); dendrograms were constructed based on Pearson product-moment correlation, and using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering.
Identification of the DGGE bands was performed in two ways. Bands 2, 3, 5, 6 and 7 were identified by V6-V8 PCR and DGGE analysis of 88 *Escherichia coli* JM109 transformant clones, containing inserts of PCR amplicons obtained from the 27f-1492r PCR from the biofilm DNA at day 14 from the two conditions, filtered and unfiltered (see below for details). The DGGE profiles of these clones were compared with the profiles of the mixed community, and matching clones, were further identified by sequencing. Band 1 was identified as follows: the band was cut from a DGGE gel which was stained with 1× SYBR gold (Invitrogen) in 200 ml 1× TAE buffer (pH 7.5), and the DNA eluted as described before (Etokebe and Spurkland, 2000). Subsequently this DNA was used for another V6-V8 PCR and DGGE to check for purity, and analyzed by cloning and sequencing.

Cloning and sequencing of the PCR-amplified products

PCR amplicons were purified using a QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). PCR products were cloned into *E. coli* JM109 using the Promega pGEM-T easy vector system (Promega, Madison, Wis.). PCRs were performed on cell lysates of ampicillin-resistant transformants, using vector-specific primers T7 and Sp6 (Table 3.1) to confirm the size of the inserts. Sequence analysis was done by BaseClear Lab services (Leiden, the Netherlands). The 16S rRNA gene sequences of the cultivated bacteria obtained in this study have been deposited in GenBank under accession numbers DQ202403-202408. The sequences of the partial 16S rRNA genes obtained in this study for the clones matching the DGGE bands have been deposited in GenBank under accession numbers DQ408658-DQ408664.

Results

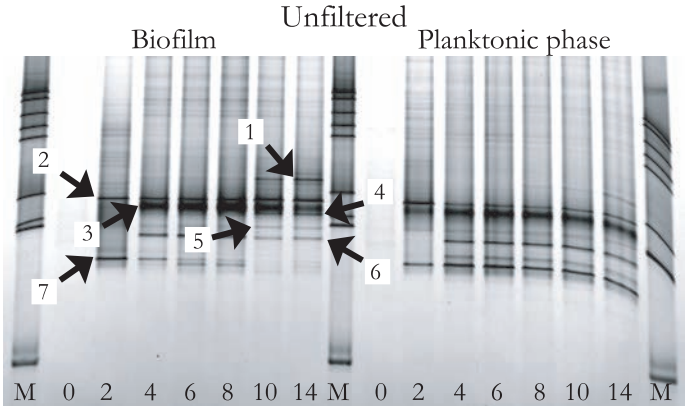
Effect of *H. vermiformis* on bacterial composition of aquatic environments

Analysis of the biofilm-associated and the planktonic microbiota by 16S rRNA gene-targeted PCR-DGGE fingerprinting revealed a succession of changes in the bacterial communities in both compartments of the BBM flasks. While biomass concentration at time point 0 was obviously too low to generate clear fingerprints, an increasing number of bands could be observed with time. Two or three dominant bands were detected after 2 days, while six to nine bands were visible from day 4 until the end of the experiment after 14 days (Fig. 3.1 A and B).

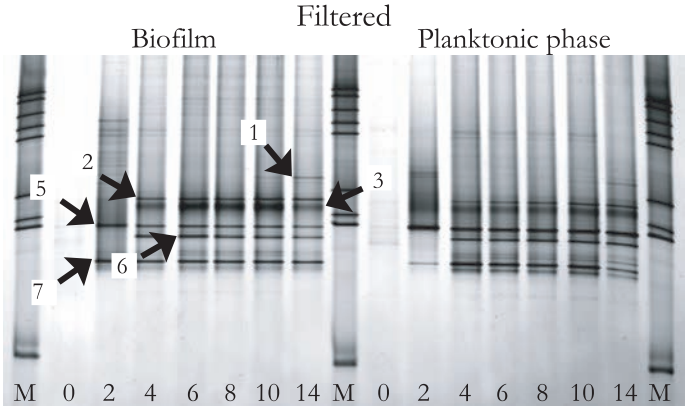
The BBM flasks that were inoculated with the filtered material did not contain *H. vermiformis* and therefore not sustained proliferation of *L. pneumophila* (Kuiper *et al.*, 2004). The bacterial communities in both the biofilm and the planktonic phase were rather similar, with the exception of samples taken after 0 and 2 days (Fig. 3.1B). While profiles of biofilm- and planktonic phase-derived samples formed separate sub-clusters, they were closely related with an average similarity of 82.6 ± 5.6% (Fig. 3.1C). For the bacterial composition of the biofilm and the planktonic phase in the BBM flasks with the unfiltered inoculum, however, this was not the case. In fact, profiles from the planktonic phase in the flasks with the unfiltered inoculum clustered with those derived from biofilm and planktonic phase in the flasks with the filtered inoculum (average similarity of 66.6 ± 6.8%), rather than with those from the biofilm in the same flask (average similarity of 39.8 ± 11.0%, Figure 3.1C). This is most likely

Figure 3.1 (right page). DGGE profiles of the bacterial communities of the biofilm and planktonic phase in an aquatic environment in the presence of *L. pneumophila* and *H. vermiformis* (**A**), and in the absence of *H. vermiformis* (**B**) and the corresponding dendrogram (**C**). The numbers shown at the bottom in A and B, and in C (t), indicate time of sampling in days after inoculation, M indicates the marker. Arrows and numbers within the gel images indicate the identified bands of the DGGE profiles.

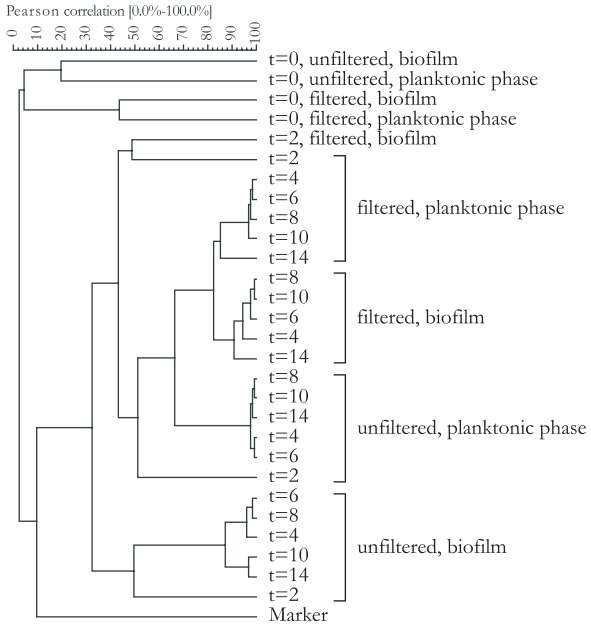
A


Unfiltered
Biofilm
Planktonic phase
1
2
3
4
5
6
7
M 0 2 4 6 8 10 14 M 0 2 4 6 8 10 14 M

B


Filtered
Biofilm
Planktonic phase
1
2
3
5
6
7
M 0 2 4 6 8 10 14 M 0 2 4 6 8 10 14 M

C


Pearson correlation [0.0%-100.0%]
0 10 20 30 40 50 60 70 80 90 100
t=0, unfiltered, biofilm
t=0, unfiltered, planktonic phase
t=0, filtered, biofilm
t=0, filtered, planktonic phase
t=2, filtered, biofilm
t=2
t=4
t=6
t=8
t=10
t=14
filtered, planktonic phase
t=8
t=10
t=6
t=4
t=14
filtered, biofilm
t=8
t=10
t=14
t=4
t=6
t=2
unfiltered, planktonic phase
t=6
t=8
t=4
t=10
t=14
t=2
unfiltered, biofilm
Marker

due to the extra band (number 4, *L. pneumophila*) and decreased intensities of band numbers 6 and 7 in the biofilm in the flasks with the unfiltered inoculum (Figure 3.1A). Furthermore, the major bands of the DGGE profiles of the biofilm communities were identified (Bands 1-7, Figure 3.1A, and Table 3.2).

Cultivable bacteria in a *L. pneumophila* containing biofilm

Six strains (M1-M6) with different colony appearances when cultivated on R2A medium were isolated from the biofilm in the flasks with the unfiltered inoculum. Partial 16S rRNA gene sequences were analyzed and phylogenetic identity of the isolates was determined using BLAST analysis (McGinnis and Madden, 2004) (Table 3.3).

The cultivated bacteria were compared with the dominant bacterial populations as determined by DGGE (Figure 3.2). Interestingly, PCR products from isolates M1 and M6 migrated to positions of predominant bands in the DGGE profiles of the mixed microbial communities of the biofilms (bands 6 and 2 respectively, Figure 3.1A). Comparison of sequences of isolate M1 and band 6 with Clustal W (Thompson *et al.*, 1994) indicated exact matching (100%) over the 402 bp of overlapping sequence. For M6 and band 2, 9 of the 1329 bp were different and one gap on each sequence was present, resulting in a similarity of >99%. Both isolate M2 as well as band 3 were

Table 3.2. DGGE bands identified from the samples originating from the biofilm. Identification of the closest relatives was done by using BLAST function (McGinnis and Madden, 2004). For bands most closely related to unidentified organisms, the closest cultured relative is also provided. Accession numbers for sequences of closest relatives are given in parentheses.

No.	GenBank accession number	Length (bp)	Closest relative	Identity (%)	Sequence compared (#bp)
1	DQ408658	427	Uncultured bacterium clone SM1H07 (AY094624)	96	427
			Acidobacteriaceae bacterium Gsoil 1619 (AB245338)	92	427
2	DQ408659	1503	*Pseudoxanthomonas mexicana* strain AMX 26B (AF273082)	99	1504
3	DQ408660	1012	*Acidovorax* sp. UFZ-B517 (AF235010)	99	1012
4	DQ408661	1502	*Legionella pneumophila* strain Paris (CR628336)	99	1503
5	DQ408662	1488	Arsenite-oxidizing bacterium NT-6 (AY027499)	99	1488
			Hydrogenophaga sp. PG-10 (AY566583)	97	1483
6	DQ408663	558	*Phenylobacterium lituiforme* strain FaiI3 (AY534887)	97	543
7	DQ408664	1489	Uncultured bacterium clone B3NR69D12 (AY957927)	99	1489
			Limnobacter thiooxidans strain CS-K2 (AJ289885)	99	1489

Table 3.3. Closest relatives of the bacteria cultured from a 6 days old biofilm grown on PVCp in tap water with *L. pneumophila* and *H. vermiformis*. For isolates most closely related to uncultured organisms, the closest cultured relative is also presented. Accession numbers for sequences of closest relatives are given in parentheses.

No.	GenBank accession number	Length (bp)	Closest relative	Identity (%)	Sequence compared (#bp)
M1	DQ202403	1301	Uncultured bacterium clone B3NR56D1 (AY957905)	98	1301
			Caulobacter sp. A1 (AF361188)	94	1254
M2	DQ202404	1355	Uncultured bacterium clone BANW427 (DQ264427)	99	1355
			Acidovorax sp. UFZ-B517 (AF235010)	99	1350
M3	DQ202405	1298	Beta proteobacterium MBIC3293 (AB002678)	99	1290
			Roseateles depolymerans 61A (DSM11813) (AB003624)	97	1295
M4	DQ202406	1250	*Agrobacterium tumefaciens* isolate C4 (AF508093)	100	1250
M5	DQ202407	1345	Uncultured bacterium clone 167ds20 (AY212618)	98	1346
			Flavobacterium sp. WB2.1-3 (AM167557)	94	1346
M6	DQ202408	1329	*Pseudoxanthomonas mexicana* strain AMX 26B (AF273082)	99	1330

identified as most closely related to *Acidovorax* sp. (Table 3.2 and 3.3). However, the PCR product of M2 did not migrate to the same position as predominant band 3. Comparison by ClustalW revealed one mismatch between the two sequences and one gap on the sequence of band 3. These results showed that the cultured isolates M1 and M6 were also predominant in the culture-independent DGGE profile (bands 6 and 2) and that isolate M2, although not representing a predominant band in the DGGE

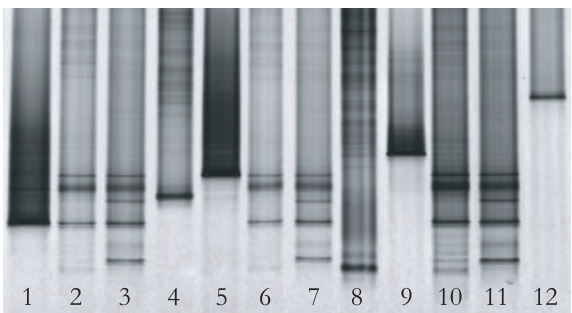


Figure 3.2. DGGE profile of the dominant cultivable bacteria compared with the mixed bacterial community. Lanes: **1**, isolate M1; **2, 6, 10,** biofilm of the flasks with the unfiltered inoculum after 6 days; **3, 7, 11,** biofilm of the flasks with the filtered inoculum after 6 days; **4**, isolate M2; **5**, isolate M6; **8**, isolate M3; **9**, isolate M4; **12**, isolate M5.

profile, is highly similar to a predominant band in the culture-independent DGGE profile.

Discussion

The BBM system, which was used in chapter 2 (Kuiper *et al.*, 2004), was also applied in this study. With this system, two types of environments were created in the Erlenmeyer flasks. The first environment, containing *L. pneumophila* and *H. vermiformis*, was created by using an unfiltered inoculum originating from a tap water system with both microorganisms as indigenous components. The second contained the same inoculum, but filtered (cellulose nitrate, 3.0-μm pore size), which removed the amoeba and thus the *L. pneumophila* growth promoting agent, thereby prohibiting growth of *L. pneumophila*, although it was initially present (Kuiper *et al.*, 2004).
The temporal development of bacterial community composition of the biofilm and the planktonic phase of both environments was followed during 14 days. In this period the bacterial community changed in time (Fig. 3.1A and B) in both environments and *L. pneumophila* proliferation was observed in the flasks with the unfiltered inoculum (Fig. 2.1B and Fig 3.1A). Furthermore, *H. vermiformis* proliferated exponentially between day 4 and day 10, and encysted afterwards in the flasks with the unfiltered inoculum (Fig. 2.1D and Table 2.2). After 14 days the number of predominant bacterial populations was highest in both the biofilm and the planktonic phase for both environments. However, it may be expected that the biofilm would have further developed, if the experiment had been prolonged, since a 14-day-old biofilm can be regarded as very young according to Martiny *et al.* (2003). A total of six to nine dominant operational taxonomic units (OTU, defined by DGGE bands) were established in the two environments. This number is low compared to the 17 OTUs previously found in young biofilms (Martiny *et al.*, 2003), but is only 3 OTUs less then the 12 OTUs found with DGGE analysis in drinking water (Farnleitner *et al.*, 2004). These differences might be due to differences in experimental set-up, as the batch system used in the present study was inoculated only once, at the beginning of the experiment, with a mixed microbial community. There was no continuous water flow as in the biofilm system described in the study by Martiny *et al.* (2003), and Farnleitner *et al.* (2004) used water samples from operational tap water systems.
The bacterial composition of the planktonic phase in the Erlenmeyer flasks with *L. pneumophila* and *H. vermiformis* was significantly more related to the biofilm and planktonic phase in the flasks without *H. vermiformis* than to its biofilm counterpart. Most probably, this was due to (i) the presence of *L. pneumophila* as dominant group and (ii) the decrease of populations corresponding to bands 6 and 7 in the DGGE profiles of the biofilm in the flasks with the unfiltered inoculum. The organisms representing bands 6 and 7, which were most closely related to *Phenylobacterium lituiforme* and *Limnobacter thiooxidans*, respectively, may have served as food source for *H. vermiformis*, since the intensity of these bands decreased during the experiment, while the

concentration of *H. vermiformis* increased (Fig. 3.1A and 2.1D). Furthermore the intensities of these bands were not reduced in the biofilm of the flask without *H. vermiformis* (Fig. 3.1B). *Phenylobacterium lituiforme* is a moderate thermophile which was first isolated from a 42°C water sample collected from the Great Artesian Basin in Australia. The bacterium grew best with yeast extract as the sole carbon and energy source (Kanso and Patel, 2004). *Limnobacter thiooxidans*, a thiosulfate-oxidizing bacterium, was first isolated from sediment of the littoral zone of a fresh water lake in Germany. After an enrichment procedure, the bacterium can now be cultivated on R2A agar (Spring *et al.*, 2001). *H. vermiformis* is able to consume other bacteria as well, since isolate M2, which was identified as an *Acidovorax* sp., was successfully used as model-prey-bacterium in chapter 6 of this thesis.

Generally, cultivation on solid media detects only a small fraction of the total microbial community in a water sample (Amann *et al.*, 1994). This explains why the DGGE profiles of the cultivated bacteria were only partly in accordance with the dominant bands of the DGGE profiles of the total bacterial community. In this study only R2A was used as medium for cultivation. If the objective of this study had been to obtain as many cultured isolates as possible, other media should have been included. Rather, our objective was to obtain easily cultivable bacteria that could be used as model bacterium, which is described in chapter 6. Nevertheless two isolates (M1 and M6) were also identified as dominant populations by cultivation-independent DGGE profiling of the mixed microbial communities of the biofilm (Fig. 3.2). Furthermore, band 4 (*L. pneumophila*) was also cultivable, although not on R2A medium, but on the BCYE medium.

Phylogenetic identification of the six isolates (M1-M6, Table 3.3) and the seven dominant bands (Fig. 3.1A, Table 3.2) in the biofilm containing *L. pneumophila* and *H. vermiformis* by 16S rRNA gene sequence analysis, revealed that the sources of the closest identified relatives were almost all water related (Kanso and Patel, 2004, Pernthaler *et al.*, 2001, Thierry *et al.*, 2004), suggesting that these bacteria are common components of the microbial community of water.

While both isolate M2 as well as band 3 were identified as most closely related to *Acidovorax* sp. and only one mismatch between the two sequences and one gap on the sequence of band 3 were observed upon comparison by ClustalW, indicating that different genotypes of *Acidovorax* sp. are present, DGGE migration was slightly different. This can most probably be attributed to only one mismatch (no gap) within the V6-V8 region used for DGGE analysis (Figure 3.2). With DGGE it is indeed possible to separate sequences which differ in only one base pair (Myers *et al.*, 1987, Sheffield *et al.*, 1989).

This study confirms that the presence of a suitable host is essential for the proliferation of *L. pneumophila*. Furthermore, this host, viz. *H. vermiformis* should be able to graze on the biofilm containing bacterial species which are suitable food sources for protozoa. Biofilms in tap water distributing systems are known for their species richness, independent of the presence of a disinfectant residual in the distributing system (Keevil

et al., 1995), and are therefore potentially excellent feeding grounds for protozoa. The results indicate that most probably not the biofilm composition but the concentraion of suitable prey bacteria in the biofilm is most important for *L. pneumophila* proliferation in water distributing systems. It is therefore recommended that biofilm formation should be limited. This can be achieved by the reduction of the biofilm formation potential of water and materials and by the prevention of a rapid biomass development.

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CHAPTER 4

Quantitative detection of the free-living amoeba *Hartmannella vermiformis* in surface water using real-time PCR

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Abstract

A real-time PCR-based method targeting the 18S rRNA gene was developed for the quantitative detection of *Hartmannella vermiformis*, a free-living amoeba, which is a potential host for *Legionella pneumophila* in warm water systems and cooling towers. The detection specificity was validated using genomic DNA of the closely related *H. abertawensis* as negative control and sequence analysis of amplified products of environmental samples. Real-time PCR detection of serially diluted DNA extracted from *H. vermiformis* was linear for microscopic cell counts between 1.14×10^{-1} and 1.14×10^{4} cells per PCR reaction. The genome of *H. vermiformis* harbours multiple copies of the 18S rRNA gene and an average number of 1330 ± 540 copies per cell was derived from the real-time PCR calibration curves for cell suspensions and plasmid DNA. No significant differences were observed between the 18S rRNA gene copy numbers in trophozoites and cysts of strain ATCC 50237, nor between this strain and strain KWR-1. The developed method was applied to water samples (200 ml) collected from a variety of lakes and rivers serving as sources for drinking water production in the Netherlands. Detectable populations were found in 21 of the 28 samples, with concentrations ranging from 5 to 75 cells/l. A high degree of similarity (≥98%) was observed between sequences of clones originating from the different surface waters and between these clones and the reference strains. Hence, *H. vermiformis*, highly similar to strains serving as host for *L. pneumophila*, is a common component of the microbial community in fresh surface water.

Introduction

Hartmannella vermiformis, a free-living amoeba, belonging to the class Lobosea, the subclass Gymnamoebia, order Euamoebida, family Hartmannellidae, was first described by Page in 1967 (Page, 1967). Other amoebae genera in the family Hartmannellidae are *Cashia*, *Glaeseria* and *Saccamoebae* (Page, 1988). *H. vermiformis* is widespread in nature and has been isolated from soil, fresh water, air and a variety of engineered water systems (Breiman *et al.*, 1990, Page, 1974, Page, 1967, Rohr *et al.*, 1998, Walker *et al.*, 1986). Two distinct life-cycle forms are known for *H. vermiformis*, viz. the trophozoite, an active feeding cell that also multiplies, and cysts which are inactive dormant cells (Page, 1976). *H. vermiformis*, as other aquatic protozoa, plays an important role in nutrient cycling in ecosystems, since free-living amoebae take part in the nitrogen mineralization processes (Clarholm, 1985, Elliott *et al.*, 1979). The organism also has direct and indirect public health significance. *H. vermiformis* has been isolated from the cerebrospinal fluid of a patient with meningoencephalitis and bronchopneumonia (Centeno *et al.*, 1996). Furthermore, it has been suggested that *H. vermiformis* can cause keratitis, although this is questioned by other investigators (Aitken *et al.*, 1996, De Jonckheere and Brown, 1998, Inoue *et al.*, 1998, Kennedy *et al.*, 1995). The indirect public health significance of the organism is related to its role as host for

Legionella pneumophila, the causative agent of Legionnaires' disease (Fields *et al*., 1990b, Fields *et al*., 1989, Kuchta *et al*., 1998, Wadowsky *et al*., 1988, Wadowsky *et al*., 1991). Traditionally, cultivation methods are applied for the detection of protozoa in aquatic habitats. Cultivated protozoa can subsequently be classified based upon their morphological characteristics and by using biochemical and immunological methods (Szénasi *et al*., 1998). To circumvent the need for cultivation, molecular tools for protozoa detection have been developed during the past decade, mainly using ribosomal RNA-targeted fluorescent in situ hybridization (FISH) with oligonucleotide probes. Probes were designed for the specific detection of *Legionella*-growth promoting free-living amoebae *Acanthamoeba*, *Naegleria* and *Hartmannella*, allowing simultaneous detection and classification of amoebae in situ (Grimm *et al*., 2001, Stothard *et al*., 1999). These techniques are time consuming, and therefore PCR-based detection methods for the free-living amoebae *Acanthamoebae* and *Naegleria* have been developed (Kilvington and Beeching, 1995, Schroeder *et al*., 2001, Vodkin *et al*., 1992). For the detection of *H. vermiformis*, however, such a method is still lacking. We developed a real-time PCR-based method to enable investigations on the contribution of *H. vermiformis* to the occurrence, persistence and proliferation of *L. pneumophila* in engineered water systems. The method targets a fragment of the 18S rRNA gene for the specific detection and quantification of *H. vermiformis* in environmental samples. The 18S rRNA gene was selected as target molecule, because its sequence is highly conserved and repeated in tandem in high copy numbers, thereby increasing the sensitivity of the method (Long and Dawid, 1980). The copy number of the 18S rRNA gene in *H. vermiformis* was determined in trophozoites and cysts. Subsequently, the developed method was applied to surface water types serving as sources for the production of drinking water in the Netherlands, as a first step in elucidating the distribution of *H. vermiformis* in such fresh water environments.

Materials and methods

Strains of *Hartmannella* and culture conditions

Three *Hartmannella* strains were used in this study. *H. vermiformis* ATCC 50237 (strain CDC-19) was axenically cultivated in modified PYNFH-medium (Fields *et al*., 1990b). *H. abertawensis* CCAP 1534/9, obtained from the UK National Culture Collection, was cultivated in MY75S medium without bacteriological agar (Anonymous, 2001) but suspended with *Escherichia coli*. *H. vermiformis* KWR-1 (Kuiper *et al*., 2004) was cultivated in Prescott and James's medium including trace elements (PJV-medium) suspended with heat-killed *E. coli* (Anonymous, 2001, Vishniac and Santer, 1957).

Primer design for *H. vermiformis*

Using the ARB software package (Ludwig *et al*., 2004), 18S rRNA gene-targeted forward (Hv1227F, 5'-TTA CGA GGT CAG GAC ACT GT-3') and reverse (Hv1728R, 5'-GAC CAT CCG GAG TTC TCG-3') primers were designed for the

specific detection of *H. vermiformis*. The primers were checked against all available nucleic acid sequences in the NCBI GenBank database using the BLAST search program (Altschul *et al.*, 1997). Furthermore, the number and position of mismatches of the primers with the 18S rRNA gene sequences of the most closely related amoebae, were assessed using ClustalW (Chenna *et al.*, 2003). For assessing the number and position of mismatches of the developed primers with the closely related *H. abertawensis*, the 18S rRNA gene sequence of this amoeba was determined in this study, using PCR, cloning and sequence conditions that were described previously (Kuiper *et al.*, 2004).

Real-time PCR assay for *H. vermiformis*

Real-time PCR assays were performed in 96-well plates in an I-cycler iQ multi-color real-time PCR detection system (Biorad, Veenendaal, the Netherlands) with a total reaction volume of 50 µl per well. Each reaction contained 25 µl iQ SYBR green supermix (Biorad), 5 µl bovine serum albumin (4 mg/ml, Roche diagnostics, Almere, the Netherlands), each primer at a concentration of 0.2 µM, and 10 µl template DNA. The thermal cycling conditions included predenaturation at 95°C for 3 min; 40 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 30 s, and extension at 72°C for 40 s, then a final extension step at 72°C for 10 min. The fluorescence intensity of SYBR green was measured automatically during the annealing steps. At the end of each run a melting curve analysis was performed. Experiments were performed with undiluted and tenfold diluted template DNA and in duplicate.

For quantification a cell-based calibration curve of *H. vermiformis* was included in each PCR reaction. This cell-based calibration curve was constructed by preparing tenfold serial dilutions of DNA extracted from a one-week-old suspension of *H. vermiformis* ATCC 50237. The concentration of *H. vermiformis* in this suspension was determined by direct cell counting as described below. The concentration was $1.14 \times 10^4 \pm 2.79 \times 10^3$ cells per PCR reaction for the undiluted DNA extract.

Genomic copy number of 18S rRNA gene in *H. vermiformis*

To determine the 18S rRNA gene copy number in *H. vermiformis*, an internal fragment of this gene (from positions 1227 to 1728, according to Weekers *et al.* [1994]) was cloned in a pGem®-T easy vector (Promega, Madison, Wis.). Recombinant plasmid DNA was purified in duplicate, using a Qiaprep spin miniprep kit (Qiagen, Hilden, Germany). After purification, plasmid DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del.). The number of construct copies in the plasmid solution was calculated, based on plasmid and insert sizes of 3001 and 502 bp, respectively. A plasmid-based calibration curve was generated with tenfold serial dilutions of plasmid containing the 18S rRNA gene fragment sequence of the target; to control pipetting steps, three tenfold serial dilutions were prepared and concentrations were checked by real-time PCR. This plasmid-based calibration curve, with a concentration of 1.25×10^7 gene copies per

PCR reaction for the dilution with the highest copy number, was used for determining the copy number of the 18S rRNA gene in *H. vermiformis*.

Enumeration of *H. vermiformis* cells

The concentration and state (trophozoite or cyst) of *H. vermiformis* was determined in triplicate, by filtering an appropriate volume of the *H. vermiformis* suspensions over a 1.2-µm pore size RTTP Isopore Membrane (Millipore, Bedford, Mass.) in a vacuum not exceeding 0.3 bar. The cells were stained with acridine orange as described by Hobbie *et al.* (1977), and fluorescence was detected by use of a Leica DMRXA fluorescence microscope. A total of one hundred random fields (each field with a surface area of 9.604×10^{-3} mm^2) per sample were analyzed.

DNA extraction efficiency

Suspensions of *H. vermiformis* KWR-1, *H. vermiformis* ATCC 50237 and *H. abertawensis*, and environmental surface water samples, were filtered over a 1.2-µm pore size RTTP Isopore Membrane (Millipore) in a vacuum not exceeding 0.3 bar. DNA extraction of the filter-retained cells was done using the FastDNA spin kit for soil (BIO 101, Carlsbad, Cal.) following the instructions supplied by the manufacturer.

Three essential steps in the applied DNA extraction method can be distinguished: (i) concentration of the organism by filtration, (ii) cell disruption by bead beating, and (iii) DNA isolation and purification. The recovery efficiencies in these three different steps were determined. The influence of the filtration step was determined in triplicate by comparing DNA extractions with and without the filtration step. Two pure cultures of *H. vermiformis* ATCC 50237 suspensions were used, one culture containing 95.8 ± 2.9% trophozoites, the other one containing 81.9 ± 9.6% cysts. These two suspensions were also used for determining the influence of the cell disruption step. Direct cell counts of these suspensions (as described above) were performed before and after bead beating. To determine the loss of DNA due to the FastDNA spin kit for soil (third step), serial tenfold dilutions of DNA isolated from a *H. vermiformis* ATCC 50237 cell culture, were prepared and used as the starting point for a second DNA extraction with the FastDNA spin kit for soil (in duplicate). All DNA samples were analyzed in duplicate by real-time PCR with Hv1227F and Hv1728R primers as described above (including a calibration curve).

In order to determine the influence of the matrix on the DNA recovery, 200 ml DNA free water, 200 ml PYNFH medium (sterile) and samples (200 ml) from three different surface water sources (Rhine, Lek Canal and Lake IJssel) were spiked with 1.1×10^5 *H. vermiformis* cells (81.9 ± 9.6% cyst). For determining the influence of the DNA purification step, these samples were spiked after filtration with DNA ($7.03 \times 10^6 \pm 1.02 \times 10^6$ 18S rRNA gene copies) obtained from a pure *H. vermiformis* cell culture. DNA was isolated in duplicate from both the spiked and the unspiked samples. The DNA samples were analyzed in duplicate by real-time PCR with Hv1227F and Hv1728R primers as described above.

Reproducibility of DNA extraction method

The growth experiment performed for obtaining information about differences of the 18S rRNA gene copy number in the two life cycle stages of *H. vermiformis* KWR-1 in a biofilm-batch system (described below), provided also information about the reproducibility of the DNA extraction. Samples for analysis were taken from the culture at the different time points (2, 4, 7, 10, 14 and 20 days of incubation) at which the concentration of *H. vermiformis* KWR-1 was determined by direct cell counting and real-time PCR. Cell suspensions were four times tenfold diluted, and subsequently DNA was extracted following the procedure described above (designated as cell dilutions). In addition, serial tenfold dilutions were prepared for DNA extracted from the undiluted cell suspension (designated as DNA dilutions). The concentrations of the cell dilutions and the DNA dilutions were determined by real-time PCR, and used to assess the linearity of the method.

Influence of *H. vermiformis* growth phase on 18S rRNA gene copy number

To evaluate the effect of life-cycle stage on 18S rRNA gene copy number in *H. vermiformis* KWR-1 and *H. vermiformis* ATCC 50237, the two strains were cultivated in a biofilm-batch model system (Kuiper *et al.*, 2004, Van der Kooij *et al.*, 2002). Autoclaved Erlenmeyer flasks, containing tap water with pieces of plasticized polyvinyl chloride (PVCp), supplemented with nitrate (KNO_3) and phosphate (KH_2PO_4) from separately autoclaved stock solutions at a final concentration of 59.3 μM and 11.0 μM, respectively, were used. The Erlenmeyer flasks for *H. vermiformis* KWR-1 were inoculated with a mixed microbial community, originating from a warm water system in the Netherlands, which was filtered over a 1.2-μm pore size cellulose nitrate filter (Sartorius, Goettingen, Germany) and with *H. vermiformis* KWR-1. The Erlenmeyer flasks for *H. vermiformis* ATCC 50237 were inoculated with a bacterial strain (*Acidovorax* sp. originating from a tap water system, cultivated on R2A medium and suspended in sterilized tap water) serving as food source and *H. vermiformis* ATCC 50237. The Erlenmeyer flasks were incubated under static conditions at 37°C for 21 days. The concentration and the state of *H. vermiformis* cells were analyzed at different time points during the experiments, from the biomass attached to PVCp. The biofilm microbial community was removed from the material pieces by six 2-min sonication steps in 10 ml of sterilized water (Branson 5510, Bransonic Ultrasonic Cleaner, Danbury, CT) at a frequency of 40 kHz and an average power input of 0.015 W/ml.

H. vermiformis concentrations were determined by real-time PCR and direct cell counting. In the real-time PCR assay both the cell-based calibration curve and the plasmid-based calibration curve were included. The state of the amoeba was determined by direct cell counting using an epifluorescence microscope, as described above.

Surface water sampling and DNA extraction

A total of twenty-eight samples were collected from different surface water types located all across the Netherlands. Most of these water types are used for drinking water production and included rivers (Meuse, Rhine, Waal and IJssel), open reservoirs for water storage, (artificial) lakes, and a few ditches in National Parks. The samples (1 L) were collected at a depth between 30 and 100 cm, stored on ice, and analyzed within 24 hours. Water samples (200 ml) were filtered over one to three 1.2-µm pore size RTTP Isopore Membrane(s) (Millipore), dependent on silting. DNA extraction of the filter-retained biomass was done using the FastDNA spin kit for soil (BIO 101).

Cloning and sequencing of PCR-amplified products

PCR amplicons obtained by *H. vermiformis*-targeted real-time PCR were purified with the MinElute™ PCR purification kit according to the manufacturer's instructions (Qiagen). PCR products were cloned into *E. coli* XL-1 Blue competent cells (Stratagene, Cedar Creek, TX) by using the Promega pGEM-T easy vector system (Promega). PCR was performed on cell lysates of ampicillin-resistant transformants by using vector specific primers T7 and Sp6 (Promega) to confirm the size of the inserts. Sequence analysis was done by BaseClear Lab services (Leiden, the Netherlands).

Phylogenetic analysis

The determined 18S rRNA gene sequences originating from the surface waters were aligned by using the ARB software package (Ludwig *et al.*, 2004). Phylogenetic trees were constructed by different methods and by using different filters as implemented in the ARB software package.

Statistical analysis

Microsoft® Excel 2000 was used for determining standard deviations, average values and regression lines. The standard deviations of the DNA extraction efficiencies were calculated using the equations given in the third edition of an "Introduction to the theory of statistics" (Mood *et al.*, 1974). Wilcoxon's two-sample test was used for determining a difference of the 18S rRNA gene copy numbers between the two *H. vermiformis* strains ATCC 50237 and KWR-1.

Nucleotide sequence accession numbers

Environmental 18S rRNA gene sequences obtained in this study have been deposited in GenBank under accession numbers DQ190242-DQ190273. The sequence of the *H. abertawensis* 18S rRNA gene has been deposited in GenBank under accession number DQ190241.

Results

Specificity of the PCR assay

Using the newly designed primers Hv1227F and Hv1728R specific amplification of a 502-bp fragment of the 18S rRNA gene of the freshwater amoeba *H. vermiformis* was observed, while no amplification was found for *H. abertawensis*. BLAST search showed that the sequences of selected primers matched exactly and only with 18S rRNA gene sequences of *H. vermiformis*. Furthermore, it was found that the forward primer had 8, 4 and 7 mismatches and the reverse primer had 6, 8 and 6 mismatches with the most closely related species, according to Page (1988): *Glaeseria mira* (Fahrni *et al.*, 2003), *Saccamoeba limax* (Amaral Zettler *et al.*, 2000), and *H. cantabrigiensis* (Fahrni *et al.*, 2003), respectively. A comparison with the sequence of *H. abertawensis*, which was obtained in this study, revealed 3 mismatches for the forward primer, while 7 mismatches were found for the reverse primer.

Melting curve analysis was performed after each real-time PCR reaction. This analysis showed that the melting temperature of the obtained amplicon was always 88.3 ± 0.6°C, and no other peaks were present in the melting curve, which implies a high specificity and no primer dimers. Finally, the primer specificity was tested in practice by amplification from DNA extracted from different environmental surface waters, followed by sequencing and phylogenetic analysis of the obtained amplicons. All sequences showed the highest similarity (≥98%) with the 18S rRNA gene of *H. vermiformis*, indicating that primers are indeed specific for *H. vermiformis*.

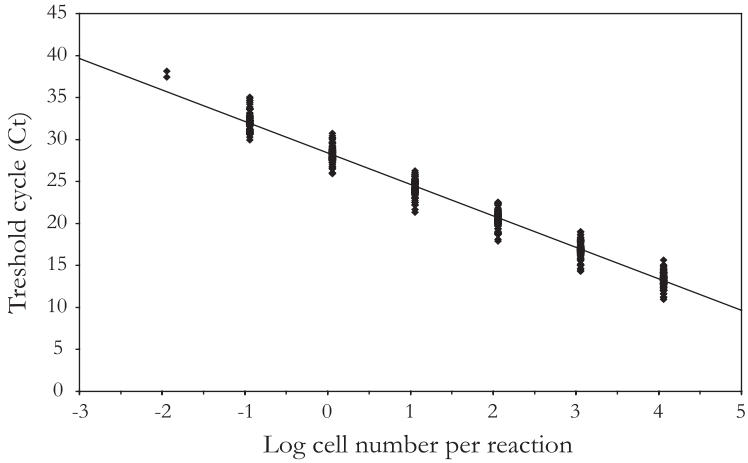


Figure 4.1. Calibration curve for real-time PCR, using primer pair Hv1227F and Hv1728R, with *H. vermiformis* ATCC 50237 grown in modified PYNFH medium. The indicated line, derived from 42 real-time PCR reactions, is defined by an average slope of -3.75 ± 0.18 and an intercept of 28.40 ± 0.95. For each concentration all obtained data points are given. At log cell number -1.94 only two analyses were conducted.

Cell-based calibration curve

A cell-based calibration curve was constructed with 10-fold serial dilutions of DNA obtained from a one-week-old culture of *H. vermiformis* ATCC 50237 (76 ± 19% trophozoites and 24 ± 6% cysts) with an initial concentration of $1.14 \times 10^4 \pm 2.79 \times 10^3$ cells per PCR reaction for the undiluted DNA extract. Application of the real-time PCR assay with primer pair Hv1227F and Hv1728R always yielded a linear relationship between the threshold cycles and the log of the starting concentration (Fig. 4.1). Target DNA was detected at concentrations as low as the equivalent of 1.14×10^{-2} cells per 50 µl of reaction mixture (Fig. 4.1). The ability to detect these low numbers of cells can be attributed to the fact that in most eukaryotes the 18S rRNA gene is repeated in tandem in a high copy number (Long and Dawid, 1980). The PCR efficiency of each real-time PCR reaction, calculated with $E=e^{\ln 10/-s}-1$ (with E is the efficiency and s is the slope of the calibration curve) which was implemented in the software supplied with the I-cycler iQ multi color real-time PCR detection system (version 3.1), gave an average value of 84.3 ± 5.3% for the 51 reactions that were performed.

Recovery efficiencies of the analytical procedure

Application of filtration for retention of cells from a trophozoite suspension resulted in 14.9 ± 8.6% (n=2) loss of DNA. No loss (-0.4 ± 16.3%) was observed with a cyst suspension. Cell disruption by bead beating resulted in an efficiency of 99.6 ± 0.1% (n=3) with the trophozoite cells and 96.0 ± 5.6% (n=3) for the cyst suspension. These values are not significantly different. The DNA concentrations obtained by real-time PCR before and after a second DNA extraction were compared to determine the efficiency of only the kit, not taking into account the influence of filtration and cell disruption by bead beating. An average efficiency of 54.7 ± 11.7% was calculated from the different determinations using DNA free water at a range of DNA concentrations (Table 4.1). However, the matrix may influence the DNA purification step and therefore DNA was spiked after filtration in different matrices (surface water, PYNFH medium, and DNA free water). These DNA recoveries in relation to the original spike, ranged from 60.8 ± 9.0% to 75.4 ± 14.0% (Table 4.1). From the three different steps (filtration, bead beating and the influence of the matrix) an overall average DNA

Table 4.1. Efficiency of DNA extraction using the FastDNA spin kit for soil, excluding filtration and cell disruption, in different dilutions in DNA free water (n=2) and in a few matrices (n=3). The values are expressed as recovery percentages of the spiked DNA.

Dilution in DNA free water	DNA extraction efficiency (%)	Matrix (dilution 10^{-1})	DNA extraction efficiency (%)
10^0	48.8 ± 17.7	Lake IJssel	72.0 ± 13.9
10^{-1}	51.8 ± 16.2	Lek Canal	72.9 ± 14.6
10^{-2}	54.5 ± 11.4	River Rhine	75.4 ± 14.0
10^{-3}	74.5 ± 24.2	Modified PYNFH medium	60.8 ± 9.0
10^{-4}	44.0 ± 8.7	DNA free water	65.0 ± 7.7

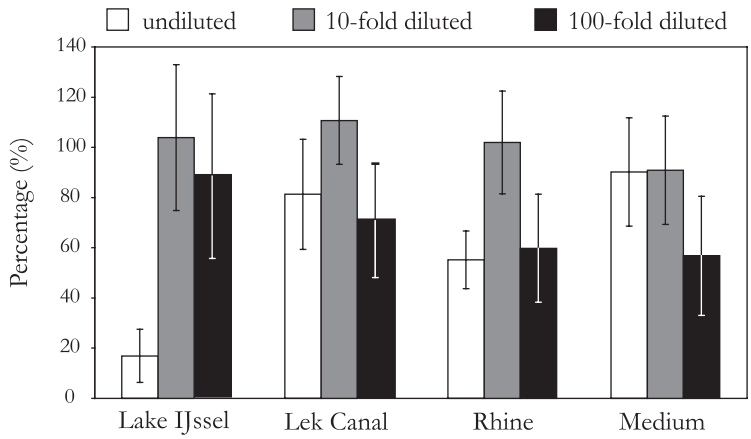


Figure 4.2. Recovery of *H. vermiformis* ATCC 50237 cells spiked in different types of surface water and in the PYNFH growth medium. DNA isolations and real-time PCR reactions were all performed in duplicate. The indicated dilutions are DNA dilutions. Bars indicate the standard deviations.

extraction efficiency of 58.7 ± 7.2% and 66.7 ± 7.5% was established by extrapolating to a 100% trophozoite and a 100% cyst suspension, respectively. Since these values are not significantly different, an average efficiency of 62.7 ± 5.7% was used for further calculations.

Samples of surface water and sterile PYNFH medium were spiked with *H. vermiformis* cells, grown as described above, to determine the effect of the matrix during the complete procedure. With this experiment it is also possible to determine PCR inhibition and the effect of DNA dilution on the inhibitory compounds. The surface waters contained very low indigenous *H. vermiformis* concentrations (as determined by real-time PCR), viz. river Rhine: $1.5 \times 10^2 \pm 5.8 \times 10^1$ cells/l, Lek Canal: $6.1 \times 10^1 \pm 3.8 \times 10^1$ cells/l, and Lake IJssel: $6.6 \times 10^1 \pm 2.5 \times 10^1$ cells/l, respectively. These concentrations were 0.02 ± 0.008 % of the spike concentration and therefore did not influence the calculations. Fig. 4.2 shows the recovery percentage of the spiked *H. vermiformis* cells in different matrices in relation to the original spike. Ten-fold dilutions of DNA extract in PCR gave recoveries of about 100%, indicating that a 10-fold dilution is sufficient for removing inhibitory compounds. This finding could be confirmed with the findings for the surface water samples; in 18% of the 28 samples, inhibition of the PCR occurred in the undiluted DNA extract. When this inhibition occurred, a 100-fold diluted DNA extract was subsequently analyzed by real-time PCR. Template concentrations calculated from reactions with the 10- and 100-fold dilutions indicated that no inhibition occurred in the 10-fold diluted PCR reaction, and 10-fold dilutions were subsequently used for calculations.

Information about the reproducibility of the applied method (filtering, DNA extraction and real-time PCR) was obtained by using *H. vermiformis* cultivated in the biofilm-batch model system. At different time points, the amoebae concentrations

were determined with direct cell counts and by real-time PCR. The cell suspensions and the DNA that was obtained at the different time points were 10-fold serial diluted (the cell dilutions were subsequently used for DNA extractions) resulting in cell and DNA dilutions. The obtained concentrations for the DNA dilutions were plotted against the concentrations of the diluted cell suspensions at the time points t=7, 10, 14 and 20 (t=day; Fig. 4.3). Linear regression showed that the concentrations based on the DNA dilutions were equal to expected *H. vermiformis* concentrations based on the cell dilutions. Hence, the applied PCR method showed a high linearity, over a large range of *H. vermiformis* concentrations (-1 to 3 log cells/ml) and no changes over time were observed, indicating that the method is independent of initial cell concentrations. Furthermore, the applied method is highly reproducible, since dilution series from different days showed similar linear regression.

18S rRNA gene copy number in *H. vermiformis*

The copy number of the 18S rRNA gene on the genome may depend on the physiological state of the protozoa (Bowers *et al.*, 2000). Therefore the 18S rRNA gene copy numbers of stage-mixed suspensions of *H. vermiformis* ATCC 50237 and *H. vermiformis* KWR-1, grown in the biofilm-batch model system and in cultivation medium (only strain ATCC 50237) were determined. For this purpose, a plasmid-based calibration curve was generated, with the plasmid containing a target 18S rRNA gene fragment, and compared with the cell-based calibration curve. In these calculations the previously determined efficiency of DNA extraction was taken into

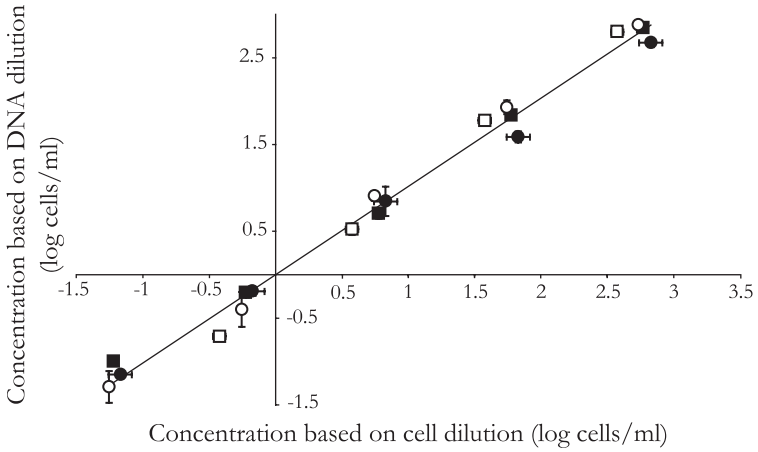


Figure 4.3. Relationship (y=1.018(±0.026)x + 0.000(±0.042)) between the concentrations of cells in dilutions of cell suspensions, analyzed by direct microscopic cell counting, and the concentrations of cells in DNA dilutions, each of which analyzed with real-time PCR. Samples were collected at different time points (t=7 (□), t=10 (■), t=14 (○), and t=20 (●)) from a biofilm-batch model system experiment. Bars indicate the standard deviations.

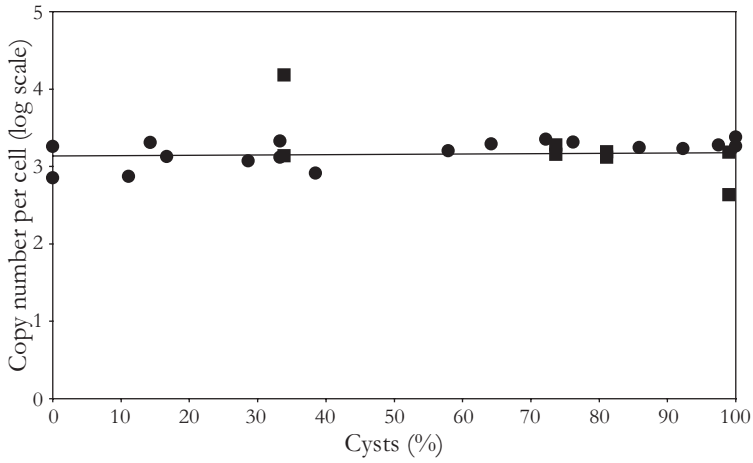
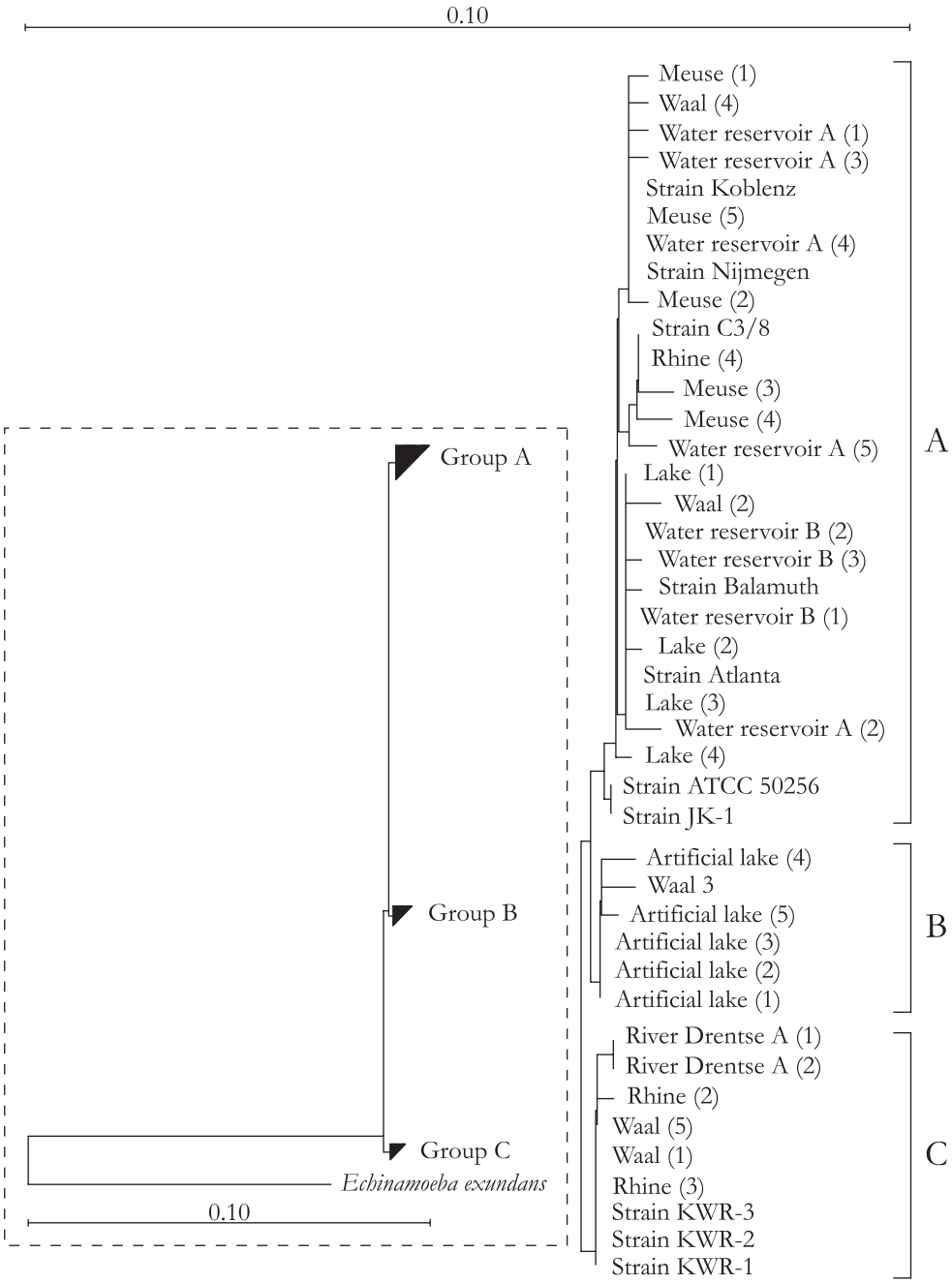


Figure 4.4. The 18S rRNA gene copy numbers in *H. vermiformis* ATCC 50237 (●) and KWR-1 (■) in relation to its maturation state (% of cysts). An average DNA extraction efficiency of 62.7% was used in the calculations. The equation of the indicated trendline is y=0.00039(±0.0016)x + 3.1377(±0.106), with R^2 = 0.0025.

account. The numbers of copies of the 18S rRNA gene were not significantly affected by the state of the *H. vermiformis* ATCC 50237 cells (Fig. 4.4). A *H. vermiformis* KWR-1 suspension yielded a regression line with a negative slope, however, the slope of the regression line was also not significant (data not shown). Using the Wilcoxon test, no significant difference was found between the 18S rRNA gene copy number of the two *H. vermiformis* strains, ATCC 50237 and KWR-1 (P≥0.95). The real-time PCR results of a total of 18 cell-based calibration curves of strain *H. vermiformis* ATCC 50237 and plasmid-based calibration curves generated simultaneously were used for estimating the copy number of the 18S rRNA gene. A number of 1330 ± 540 copies per cell was derived from these data, taking into account the observed DNA extraction efficiency.

Figure 4.5 (right page). Phylogenetic tree of different *H. vermiformis* sequences (502/503 bp) amplified with the specific primers described in this study. Trees were calculated, using different methods as implemented in the ARB software package (Ludwig *et al.*, 2004) and yielded the same topology, as the neighbour joining tree shown in the figure. However, bootstrap values of the tree were low. The overall tree is shown at the left with *Echinamoeba exundans* (Amaral Zettler *et al.*, 2000), as outgroup. The bar within this box indicates 10% sequence divergence. The rest of the figure provides an expanded view of the three subgroups, with the bar at the top of the figure indicating 10% sequence divergence. The numbers between brackets indicate the clone number. Strains Koblenz (X75514), Nijmegen (X75515) and Atlanta (X75513) were previously sequenced by Weekers *et al.* (1994), strain Balamuth (ATCC 30966, M95168) by Gunderson *et al.* (1994), strain C3/8 (AF426157) by Walochnik *et al.* (2002), strains JK-1 and ATCC 50256 by Kuchta *et al.* (1998) and strains KWR-1, -2 and -3 by Kuiper *et al.* (2004).


0.10
Meuse (1)
Waal (4)
Water reservoir A (1)
Water reservoir A (3)
Strain Koblenz
Meuse (5)
Water reservoir A (4)
Strain Nijmegen
Meuse (2)
Strain C3/8
Rhine (4)
Meuse (3)
Meuse (4)
Water reservoir A (5)
Lake (1)
Waal (2)
Water reservoir B (2)
Water reservoir B (3)
Strain Balamuth
Water reservoir B (1)
Lake (2)
Strain Atlanta
Lake (3)
Water reservoir A (2)
Lake (4)
Strain ATCC 50256
Strain JK-1
A
Artificial lake (4)
Waal 3
Artificial lake (5)
Artificial lake (3)
Artificial lake (2)
Artificial lake (1)
B
River Drentse A (1)
River Drentse A (2)
Rhine (2)
Waal (5)
Waal (1)
Rhine (3)
Strain KWR-3
Strain KWR-2
Strain KWR-1
C
Group A
Group B
Group C
Echinamoeba exundans
0.10

Distribution and genetic diversity of *H. vermiformis* in surface water in the Netherlands
H. vermiformis was observed in 75% of the 28 surface water samples collected in autumn (water temperature 11.7±1.3°C). The concentrations ranged from 5.0 (detection limit) to 75 cells/l with a median of 9.6 cells/l. PCR amplicons, originating from eight different surface waters, including drinking water sources, were subjected to cloning and sequence analysis. The sequences (502/503 bp) of 32 clones were compared by phylogenetic analysis with other sequences of different *H. vermiformis* strains (Fig. 4.5). A high degree of sequence similarity (≥98%) was obtained for *H. vermiformis* strains originating from different surface waters in the Netherlands and strains originating from Europe and Northern America. However, despite low bootstrap values, three subgroups could be distinguished (A, B and C) (Fig. 4.5).

Discussion

Primer design

Up to now laborious and time consuming methods have been used for the detection and quantification of *H. vermiformis*, which is a potential host for *L. pneumophila*. In this study, a method for the quantitative detection of *H. vermiformis* by 18S rRNA gene-targeted real-time PCR was developed. The 18S rRNA gene is repeated in tandem in high copy numbers and is highly conserved (Long and Dawid, 1980), thus potentially providing high detection sensitivity. Theoretically, a single copy gene, for example the actin-encoding gene, may provide a better target for quantification, but sequences of this gene were not available for *H. vermiformis*. On the other hand, extensive application of ribosomal RNA as the prime marker for microbial detection and identification in ecological and diagnostic studies has resulted in large sequence databases, including at least 18 entries of 18S rRNA gene sequences of *H. vermiformis* strains, providing a sound basis for the design of specific primers. Hence, estimation of the potential copy number variation with life-cycle stage was needed to obtain an accurate method. Furthermore, the presence of highly variable segments within the 18S rRNA gene fragment amplified by the primers used in this study allows further identification of *H. vermiformis* microdiversity, by cloning and sequence analysis of amplicons retrieved from environmental samples.

PCR efficiency, reproducibility and inhibition

The developed real-time PCR assay showed a high PCR efficiency (84.3%), and a high reproducibility (100%). Furthermore, the method is highly sensitive, as evidenced by the detection of concentrations of 18S rRNA gene fragments, corresponding to less then a single cell (Fig. 4.1). Complex matrices, such as surface waters, may contain organic and inorganic compounds, which interfere with several steps in the isolation and amplification protocols, including cell lysis and polymerase activity during the amplification of target DNA. Such compounds may even degrade the DNA (Wilson, 1997). The FastDNA spin kit for soil used for DNA extraction in this study was able to

eliminate most of the inhibitory compounds. However, in 18% of the surface water samples inhibition in the real-time PCR occurred when undiluted DNA was analyzed, but the 10- and 100-fold dilutions showed no inhibition. To achieve reliable quantification of *H. vermiformis* in surface water samples, we found that a 10-fold diluted DNA extract in all cases was sufficiently pure, even in cases where inhibitory compounds negatively affected amplification efficiency from the original DNA extract (Fig. 4.2). This procedure therefore is recommended for further applications.

DNA extraction efficiency

The here-developed real-time PCR assay was used for the detection and quantification of *H. vermiformis* in environmental samples. However, to be quantitative, the method requires a reproducible and efficient recovery at all stages of the analytical procedure. The efficiency of the entire procedure, including filtering of the samples and DNA extraction, using the FastDNA spin kit, was established on 62.7 ± 5.7%. This efficiency is lower than the 92-96% extraction efficiency reported for bacterioplankton in seawater, using another DNA extraction method (Boström *et al.*, 2004). Nevertheless, the efficiency obtained in this study is high compared with the 20-61% efficiencies obtained in other studies (Fuhrman *et al.*, 1988, Weinbauer *et al.*, 2002). Furthermore, the applied procedure for DNA extraction is highly reproducible and independent of initial cell concentration, since the concentrations derived from the DNA dilutions are proportional with the cell dilutions (Fig. 4.3). A cell-based calibration curve, which was obtained using the same procedure, was included in each PCR reaction and the DNA extraction efficiency was used for the estimation of the copy number of the 18S rRNA gene in *H. vermiformis*.

Table 4.2. Number of ribosomal RNA gene copies as reported for unicellular eukaryotes (amoebae, protozoa and dinoflagellates)

Species	Number of rRNA gene copie per haploid genome	Reference
Alexandrium minutum	1084 ± 120.3	(Galluzzi *et al.*, 2004)
Babesia bovis	3	(Dalrymple, 1990)
Cryptosporidium parvum	5	(Le Blancq *et al.*, 1997)
Cyclospora cayetanensis	2-20	(Varma *et al.*, 2003)
Dictyostelium discoideum	200	(Long and Dawid, 1980)
Euglena gracilis	1000	(Long and Dawid, 1980)
Hartmannella vermiformis	1330 ± 540	This study
Leishmania donovani	170	(Long and Dawid, 1980)
Physarum polycephalum	280	(Long and Dawid, 1980)
Tetrahymena pyriformis	290	(Long and Dawid, 1980)
Tetrahymena thermophila	600	(Long and Dawid, 1980)
Theileria parva	2	(Kibe *et al.*, 1994)
Toxoplasma gondii	110	(Guay *et al.*, 1992)

Copy number of 18S rRNA gene

In most eukaryotes, rRNA genes are organized in tandemly repeated units (Long and Dawid, 1980). Furthermore, it is known that the DNA content can vary substantially from species to species but also between growth phases. For example the dinoflagellate *Alexandrium minutum* is generally haploid (*n*), only the resting cysts (planozygotes) being diploid (*2n*) (Galluzzi *et al.*, 2004). Therefore it has been suggested that the copy number of the 18S rRNA gene might vary during the growth cycle of protozoa (Bowers *et al.*, 2000). Our results with *H. vermiformis*, strains ATCC 50237 and KWR-1, indicate that the copy number of the 18S rRNA gene in cysts did not differ significantly from that in trophozoites. Furthermore, no significant difference was observed in copy numbers between the two strains (ATCC 50237 and KWR-1). The estimated copy number for the 18S rRNA gene on the genome of *H. vermiformis* (1330 ± 540 copies/cell) exceeds the values reported for other unicellular eukaryotes (amoebae, protozoa and dinoflagellates; Table 4.2), but is well within the range of about 50-10,000 found for other eukaryotic cells (Long and Dawid, 1980).

***H. vermiformis* in surface water**

H. vermiformis was observed in 75% of the samples from fresh water environments, used for water supply and cooling purposes in the Netherlands, at concentrations ranging from 5 to 75 cells/l. In contrast, only 2% of 330 samples collected from the James River in Virginia (USA) were *Hartmannella*-positive and the organism was mainly found in samples collected in late summer and autumn (Ettinger *et al.*, 2003). In the present study all samples were taken in late autumn (water temperature 11.7 ± 1.3°C). The difference in detection rate can partly be attributed to the high sensitivity of the method used in this study with a detection limit of 5 cells/l. The amoebae of the genus *Hartmannella* observed in the James River were more associated with the sediment than with the water column (Ettinger *et al.*, 2003). Hence, analysis of water samples only, as done in our survey, may give an underestimation of the positive sites. *H. vermiformis* has also frequently been found in samples collected from natural waters (rivers, 36.4%; lakes, 16.7%) and from man-made environments (artificial lake, 20%; swimming pools, 6.3%) in Bulgaria (Tsvetkova *et al.*, 2004) and in water at different stages of water treatment in Germany (Michel *et al.*, 1995). Furthermore, *H. vermiformis* has been observed as the predominant amoeba in warm tap water (Breiman *et al.*, 1990, Rohr *et al.*, 1998). These reports and our observations indicate that these amoebae are a common component of natural fresh water environments and water installations. The majority of the surface water types included in this study, serves as source for drinking water production in the Netherlands. Cysts may survive and proliferate in different steps of water treatment, may enter the distributing system and subsequently may multiply in biofilms attached to the pipe walls.

The high degree (≥98%) of sequence similarity between *H. vermiformis* strains originating from different surface waters in the Netherlands and the reference strains indicates that the observed organisms may be potential hosts for *Legionella*. Our

phylogenetic analysis did not support the previously suggested separation of European strains from strains originating from North America (Kuchta *et al.*, 1998, Weekers *et al.*, 1994). This supports other findings, in which the whole 18S rRNA gene was taken into account (Kuiper *et al.*, 2004, Walochnik *et al.*, 2002). Despite the high sequence similarity, three subgroups of *H. vermiformis* could be distinguished. Elucidation of the ecophysiological characteristics of these different subgroups requires further research.

In conclusion, the newly developed real-time PCR assay is specific and sensitive for the detection of *H. vermiformis* in environmental samples. The genomic 18S rRNA gene copy number in *H. vermiformis* which was estimated at 1330 ± 540, did neither differ significantly between the two stages of the life cycle of the amoeba, nor between two different strains. Further application of the method for quantitative detection of *H. vermiformis* in natural and in engineered water systems will provide information about conditions favoring the growth of this organism, and also the proliferation of *L. pneumophila*, in these environments.

Acknowledgments

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CHAPTER 5

Relationships between *Legionella pneumophila*, *Hartmannella vermiformis* and selected water quality parameters in surface water, cooling towers and warm water systems

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Abstract

Legionella pneumophila multiplies in fresh water environments only within a eukaryotic host. One of these hosts, *Hartmannella vermiformis*, is frequently detected in man-made water systems in the presence of *L. pneumophila*, suggesting that its presence is a precondition for the proliferation of *L. pneumophila*. *H. vermiformis* was observed in 92 (81.4%) of all water samples (n=113), collected from different aquatic environments. *L. pneumophila* was detected in 43 (38%) of all samples. No statistically significant correlation was observed between the presence of *L. pneumophila* and *H. vermiformis*, with *L. pneumophila* being absent in 54 samples containing *H. vermiformis* (n=92) and *H. vermiformis* being absent in 5 samples in which *L. pneumophila* had multiplied during incubation at 37°C (n=15). However, 19 (68%) of the 28 water samples originating from cooling towers contained both organisms and a significant positive correlation between the log concentrations of *L. pneumophila* and *H. vermiformis* was found (P=0.023). Another significant positive correlation was demonstrated between total *Legionella* spp. concentrations and *H. vermiformis* in surface water samples (P=0.007, n=21). The almost ubiquitous presence of *H. vermiformis* implicates that proliferation of *L. pneumophila* or *Legionella* spp. is not limited by the absence of a potential host. Other factors with positive correlations to increased concentrations of *L. pneumophila* or *Legionella* spp. were temperature, ATP, heterotrophic plate count and dissolved organic carbon.

Phylogenetic analysis of *H. vermiformis*-related sequences retrieved from the different environments showed 3 groups, based on a 502/503 bp fragment of the 18S rRNA gene. One of these groups could be divided in three subgroups. All *H. vermiformis* (sub)groups contained sequences retrieved from water samples in which *L. pneumophila* was detected, suggesting that all observed genotypes are potential hosts for *L. pneumophila*. Interestingly, samples which harboured more then one phylotype of *H. vermiformis* showed a significant correlation with the presence of *L. pneumophila*. Furthermore, it was shown that samples retrieved from cooling towers contained significantly more phylotypes than samples retrieved from surface waters or warm water systems.

Introduction

Legionella pneumophila is widespread in natural fresh water environments, despite its fastidious nature and has also frequently been observed in engineered systems such as warm water distributing systems, cooling towers and wastewater treatment plants. The multiplication of the organism in water systems poses a potential human health risk when aerosolisation can occur (Fliermans *et al.*, 1981, Steinert *et al.*, 2002).

In vitro, legionellae can only grow in complex media with supplements of cysteine and iron salts (Edelstein, 1981). For their multiplication *in vivo* in aquatic environments, protozoa are required. In several *in vitro* studies, using cocultures, legionellae have been

shown to multiply within 14 species of amoebae (*Acanthamoeba castellanii, A. culbertsoni, A. palestinensis, A. polyphaga, A. royreba*, *Hartmannella cantabrigiensis*, *H. vermiformis*, *Naegleria fowleri*, *N. gruberi*, *N. jadini*, *N. lovaliensis*, *Balamuthia mandrillaris*, *Echinamoeba exundans*, and *Vahlkampfia jugosa*), three species of ciliated protozoa (*Tetrahymena pyriformis*, *T. thermophila*, and *T. vorax*), and one species of slime mold (*Dictyostelium discoideum*) (Fields *et al.*, 2002, Miyamoto *et al.*, 2003, Shadrach *et al.*, 2005). In fresh water environments the intracellular proliferation of *Legionella* has been observed with *Acanthamoeba* spp., *Naegleria* spp. and *Hartmannella* sp. (Harf and Monteil, 1988). These three genera and strains of the genera *Echinamoeba*, *Saccamoeba*, and *Vahlkampfia* have also been isolated from *Legionella*-contaminated plumbing systems (Rohr *et al.*, 1998).

Hartmannella spp. were identified for the first time as the protozoan host for *L. pneumophila* in a warm water system in 1988 (Wadowsky *et al.*, 1988). Since then many studies have shown the presence of *Hartmannella vermiformis* in similar aquatic habitats as *Legionella* bacteria, such as soil, fresh water, air samples and different engineered water systems (Breiman *et al.*, 1990, Page, 1967, Walker *et al.*, 1986) and laboratory experiments have confirmed its role as host for *L. pneumophila* (Kuiper *et al.*, 2004, Murga *et al.*, 2001, Wadowsky *et al.*, 1991). Of all environments analyzed, *H. vermiformis* was most strongly correlated with the presence of *L. pneumophila* in water systems (Breiman *et al.*, 1990, Rohr *et al.*, 1998). Based on partial 18S rRNA gene sequences retrieved from different fresh water environments, we identified three subgroups of *H. vermiformis* that could be distinguished, despite an overall high sequence similarity (Chapter 4). Nevertheless, the ecophysiological characteristics of these different subgroups remain unknown.

Besides the necessity for a suitable host, other environmental factors favouring the survival and growth of *L. pneumophila* in water systems include: a water temperature between 25 and 45°C, increased biomass concentrations, and the presence of biofilms and sediments (Colbourne *et al.*, 1984, Rogers *et al.*, 1994a, Van der Kooij *et al.*, 2002 and 2005).

Hence, the objectives of this study were (i) to determine the significance of *H. vermiformis* as the natural host for *L. pneumophila* in aquatic environments, (ii) to assess selected water quality parameters as growth promoting factors for *Legionella* spp. and/or *H. vermiformis* in these environments and (iii) to establish whether intracellular proliferation of *Legionella* spp. is a feature characteristic of *H. vermiformis* in general, or whether it is restricted to specific subgroups.

Materials and methods

Experimental set-up and sample collection

A total of 80 samples originating from surface waters (SW, 28 samples), warm water systems (WWS, 19 samples), cooling towers (CT, 28 samples) and the effluent of wastewater treatment plants (WWTP, 5 samples) were examined. Different parameters were included in the analyses: adenosine triphosphate (ATP), dissolved organic carbon

(DOC), heterotrophic plate count (HPC), amount of thermotolerant coliform bacteria (TTCB), *L. pneumophila* and *H. vermiformis* concentrations. The SW and WWTP samples were also examined for *L. pneumophila* and *H. vermiformis* concentrations after incubation for 10 days at 37°C. The SW and WWTP samples (before and after incubation) were also examined for *Legionella* spp. concentrations.

Sample collection. SW samples were collected in 1-liter Schott flasks at a depth between 30 and 100 cm below the surface. WWTP samples were taken from the effluent current. WWS and CT samples were collected according to standardised procedures (NEN 6559 and NPR 6569). After measuring the temperature using a calibrated digital thermometer (Portec P9007, Wrestlingworth, Sandy, UK) the flasks were put on ice and samples were analysed within 24 hours.

Analytical methods

Adenosine triphosphate (ATP) is an energy-rich compound, which is present in all living organisms. The measurement of ATP is based on the production of light in the luciferine-luciferase assay. ATP was released from suspended cells with nucleotide-releasing buffer (NRB, Celsis International B.V., Landgraaf, the Netherlands). The intensity of the emitted light was measured in a luminometer (Celsis Advance™) calibrated with solutions of free ATP (Celsis) in autoclaved tap water following the procedure as given by the manufacturer.

Dissolved organic carbon (DOC) concentrations were determined following a standard procedure (ISO 8245).

H. vermiformis concentrations. Water samples (varying in volume from 0.5 to 1 litre) were filtered over one to three 1.2-µm pore size RTTP Isopore Membranes (Millipore, Bedford, Mas.), depending on silting. DNA extraction from the filter-retained biomass was done using the FastDNA spin kit for soil (BIO 101, Carlsbad, Cal.) following the instructions supplied by the manufacturer. Real-time PCR assays were performed in 96-well plates in an I-cycler iQ Multi-Color Real-time PCR detection system (Biorad, Veenendaal, the Netherlands) as described previously (Chapter 4). Experiments were performed in duplicate, with undiluted and tenfold diluted template DNA. A hundredfold diluted template DNA was analyzed for samples where inhibition occurred with the undiluted template. For quantification a cell-based calibration curve of *H. vermiformis* ATCC 50237 was included in each PCR reaction. This curve was generated by using tenfold serial dilutions of concentrated DNA from a *H. vermiformis* ATCC 50237 suspension ($1.14 \times 10^4 \pm 2.79 \times 10^3$ cells per PCR reaction for the undiluted DNA extract).

Heterotrophic plate counts (HPC). HPC values were determined by plating an appropriate dilution of the samples on R2A medium (Difco, BD, Sparks, MD). Volumes of 0.05 ml were spread on duplicate plates, using an Eddy Jet spiral plater (IUL Instruments, Barcelona, Spain) and colonies were counted after 10 days of incubation at 25°C.

L. pneumophila and *Legionella* spp. (the latter only for SW and WWTP) concentrations were determined by real-time PCR. The water samples were filtered over one to three

0.2-μm pore size GTTP Isopore Membrane (Millipore), depending on silting. The volumes filtered were: 10 ml for SW and WWTP, 500 ml for WWS and 350 ml for CT samples. DNA extraction of the filter-retained biomass was done using the FastDNA spin kit for soil (BIO 101). Real-time PCR assays were performed in 96-well plates in an I-cycler iQ Multi-Color Real-time PCR detection system (Biorad) as described elsewhere (Wullings *et al.*, 2005, Wullings and Van der Kooij, 2006). The standard curve was generated by using tenfold serial dilutions of concentrated DNA from a *L. pneumophila* ATCC 33152 colony suspension (7×10^4 cells/ml). Real-time PCR experiments were performed using tenfold dilutions of the original DNA extract as template and in duplicate.

Thermotolerant coliform bacteria (TTCB). Feacal contamination of the different water types was determined by analysing the concentrations of thermotolerant coliform bacteria and *Escherichia coli*. This was achieved by filtering an adequate amount of water (0.1 to 100 ml) over a 0.45-μm pore size cellulose nitrate filter (Sartorius, Goettingen, Germany). The filters were subsequently placed on membrane lauryl sulphate agar (Oxoid) and incubated for 5 h at 25°C, followed by an incubation at 44°C for 22 h. Suspected colonies were confirmed by subculturing a representative number in brilliant green bile (2%) broth (Oxoid) with Durham tubes, for detection of gas formation (incubation at 44°C). The same colonies were also subcultured in tryptone soy broth (incubation at 44°C) (Oxoid) and examined for the production of indole by addition of 0.2 ml of Kovacs' reagent.

Phylogenetic analysis of *H. vermiformis*

PCR amplicons obtained by 18S rRNA gene targeted *H. vermiformis* specific real-time PCR were purified with the MinElute™ PCR purification kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). PCR products were cloned into *E. coli* XL-1 Blue competent cells (Stratagene, Cedar Creek, Tex.) by using the Promega pGEM-T easy vector system (Promega, Madison, Wis.). PCR was performed on cell lysates of ampicillin-resistant transformants by using vector specific primers T7 and Sp6 (Promega) to confirm the size of the inserts. Two to seven clones per source were sequenced and sequence analysis was done by BaseClear Lab services (Leiden, the Netherlands).

Environmental 18S rRNA gene sequences obtained in this study have been deposited in GenBank under accession numbers DQ407525-DQ407609. The sequences of the clones from SW samples before incubation have been published previously (Chapter 4), under accession numbers DQ190242-DQ190273. These sequences are indicated in this study as SW 1.1-1.3, SW 2.1-2.4, SW 3.1-3.2, SW 4.1-4.5, SW 5.1-5.3, SW 6.1-6.5, SW 7.1-7.5, and SW 8.1-8.5, respectively.

The 18S rRNA gene sequences originating from the environmental samples were aligned with reference sequences by using the ARB software package (Ludwig *et al.*, 2004). Phylogenetic trees were constructed by different methods and by using different filters as implemented in the ARB software package.

Statistical analysis

Odds ratios with 95% confidence intervals were calculated for determining correlations between the presence/absence of *L. pneumophila* and/or *H. vermiformis* in the samples originating from the four fresh water environments, for the presence/absence of *Legionella* spp. and/or *H. vermiformis* in the SW samples, and for the presence/absence of *L. pneumophila* in water samples containing one or more than one phylotype of *H. vermiformis*. A correlation is considered significant when the odds ratio exceeds 1.0 and the 95% confidence interval does not include 1.0. A test for homogeneity was conducted to determine statistically if the prevalence of one or more phylotypes of *H. vermiformis* correlated with the origin of the samples (SW, CT or WWS).

Correlation coefficients were calculated for the different background parameters (ATP, HPC, DOC, TTCB, and temperature) in relation with the log transformed concentrations of *L. pneumophila* and/or *Legionella* spp. and *H. vermiformis*. For these parameters (except for DOC and temperature) the log transformed concentrations were used, since these transformed concentrations can be considered normally distributed. Furthermore, correlation coefficients were calculated for log concentrations of *H. vermiformis* and *L. pneumophila* and/or *Legionella* spp., for samples where *H. vermiformis* and either *Legionella* spp. or *L. pneumophila* were both present. All correlation coefficients were only tested on a positive correlation using a *t*-test:

$$\underline{t} = \frac{\underline{r}\sqrt{n-2}}{\sqrt{1-\underline{r}^2}}$$

with $\underline{t}$ = *t*-testing value; $\underline{r}$ = the correlation coefficient; n = the number of observations. It should be noted that linear correlation was used, although the relations may not be linear. This provides a global indication of the correlation. Visual inspection of the variance data is also crucial.

Results

Odds ratios and correlation coefficients

L. pneumophila was detected in only one SW and in one WWTP sample (4 and 20% respectively) at concentrations of 2.5×10^3 and 1.5×10^5 cells/l, respectively. Incubation of the samples at 37°C promoted the growth of *L. pneumophila* to 39% of the SW and 100% of the WWTP samples (Table 5.1), with concentrations ranging from 1.0×10^3 to 1.2×10^8 cells/l. *L. pneumophila* was found in 7 (37%) of the WWS samples at concentrations ranging from 2.4×10^1 to 1.2×10^4 cells/l and in 19 (68%) of the CT samples at concentrations ranging from 4.9×10^1 to 1.1×10^4 cells/l. *L. pneumophila* proliferated in 10 (36%) of the 28 SW samples incubated at 37°C. Overall, *L. pneumophila* was observed in 43 (38%) of all samples investigated and *Legionella* spp. was found in 96% of the SW samples and 100% of the WWTP samples. *H. vermiformis* was observed in 92 (81%) of all samples tested. The amoeba was found in those SW

Table 5.1. Numbers and percentages of environmental samples containing *L. pneumophila* (LP) and/or *H. vermiformis* (HV)

Environment	No. positive (%)				
	LP	HV in LP positive	HV	LP in HV positive	LP and HV positive
SW-bi[a] (n=28)	1 (4)	1 (100)	21 (75)	1 (5)	1 (4)
SW-ai[a] (n=28)	10 (36)	6 (60)	17 (61)	6 (35)	6 (21)
WWTP-bi[a] (n=5)	1 (20)	1 (100)	5 (100)	1 (20)	1 (20)
WWTP-ai[a] (n=5)	5 (100)	4 (80)	4 (80)	4 (100)	4 (8)
WWS (n=19)	7 (37)	7 (100)	17 (89)	7 (41)	7 (37)
CT (n=28)	19 (68)	19 (100)	28 (100)	19 (68)	19 (68)
Overall (n=113)	43 (38)	38 (88)	92 (81)	38 (41)	38 (34)

[a] bi: before incubation, ai: after incubation

and WWTP samples where *L. pneumophila* was detected prior to incubation and in all the *L. pneumophila*-positive CT and WWS samples (Table 5.1). *L. pneumophila* multiplied in 10 SW samples incubated at 37°C but *H. vermiformis* was only observed in 6 of these samples. In the other 4 samples *H. vermiformis* was not detected, or even declined during incubation (Fig. 5.1A). *L. pneumophila* also multiplied in 5 WWTP samples, with *H. vermiformis* present in 4 samples (Fig. 5.1B). These observations show that growth of *L. pneumophila* is not strictly dependent on the presence of *H. vermiformis*. Odds ratios calculated for all 113 samples (28 SW and 5 WWTP samples before and after incubation, 28 CT samples, and 19 WWS samples) together and for each environment separately, revealed no significant correlation, indicating that the presence/absence of *L. pneumophila*/*Legionella* spp. did not strictly depend on the presence/absence of *H.*

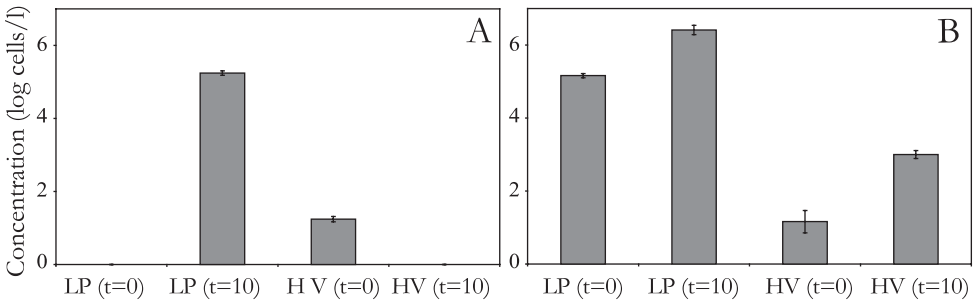

Figure 5.1. Examples of concentrations of *L. pneumophila* and *H. vermiformis*, before (t=0) and after (t=10) incubation at 37°C for ten days. No concentrations are indicated if these were below the detection limit (1.0×10^3 cells/l for *L. pneumophila* and 5 cells/l for *H. vermiformis*). **A.** SW sample (originating from the Twente-canal near Enschede, the Netherlands) with increase of *L. pneumophila*, but decrease of *H. vermiformis* after incubation. **B.** WWTP sample (originating from WWTP Ede, the Netherlands) with increase of both *L. pneumophila* and *H. vermiformis* concentrations after incubation.

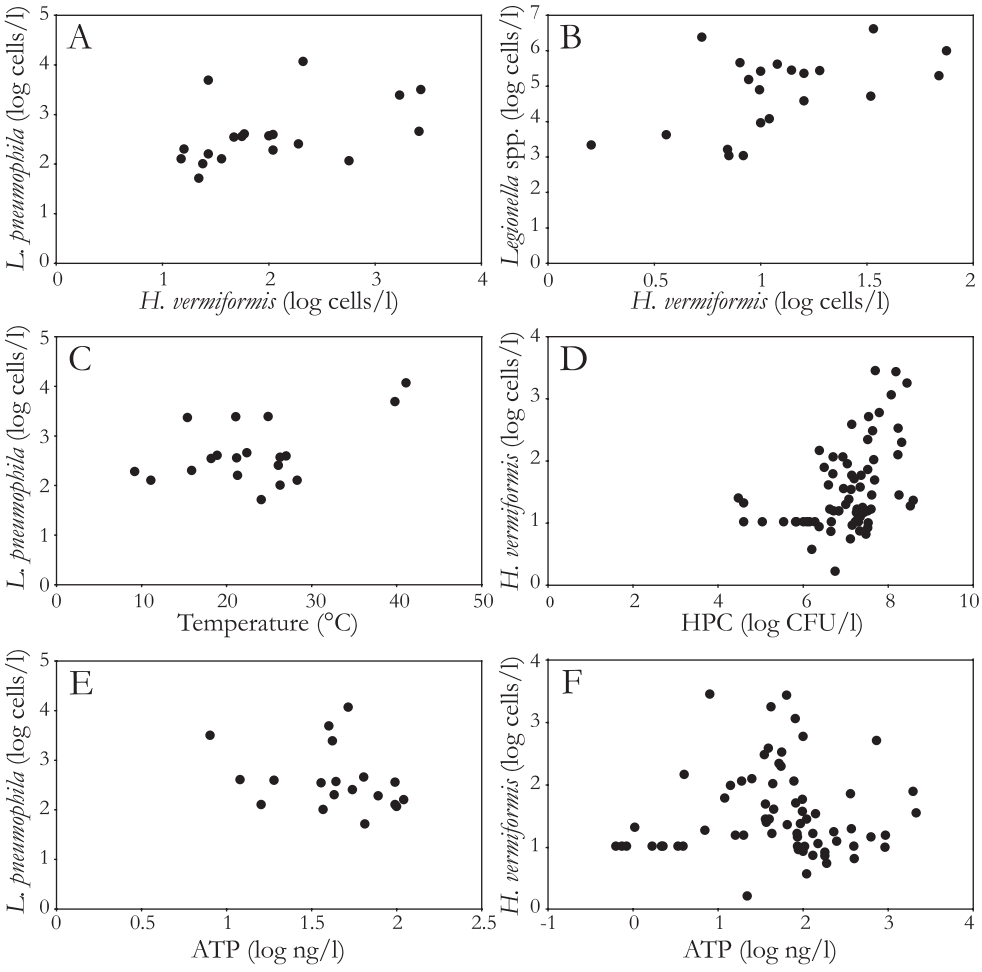

Figure 5.2. Scatter plots for *L. pneumophila*, *Legionella* spp. and *H. vermiformis* for various factors. **A.** *L. pneumophila* and *H. vermiformis*, originating from CT samples (P=0.023). **B.** *Legionella* spp. and *H. vermiformis*, originating from SW samples before incubation (P=0.007). **C.** *L. pneumophila* and temperature, originating from CT samples (P=0.011). **D.** *H. vermiformis* and HPC, originating from all samples (P=0.000). **E.** *L. pneumophila* and ATP, originating from CT samples (P>0.05, r =-0.310). **F.** *H. vermiformis* and ATP, originating from all samples (P>0.05, r =0.074).

vermiformis. However, for samples in which both organisms were present, a significant positive correlation was observed between the concentrations of *L. pneumophila* and *H. vermiformis* in the CT samples (P=0.023, Fig. 5.2A, Table 5.2) and between *Legionella* spp. and *H. vermiformis* in the SW samples before incubation (P=0.007, Fig. 5.2B, Table 5.2). When the concentrations of *H. vermiformis*, *L. pneumophila* or *Legionella* spp. were correlated to the different background parameters, only a few significant positive correlations were found (Table 5.2). For SW samples (before incubation) significant

Table 5.2. Significant positive correlation coefficients between *H. vermiformis* (HV), *L. pneumophila* (LP), *Legionella* spp. (LS) log concentrations and background parameters. The critical value given is the 95% confidence interval and is dependent on the degrees of freedom.

Sample origin	Combination	r	t	Critical value	P
SW (before incubation)	LS and ATP	0.635	4.115	1.708	0.000
	LS and HPC	0.413	2.269	1.708	0.016
	LS and DOC	0.487	2.733	1.711	0.006
	LS and HV	0.530	2.727	1.729	0.007
	HV and ATP	0.695	4.214	1.729	0.000
WWS	HV and ATP	0.830	5.772	1.753	0.000
	HV and HPC	0.684	3.633	1.753	0.001
CT	LP and Temp	0.521	2.514	1.740	0.011
	LP and HV	0.462	2.151	1.740	0.023
	HV and HPC	0.369	2.026	1.706	0.027
All samples (except for the incubated samples)	HV and HPC	0.440	4.073	1.667	0.000
	HV and DOC	0.323	2.815	1.667	0.003

positive correlations were found for *Legionella* spp. with ATP (P=0.000), HPC (P=0.016) and DOC (P=0.006), and for *H. vermiformis* with ATP (P=0.000). In the CT samples significant positive correlations were found for *L. pneumophila* with temperature (P=0.011) and for *H. vermiformis* with HPC (P=0.027), and in the WWS samples for *H. vermiformis* with HPC (P=0.001), and ATP (P=0.000, Fig. 5.2C). Neither of the combinations analyzed for data from the WWTP samples showed any significant correlation, which is most probably due to the low number of samples (n=5). When correlation coefficients were determined for all samples together (except for the incubated samples), significant positive correlations were obtained for *H. vermiformis* with DOC (P=0.003) and HPC (P=0.000, Fig. 5.2D). Visual inspection and *r*-values showed that the observed correlations are significant, but not very strong. Fig. 5.2 depicts examples of both significant and insignificant correlations.

Phylogenetic analysis of environmental *H. vermiformis* sequences

To identify the genetic diversity of *H. vermiformis* in the aquatic environment, sequences (502/503 bp) of 117 clones were compared by phylogenetic analysis with known sequences of different *H. vermiformis* strains (Atlanta, KWR-1, -2, and -3). While a high degree of sequence identity (≥97%) was found for *H. vermiformis* sequences originating from the different environments (CT, SW and WWS), three groups (A [n=73], B [n=18], and C [n=25]) could be distinguished, one of which comprised three subgroups (A1 [n=27], A2 [n=32] and A3 [n=10] and 4 outgroups). The sequences of five clones (all originating from CT samples) did not cluster within any of the three major groups. Furthermore, four clones within group A could not be assigned to one

of the three subgroups (Fig. 5.3A).
Sequences in group B and subgroup A1 originated mainly from cooling towers, while A2 and A3 were mainly retrieved from warm water systems and surface waters, respectively (Fig. 5.3B-I). All groups contained clones of *H. vermiformis* originating from

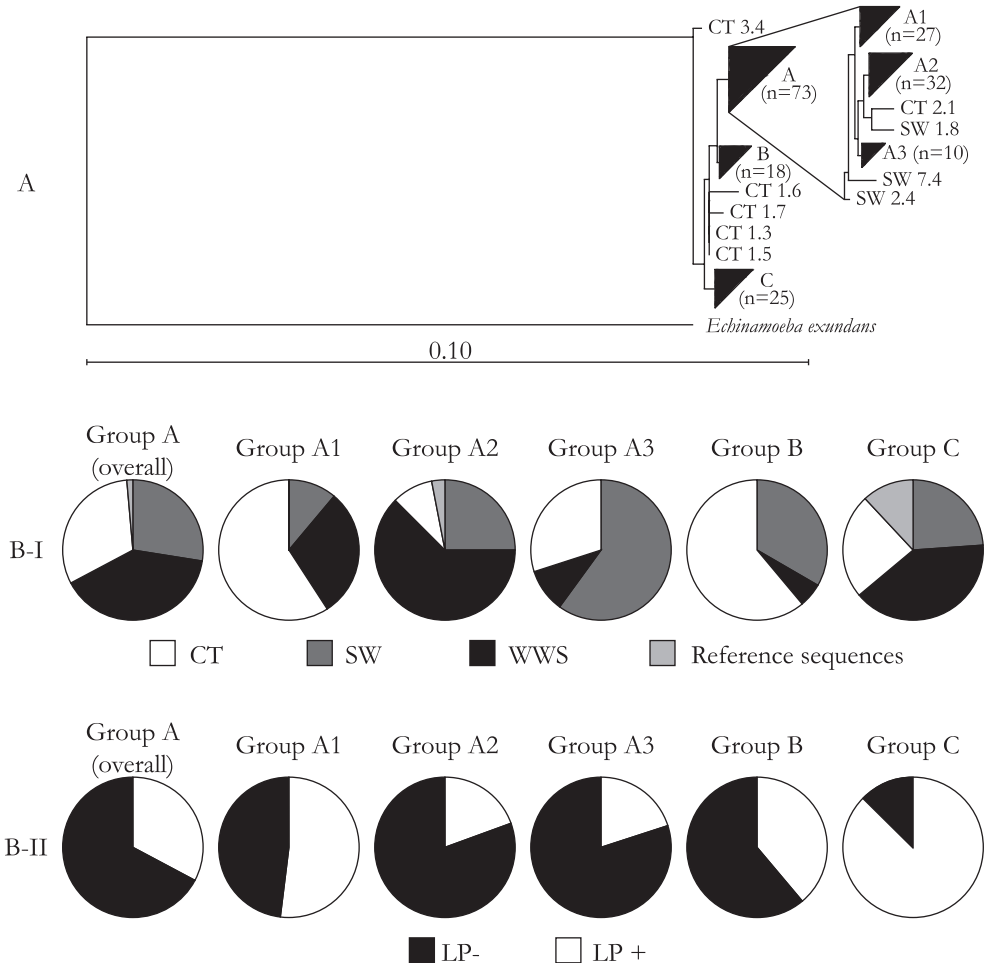

Figure 5.3. A. Phylogenetic tree of *H. vermiformis* sequences (502/503 bp) originating from different environments in the presence or absence of *L. pneumophila*. Trees were calculated using different methods as implemented in the ARB software package (Ludwig *et al.*, 2004), and yielded the same topology as the neighbour joining tree shown in the figure. Also, tree topology and grouping is similar to earlier analyses based on a smaller subset of sequences (Chapter 4). The reference bar indicates 10% sequence divergence and is applicable to the main tree as well as the sub-tree zooming into group A. Strain Atlanta (X75513) and strains KWR-1, -2, and -3 (AY502959-AY502961) were used as reference strains. *Echinamoeba exundans* (AF 293895) was used as outgroup. **B-I.** Distribution of sample origin over the different phylogenetic groups. **B-II.** Distribution of presence (LP^+) or absence (LP^-) of *L. pneumophila* in the original samples, and after incubation in the SW samples.

water samples in which also *L. pneumophila* could be detected. Nevertheless while 88% of the clones in group C (n=25) were retrieved from samples containing *L. pneumophila*, groups A2 and A3 originated mainly from those without detectable numbers of *L. pneumophila* (Fig. 5.3B-II). Subgroup A1 (n=27) mostly comprised clones of *H. vermiformis* from sources with elevated temperatures above 25°C (74%). In contrast, subgroup A3 (90%, n=10) and group C (72%, n=25) contained mainly clones from reduced temperatures (T<25°C). No clear relations between the different phylogenetic *H. vermiformis* groups and ATP, DOC, HPC, and TTCB concentrations could be demonstrated (data not shown).

Several clones (up to seven) originating from each source were used in the phylogenetic analysis for detection of different phylotypes from one source. This indicated that in 11 sources (n=23), only one type of *H. vermiformis* was observed, and only 1 source contained *L. pneumophila* (9%). In 4 sources, however, *H. vermiformis* clones belonging to all three different groups (A, B, and C) were found and three of these sources also contained *L. pneumophila* (75%). Odds ratios showed that if a source had more than one phylotype, the chance of the prevalence of *L. pneumophila* was significant (Odds ratio, 30: 95% confidence interval 2.63 to 342). Furthermore it was significantly shown with a test for homogeneity that the prevalence of more than one phylotype depended on the origin of the sample (CT, WWS or SW). Samples retrieved from cooling towers contained significantly more phylotypes than samples retrieved from warm water systems or surface waters.

Discussion

L. pneumophila and *H. vermiformis* in aquatic environments

The occurrence of *L. pneumophila* in surface water in this study (39%, including the incubation step) was lower then previously shown by Fliermans *et al.* (1981). These investigators demonstrated that *L. pneumophila* was present in 98.5% (n=67) of the surface waters tested, even though a higher detection limit of 9.1×10^3 cells/l was used as compared to a detection limit of 1.0×10^3 cells/l that was applied for the SW samples in the present study. However, the occurrence of *L. pneumophila* and *Legionella* spp. (96%) in surface waters in this study was in agreement with results of a recent study, with *L. pneumophila* and *Legionella* spp. observed in 40% and 100% of the SW samples at low temperatures, respectively (Wullings and Van der Kooij, 2006). These observations confirm the widespread presence of such bacteria in the fresh water environments. Previously, legionellae were detected in 40% of fresh water environments by culture and up to 80% by PCR (Fields, 1997). In all phases of sewage treatment legionellae were found (Palmer *et al.*, 1993), which is in agreement with the detection in 100% of the WWTP samples in this study. The correlation between the presence of *Legionella* spp. and *H. vermiformis* as observed in the present study may implicate that *H. vermiformis* serves as host for *Legionella* spp., but information about this relationship is lacking.

L. pneumophila has frequently been found in warm water systems with an incidence of 17 to 60% (Borella *et al.*, 2005, Luck *et al.*, 1993, Zacheus and Martikainen, 1994). The 37% occurrence of *L. pneumophila* obtained in this study is within this range. The percentage of positive *L. pneumophila* samples in the cooling towers (68%) is relatively high as compared to the 8.5, 26 and 52% previously found (Koide *et al.*, 1993, Türetgen *et al.*, 2005, Witherell *et al.*, 1986). In this study real-time PCR was used for the detection of *L. pneumophila*, with a detection limit of 2.9×10^1 cells/l in case of the CT samples. Cultivation was used in the studies by Witherell *et al.* (1986), and Türetgen *et al.* (2005), with detection limits of 1.3×10^2 and 1.0×10^1 CFU/l respectively, and a PCR technique was used by Koide *et al.* (1993), with a detection limit of 2.4×10^3 cells/l. The real-time PCR method used in this study may have overcome the problem of overgrowth by other bacteria on the solid media used for *Legionella* cultivation (Wellinghausen *et al.*, 2001). Hence the previous studies with culture methods may have underestimated the occurrence of *L. pneumophila.*

H. vermiformis was found in 75% of the SW, 100% of the WWTP, 89% of the WWS, and 100% of the CT samples. These percentages exceed the values previously reported for surface waters (ranging from 2 to 36.4%) and potable water sites (27%) in hospitals and indicate that *H. vermiformis* is ubiquitous in fresh water habitats, including man-made systems (Breiman *et al.*, 1990, Ettinger *et al.*, 2003, Tsvetkova *et al.*, 2004). The higher sensitivity of the applied real-time PCR assay in this study compared with the cultivation methods used in the other studies, which is among other technical reasons due to the presence of multiple 18S rRNA gene copies on the genome of *H. vermiformis*, may explain the differences in occurrence (Chapter 4).

The general presence of *H. vermiformis* potentially makes this amoeba a host for the proliferation of *L. pneumophila*, but odds ratios determined here, did not demonstrate a significant relationship in occurrence. Incubation of the SW samples supported the growth of *L. pneumophila* in 10 of the 28 samples, but in only 6 of these *L. pneumophila* positive samples, *H. vermiformis* concentrations also increased. Hence, in 4 of these *L. pneumophila* positive samples other protozoa probably acted as host for *L. pneumophila.* Four genera, *Acanthamoeba*, *Naegleria*, *Vannellla*, and *Vahlkampfia* have been frequently found in environmental sources (Grimm *et al.*, 1998, Ettinger *et al.*, 2003, Tsvetkova *et al.*, 2004) and may have acted as host for *L. pneumophila* in the SW samples where *H. vermiformis* was not detected after incubation. Nevertheless, in cases where *L. pneumophila* and *H. vermiformis* were present in cooling towers, a positive quantitative correlation was demonstrated. Similarly, the presence of *Hartmannella* species, especially *H. vermiformis*, was highly correlated with the presence of *L. pneumophila* (relative risk, 4.0; 95% confidence interval, 2.1 to 7.4) in another study (Breiman *et al.*, 1990).

These results show that *H. vermiformis* is not strictly required for the proliferation and survival of *L. pneumophila* in its natural habitat, but probably is an important host for *L. pneumophila* and also for *Legionella* spp. in these environments. This conclusion is in agreement with a study by Rohr *et al.* (1998), in which amoebae were found in 52% (n=56) of *Legionella*-contaminated warm water systems in six hospitals. These amoebae

were identified as *Hartmannella vermiformis* (65%), *Echinamoebae* spp. (15%), *Saccamoebae* spp. (12%), and *Vahlkampfia* spp. (9%).

Growth promoting factors for *L. pneumophila*, *H. vermiformis* and *Legionella* spp.
Temperature is an important factor affecting the occurrence and multiplication of *L. pneumophila* in an aquatic environment. The optimum temperature for growth of this organism is generally around 35°C (Katz and Hammel, 1987), while multiplication occurs at temperatures ranging from 20 to 43°C (Rogers *et al.* 1994a, Wadowsky *et al.*, 1985). Strains have been isolated at temperatures ranging from 6 to 63°C (Fliermans *et al.*, 1981). A positive correlation between an increase in temperature and *L. pneumophila* concentrations in biofilms was previously shown (Rogers *et al.*, 1994a). However, no significant correlation, was previously demonstrated in cooling tower systems (Kusnetsov *et al.*, 1993) which is in contrast to the results presented in this study for CT samples (positive correlation with P=0.011).
A rapid increase of the bacterial biomass concentration (ATP) may lead to a relatively strong growth of *Legionella* (Van der Kooij *et al.*, 2005), and higher ATP concentrations coincided with higher *L. pneumophila* concentrations (Kuiper *et al.*, 2004). Most likely, higher concentrations of bacterial biomass as induced by higher nutrient availability can support higher protozoan concentrations, and subsequently yield higher *Legionella* concentrations. The positive correlations between numbers of *H. vermiformis* and ATP concentrations in surface waters and warm water systems; and between *Legionella* and ATP concentrations in surface waters in this study are in agreement with this general rule. Growth-promoting organic compounds are the main driving force for such microbial growth. The DOC concentration is an indicator for nutrient availability in the aquatic environment, and in the present study, positive correlations were established between *Legionella* spp. and DOC concentrations in surface water, and between *H. vermiformis* and DOC concentrations, when samples from all environments were judged together.
Higher HPCs may indicate higher nutrient availability leading to higher protozoan concentrations, thus also explaining the positive correlations between *Legionella* spp. and HPCs in surface waters. Similarly, numbers of *H. vermiformis* and HPC were positively correlated in samples from cooling towers, warm water systems and all environments together. However, in earlier studies, significant inverse correlations between the growth of *L. pneumophila* and HPCs were found (Kusnetsov *et al.*, 1993, Toze *et al.*, 1990, Zanetti *et al.*, 2000). It has also been suggested that some microbial species present in water might play an important role in the control of *Legionella* (Paszko-Kolva *et al.*, 1991 and 1993), and bacteria such as *Pseudomonas* spp. and *Aeromonas* spp. have been shown to be capable of inhibiting *Legionella* on solid media (Toze *et al.*, 1990). Nevertheless, such processes have not been observed in aquatic environments. Furthermore, HPCs represent only a small fraction of the bacterial biomass, limiting their use in correlation studies.
In line with a previous study no correlation between the presence of *Legionella* spp. and

coliform bacteria was noticed (Palmer *et al.*, 1995). This is, however, not according to the hypothesis that high concentrations of thermotolerant coliform bacteria and *E. coli* in surface waters would be in relation with *Legionella* concentrations since water conditions conducive to the presence of large quantities of feacal coliform bacteria were correlated with the prevalence of the free-living amoeba *Vannella* (Ettinger *et al.*, 2003), which may act as host for *Legionella* spp.
Still, positive correlations are not by definition a result of a direct interaction between the two characteristics compared.

Ecophysiological characteristics of *H. vermiformis*

Genetic diversities of *H. vermiformis* in fresh water environments were determined to obtain information about the suitability of the detected *H. vermiformis* as host for *L. pneumophila*. This study showed high sequence similarities (≥97%) between the 117 *H. vermiformis* clones obtained from different fresh water environments. Nevertheless, three different groups could be distinguished, with one group (A) further divided in three subgroups. The three groups were also previously found (Chapter 4), based on sequences retrieved from surface waters. In this study, additional samples originating from cooling towers and warm water systems were included. While the three groups all contained clones coming from the three environments, group B and subgroup A1 were dominated by *H. vermiformis* clones retrieved from cooling towers, subgroup A3 by clones from surface water, and subgroup A2 by clones originating from warm water systems.
Furthermore, the prevalence of *L. pneumophila* was 88% for samples contributing to group C (Fig. 5.3). However, clones of *H. vermiformis*, originating from water samples containing *L. pneumophila*, were present in all groups, thus suggesting that all observed genotypes are potential hosts for *L. pneumophila*.
Similarly, samples with elevated temperatures above 25°C were inhabited by strains of all groups. Hence no discrimination based on temperatures can be made between the different (sub)groups. The same observation was made for concentrations of ATP, DOC, HPC, and TTCB, suggesting that also these ecophysiological characteristics cannot be used as discriminatory proxies for the different phylogenetic groups. However, only a 502-503 bp fragment of the 18S rRNA gene was used for the comparisons, which is 27.3 % of the whole 18S rRNA gene. It is therefore suggested that comparisons of sequences of the whole 18S rRNA gene might be more useful in observing specific correlations between ecophysiological characteristics and the different groups.
From 11 out of 23 environmental sources, only one sequence-type of *H. vermiformis* was retrieved; while from 4 sources clones belonging to all three groups were obtained. It was shown that the prevalence of more than one phylotype depended on the origin of the sample (CT, WWS or SW). This indicates that in some environments a variety of subtypes of *H. vermiformis* is present, while in other sources only one subtype is prevailing. It was shown that when a source contained more than one phylotype of *H.*

vermiformis, the chance of the presence of *L. pneumophila* was significantly higher. It is thus tempting to speculate that in the presence of multiple subtypes of *H. vermiformis* the chance of the presence of an available host for *L. pneumophila* will increase, which subsequently increases the possibility of the presence of *L. pneumophila*.

Conclusions

The following conclusions can be drawn from the presented study:
(1) *H. vermiformis* is a common component of the microbial community in fresh water. Hence, proliferation of *L. pneumophila* is not limited by the absence of a potential host.
(2) *H. vermiformis* is not pivotal for the proliferation and survival of *L. pneumophila* in its natural environment, but a positive quantitative correlation (P=0.023, n=19) between log concentrations as demonstrated for cooling tower samples where both microorganisms were present, indicates that it may play a significant role.
(3) The water temperature in the cooling towers had a positive correlation with the concentration of *L. pneumophila* (P=0.011, n=19). ATP, HPC and DOC concentrations had no significant correlation with the occurrence of *L. pneumophila*.
(4) ATP and DOC concentrations, and HPCs, were positively correlated with the concentrations of total *Legionella* spp. in surface waters. In addition, ATP concentrations and HPCs were positively correlated with *H. vermiformis* concentrations in CT samples.
(5) TTCB concentrations did not correlate with the presence of *L. pneumophila*, *H. vermiformis* nor *Legionella* spp.. The influence of fecal contamination in SW, WWS and CT on *L. pneumophila* is therefore not shown.
(6) Based on partial 18S rRNA gene sequences (502/503 bp, 27.3% of the whole 18S rRNA gene), *H. vermiformis* can be divided in three phylogenetic groups (one of which with three subgroups), which all represent potential hosts for *L. pneumophila*. The chance of the presence of a suitable host for *L. pneumophila* was significantly increased when all three groups were present.
(7) Neither temperature, nor concentrations of *L. pneumophila*, ATP, HPC, DOC, and TTCB can be used as ecophysiological proxies for the different (sub)groups of *H. vermiformis*.

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CHAPTER 6

Quantitative growth interactions between an *Acidovorax* sp., *Hartmannella vermiformis* and *Legionella pneumophila* in a biofilm-batch model system

Co-authored by: Hauke Smidt, Bart A. Wullings, Marcel H. Zwietering and Dick van der Kooij

Abstract

Quantitative information about conditions promoting the proliferation of *L. pneumophila* is needed to define and optimize preventive measures to limit its presence in man-made water systems. The growth of a *L. pneumophila* strain in the presence of a host protozoan (*Hartmannella vermiformis*) and a strain of *Acidovorax* serving as prey bacterium (PB) was quantified in a biofilm-batch model (BBM) system, containing pieces of growth-promoting plasticized polyvinyl chloride (PVCp) in autoclaved tap water. In the BBM system incubated under static conditions, *L. pneumophila* multiplied mainly in the biofilm, to a total concentration of 6.9 ± 0.1 log CFU/cm², even exceeding the total stationary PB concentration (6.2 ± 0.3 log CFU/cm²). Growth of *H. vermiformis* in tap water was not induced at suspended PB concentrations ≤6.5 log CFU/ml under static conditions and not at ≤7.5 log CFU/ml when shaken. Under static conditions, growing *H. vermiformis* reduced concentrations of suspended PB to 5.5 log CFU/ml, and to 3.4 log CFU/cm² in the biofilm. Logistic growth models were suited for describing growth of PB and *H. vermiformis* in the BBM system. A maximum ingestion rate of 8-9 × 10^3 PB × *H. vermiformis*$^{-1}$ × day^{-1} was derived, and a total of 6.5 × 10^3 (± 5.7 × 10^3) PB cells were consumed for each *H. vermiformis* cell division. The maximum number of *L. pneumophila* growing inside a cell of *H. vermiformis* was estimated at about 500 cells. Consequently, consumption of at least 13 PB cells is needed for one *L. pneumophila* cell. The observations confirm that proliferation of *L. pneumophila* in aquatic biofilms requires the presence of a protozoan host in combination with a sufficiently high concentration of prey bacteria.

Introduction

Legionella pneumophila is an opportunistic pathogen that can cause severe pneumonia (Fraser *et al.*, 1977), or Pontiac fever (Glick *et al.*, 1978) when individuals are exposed to aerosols of water containing this bacterium. *L. pneumophila* is present in many natural fresh water environments at elevated temperatures, and also in a variety of man-made water installations (Fliermans *et al.*, 1981, Leoni *et al.*, 2001, Tobin *et al.*, 1980, Yamamoto *et al.*, 1992). Within these environments *L. pneumophila* proliferates mainly in biofilms (Colbourne *et al.*, 1984, Rogers *et al.*, 1994a). A total of 14 species of amoebae, two species of ciliated protozoa, and one species of a slime mold have been identified as host for *L. pneumophila* in laboratory tests (Fields *et al.*, 2002, Shadrach *et al.*, 2005). Some investigators have questioned the need of protozoa for the proliferation of *L. pneumophila* in biofilms (Rogers and Keevil, 1992, Surman *et al.*, 2002), but recently, it was demonstrated that also at high concentrations of biofilm on PVCp, *L. pneumophila* only multiplied in the presence of the bacteriotrophic amoeba *Hartmannella vermiformis* (Kuiper *et al.*, 2004). *H. vermiformis* has repeatedly been identified as the amplification factor for *L. pneumophila* in laboratory tests and in tap water installations (Fields *et al.*, 1989 and 1990b, Kuchta *et al.*, 1998, Murga *et al.*, 2001, Wadowsky *et al.*,

1988 and 1991). In a biofilm composed of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and a *Flavobacterium*-like organism in a biofilm reactor, multiplication of *L. pneumophila* was induced only with the addition of *H. vermiformis* (Murga *et al.*, 2001).
Measures for limiting the multiplication of *L. pneumophila* in water installations can contribute to the prevention of legionellosis, but quantitative information on growth-promoting conditions is needed for appropriate preventive measures. Recently, a preliminary model for quantitative microbial risk assessment (QMRA) was developed for *Legionella* within tap water installations. However, more quantitative information on the relationships between *Legionella* and amoebae, biofilm detachment, and the dose-response relation of *Legionella* in men would be required for further refinement of this model (Storey *et al.*, 2004). As a first step in collecting the required information a study was conducted aiming at determining the quantitative relationships between *L. pneumophila*, protozoa and prey bacteria. For this purpose pure cultures of *H. vermiformis*, a suited bacterial prey and *L. pneumophila* respectively, were grown under defined conditions in a simplified biofilm-batch model (BBM) system.

Materials and methods

Strains

L. pneumophila serotype 1, originating from a plumbing system in the Netherlands and used in a previous study (Kuiper *et al.*, 2004), was cultivated on buffered charcoal yeast extract agar (BCYE) (Edelstein, 1981). After two passages on this medium, one colony was suspended in autoclaved tap water for use as inoculum. Heterotrophic bacteria, serving as potential prey for amoebae, were cultivated on R2A medium (Reasoner and Geldreich, 1985) (Difco, BD, Sparks, Mar.). After two passages on this medium, one colony was suspended in autoclaved tap water for use as inoculum. *Escherichia coli* K-12, cultivated in Luria-Bertani bouillon (Merck, Darmstadt, Germany), was washed and concentrated three times in Prescott and James's medium including trace elements (PJV medium), by centrifugation (3,660 ×g) (Anonymous, 2001, Vishniac and Santer, 1957). This *E. coli* K-12 suspension was heat killed (15 min, 121°C) and used as food source for *H. vermiformis* ATCC 50237 on non-nutrient agar plates (Anonymous, 2001). *H. vermiformis* ATCC 50237 (strain CDC-19) was axenically cultivated in modified PYNFH-medium (Fields *et al.*, 1990b). One-week-old cultures containing about 4.5 log cells/ml were directly used as inoculum.

Enumeration of microorganisms

The concentration of heterotrophic bacteria, including the selected prey bacterium (PB), in the planktonic or in the biofilm phase was determined at different time points during the experiments by cultivation on R2A medium. An appropriate dilution was plated on R2A medium, using the Eddy Jet spiral plater (IUL Instruments, Barcelona, Spain), followed by incubation at 30°C for 3 days. *L. pneumophila* was enumerated by plating an appropriate dilution on GVPC medium (Dennis *et al.*, 1984) (Oxoid Ltd.,

Basingstoke, Hampshire, England), also using the Eddy Jet spiral plater, followed by incubation at 37°C for 7 days. GVPC medium was used instead of BCYE, because the selected PB was able to grow on BCYE medium. *H. vermiformis* concentrations were determined either by cultivation (Kuiper *et al.*, 2004) or by real-time PCR. For the latter method, 15 ml of the planktonic phase and 25 ml of the biofilm phase was filtered over a 1.2-μm pore size RTTP Isopore Membrane (Millipore, Bedford, Mass.). DNA extraction of filter-retained cells was done using the FastDNA spin kit for soil (BIO 101, Carlsbad, Cal.) following the instructions of the manufacturer. The applied real-time PCR for the specific determination of *H. vermiformis* concentrations is described elsewhere (Chapter 4). The physiological state of the amoebae was determined by filtering 15 ml of the planktonic phase and 25 ml of the biofilm phase over a TTTP Isopore Membrane (Millipore) (2.0-μm pore size). The cells were stained with acridine orange as described by Hobbie *et al.* (1977) and were observed with epifluorescence microscopy (Axioskop 20, Zeiss, Jena, Germany). A total of one hundred random fields were analyzed. The biovolume of the trophozoite state was estimated by measuring length and width of 50 organisms using an eyepiece micrometer, and assuming an ellipsoid shape.

Biofilm-batch model (BBM) system

The BBM system, which had been developed in earlier investigations (Kuiper *et al.*, 2004, Van der Kooij *et al.*, 2002), was used in this study as the standard experimental set-up. Biofilms developed on pieces of plasticized polyvinyl chloride (PVCp) tubing (diameter, 0.80 cm; about 6.2 cm^2 of surface area) in tap water in thoroughly cleaned Erlenmeyer flasks with a volume of 1 liter and closed with a cotton wool plug. This tap water (600 ml, pH 7.8) was supplemented with nitrate and phosphate at final concentrations of 72.5 and 13.5 μM, respectively, and each flask was autoclaved (15 min, 121°C) after addition of PVCp pieces. The experimental flasks were inoculated with the test organisms at a concentration ranging from 0.4 to 2 log cells/ml and the flasks were incubated in the dark at either 30°C or 37°C without shaking, unless otherwise mentioned. The PVCp surface (S) to tap water volume (V) ratio was kept constant during the test (S/V = 0.16 cm^{-1}) by removing a proportional volume of water after the collection of a sample piece of PVCp. The attached microbial biomass was removed from the material pieces for determining the concentrations of the microorganisms in the biofilm. Removal was achieved by six 2-min sonication steps in 10 ml of sterilized water (Branson 5510, Bransonic Ultrasonic Cleaner, Danbury, CT) at a frequency of 40 kHz and an average power input of 0.015 W/ml. The use of modified R2A medium instead of tap water as growth medium in the BBM system was tested. This medium consisted of 0.5 g/l yeast extract (Oxoid), 0.3 g/l sodium pyruvate (Fluka, Buchs, Switzerland), 0.3 g/l dipotassium phosphate (Merck) and 0.05 g/l magnesium sulfate (Merck). Other differing test conditions are described with the individual experiments. Growth of the organisms as determined in the planktonic and in the biofilm phase was expressed as CFU (cells)/cm^2 of the exposed surface using the

S/V ratio. This S/V ratio was also used to express concentrations of planktonic cells in the absence of a biofilm carrier as cells/cm^2 to enable comparison.

Isolation of a prey bacterium (PB)

BBM flasks were inoculated with 0.5 ml of a mixed microbial community containing indigenous *L. pneumophila* and *H. vermiformis*, originating from a warm water plumbing system in the Netherlands. After 7 days of incubation at 37°C under static conditions 1 ml of the biofilm suspension obtained by sonication was used to inoculate freshly prepared BBM flasks. Inoculation and incubation of such flasks was repeated four times and subsequently an appropriate dilution of the biofilm microbial community was plated on R2A medium. The predominant bacteria, apparently different strains as judged by colony morphology, were isolated with the streak plate method. The 16S rRNA genes of the dominant bacteria were cloned and sequenced. The bacteria were identified by nucleotide sequence similarity searches using BLAST (Altschul *et al.*, 1997). The growth of *H. vermiformis* on the different bacterial strains was tested with a method described earlier by Weekers *et al.* (1993), at a bacterial cell density of 10^8 CFU/ml and incubation at 37°C.

Effect of shaking and biofilm carrier

Eight BBM flasks were prepared following the standard experimental set-up. The pieces of PVCp were removed from four BBM flasks after autoclaving and these flasks were autoclaved again (121°C, 15 min). This was done to allow for experiments in the presence of nutrients released from the PVCp, but in the absence of the PVCp pieces as biofilm carrier. All flasks were inoculated with the selected PB, *L. pneumophila* and *H. vermiformis* to initial concentrations of 1.3, 0.4, and 0.7 log cells/ml, respectively. The flasks were incubated at 30°C for 30 days; two flasks with pieces of PVCp and two flasks without pieces of PVCp in a shaking incubator (150 rpm), the other four flasks under static conditions. At different time points during the experiment the biofilm and the planktonic phase were analyzed for the concentrations of PB, *L. pneumophila* and *H. vermiformis* and the state of *H. vermiformis*. The *H. vermiformis* concentration was determined by real-time PCR.

Growth rates and growth levels of PB, *H. vermiformis* and *L. pneumophila* in the BBM system

The equation for logistic growth was used to describe the increase of the concentration of PB (total of planktonic and biofilm phase) in the BBM system:

$$\frac{dB}{dt} = k_{B1} \cdot B \cdot \left(1 - \frac{B}{B_{\max}}\right) \qquad (1.0)$$

where: B is the bacterial concentration (cells/cm^2), B_{max} is the maximum bacterial concentration (cells/cm^2), k_{B1} is the specific growth rate (day^{-1}), and t is the time (day). This equation can be solved analytically by:

$$B(t) = \frac{B_{\max}}{1 + \frac{B_{\max} - B_i}{B_i} \cdot e^{-k_{B1} \cdot t}} \tag{1.1}$$

where B_i is the initial bacterial concentration (cells/cm^2).
After reaching a maximum value a decline of the total PB concentration was observed in the presence of *H. vermiformis*, probably due to grazing. This decline can be described with:

$$\frac{dB}{dt} = -k_{B2} \cdot \left(B - B_{final}\right) \tag{2.0}$$

where: k_{B2} is the specific inactivation rate (day^{-1}) and B_{final} is the final total PB concentration (cells/cm^2) in the BBM system. Equation 2.0 can be solved analytically to:

$$B(t) = \left(B_{\tilde{\tau}} - B_{final}\right) \cdot e^{-k_{B2} \cdot t} + B_{final} \tag{2.1}$$

with $B_{\tilde{\tau}}$ the imaginary bacterial concentration at time zero, when it is assumed that only equation 2.0 is describing the bacterial growth (cells/cm^2). However, at time point τ, the growth of PB changes into decline, and substitution of eq. 2.1 (with t=τ) in eq. 1.1 gives eq. 3.0, which is used for fitting the data points of PB in the presence of *H. vermiformis*, when t>τ.

$$B(t) = \left(\frac{B_{\max}}{1 + \frac{B_{\max} - B_i}{B_i} \cdot e^{-k_{B1} \cdot \tau}} - B_{final} \right) \cdot e^{k_{B2} \cdot (\tau - t)} + B_{final} \tag{3.0}$$

The *H. vermiformis* concentration in the flasks can be described with an equation similar to eq. 1.1, viz.:

$$H(t) = \frac{H_{\max}}{1 + \frac{H_{\max} - H_i}{H_i} \cdot e^{-k_{H1} \cdot t}} \tag{4.0}$$

where, *H(t)* is the *H. vermiformis* concentration (cells/cm^2) as a function of time, H_{max} is the maximal *H. vermiformis* concentration (cells/cm^2), H_i is the initial *H. vermiformis* concentration (cells/cm^2) and k_{H1} is the specific growth rate (day^{-1}) of *H. vermiformis*.
The *L. pneumophila* concentration in the BBM system with all three microbial components can be described with eq. 5.0:

$$\frac{dL}{dt} = k_L \cdot L \cdot \frac{H}{H + k_{H2}} \cdot \left(1 - \frac{L}{L_{\max}}\right) \quad (5.0)$$

where, L is the *L. pneumophila* concentration (cells/cm^2), L_{max} is the maximum *L. pneumophila* concentration (cells/cm^2), k_L is the maximal specific growth rate (day^{-1}) and k_{H2} is the *H. vermiformis* concentration (cells/cm^2) at which the specific growth rate of *H. vermiformis* is ½ of the maximal value. However, if $H << k_{H2}$, then equation 5.1 is applicable:

$$\frac{dL}{dt} = k_{LH} \cdot L \cdot H \cdot \left(1 - \frac{L}{L_{\max}}\right) \quad (5.1)$$

with k_{LH} is a rate constant (day^{-1} × (cell/cm^2)$^{-1}$). Since equations 5.0 and 5.1 are both dependent on the *H. vermiformis* concentration, which is also variable, these equations cannot be solved analytically and therefore standard deviations could not be given.
The different models were fitted to the data by determining a value for the parameters that minimized the residual sum of squares (RSS) between the observed data and the values of the model. These parameters were determined by using the solver algorithm in Microsoft® Excel 2000. If a model could be analytically solved, TableCurve™ 2D was used for checking the results obtained with Microsoft® Excel 2000 en for determining the standard deviation.

Yield of *H. vermiformis* on PB

The yield of *H. vermiformis* on PB was determined in the planktonic phase in the BBM system without PVCp. Suspensions with initial PB concentrations of 9.3, 8.2, 7.5, 6.5, 5.5, 4.7, and 3.4 log CFU/ml were made, by cultivation of PB in modified R2A medium. Subsequently the cells were washed and concentrated three times in autoclaved tap water by centrifugation (3,660 ×g). *H. vermiformis* was added at an initial concentration of 1.2 log cells/ml. Also control flasks with only PB suspensions were prepared. The flasks were incubated in duplicate at 30°C without shaking. Concentrations of PB and *H. vermiformis* were determined by cultivation, respectively real-time PCR, at two-day intervals for ten days. Growth yields of the number of PB per *H. vermiformis* were derived from the changes in the concentrations of these organisms. Ingestion rates were calculated from the changes within an incubation period of 6 days, before encystation started.

Yield of *L. pneumophila* on *H. vermiformis*

One ml of a 50 ml mixed suspension in PJV medium containing one colony of *L. pneumophila* grown on BCYE medium and about 10^9 CFU/ml heat-killed *E. coli* K-12 cells, was spread on non-nutrient agar. *H. vermiformis* was inoculated in the middle of the plate and the plates were incubated for 6 days at 30°C. Thereafter, PJV medium including trace elements was added to the plates and the microorganisms were removed from the plate using a sterile swab. The obtained suspension was filtered over

a 2.0-μm pore size TTTP Isopore Membrane (Millipore) at a vacuum not exceeding 3 kPa. The samples were fixed on the filter and fluorescent in situ hybridization was applied to the filter-retained cells as described elsewhere (Kuiper *et al.*, 2004). Fluorescence was detected by use of a Leica DMRXA fluorescence microscope. A total of 50 *H. vermiformis* cells with intracellular *L. pneumophila* cells were analyzed and the number of intracellular *L. pneumophila* cells in one *H. vermiformis* cell was estimated at a magnification of 1,600.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the isolated potential prey bacteria have been deposited in GenBank under accession numbers DQ202403-202405 and DQ202408.

Results

PB selection and identification

Four bacterial isolates, M1 (98% similarity of 16S rRNA gene to *Caulobacter* sp.), M2 (99% similarity to *Acidovorax* sp.), M3 (99% similarity to a β-Proteobacterium), and M6 (99% similarity to *Pseudoxanthomonas mexicana*) were identified as the predominant fast growing bacteria in the BBM system, using several serial transfers. The *Acidovorax* strain showed the strongest growth in the BBM system with PVCp, reaching a total of 7.1 log CFU/cm^2 within 5 days of incubation. After 21 days of incubation 26% of the total number of *Acidovorax* sp. cells was present in the biofilm. The *Caulobacter* strain, the β-Proteobacterium and *Pseudoxanthomonas mexicana* attained clearly lower concentrations, viz. <2.4, 5.8 and 6.3 log CFU/cm^2, with biofilm percentages being undetectable, 12 and 4.3%, respectively.

H. vermiformis multiplied to 3.0 log cells/ml in the presence of the *Acidovorax* strain and concentrations of 1.6 and 2.4 log cells/ml were observed in the presence of the *Caulobacter* sp. and the β-Proteobacterium, respectively. No growth of *H. vermiformis* was observed in the presence of *Pseudoxanthomonas mexicana*. Based on these results the *Acidovorax* strain was selected as model prey bacterium (PB) in this study.

Growth requirements for *L. pneumophila*

Duplicate BBM flasks with PVCp pieces in either modified R2A medium or tap water were inoculated with PB, *L. pneumophila* and *H. vermiformis*, with *L. pneumophila* and *H. vermiformis*, with PB and *L. pneumophila* and with only *L. pneumophila* at the same initial concentrations (2.0, 2.3 and 1.4 log cells/cm^2, for PB, *L. pneumophila* and *H. vermiformis* respectively) and incubated at 30°C under static conditions. Total PB concentrations (8.3 ± 0.03 log CFU/cm^2) on day 45 in modified R2A medium exceeded those in tap water (6.6 ± 0.6 log CFU/cm^2), when all three microbial components were inoculated. The *H. vermiformis* concentrations in tap water and modified R2A medium were comparable (3.0 ± 0.3 and 3.2 ± 1.8 log cells/cm^2, respectively), while *L. pneumophila* was not detected in the modified R2A medium and reached concentrations of 6.1 ±

0.1 log CFU/cm² in tap water. *L. pneumophila* also did not grow in the absence of PB and *H. vermiformis* in tap water with PVCp pieces, and in modified R2A medium, nor with only PB or *H. vermiformis*. *H. vermiformis* and *L. pneumophila* were also incubated at initial concentrations of 1.2 and 1.6 log cells/ml in 500 ml of modified PYNFH medium (Fields *et al.*, 1990b), contained in one-liter Erlenmeyer flasks without biofilm support and without PB. *H. vermiformis* attained a concentration of 5.4 ± 0.1 log cells/ml after 20 days of incubation, but no growth of *L. pneumophila* was observed. Hence, PB and *H. vermiformis* are both needed in the BBM system, and tap water with PVCp was selected for further experiments.

Effect of shaking and PVCp

Four different conditions (shaking or non-shaking, with or without PVCp) were applied to the BBM system incubated in duplicate at 30°C. The growth of PB, *H. vermiformis* and *L. pneumophila* in the BBM flasks was described, using the sum of the microbial concentrations in the planktonic phase and in the biofilm. Concentrations in the flasks with PVCp and without PVCp, but with the nutrients released by this material, were converted to number of cells per cm² using the (imaginary) S/V ratio. PB reached maximum total concentrations of 7.4 ± 1.4 and 7.3 ± 0.03 log CFU/cm² within 60 hours of incubation in the flasks with PVCp, and without and with shaking, respectively (Fig. 6.1). Converted maximum PB concentrations without PVCp for the non-shaken and shaken flasks (7.3 ± 0.03 and 6.7 ± 1.6 log CFU/ cm², respectively), were not significantly different from those with PVCp. Without shaking, *L. pneumophila* and *H. vermiformis* proliferated in the flasks with and without pieces of PVCp. In the unshaken flasks with PVCp the final concentrations of *L. pneumophila* (6.9 ± 0.1 log

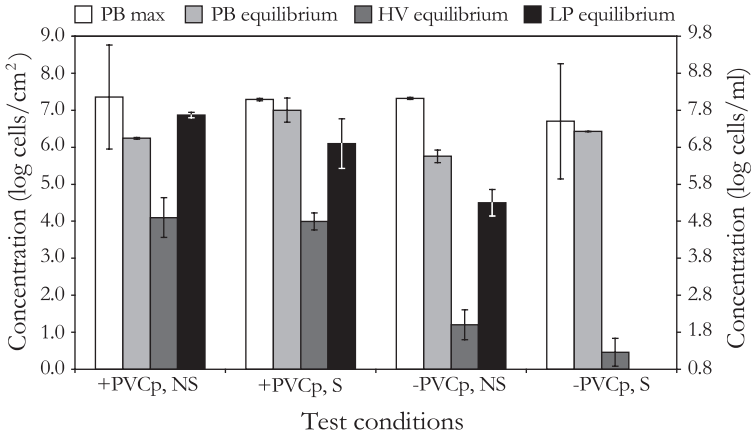

Figure 6.1. Concentrations of PB after 2.5 days of incubation (PB max) and after 30 days of incubation (PB equilibrium) and final concentrations of *H. vermiformis* and *L. pneumophila* (HV equilibrium, LP equilibrium, respectively). Test conditions are indicated, with NS non-shaking, and S shaking. Concentrations are indicated at the primary Y-axis in log cells/cm², and at the secondary Y-axis in log cells/ml, using the S/V ratio (0.16 cm^{-1}).

Table 6.1. State of protozoa, as determined by direct cell counting using acridine orange, in a BBM system with and without PVCp, with and without shaking (all experiments were performed in duplicate)

Time point (day)[a]	% of protozoa in indicated state[b]					
	BBM system with PVCp, without shaking		BBM system with PVCp, with shaking		BBM system without PVCp, without shaking	
	Troph.[c]	Cyst	Troph.	Cyst	Troph.	Cyst
7	55.8 ± 34.3	44.4 ± 34.1	100.0 ± 0.0	0.0 ± 0.0	85.6 ± 20.3	14.4 ± 20.3
8	70.2 ± 2.1	28.6 ± 2.8	87.0 ± 18.3	12.6 ± 17.8	45.0 ± 23.8	55.7 ± 24.0
9	38.0 ± 1.9	62.1 ± 2.6	60.3 ± 56.2	38.2 ± 54.1	7.0 ± 2.2	90.6 ± 6.9
10	6.2 ± 2.4	94.1 ± 3.2	50.2 ± 29.4	51.1 ± 28.6	20.1 ± 20.8	79.7 ± 22.2
12	2.7 ± 2.7	98.5 ± 0.9	5.2 ± 1.7	96.0 ± 1.3	14.3 ± 1.6	84.4 ± 2.1
16	0.4 ± 0.2	99.9 ± 0.2	6.9 ± 5.3	93.8 ± 5.3	2.7 ± 3.8	97.8 ± 3.1
30	1.4 ± 2.0	99.1 ± 1.3	2.8 ± 0.3	97.5 ± 0.8	4.0 ± 5.7	96.7 ± 4.7

[a] *H. vermiformis* was first detected at day 7. Before that day, concentrations were below the detection limit (n<1.1 log *H. vermiformis* cells/ml and n<2.0 log *H. vermiformis* cells/cm^2)

[b] The results of the BBM system without PVCp and with shaking are not given, since the concentrations remained below or around the detection limit

[c] Troph., trophozoite

CFU/cm^2) and *H. vermiformis* (4.1 ± 0.5 log cells/cm^2) were clearly higher than in the unshaken flasks without PVCp (converted concentrations: *L. pneumophila* 4.5 ± 0.4 log CFU/cm^2 and *H. vermiformis* 1.2 ± 0.4 log cells/cm^2, respectively) and *L. pneumophila* even exceeded the final PB concentrations in the unshaken flasks with PVCp. *L. pneumophila* and *H. vermiformis* multiplied in the flasks with PVCp, when shaking conditions were applied, however no growth of *L. pneumophila* nor *H. vermiformis* was noticed in the shaking flasks in the absence of PVCp (Fig. 6.1). PB reached a stationary phase after about 10 to 15 days with total converted concentrations ranging from 5.8 to 7.0 log CFU/cm^2. In the stationary phase more than 97% of PB was present as planktonic cells; final PB biofilm concentrations in the unshaken flasks with PVCp (3.9 ± 0.5 log CFU/cm^2) were not significantly different from final PB concentrations in the biofilm in the shaken flasks with PVCp (4.4 ± 1.1 log CFU/cm^2). *H. vermiformis* was almost equally present in biofilm and planktonic phase, and *L. pneumophila* was mainly present in the biofilm (70%). More than 95% of the amoebae had changed from the trophozoite form at day 7 and 8 to the cyst form at day 12 in the flasks with PVCp (Table 6.1) and also *H. vermiformis* concentrations reached a stationary phase, indicating that predation of PB had ceased.

The ability of *H. vermiformis* and *L. pneumophila* to grow in the absence of an added biofilm carrier was confirmed with an experiment in a one-liter measuring cylinder (diameter 6.15 cm) containing 600 ml of tap water supplemented with nitrate (72.5 μM), phosphate (13.5 μM) and nutrients released from PVCp during autoclaving. The concentrations of these organisms at the bottom of the cylinder were much higher than those in the upper layers (Fig. 6.2). *H. vermiformis* concentrations in the upper layers

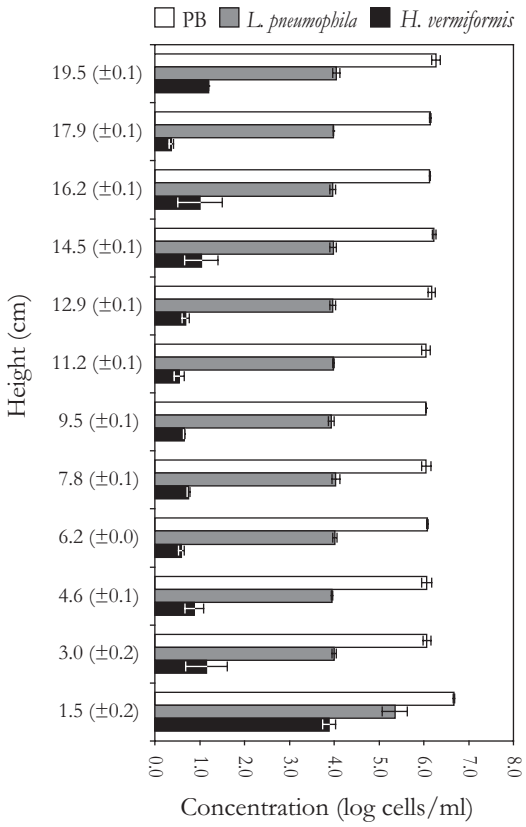

Figure 6.2. Distribution of PB, *L. pneumophila* and *H. vermiformis* in measuring cylinders prepared according to BBM flasks, without PVCp, but with the nutrients released from the PVCp, after 13 days of incubation at 30°C, without shaking. Each column represents the mean concentration from two experiments, and bars indicate standard deviations.

(0.9±0.3 log cells/ml) were not significantly higher then the inoculated amount (1.3 log cells/ml), indicating that no growth of *H. vermiformis* occurred in these upper layers, or that sedimentation of *H. vermiformis* cells occurred.

Growth rates and growth levels of PB, *H. vermiformis* and *L. pneumophila* in the BBM system

PB multiplied exponentially and attained a final total concentration of 7.6 ± 0.1 log CFU/cm² at day 5 in the absence of *L. pneumophila* and *H. vermiformis* (Fig. 6.3A, Table 6.2), with 4.4 ± 0.6 log CFU/cm² present in the biofilm. PB also multiplied exponentially in the presence of *H. vermiformis*, but the bacterial concentration declined to a final total concentration of 6.3 ± 0.2 log CFU/cm² (and 3.4 ± 0.4 log CFU/cm² in the biofilm), with a specific inactivation rate (k_{b2}), after reaching its maximum concentration after 4 days (Fig. 6.3B, Table 6.2). The exponential growth rate of *H.*

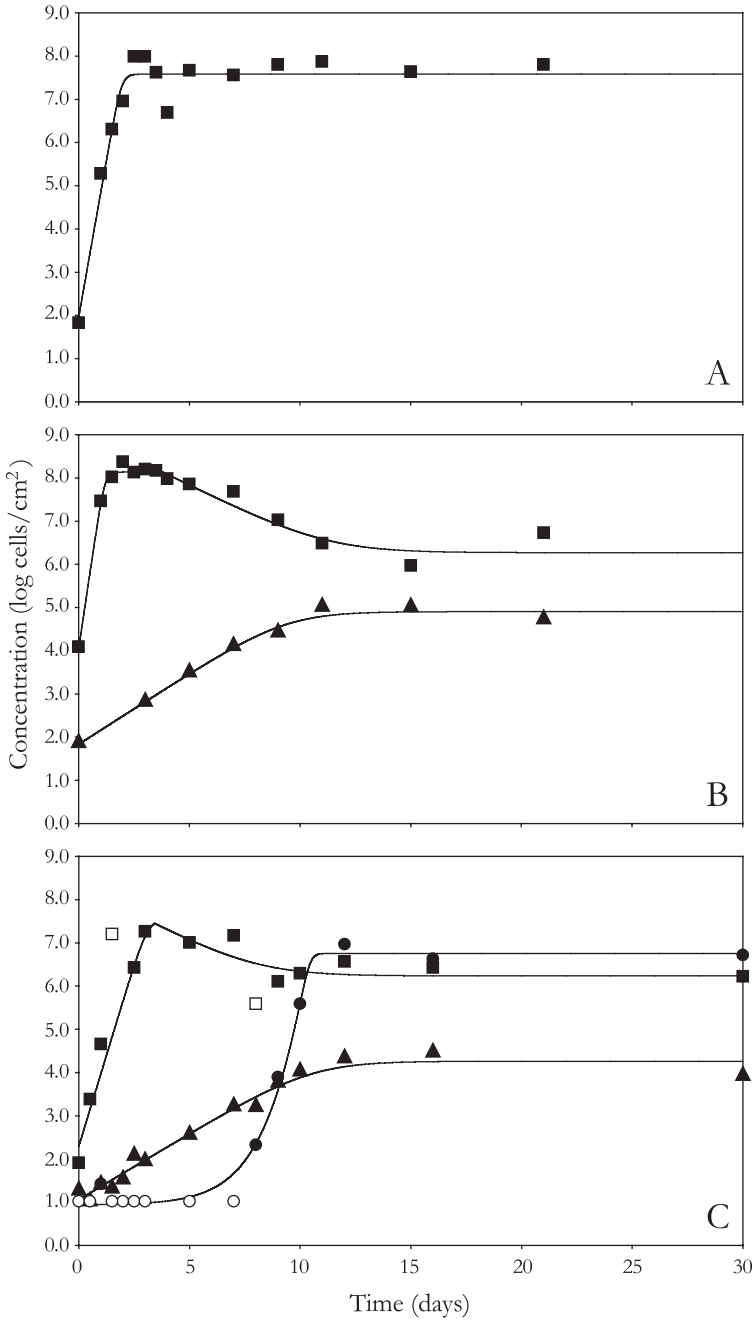
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
A
B
C
Concentration (log cells/cm2)
0
5
10
15
20
25
30
Time (days)

Figure 6.3 (left page). Data points with fitted models. Experiments were performed in BBM flasks with PVCp, and non-shaking conditions**. A**. Growth of PB (■) in BBM flasks with only PB. **B**. Growth of PB (■) and *H. vermiformis* (▲) in BBM flasks inoculated with both microorganisms. **C**. Growth of PB (■,□), *H. vermiformis* (▲) and *L. pneumophila* (●,○) in BBM flasks inoculated with these three microorganisms. Open squares represent data points which were not incorporated in model fitting using equations 1.1 and 3.0, open circles represent data points that are presented at the detection limit, since concentrations determined were below the detection limit. Equations 1.0-5.1 are used for fitting the models to the data points. Parameter values are given in Table 6.2.

vermiformis (k_{H1}) in the flasks with PB (Fig. 6.3B, Table 6.2) was not significantly different from k_{H1} in the flasks in the presence of PB and *L. pneumophila* (Fig. 6.3C, Table 6.2). The addition of *L. pneumophila* to the system also did not apparently influence the final total PB concentration (6.2 ± 0.3 log CFU/cm² and 3.9 ± 0.5 log CFU/cm² for only the biofilm). The final *H. vermiformis* concentrations were lower, although no decline of *H. vermiformis* was observed after maximum *L. pneumophila* concentrations were reached. *L. pneumophila* multiplied to concentrations of 6.8 log CFU/cm², after a lag phase of about 1 week and even exceeded the total final PB concentration (Fig. 6.3C).

Growth yield and ingestion rate of *H. vermiformis* on PB

No growth of *H. vermiformis* was observed in the planktonic phase in autoclaved tap water supplemented with PB at cell densities of 9.3 and ≤6.5 log CFU/ml. Initial cell densities of 8.2 and 7.5 log CFU/ml clearly promoted growth of *H. vermiformis* (Fig. 6.4) and the ingestion rates ($2.0 \times 10^3 \pm 8.6 \times 10^2$ and $5.2 \times 10^2 \pm 6.7 \times 10^1$ PB × *H. vermiformis*$^{-1}$ × d^{-1}, respectively) were calculated from concentration changes during the first 6 days of incubation, hence prior to cyst formation. Also growth yields (8.2×10^5 and 3.2×10^4 *H. vermiformis*/PB) were calculated, indicating that $7.6 \times 10^3 \pm 6.4 \times 10^3$ PB cells were used for each *H. vermiformis* cell division. The growth yield of *H.*

Table 6.2. Values of the parameters in Fig. 6.3 with their standard deviations for cases where the models could be solved analytically

Characteristics	Parameter	Figure 6.3A	Figure 6.3B	Figure 6.3C
Initial concentrations	B_i (log cells/cm^2)	1.96 ± 0.37	4.05 ± 0.25	2.28 ± 0.30
	H_i (log cells/cm^2)	-	1.83 ± 0.13	1.06 ± 0.11
Specific rates	k_{b1} (day^{-1})	6.66 ± 0.78	7.92 ± 0.91	3.88 ± 0.50
	k_{b2} (day^{-1})	-	0.58 ± 0.15	0.61 ± 0.63
	k_{H1} (day^{-1})	-	0.76 ± 0.06	0.70 ± 0.05
Maximum and final concentrations	B_{max} (log cells/cm^2)	7.58 ± 0.12	8.14 ± 0.11	7.61 ± 1.53
	B_{final} (log cells/cm^2)	-	6.27 ± 0.19	6.23 ± 0.25
	H_{max} (log cells/cm^2)	-	4.90 ± 0.10	4.26 ± 0.13
	L_{max} (log cells/cm^2)	-	-	6.75
Model parameters	τ (day)	-	3.80 ± 0.92	3.39 ± 1.67
	k_{LH} (cm^2 × day^{-1} × cell^{-1})	-	-	7.82×10^{-4}

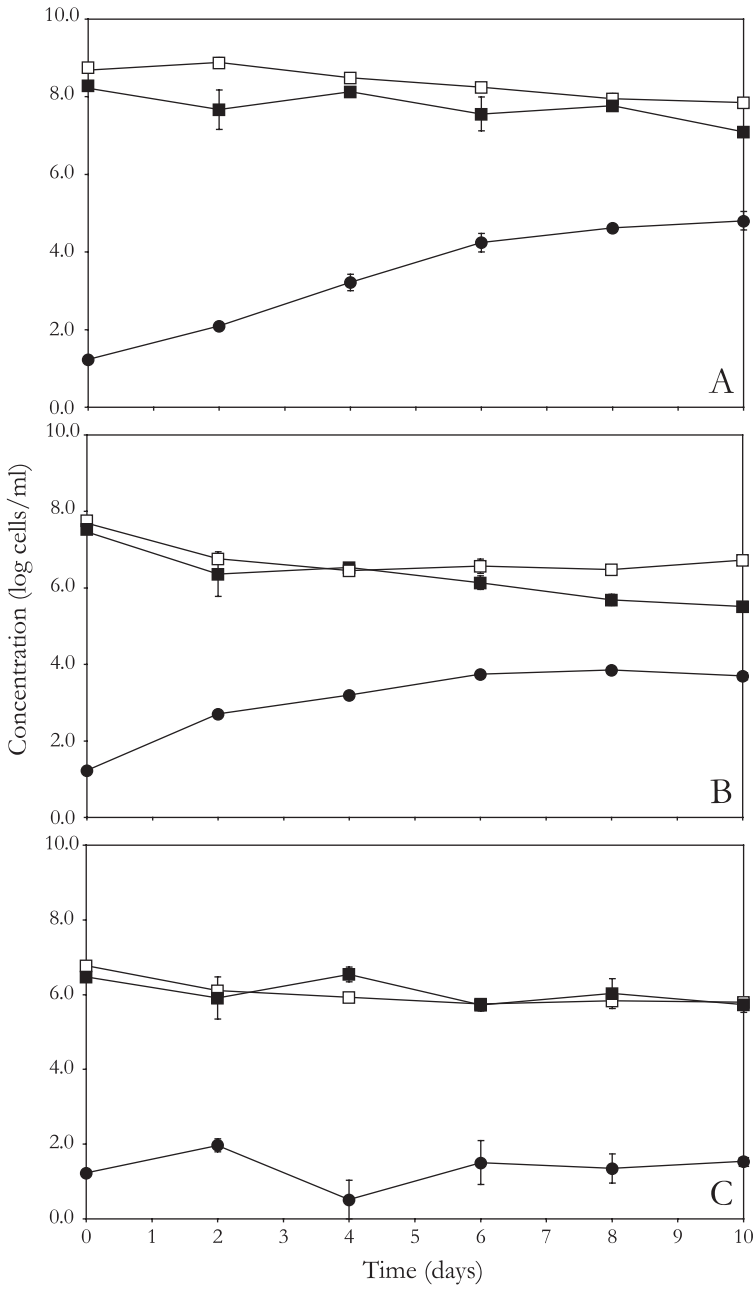
10.0
8.0
6.0
4.0
2.0
0.0
A
B
C
Concentration (log cells/ml)
0
1
2
3
4
5
6
7
8
9
10
Time (days)

Figure 6.4 (left page). Concentrations of PB (■) during growth of *H. vermiformis*, when a known initial bacterial concentration was applied in the flasks, control of the PB (□) concentration, when no *H. vermiformis* is present, and *H. vermiformis* (●) concentrations during incubation with a known PB concentration. Each point represents the mean concentration from two experiments, and bars indicate the standard deviations. **A**. Initial bacterial cell density of 8.2 log CFU/ml. **B**. Initial bacterial cell density of 7.5 log CFU/ml. **C**. Initial bacterial cell density of 6.5 log CFU/ml. Initial bacterial cell densities <6.5 log CFU/ml proceeded comparable.

vermiformis on PB, as calculated from the concentration changes between t=4, t=6.5, and t=9 shown in Fig. 6.3B, corresponds to $5.8 \times 10^3 \pm 6.5 \times 10^3$ PB cells for one *H. vermiformis* cell division. The average growth yield, obtained from these different experiments, viz. $6.5 \times 10^3 \pm 5.7 \times 10^3$ PB cells/*H. vermiformis*, was used for further calculations.

Yield of *L. pneumophila* on *H. vermiformis*

The number of intracellular *L. pneumophila* visible in *H. vermiformis* cells grown on heat-killed *E. coli*, ranged from 1 cell per amoeba to a maximum of about 200 cells per amoeba. The biovolume of intracellularly grown *L. pneumophila* cells, was estimated at 0.11 ± 0.05 μm^3, assuming a cylindrical shape and using the dimensions of the mature intracellular form of *L. pneumophila* in *H. vermiformis* (Abu Kwaik, 1996, Fields, 1996, Greub and Raoult, 2003). The biovolume of *H. vermiformis*, with an average length and width of 13.8 ± 3.6 and 5.1 ± 1.6 μm, respectively, was estimated at 192 ± 113 μm^3. Hence, $1.7 \times 10^3 \pm 1.2 \times 10^3$ *L. pneumophila* cells correspond to the average volume of one *H. vermiformis* cell. However, mature intracellular forms of *L. pneumophila* are not densely packed and only about 50% of the *H. vermiformis* cell volume is filled (Greub and Raoult, 2003). Therefore, the number of *L. pneumophila* cells in an infected *H. vermiformis* cell just before lysis, was estimated at 865 ± 620 bacteria. The rounded mean of the observed maximum number of *L. pneumophila* cells in *H. vermiformis* cells and the estimated number, viz. 500 cells, was used for further estimations. From this value and the yield of *H. vermiformis* on PB, it was derived that a minimum of 13 ± 11 PB cells were consumed for the growth of one *L. pneumophila* cell.

Discussion

Model system

Quantitative information about conditions affecting the growth of *Legionella* in aquatic environments is needed for defining preventive control measures aiming at efficient limitation of its growth in water installations. Therefore, a biofilm-batch test as previously developed (Kuiper *et al.*, 2004, Van der Kooij *et al.*, 2002) was optimized to a BBM system for collecting quantitative information on the relationship between a strain of *L. pneumophila*, a protozoan host (*H. vermiformis*) and a suited bacterial prey (PB). Isolate M2 used in this study as PB was most closely related (99%) with an uncultured bacterium clone BANW427 (DQ264427) isolated from subsurface

groundwater and with *Acidovorax* sp. UFZ-B517 (AF235010) isolated from a chlorobenzene contaminated aquifer system. *Acidovorax* spp. have also been observed on the inner surface of bottles containing non-carbonated mineral water, on copper pipes in plumbing systems, and on pipes of PVC and cast iron used for water supply, indicating its biofilm forming potential, and was isolated from water treatment plants (Critchley *et al.*, 2003, Jayasekara, 1999, Norton and LeChevallier, 2000). Hence, *Acidovorax* may serve as a natural food source for amoebae in tap water systems.
The *L. pneumophila* strain proliferated in the BBM system only when also PB and *H. vermiformis* were present, thus confirming that the presence of a protozoan host consuming prey bacteria is an essential growth requirement. However, no growth of *L. pneumophila* was observed in modified R2A medium, despite the presence of PB and high *H. vermiformis* concentrations (3.2 ± 1.8 log cells/cm^2). The high PB concentrations (8.3 ± 0.03 log CFU/cm^2) may have prevented the uptake of *L. pneumophila* by *H. vermiformis*, as described for *Pseudomonas aeruginosa* cells with *Acanthamoeba polyphaga* (Qureshi *et al.*, 1993). However, it can not be excluded that oxygen limitation or another water quality parameter played a role in limiting the activity of *H. vermiformis*. *L. pneumophila* also did not multiply in modified PYNFH medium, in which *H. vermiformis* was axenically cultivated to concentrations of 5.4 ± 0.1 log cells/ml. *L. pneumophila* may not have survived inoculation (0.6 log cells/ml) as was previously shown in axenic cultures of *Acanthamoeba royreba* and *Naegleria lovaniensis*, where 99.9% of inoculated *L. pneumophila* was destroyed (Tyndall and Domingue, 1982). Another hypothesis is that the concentration of *L. pneumophila* was too low to be detected for *H. vermiformis*. Based on these findings, the intracellular proliferation of *L. pneumophila* was studied in a BBM system containing tap water and PVCp as biofilm carrier and as nutrient source for the selected PB with *H. vermiformis* as the protozoan host.

Interactions between growth of PB and *H. vermiformis*
PB multiplied rapidly in the BBM system under all test conditions. The growth rates ranged from 3.9 ± 0.5 to 7.9 ± 0.9 day^{-1}. The doubling times (2.1 to 4.3 h) were slightly shorter than the value of 5.5 h reported for *Acidovorax* strain OY-107 in artificial ground water (Mailloux and Fuller, 2003). The difference might have been due to the availability of the nutrients provided by the PVCp. The final concentrations of PB in the presence of *H. vermiformis* declined to values of 6.6 (static) and 7.8 (shaking) log CFU/ml, *H. vermiformis* encysted and its growth ceased (Fig. 6.1, Table 6.1). A PB concentration of about 7.5 log CFU/ml did not induce growth of *H. vermiformis* under shaking conditions in the BBM system without biofilm carrier (Fig. 6.1). However, under static conditions without PVCp growth of *H. vermiformis* was observed at this PB concentration, which subsequently declined to a level of 5.5 log CFU/ml (Fig. 6.4B). PB levels were reduced by *H. vermiformis* to about 6 log CFU/ml under static conditions, but growth of *H. vermiformis* was not induced at PB levels ≤6.5 log CFU/ml (Fig. 6.4). Hence, the threshold concentration for growth of *H. vermiformis* on suspended *Acidovorax* bacteria under static conditions is around 6.5 log CFU/ml. The

amoebae probably are present at the bottom and the wall of the culture vessels, acquiring the suspended prey bacteria. Multiplication of *H. vermiformis* under shaking conditions in the presence of a high concentration of suspended bacteria (9 log CFU/ml) has been reported (Weekers *et al.*, 1993), but in the present study no growth of *H. vermiformis* was observed at an initial PB concentration of 9.3 log CFU/ml under static conditions. Under static conditions the high level of bacteria might have reduced the oxygen concentration, thus limiting the activity of *H. vermiformis*. High bacterial cell densities can also negatively affect the growth of amoebae, due to the release of inhibitory metabolites and the extensive change of pH (Barbeau and Buhler, 2001, Qureshi *et al.*, 1993, Wang and Ahearn, 1997).

The PB strain was selected based on the high percentage present in the biofilm (26%), but only 0.5% of PB (3.9 ± 0.5 log CFU/cm^2) was present in the biofilm with *L. pneumophila* and *H. vermiformis* in the stationary phase. This difference can be attributed to grazing of *H. vermiformis* on the bacteria in the biofilm. The final PB concentration in the biofilm (3.4 ± 0.4 log CFU/cm^2) was much lower than the final biofilm concentration (about 4.4 log CFU/cm^2) reported for *H. vermiformis* grazing on a bacterial biofilm containing *P. aeruginosa*, *K. pneumoniae* and a *Flavobacterium* sp. (Murga *et al.*, 2001). It might be that a biofilm containing several bacterial species sustains amoebic grazing better than a single-species biofilm. When the threshold concentrations for suspended bacteria are converted to CFU/cm^2, assuming a biofilm thickness of 13.8 μm, which was the observed mean length of *H. vermiformis*, a threshold concentration of the prey that sustains amoebic grazing in biofilms can be estimated at around 3.6 log CFU/cm^2. The biofilm concentration of PB (4.4 ± 0.6 log CFU/cm^2) in the BBM flasks without *L. pneumophila* and *H. vermiformis* was higher then this threshold concentrations and could thus promote growth of *H. vermiformis* if this organism was also inoculated in the BBM system.

The obtained threshold value of PB is in agreement with the utilization of suspended bacteria reported for a number of combinations of protozoa and bacteria. *Hartmannella*, *Naegleria*, nor *Vahlkampfia* did multiply when the concentration of *Rhizobium meliloti* dropped below 6 to 7 log CFU/ml (Danso and Alexander, 1975). Critical values of 5 to 7 log bacterial cells/ml were found for ciliates (Habte and Alexander, 1978b) and it has been suggested that flagellates are unable to reduce bacterial densities below 0.5-2 × 10^6 cells/ml (Fenchel, 1982). The coexistence of protozoan predators and bacterial prey depends on the ability of the bacteria to multiply under the prevailing conditions (Habte and Alexander, 1978b). In the BBM system, multiplication of PB may have continued in the presence of PVCp, but such multiplication obviously was not sufficient to sustain multiplication of *H. vermiformis*, as is demonstrated by complete encystations (Fig. 6.3). Although it is estimated that 99% of microbial activity in fresh water exists within surface-associated communities (Bryers and Characklis, 1982), consumption of suspended bacteria might play a role in natural aquatic environments, where concentrations of suspended bacteria are relatively high. In man-made systems, like warm water installations, biofilm grazing may be

more important. In the BBM system with a mixed microbial community originating from tap water, *H. vermiformis* was mainly present in the biofilm rather than in the planktonic phase (Kuiper *et al.*, 2004). The amoeba *Acanthamoeba castellanii* has the capacity to graze on mixed biofilm communities and to become integrally associated with the microbial biofilm community (Huws *et al.*, 2005). In riverbanks, cells of *Hartmannella* were mainly detected in the sediment rather than in the water column (Ettinger *et al.*, 2003).

An average ingestion rate of 8.2-8.9 $\times 10^3$ PB $\times$ *H. vermiformis* $^{1} \times$ day ^{1} was derived from the observed yield and growth rate of *H. vermiformis* in the BBM system. This ingestion rate is slightly higher than the value calculated from the declining PB concentration and the growth of *H. vermiformis* (Fig. 6.4). The observed ingestion rates are close to those reported for other protozoa e.g. 48-408 bacteria per day for heterotrophic nanoflagellates (HNAN) in a stratified fresh water lake (Bloem *et al.*, 1989) and up to 6.1 $\times 10^3$ bacteria $\times$ HNAN$^{-1} \times$ day ^{1} in batch cultures (Fenchel, 1982). An ingestion rate of 5.8 $\times 10^3 \pm 7.0 \times 10^2$ *E. coli* cells $\times$ amoeba$^{-1} \times$ day^{-1} was found for the closely related *Hartmannella cantabrigiensis*, when the cells were attached to a surface (Heaton *et al.*, 2001). Given the typical characteristic of amoebae to graze on surfaces, it is expected that ingestion rates are highest in the biofilm. On the other hand, attachment to the surface may hamper uptake (Parry, 2004). In mixed microbial communities, grazing by protozoa may contribute to changes in the biofilm composition, thus leading to less favorable conditions for their proliferation with increasing biofilm age.

The number of PB needed for each protozoan cell division, which is another characteristic for the interaction between PB and *H. vermiformis* was estimated at 6.5 $\times 10^3 \pm 5.7 \times 10^3$ PB/*H. vermiformis*. This value exceeds the number of *R. meliloti* cells (600 to 3600) needed for the production of one *Hartmannella* sp. cell (Danso and Alexander, 1975) but is in the range of yields reported for grazing of *H. vermiformis* on the soil bacteria *Agrobacterium tumefaciens*, *Arthrobacter simplex*, *Bacillus megaterium*, *Bacillus subtilis*, *Klebsiella aerogenes* and *Pseudomonas fluorescens* ($2.2 \times 10^3 - 6.5 \times 10^3$ PB/*H. vermiformis*) (Weekers *et al.*, 1993). Only about 450 *E. coli* K-12 cells were needed for the production of one *H. vermiformis* cell (Weekers *et al.*, 1993) indicating that certain bacteria are a very good food source, possibly in combination with a larger cell size. These reports and the observations with the other isolates in this study (*Caulobacter* sp. and a β–Proteobacterium) indicate that yield of *H. vermiformis* in natural environments, with a variety of prey bacteria, may differ from the yield observed for the *Acidovorax* strain used in this study. Certain aquatic bacteria, e.g. *Pseudoxanthomonas mexicana*, may even not serve at all as food source for *H. vermiformis* or other amoebae.

Interactions between growth of *L. pneumophila* and *H. vermiformis*

Growth of *L. pneumophila* only occurred in the presence of growing *H. vermiformis*. *L. pneumophila* proliferated within *H. vermiformis*, but the growth rates of *H. vermiformis* in the BBM flasks with or without *L. pneumophila* were not significantly different (0.76 ±

0.06 and 0.70 ± 0.05 day^{-1}, respectively). No decline in *H. vermiformis* concentrations during and after the exponential growth of *L. pneumophila* occurred, which can be explained by encystment. However, final *H. vermiformis* concentrations were significantly different in the BBM flasks with and without *L. pneumophila* (4.9 ± 0.1 and 4.3 ± 0.1 log cells/cm^2). This difference is most probably due to the higher B_{max} concentrations in the BBM flasks without *L. pneumophila* (Table 6.2). The growth rates found for *H. vermiformis* were only 1.5-fold lower than those of naked amoebae in their natural environment (Arndt, 1993).

The maximum number of *L. pneumophila* proliferating intracellularly within *H. vermiformis* is difficult to determine experimentally. Direct counting using microscopy most probably underestimated actual numbers, because examination of the centre of the *H. vermiformis* cells is hampered. Moreover lysis of infected cells followed by cultivation would only give an estimate of the average numbers in cells just infected and cells just prior to collapse. Furthermore, the calculation based on cell volumes most probably provides an overestimation, since *H. vermiformis* will lyse before they are completely filled with *L. pneumophila*. Finally, the size of the *H. vermiformis* cells shows variations. Hence, a value between the maximum number of counted *L. pneumophila* cells and the number of calculated cells is considered as best approximation. From the estimated value of 500 *L. pneumophila* cells/*H. vermiformis* and the number of PB cells needed for one *H. vermiformis* cell division, we calculated that the consumption of at least 13 PB cells is needed for the proliferation of one *L. pneumophila* cell. This is only valid in the presence of an active host and a PB density exceeding the threshold concentration. The final *L. pneumophila* concentration (6.9 ± 0.1 CFU/cm^2) in the BBM flasks with PVCp and incubated under non-shaking conditions, exceeded the final PB concentrations (6.2 ± 0.3 CFU/cm^2) in these flasks (Fig. 6.1). These observations reinforce that *Acidovorax* sp. is a suited prey bacterium, also in modeling experiments.

Practical significance

The results obtained in this study demonstrate that very high numbers of *L. pneumophila* may be attained in the presence of a suitable host and a prey bacterium. The *L. pneumophila* to PB ratio indicates that *L. pneumophila* may constitute a significant proportion of a bacterial community when prey bacteria predominate. The isolation of the *Acidovorax* strain in this study from a rapidly developing biofilm suggests that rapid growth may enhance growth of suited prey bacteria. A rapid growth of *Legionella* has also been observed in a batch test, directly after incubation (Wadowsky *et al.*, 1991) and in an experimental tap water installation in the phase of a rapid increase of biomass after heat treatment (Van der Kooij *et al.*, 2005). Growth of *H. vermiformis* and *L. pneumophila* does not occur when the PB level is below a threshold value. Hence limiting bacterial growth to a level below this threshold and prevention of a rapid biofilm development may be effective in limiting *L. pneumophila* proliferation. Keeping the biofilm concentration below this threshold value in man-made water systems requires a far reaching removal of growth-promoting compounds from the water and the use

of materials which do not release compounds enhancing bacterial multiplication.

Acknowledgment

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CHAPTER 7

General discussion

Introduction

Legionella pneumophila is widespread in natural fresh water environments and man-made water systems at elevated temperatures (Fliermans *et al*., 1981, Leoni *et al*., 2001, Tobin *et al*., 1980, Yamamoto *et al*., 1992). Exposure to this organism via aerosols can lead to a serious pneumonia, the so-called Legionnaires' disease. A large outbreak in the Netherlands among visitors of a flower show, with an unchlorinated whirlpool as identified source of amplification, and the detection of the organism in installations for warm tap water, were the reasons for the Dutch government to introduce preventive legislation and many instructive documents (Tijdelijke regeling *Legionella* preventie, 2000). Risk analysis of installations for warm tap water, in combination with corrective measures such as prevention of stagnation and a hot water temperature above 60°C, are the main components of the policy to limit multiplication of *Legionella* in such systems. Still, a total of 275 cases were reported in the Netherlands in 2005 (Anonymous, 2006). Also in other countries in Europe, Northern America and Asia, community-acquired cases of legionellosis and outbreaks continue to occur at an average incidence rate of about 10 cases per million persons. More information about the effects of environmental conditions on the proliferation of *Legionella* in man-made water systems may contribute to define more effective preventive measures. Therefore, the interactions between legionellae and other microbiota, viz. bacteria and protozoa were studied.

L. pneumophila proliferates in biofilms and can be observed in the planktonic phase (Colbourne *et al*., 1984). Biofilm matrices are known to provide shelter and a gradient of nutrients. Although the high nutritional demands of *L. pneumophila* are satisfied in the intracellular environment, which is found in different protozoa (Fields *et al*., 2002, Rowbotham, 1980a), the complex composition of biofilms have led some researchers to conclude that biofilms can support the multiplication of legionellae outside a host cell (Rogers and Keevil, 1992, Surman *et al*., 2002). The need for protozoa for the proliferation of *L. pneumophila* in aquatic habitats is therefore still not fully understood. The objectives of the present study were therefore (i) to determine whether specific bacteria and/or protozoa are needed for growth of *L. pneumophila* in biofilms present in tap water installations, (ii) to collect quantitative information about the relationships between *L. pneumophila* and required organism(s) in fresh water environments used for drinking water production, and (iii) to obtain quantitative information on the proliferation of *L. pneumophila* in aquatic biofilms under conditions resembling those in installations for warm tap water. The collected information might be used to define preventive measures aiming at limiting the proliferation of *Legionella* by limiting the growth of prey bacteria for protozoa serving as host for *L. pneumophila*.

L. pneumophila needs other organisms for proliferation in environmental biofilms

Experiments conducted in a biofilm-batch model system clearly demonstrated that *L. pneumophila* multiplied in trophozoites of *Hartmannella vermiformis* present in aquatic biofilms developing on plastic materials in contact with tap water. Different materials (unplasticized PVC [PVCu] and plasticized PVC [PVCp]) supported biofilm growth to a different extent (as measured by total ATP concentrations), which in turn yielded higher cell numbers of *L. pneumophila*. The proliferation of *L. pneumophila* was due to its intracellular multiplication in *H. vermiformis* in the presence of a high concentration of heterotrophic bacteria in the biofilm. No multiplication of *L. pneumophila* was observed under similar conditions, in the absence of *H. vermiformis* (Chapter 2).
Hence, the presence of a suitable host is essential for the proliferation of *L. pneumophila*. However, the host, in this case *H. vermiformis*, should graze on the biofilm. Therefore bacterial populations, which may serve as food for protozoa, should be present in the biofilm. Bacteria most probably serving as prey for *H. vermiformis* in the biofilm-batch model system with a mixed inoculum, were identified as *Limnobacter thioxidans* and *Phenylobacterium lituiforme* (Chapter 3), but also *Acidovorax* sp., *Caulobacter* sp., and a β-Proteobacterium could act as prey bacterium for *H. vermiformis* (Chapter 6). Biofilms in tap water distributing systems show a great microbial diversity, independent of the presence of a disinfectant residual in the system (Keevil *et al.*, 1995), and thus are therefore excellent feeding grounds for protozoa.

Detection of *H. vermiformis* and its relationship with *L. pneumophila*

H. vermiformis is widespread in nature, and has been isolated from soil, fresh water, air samples and different engineered water systems (Breiman *et al.*, 1990, Page, 1967 and 1974, Rohr *et al.*, 1998, Walker *et al.*, 1986). The amoeba has frequently been observed as an important host for *L. pneumophila* (Fields *et al.*, 1989 and 1990b, Kuchta *et al.*, 1998, Kuiper *et al.*, 2004, Wadowsky *et al.*, 1988 and 1991). However, detection methods for this amoeba, which have been used until now, viz. cultivation and microscopy, are time-consuming. To enable investigations on the contribution of *H. vermiformis* to the occurrence, persistence and proliferation of *L. pneumophila* in natural and engineered water systems, an 18S rRNA gene targeted real-time PCR method for the cultivation-independent and specific detection and quantification of *H. vermiformis* was developed (Chapter 4). This method was subsequently applied to water samples obtained from surface waters, cooling towers, warm tap water systems and waste water treatment plants.
H. vermiformis was observed in most samples, demonstrating that this organism is commonly present in fresh water environments, both natural and man-made. Hence, growth of *Legionella* is not restricted by the absence of a suitable protozoan host. A significant positive correlation was found between the concentrations of *H. vermiformis* and *L. pneumophila* in samples retrieved from cooling towers (Chapter 5),

demonstrating the importance of *H. vermiformis* as a host for *L. pneumophila* in many fresh water environments. Furthermore, *H. vermiformis* was also observed at water temperatures too low for multiplication of *L. pneumophila*. In a number of samples *L. pneumophila* proliferated in the absence of *H. vermiformis*, indicating that other protozoa also contribute to the occurrence, persistence and proliferation of *L. pneumophila* in fresh water environments.
The real-time PCR method, developed for the specific detection and quantification of *H. vermiformis*, proved also useful as a starting point for a more detailed assessment as to what extent phylogenetic *H. vermiformis* microdiversity could be linked to the ability to serve as host for *L. pneumophila*. Sequence analysis of the amplified 18S rRNA gene fragment (502/503 bp), revealed that *H. vermiformis* strains can be divided in three groups (with one group further divided in three subgroups). All (sub)groups of *H. vermiformis* also comprised sequences retrieved from water samples in which *L. pneumophila* was detected, suggesting that all observed genotypes are potential hosts for *L. pneumophila*. Environments harbouring different phylotypes of *H. vermiformis* showed a higher tendency to contain *L. pneumophila*, suggesting that in the presence of multiple subtypes of *H. vermiformis* the chance of the presence of an available host for *L. pneumophila* will increase, which subsequently increases the possibility of the presence of *L. pneumophila*.

Acidovorax sp., *L. pneumophila* and *H. vermiformis* growth interactions

Defining preventive control measures for limiting *L. pneumophila* requires quantitative information about conditions affecting proliferation of *L. pneumophila* in man-made water systems. Therefore, a biofilm-batch model system for the proliferation of *L. pneumophila* was developed (Chapter 6), to study the proliferation of *L. pneumophila* in autoclaved tap water with two essential microbial components: a host protozoan (*H. vermiformis*) and a bacterial strain (*Acidovorax* sp.) serving as prey. *L. pneumophila* multiplied inside *H. vermiformis* that was grazing on the biofilm, formed by *Acidovorax* sp. on the PVCp biofilm carrier. It has been shown previously that after intracellular multiplication *L. pneumophila* will disperse into the environment, entering new suitable host cells. Furthermore, we found that *H. vermiformis* could only proliferate at an *Acidovorax* sp. concentration of around 6.5 log CFU/ml. When *Acidovorax* sp. concentrations dropped below this threshold concentration due to the grazing by *H. vermiformis*, the amoebae changed from their active (trophozoite) to their inactive (cyst) form. The interactions between prey bacteria, host protozoa and *Legionella* are summarized in a schematic overview (Fig. 7.1).
Microbial biofilms in tap water distributing systems are known for their species richness, independent of the presence of a disinfectant residual in the distributing system (Keevil *et al.*, 1995). *Legionella* species may represent up to 7% of biofilms grown in treated water (Schwartz *et al.*, 1998) and a large diversity of yet-uncultured legionellae are common members of the microbial communities in treated tap water in the

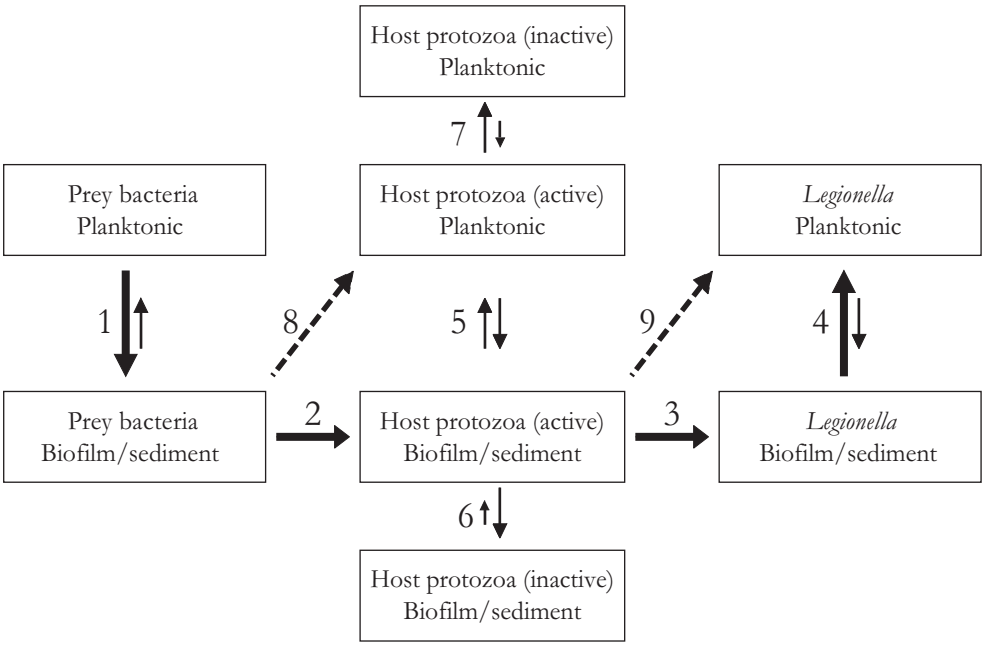

Figure 7.1. Schematic overview for the proliferation of *L. pneumophila* in water systems. **1.** Prey bacteria are present in the planktonic phase and colonize the surface, and/or form sediment at the bottom of pipes and reservoirs. **2.** Protozoan hosts multiply in the biofilm and/or the sediment, grazing on prey bacteria. **3.** Intracellular proliferation of *L. pneumophila* occurs in protozoan hosts, which are mainly present in the biofilm/sediment. **4.** After intracellular proliferation *L. pneumophila* may leave the biofilm, looking for other suitable hosts. **5.** Coincidently, protozoa may leave the biofilm, due to disturbance of static conditions. **6.** Trophozoite forms of the protozoan host in the biofilm/sediment will change to cysts, due to decline of the prey bacterial concentration. **7.** Trophozoite forms of the protozoan host in the planktonic phase will change to cysts, due to decline of the prey bacterial concentration. **8.** Unknown relationship between the grazing of planktonic protozoan hosts on biofilm bacteria; the contribution of this grazing to the protozoan host concentration in the planktonic phase is most likely negligible. **9.** Unknown relation between the intracellular proliferations of *L. pneumophila* in protozoan hosts present in the biofilm followed by direct distribution of *L. pneumophila* cells in the planktonic phase after lysis.

Netherlands (Wullings and van der Kooij, 2006). *H. vermiformis* has been observed as the predominant amoeba in warm tap water (Breiman *et al.*, 1990, Rohr *et al.*, 1998), however also other protozoa, namely *Acanthamoebae* spp., *Echinamoeba* spp., *Naegleria* spp., *Saccamoeba* spp., *Vahlkampfia* spp., *Vanella* spp. and *Tetrahymena* spp. were detected in water distributing systems (Barbeau and Buhler, 2001, Bick, 1972, Breiman *et al.*, 1990, Harf and Monteil, 1988, Henke and Seidel, 1986, Michel and Borneff, 1989, Rohr *et al.*, 1998). These findings imply that other bacteria may act as prey for *H. vermiformis* or other protozoa; other protozoa may act as host for *L. pneumophila*, and other legionellae potentially compete with *L. pneumophila* for a suitable host. All these variable conditions might result in other growth characteristics in the schematic

overview of the proliferation of *L. pneumophila* in water distributing systems, and future research is needed for the elucidation of the components of the presented model (Fig. 7.1).

Experiments with the biofilm-batch model system revealed that a threshold concentration of around 6.5 log CFU/ml of the model prey, *Acidovorax* sp., is needed for the proliferation of *H. vermiformis* in tap water (Chapter 6). However concentrations of suspended bacteria in tap water installations generally are relatively low and therefore, the bacteria present in biofilms are more important in relation to *L. pneumophila* proliferation (Keevil *et al.*, 1995). The above-mentioned volumetric threshold concentration corresponds to a threshold concentration of around 3.6 log CFU/cm^2. Based on heterotrophic plate counts (HPCs), biofilm concentrations in a model water system ranged from 4.2 to 4.7 log CFU/cm^2, depending on the material of the biofilm carrier (Van der Kooij *et al.*, 2005). These values were relatively low in comparison to those observed in other model systems (5-6 log CFU/cm^2) (Lehtola *et al.*, 2004, Rogers *et al.*, 1994b, Volk and LeChevallier, 1999). The threshold concentration of *Acidovorax* sp. for *H. vermiformis* was lower than this range of biofilm concentrations, indicating that biofilm concentrations in water distributing systems can support protozoa proliferation. However, other protozoa and other prey bacteria may also be involved in the grazing of the biofilm in water distributing systems, resulting in other threshold concentrations. Furthermore, HPC values of bacteria in natural (mixed) microbial communities in biofilms, are only a small fraction (<1%) of the total number, as the large majority is not accessible to currently used methods for cultivation (Momba *et al.*, 2000).

Preventive measures against *Legionella*

Starvation, disinfection, or a combination?

In the Netherlands, drinking water is distributed without a disinfectant residual, and application of chemical disinfection in tap water installations is strictly limited to cleaning operations (Waterleidingbesluit, 2004). Biofilm formation in drinking water distribution systems is limited by a far reaching removal of biodegradable, growth-promoting compounds from water by the application of multiple barrier water treatment (Van der Kooij *et al.*, 1999). This approach is expected also to limit biofilm formation in tap water installations. However, *Legionella* has been observed in many installations of warm tap water exceeding the *Legionella* spp. concentrations which are regarded as safe (100 CFU/l) (Waterleidingbesluit, 2004). This presence of *Legionella* in tap water installations is most probably due to dead ends in installations, the use of materials promoting biofilm formation, and long resident times and stagnation in the water pipes. Application of a disinfectant can therefore contribute to the eradication of *Legionella*. The present study was conducted to elucidate relationships between biofilm formation processes and the proliferation of *Legionella* to collect information enabling to improve preventive measures. It was shown that increased biofilm concentrations

led to increased *L. pneumophila* concentrations due to intracellular proliferation, which could only occur if the bacterial threshold concentration was achieved. For applying an effective disinfectant to water distribution systems for the eradication of *Legionella* bacteria, it should be noted that cells of *Legionella* residing within biofilms are better protected against disinfectants than planktonic bacteria. This can be most probably attributed to the need for the disinfectant to penetrate the biofilm in order to reach the target bacteria (Green, 1993, Wright *et al.*, 1991). Furthermore, *Legionella* within protozoa are more protected against disinfectants, and it is even more difficult to kill *Legionella*, when they are associated with amoebic cysts (Donlan *et al.*, 2005, Kilvington and Price, 1990, Kuchta *et al.*, 1998).

For the distribution of safe drinking water around the world, a variety of disinfectant methods are applied, based on physical (e.g. UV irradiation), thermal (e.g. heat pasteurization), and chemical (e.g. chlorination) techniques. Also for the disinfection of tap water, considering the eradication of *Legionella*, these three techniques are used and will therefore be briefly discussed here. The advantages and limitations of the techniques which are mainly applied in tap water installations are summarized in table 7.1 (Kim *et al.*, 2002, Lin *et al.*, 1998).

While physical disinfection methods are not widely used in hot water systems, UV irradiation is the main physical treatment used. Sources for UV lights are typically installed near peripheral outlets (e.g. shower heads or faucets) to prevent the formation of *Legionella*-containing-aerosols. However, UV irradiation alone, applied in a water system is insufficient to control *Legionella*, because this organism proliferates in biofilms which are not affected by UV irradiation (Liu *et al.*, 1995, Schulze-Robbecke *et al.*, 1990).

Chemical disinfection methods can be divided in three groups: metal ions (copper/ silver ionization), oxidizing agents and non-oxidizing organic agents. Copper-silver ionization is a disinfection technology that has been used with increasing frequency to control *Legionella* spp. in hospital hot water systems (Lin *et al.*, 1996, 1998, and 2002). Several oxidizing agents have been widely and successfully used to disinfect potable water. They are molecular halogens (e.g. chlorine), chlorine dioxide, chloramines and ozone. Chlorination is the most widespread technique, but chlorine dioxide is also commonly used in the USA (Lykins *et al.*, 1990) and European countries (Haberer, 1994). Nevertheless, since the recognition of the formation of harmful chlorination by-products the popularity of the use of monochloramine has significantly increased. Many organic disinfectants have been used against *Legionella*. They include heterocyclic ketones, guanidines, halogenated amides, halogenated glycols, thiocarbamates, amines, aldehydes, thiocyanates, and organo-tin compounds. These non-oxidizing agents however are mainly used in cooling water systems and not in potable water systems, due to toxic residuals and by-products (Kim *et al.*, 2002).

Raising the hot water temperature was the first method successfully used for disinfection (Best *et al.*, 1983, Fisher-Hoch *et al.*, 1981). The "superheat-and-flush" method can be used as an emergency procedure during an outbreak of Legionnaires'

Table 7.1. Disinfection methods with their respective advantages and limitations (Domingue *et al.*, 1988, Donlan *et al.*, 2000, Hamilton *et al.*, 1996, Kim *et al.*, 2002, Lin *et al.*, 1996 and 1998, Muraca *et al.*, 1987, Rook, 1974, Schulze-Robbecke *et al.*, 1990, White, 1972)

Disinfection method	Advantages	Limitations
Copper/silver ionization	Easy installation and maintenance High water temperatures do not affect efficacy Residual disinfectant throughout the system *Legionella* cells are killed, rather than suppressed	Elevated pH (≥8.0) reduces efficacy Regular removal of scale from electrodes Excessively high ion levels give water a blackish colour Environmental concern
Chlorine	Residual disinfectant throughout the system	Corrosiveness and damaging of pipes *Legionella* are suppressed rather than killed Formation of undesirable by-products (carcinogenic, taste/odour)
Chlorine dioxide	Residual disinfectant throughout the system Removes phenols better than chlorine More effective in eradication of *L. pneumophila* than chlorine	Corrosiveness and damaging of pipes *Legionella* are suppressed rather than killed Formation of carcinogenic compounds
Monochloramine	Residual disinfectant throughout the system Better penetration in biofilm than free chlorine	Corrosiveness and damaging of pipes Nitrification, toxicity for fish
Ozone	More effective at inactivating *L. pneumophila* than chlorine Inactivation by both gaseous and dissolved ozone	No residual effect, since ozone dissipates in water much quicker than chlorine Formation of biodegradable by-products
UV irradiation	Easy to install and not harmful for water or plumbing No formation of disinfectant by-products	No disinfectant residual Accumulation of scale on the quartz glass tubes
Superheat-and-flush	No special equipment required Minimal costs	Method is time-consuming Disinfection only temporal, recolonization of *L. pneumophila* Requires safety measures
Instantaneous heating system	Elimination of large volume water heaters, possible sources for *Legionella* spp.	No residual protection Heat treatment limited to incoming water No complete eradication of *Legionella*

disease or intermittently to suppress widespread *Legionella* contamination. With this method, the temperature of the hot water tank is elevated to 70°C, and then all water outlets, faucets and showerheads are flushed for 30 min (Lin *et al.*, 1998). Another thermal disinfection method is the instantaneous heating system, where water is flash-heated to a temperature >88°C and then blended with cold water to achieve the desired temperature (Lin *et al.*, 1998).
Since the legionellosis outbreak in 1999 in Bovenkarspel in the Netherlands (Den Boer *et al.*, 2002), increased regulations became effective (Waterleidingbesluit, 2001, Waterleidingbesluit, 2004). Water distributing systems in the Netherlands, that pose a potential human health risk due to the identification of *L. pneumophila* in the systems, have to be disinfected using thermal disinfection. If thermal disinfection is not possible, disinfection methods without addition of a compound to the water installations are recommended (ultra filtration, UV-irradiation and pasteurization) (Waterleidingbesluit, 2004).

Concluding remarks and recommendations

The results presented in this thesis demonstrate that *L. pneumophila* can only proliferate in biofilms intracellularly in a suitable protozoan host. *H. vermiformis* is such a suitable host, which is frequently found co-occurring with *L. pneumophila*. However, *H. vermiformis* can only act as host for *L. pneumophila* if it proliferates, and thus depends on the presence of sufficient and appropriate prey bacteria. Biofilms in tap water systems show high bacterial diversities and an appropriate prey bacterium is therefore most probably always present. Eradication of *Legionella* from tap water systems requires biofilm concentrations to levels below the threshold value of prey bacteria needed for proliferation of protozoa (around 3.6 log CFU/cm^2). Removal of biodegradable compounds from the water and selection of materials that do not support biofilm formation in combination with the maintenance of a disinfectant residual are crucial in this approach. Hence, further studies are needed to elucidate the relationship between biofilm concentration, biofilm composition and the growth promoting properties (biological instability) of tap water and materials in contact with tap water.
The most effective way for limiting *L. pneumophila* proliferation and survival in water distributing systems may be a multiple barrier approach, which includes the use of a suitable control method, the supply of biological stable low nutrient water, and the application of materials with a low biofilm formation potential.

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SUMMARY

Occurrence of *Legionella pneumophila* and *Hartmannella vermiformis* in fresh water environments and their interactions in biofilms

Summary

Legionella pneumophila is widespread in natural fresh water environments and is also frequently found in man-made water systems, despite its fastidious nature. Exposure to this organism via aerosols can lead to a serious pneumonia, the so-called Legionnaires' disease. In 1999, one of the worst outbreaks of this disease occurred in the Netherlands, due to the proliferation of *L. pneumophila* in a whirlpool spa at display at a flower show in Bovenkarspel. This outbreak caused much concern and dispute, and therefore the Dutch government introduced legislation and instructive documents, aiming at preventing the growth of *Legionella* in man-made water systems. However, observations in practice revealed that control measures were not always effective and community-acquired cases of legionellosis continue to occur in the Netherlands. In other countries in Europe, in Northern America and in Asia, legionellosis is also reported as a water-related public health problem, and causes large outbreaks. Therefore, more information on how and to what extent environmental conditions affect the proliferation of *L. pneumophila* in man-made water systems is needed to define more effective control measures. For this reason, the interactions between legionellae and other microbiota, viz. bacteria and protozoa in water systems were studied.

Biofilms and protozoa are known to play a major role in the proliferation of *L. pneumophila*. Biofilm matrices provide shelter and a gradient of nutrients; protozoa may act as host for *L. pneumophila*, since multiplication of *L. pneumophila* was demonstrated in a variety of protozoan genera (e.g. *Acanthamoeba*, *Naegleria*, *Hartmannella* and *Tetrahymena*). The complex composition of biofilms has led some researchers to conclude that biofilms can support the multiplication of legionellae outside a host. The need for protozoa for the proliferation of *L. pneumophila* in aquatic habitats is therefore still not fully understood.

A biofilm-batch model (BBM) system was used for the development of different concentrations of aquatic biofilm by using polyvinylchloride, either unplasticized (PVCu) or plasticized (PVCp), as a biofilm carrier and as a source of energy and carbon (**Chapter 2**). The BBM system contained autoclaved tap water, PVC pieces and the inoculum. The inoculum, a mixed microbial community including indigenous *L. pneumophila*, originating from a tap water system, was added either unfiltered or filtered (cellulose nitrate, 3.0-μm pore size). Both the attached (biofilm) and the suspended biomasses were examined for their total concentrations of ATP, for cultivable *L. pneumophila*, and for the abundance of protozoa. *L. pneumophila* grew to high numbers only in the BBM system with the unfiltered inoculum. Filtration did not affect biofilm formation as determined by ATP but obviously removed the growth-supporting factor. The free-living amoeba *Hartmannella vermiformis* was detected in all flasks in which *L. pneumophila* proliferated, whereas no protozoa were detected in the BBM system with the filtered inoculum. Fluorescent in situ hybridization clearly demonstrated the intracellular multiplication of *L. pneumophila* in trophozoites of *H.*

vermiformis. These observations and calculations revealed that the intracellular growth of *L. pneumophila* in *H. vermiformis* was most probably the mechanism of proliferation for *L. pneumophila* in biofilms on PVCp, which contained a high concentration of heterotrophic bacteria.

Culture-independent microbial community analysis by 16S ribosomal RNA-targeted denaturing gradient gel electrophoresis (DGGE) showed that both the biofilm and the suspended biomass in the BBM systems with the unfiltered as well as with the filtered inoculum were composed of a variety of bacterial populations (**Chapter 3**). However, the bacterial composition of the suspended biomass in the BBM system with the unfiltered inoculum, thus with *H. vermiformis* and *L. pneumophila*, were more closely related to the biofilm and suspended biomass of the BBM system with the filtered inoculum than to its biofilm counterpart. This was due to (i) the presence of *L. pneumophila* as dominant group in the biofilm of the environment with *H. vermiformis* and (ii) the decrease in intensity of two bands in the DGGE profile of the biofilm in the flasks with the unfiltered inoculum, representing a *Phenylobacterium lituiforme*-related and a *Limnobacter thiooxidans*-related population. The decrease in the abundance of these bacteria was attributed to selective grazing of *H. vermiformis* on the biofilm. This indicated preferential grazing of *H. vermiformis* on several specific bacteria in a multi-species biofilm. It is suggested that several bacterial species, and not just one specific organism, may serve as food source for *H. vermiformis* in natural environments. Biofilms in tap water distributing systems show a great microbial diversity and thus are excellent feeding grounds for protozoa.

H. vermiformis, found as host for *L. pneumophila* in the BBM system and also in other studies, is widespread in nature, and has been isolated from soil, fresh water, air samples and engineered systems. Since detection methods for this amoeba, which have been used until now, are time-consuming, an 18S rRNA gene-targeted real-time PCR method for the cultivation-independent and specific detection and quantification of *H. vermiformis* was developed (**Chapter 4**). This method enables investigations on the contribution of *H. vermiformis* to the occurrence, persistence and proliferation of *L. pneumophila* in engineered water systems and was therefore applied to water samples obtained from surface waters, cooling towers, warm tap water systems and waste water treatment plants (**Chapter 5**). These results, in combination with the quantification of *L. pneumophila* demonstrated that (i) *H. vermiformis* is commonly present in fresh water environments, both natural and man-made; indicating that growth of *Legionella* is not restricted by the absence of a suitable protozoan host, and (ii) a significant positive correlation between the log concentrations of *H. vermiformis* and *L. pneumophila* occurred in water samples retrieved from cooling towers, demonstrating the importance of *H. vermiformis* as host for *L. pneumophila* in these environments. However, in a number of samples *L. pneumophila* proliferated in the absence of *H. vermiformis*, indicating that other protozoa also contribute to the occurrence, persistence and proliferation of *L. pneumophila* in fresh water environments. Defining preventive control measures for limiting *L. pneumophila* requires quantitative

information about conditions affecting proliferation of *L. pneumophila* in man-made water systems. In a BBM system containing PVCp and autoclaved tap water, the growth of *L. pneumophila* was quantified, only in the presence of a protozoan host (*H. vermiformis*) and an *Acidovorax* sp. strain serving as prey bacterium (PB) for *H. vermiformis*, and thus without a mixed microbial community (**Chapter 6**). It was shown that *L. pneumophila* multiplied mainly in the biofilm, however, the bacterium could also grow without a biofilm carrier if the PB concentration was above a threshold concentration. This threshold concentration depended on conditions applied during incubation at 30°C. Shaking conditions required a threshold concentration of around 7.5 log CFU/l, while under static conditions a threshold concentration of around 6.5 log CFU/ml was observed. However, in tap water installations concentrations of suspended bacteria are relatively low, and therefore the bacteria in biofilms are more important in relation to *L. pneumophila* proliferation. The above mentioned volumetric threshold concentration for PB under static conditions corresponds to a PB biofilm concentration of around 3.6 log CFU/cm^2, a concentration that is normally found in tap water installations.
In conclusion, *L. pneumophila* can only proliferate in biofilms intracellularly in a suitable protozoan host. *H. vermiformis* is such a suitable host, which is frequently found co-occurring with *L. pneumophila*. However, *H. vermiformis* can only act as host for *L. pneumophila* if it proliferates, and thus depends on the presence of sufficient and appropriate prey bacteria. Biofilms in tap water systems show high bacterial diversity, and an appropriate prey bacterium is therefore most probably always present. Eradication of *Legionella* from tap water systems requires the reduction of the prey bacteria concentration in biofilms to levels below the threshold value needed for proliferation of protozoa. The most effective way for limiting *L. pneumophila* proliferation and survival in man-made water systems may be a multiple barrier approach, which includes the supply of biologically stable low nutrient water, the application of construction materials with a low biofilm formation potential and the use of an appropriate control method.

SAMENVATTING

Het voorkomen van *Legionella pneumophila* en *Hartmannella vermiformis* in zoetwater milieus en hun interacties in biofilms

Samenvatting

Legionella pneumophila komt, ondanks zijn hoge voedingseisen, algemeen voor in oppervlaktewater en wordt daarnaast regelmatig gevonden in kunstmatige watersystemen. Blootstelling aan deze bacterie via aerosolen kan leiden tot een ernstige longontsteking, de zogenaamde veteranenziekte. In 1999 vond in Nederland een van de grootste uitbraken in de geschiedenis van deze ziekte plaats, doordat *L. pneumophila* in grote aantallen aanwezig was in een whirlpool die getoond werd bij een bloemententoonstelling in Bovenkarspel. Deze uitbraak bracht grote commotie teweeg en daarom heeft de Nederlandse regering preventieve maatregelen genomen en is een wetsvoorstel ter bestrijding van *Legionella* ingediend. Echter, praktijkwaarnemingen lieten zien dat de genomen maatregelen niet altijd effectief waren en nog steeds worden er gevallen van de veteranenziekte in Nederland geregistreerd. Ook in andere Europese landen en in Noord-Amerika en Azië worden nog regelmatig uitbraken van de veteranenziekte gerapporteerd. Meer informatie over de effecten van omgevingsfactoren op de vermenigvuldiging van *L. pneumophila* in kunstmatige watersystemen zal bijdragen aan het formuleren van effectievere maatregelen ter bestrijding van *L. pneumophila*. Daarom zijn de interacties tussen *Legionella*-bacteriën en andere micro-organismen, namelijk bacteriën en protozoa, bestudeerd.

Biofilms en protozoa spelen een belangrijke rol bij de vermeerdering van *L. pneumophila*. Biofilms (lagen bacteriën die omgeven zijn door een zelfgeproduceerd slijm en zijn vastgehecht op een oppervlak) geven de bacterie bescherming en bovendien is in biofilms een groot aantal voedingsstoffen aanwezig. Protozoa (eencellige eukaryotische micro-organismen) kunnen als gastheer dienen voor *L. pneumophila*, aangezien groei van *L. pneumophila* is aangetoond in verschillende protozoa geslachten, zoals *Acanthamoeba*, *Naegleria*, *Hartmannella* en *Tetrahymena*. De complexe samenstelling van biofilms heeft een aantal onderzoekers doen concluderen dat biofilms de vermeerdering van *L. pneumophila* buiten een gastheer kunnen ondersteunen. Het is dan ook nog steeds niet duidelijk of *L. pneumophila* protozoa nodig heeft voor groei in zijn natuurlijke omgeving.

In dit onderzoek is een biofilm-batch model (BBM) systeem toegepast om verschillende biofilmconcentraties te ontwikkelen (**Hoofdstuk 2**). Hard en zacht polyvinylchloride (respectievelijk PVCu en PVCp) is hiervoor gebruikt, beide materialen dienden zowel als biofilmdrager en als energie- en koolstofbron. Het BBM-systeem bevatte naast de PVC stukjes geautoclaveerd drinkwater en entmateriaal. Dit entmateriaal bestond uit een gemengde microbiële cultuur en was oorspronkelijk afkomstig van een drinkwater systeem waarin *L. pneumophila* was gedetecteerd. Het entmateriaal is zowel gefiltreerd (filter met een poriegrootte van 3.0-µm) als ongefilterd toegevoegd aan verschillende BBM systemen. Van zowel de biofilm als de gesuspendeerde micro-organismen zijn de ATP (adenosine trifosfaat, deze stof is alleen aanwezig in levende [micro-]organismen en is daardoor een maat voor de actieve biomassa), *L. pneumophila* en protozoaconcentraties bepaald. Het bleek dat *L.*

pneumophila alleen kon groeien in de BBM systemen waaraan het ongefiltreerde entmateriaal was toegevoegd. Blijkbaar is door het filtreren van het entmateriaal de groei bevorderende factor van *L. pneumophila* verwijderd. Echter, filtratie had geen invloed op de totale activiteit van de biomassa (zowel biofilm als gesuspendeerde bacteriën), aangezien de ATP concentraties vergelijkbaar waren in de twee verschillende systemen. De amoebe (een protozo) *Hartmannella vermiformis* werd gedetecteerd in de BBM systemen waarin *L. pneumophila* gegroeid was, terwijl er geen protozoa werden gedetecteerd in de BBM systemen met het gefiltreerde entmateriaal. Daarnaast is met de fluorescente in situ hybridisatie methode (FISH) duidelijk aangetoond dat *L. pneumophila* intracellulair vermenigvuldigde in actieve vormen van *H. vermiformis*. Deze waarnemingen en berekeningen maken duidelijk dat de intracellulaire groei van *L. pneumophila* in *H. vermiformis* hoogstwaarschijnlijk de enige manier is van vermeerdering van *L. pneumophila* op PVCp, dat hoge microbiële biofilm concentraties bevat.
Een cultuuronafhankelijke methode (denaturerende gradiënt gel electroforese [DGGE]), die de dominante bacterie groepen laat zien in de vorm van een streepjespatroon, maakte duidelijk dat zowel de biofilm als de gesuspendeerde biomassa in de BBM-systemen met het ongefiltreerde en met het gefiltreerde entmateriaal was samengesteld uit verschillende bacteriële stammen (**Hoofdstuk 3**). Echter, de bacteriële samenstelling van de gesuspendeerde biomassa in het BBM systeem met het ongefiltreerde entmateriaal, dus met *L. pneumophila* en *H. vermiformis*, was beter te vergelijken met de samenstelling van de biofilm en de gesuspendeerde biomassa in het BBM systeem met het gefiltreerde entmateriaal, dan met de samenstelling van de biofilm in hetzelfde systeem. Dit komt door (i) de aanwezigheid van *L. pneumophila* als dominante groep in de biofilm in het BBM systeem met het ongefiltreerde entmateriaal en (ii) door de afname van de dominantie van twee banden in het DGGE patroon van het BBM systeem met het ongefiltreerde entmateriaal, die overeenkomen met een *Phenylobacterium lituiforme*-gerelateerde en een *Limnobacter thiooxidans*-gerelateerde bacterie populatie. Deze afname wordt toegekend aan het grazen van *H. vermiformis* op de biofilm. Daarnaast laat het zien dat *H. vermiformis* graast op verschillende dominante bacterie groepen in een biofilm bestaande uit meerdere bacterie soorten en niet op één specifieke bacterie populatie. Aangezien biofilms in drinkwater systemen een hoge mate aan microbiële diversiteit laten zien, zijn deze biofilms uitstekende voedselbronnen voor protozoa.
H. vermiformis, de gastheer voor *L. pneumophila* in het BBM systeem, is ook regelmatig als gastheer voor *L. pneumophila* gevonden in andere onderzoeken. Deze amoebe komt veel in de natuur voor, en is o.a. geïsoleerd uit de bodem, oppervlaktewater, lucht, en kunstmatige watersystemen. Aangezien de detectiemethoden die tot nu toe voor deze amoebe gebruikt zijn erg tijdrovend waren is een cultuuronafhankelijke specifieke en kwantitatieve methode voor de detectie van *H. vermiformis* ontwikkeld (**Hoofdstuk 4**). Deze moleculair-biologische methode, gebaseerd op een kwantitatieve polymerase ketting reactie (PCR), geeft binnen een halve dag uitsluitsel over de aanwezigheid en concentratie van *H. vermiformis* in watermonsters. Met deze methode kan onderzocht

worden wat de bijdrage is van *H. vermiformis* op het voorkomen, het overleven en de vermeerdering van *L. pneumophila* in watersystemen, en daarom is de methode toegepast op watermonsters afkomstig van oppervlaktewater, koeltorens, warmwatersystemen en rioolwater zuiveringsinstallaties (**Hoofdstuk 5**). De verkregen resultaten, in combinatie met de *L. pneumophila* concentraties van dezelfde monsters, lieten zien dat (i) *H. vermiformis* algemeen voorkomt in de onderzochte milieus. Dit duidt erop dat de groei van *Legionella* niet belemmerd wordt door de afwezigheid van een geschikte gastheer, en dat (ii) er een significante positieve correlatie bestaat tussen de logaritmische concentraties van *H. vermiformis* en *L. pneumophila* welke voorkomen in watermonsters afkomstig van koeltorens, wat het belang laat zien van *H. vermiformis* als gastheer voor *L. pneumophila* in zoetwatermilieus. Echter, in een aantal watermonsters waarin *L. pneumophila* gegroeid was, werd *H. vermiformis* niet gedetecteerd; andere protozoa hebben dus ook bijgedragen aan het voorkomen, het overleven en de vermeerdering van *L. pneumophila* in zoetwater milieus.
Om preventieve maatregelen te kunnen nemen om het voorkomen van *L. pneumophila* in kunstmatige watersystemen te verlagen, is meer kwantitatieve informatie nodig over de condities die van invloed zijn op de vermeerdering van *L. pneumophila* in deze systemen. Daarom is de groei van *L. pneumophila* gekwantificeerd in een BBM-systeem, dat naast geautoclaveerd water en stukjes PVCp, alleen *H. vermiformis* als gastheer en een *Acidovorax* stam als prooibacterie (PB) bevatte (**Hoofdstuk 6**). De resultaten van dit onderzoek lieten zien dat *L. pneumophila* voornamelijk vermeerderde in de biofilm. Echter, *L. pneumophila* kon ook groeien zonder biofilmdrager (dus zonder PVCp) mits de PB concentratie voldoende hoog was. Het bleek dat de PB concentratie een drempelwaarde moest hebben voordat *H. vermiformis*, en daardoor ook *L. pneumophila*, kon groeien. Deze drempelwaarde was lager in de BBM systemen die geïncubeerd waren onder statische omstandigheden dan in de BBM-systemen geïncubeerd in een schudstoof (ongeveer 6.5 en 7.5 log KVE/ml voor statisch respectievelijk schudden). In de praktijk zal in drinkwaterinstallaties deze bacterie concentratie in de bulkvloeistof niet bereikt worden, omdat in deze installaties de bacteriën zich voornamelijk in de biofilm bevinden. De zojuist genoemde volumetrische drempelwaarde voor de statische experimenten komt overeen met ongeveer 3.6 log KVE/cm^2 in biofilms, een dergelijke concentratie wordt algemeen gevonden in drinkwaterinstallaties.
Concluderend, *L. pneumophila* kan in biofilms alleen vermeerderen als intracellulaire parasiet in een gastheer. *H. vermiformis* is zo'n gastheer en deze amoebe wordt regelmatig samen met *L. pneumophila* gedetecteerd. Echter, *H. vermiformis* kan alleen als gastheer voor *L. pneumophia* optreden indien deze amoebe zich vermenigvuldigt, en hangt daardoor af van de aanwezigheid van voldoende en geschikte prooibacteriën. Biofilms in waterleiding installaties hebben over het algemeen een hoge bacteriële diversiteit, en daardoor zijn er bijna altijd geschikte prooibacteriën aanwezig voor protozoa. Om *Legionella* bacteriën uit de waterleidingen te kunnen weren is het noodzakelijk om de biofilm concentraties te verlagen tot niveaus beneden de drempelwaarde, nodig voor groei van protozoa. Dit kan bereikt worden door een zgn. "multi-barrier" benadering,

hiermee wordt een combinatie van methoden bedoeld, zoals het gebruiken van geschikte controlemethoden, het leveren van biologisch stabiel water met een lage concentratie aan voedingsstoffen en het toepassen van materialen voor de waterleidingen die een remmend effect hebben op de biofilmvorming.

Dankwoord

Dankwoord

Zoals ik in een van mijn stellingen al aangeef, kun je het schrijven van een proefschrift goed vergelijken met het zwemmen van een marathon. Niet alleen duren beide altijd erg lang en moet er op het eind nog vaak gesprint worden, er zijn meer overeenkomsten. Als voorbeeld zal ik de IJsselmeermarathon geven, die ik in het verleden heb gezwommen. Zo'n marathon zwem je niet zomaar, het vereist flink veel voorbereiding en trainingsarbeid. Trainingen doe je soms alleen, maar meestal met teamgenoten. De trainingen worden gemaakt door de hoofdtrainer. Hij zet de grote lijnen uit en houdt overzicht over je vorderingen. Naast de hoofdtrainer wordt je ook begeleid door enthousiaste hulptrainers, die je scherp houden. Zo werk je gezamenlijk naar een doel: de finish. Maar uiteindelijk ben jij degene die 'alleen' aan de start staat. In Stavoren spring je in het water, nog 22 km te gaan tot de finish. Je wordt begeleid door een boot met aan boord je coach, de schipper en een jurylid. De schipper bepaalt de koers, je coach geeft informatie, aanmoedigingen en zorgt voor je voeding, en het jurylid controleert of alles volgens de regeltjes gebeurt. Het begin gaat goed, maar gedurende de race ga je door diepe dalen. In de haven van Medemblik staan familie, vrienden en enthousiastelingen op de kant je aan te moedigen bij de laatste loodjes tot de finish!!! Je hebt het gehaald, je doel is bereikt! Die finish heb ik nu ook bereikt met mijn proefschrift, en hoewel ik het weliswaar 'alleen' heb gedaan, had ik het niet gered zonder de hulp van velen, en die wil ik dan ook in dit stuk bedanken.

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... de finish

Curriculum vitae

Melanie Winetta Kuiper werd geboren op 21 juni 1976 te Steenwijkerwold. Hier volgde zij de lagere school. In 1988 ging zij naar de RSG "Jan Hendrik Tromp Meesters" te Steenwijk, waar zij in 1994 haar Gymnasium β diploma behaalde. In datzelfde jaar ging zij Levensmiddelentechnologie studeren aan de Wageningen Universiteit. In maart 2000 studeerde ze af in de richting levensmiddelenleer. Ze heeft hiervoor twee afstudeeronderzoeken gedaan: levensmiddelenmicrobiologie en kwaliteitskunde. Het onderzoek bij de leerstoelgroep Levensmiddelenmicrobiologie was gericht op de ontwikkeling van probiotica voor huisdieren (honden en katten). Tijdens het onderzoek bij de leerstoelgroep Bedrijfskunde heeft ze het supply chain quality management (SCQM) model getest op de biologische varkensvlees keten. Aan het einde van haar studie heeft ze stage gelopen bij het New Zealand Dairy Research Institute (Palmerston North), waar ze onderzoek gedaan heeft naar de effecten van pre- en/of probiotica op de bacteriële samenstelling van de darmflora bij de mens. Vanaf februari 2001 werkte ze als assistent in opleiding (AIO) bij het laboratorium voor Microbiologie en de leerstoelgroep Levensmiddelenmicrobiologie van de Wageningen Universiteit en bij Kiwa Water Research N.V. te Nieuwegein. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Vanaf half april 2006 is ze werkzaam als adviseur bij de Grontmij in De Bilt.

List of publications

Kuiper, M.W., Broeckx, P.-B., Valster, R.M., Wullings, B.A., Smidt, H., Zwietering, M.H., Van der Kooij, D., 2006. Relationships between *Legionella pneumophila*, *Hartmannella vermiformis* and selected water quality parameters in surface water, cooling towers and warm water systems. *In preparation*.

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Kuiper, M.W., Wullings, B.A., Boonstra, H., Valster, R.M., Smidt, H., Van der Kooij, D., 2004. Quantitative detection of *Hartmannella vermiformis* in different aquatic environments using a newly developed real-time PCR method. 10th International symposium on microbial ecology ISME-10, Abstract book page 337.

Abbreviations

ATP	Adenosine triphosphate
BBM	Biofilm-batch model
CFU	Colony forming units
CT	Cooling towers
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
EPS	Extracellular polymeric substances
FISH	Fluorescent in situ hybridization
HPC	Heterotrophic plate count
HV	*Hartmannella vermiformis*
KVE	Kolonie vormende eenheden
LP	*Legionella pneumophila*
LS	*Legionella* spp.
OTU	Operational taxonomic unit
PB	Prey bacterium
PCR	Polymerase chain reaction
PJV	Prescott and James's medium including trace elements
PVCp	Plasticized polyvinyl chloride
PVCu	Unplasticized polyvinyl chloride
QMRA	Quantitative microbial risk assessment
RNA	Ribonucleic acid
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RSS	Residual sum of squares
S	Surface
SW	Surface waters
TTCB	Thermotolerant coliform bacteria
UPGMA	Unweighted pair group method with arithmetic mean
UV	Ultraviolet
V	Volume
WWS	Warm water systems
WWTP	Waste water treatment plants

Addendum

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