Free-living protozoa in drinking water supplies: community composition and role as hosts for *Legionella pneumophila*

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Abstract

Free-living protozoa, which feed on bacteria, play an important role in the communities of microorganisms and invertebrates in drinking water supplies and in (warm) tap water installations. Several bacteria, including opportunistic human pathogens such as *Legionella pneumophila*, are able to survive and replicate within protozoan hosts, and certain free-living protozoa are opportunistic human pathogens as well. However, not much is known about the occurrence of these organisms in drinking water supplies in relation to water quality and other environmental conditions. This lack of knowledge may in part be attributed to the limitations of microscopic techniques and cultivation methods for detection and identification of protozoa. In the investigations described in this thesis, molecular methods were applied to identify communities of free-living protozoa in drinking water supplies and to elucidate the effects of environmental conditions on the growth of these organisms. Molecular methods were also used to detect and identify protozoan hosts, both known and yet-undescribed, for *L. pneumophila*. The eukaryotic communities were studied using terminal restriction fragment length polymorphism (T-RFLP), clone library analyses of partial 18S rRNA gene fragments, and qPCR assays for *Acanthamoeba* spp. and *Hartmannella vermiformis*.

In two groundwater supplies in the Netherlands (T $<$ 18°C) and three supplies in the Caribbean (T $\sim$ 30°C) using seawater as source, highly diverse eukaryotic communities were observed, including free-living protozoa, fungi, and metazoa. A total of 127 operational taxonomic units (OTUs, each OTU contains sequences with $\geq$ 99% similarity) related to free-living protozoa were obtained from the supplies in the Netherlands and 59 OTUs were identified from the supplies in the Caribbean. Free-living protozoa related to the phyla Amoebozoa, Cercozoa, Choanozoa, Ciliophora and Stramenopiles were observed in both regions, whereas the phyla Euglenozoa and Myzozoa were only found in the supplies in the Netherlands. *H. vermiformis*, a described host for *L. pneumophila* was observed in all five supplies, and was a predominant protozoan in the treated water and in the distribution system in one of the examined supplies. *Acanthamoeba* spp. were detected at low concentrations in only a few samples.

In one drinking water supply in the Caribbean, the concentration of *H. vermiformis* correlated with the concentration of *Legionella* spp., and clones related to Amoebozoa predominated the protozoan community. These observations, in combination with the low turbidity and the varying ATP concentrations, indicated that biofilms in this system promoted growth of amoebae and also *Legionella* spp. Ciliophora represented 25% of the protozoan OTUs in another Caribbean supply with elevated ATP concentrations

and turbidity, related to corroding cast-iron pipes. Thus, sediments seem to favor growth of ciliates. Cercozoan types predominated in one of the Caribbean supplies with low ATP concentrations ($<$ 1 ng liter^{-}1) and turbidity ($<$ 1 NTU) in most samples of distributed water. Growth of *H. vermiformis* was also observed in a drinking water supply in the Netherlands with a high concentration of natural organic matter (NOM) combined with an elevated concentration of active biomass (ATP), whereas no growth of this amoeba was observed in the groundwater supply with a low concentration of ATP ($<$ 1 ng liter^{-1}) and NOM ($<$ 0.5 mg C liter^{-1}).

A biofilm batch test (BBT) was applied to amplify and subsequently identify protozoan hosts for *L. pneumophila*. In this test 21 freshwater samples, with added polyethylene cylinders to promote biofilm formation, were inoculated with *L. pneumophila* and subsequently incubated in duplicate at 37°C. Growth of *L. pneumophila* was observed in 16 of 18 water types when the host protozoan *H. vermiformis* had been added. Indigenous *H. vermiformis* was indicated as host in 12 of the 19 BBT flasks with growth of *L. pneumophila*. In none of the water samples incubated at 37°C in the BBT system, growth of *Acanthamoeba* spp. was observed. In several flasks with growth of *L. pneumophila*, the protozoa *Diphylleia rotans*, *Echinamoeba thermarum* and *Neoparamoeba* sp. were identified as candidate hosts. *In vitro* studies are needed to confirm their role as hosts for *L. pneumophila*.

To assess the effects of water temperature on indigenous free-living protozoan communities, four water types were incubated in a BBT system at 20, 30, 37 and 42°C. Only two of the 53 OTUs related to free-living protozoa obtained from these freshwater types at day 0 were also detected after incubation. Multiplication of indigenous *Acanthamoeba* spp. was observed at 20 and 30°C, with only limited growth in one flask at 37°C. Indigenous *H. vermiformis* multiplied at 20, 30 and 37°C, but at 42°C proliferation was only observed in water collected from the river Rhine. Growth of organisms related to Amoebozoa and Stramenopiles was observed at all four temperatures, whereas Cercozoan and Euglenozoan types predominated in the BBT system incubated at 37 and 30°C, respectively. The Amoebozoan *Arachnula* sp. was identified as candidate host for *L. pneumophila* at 42°C, but *in vitro* studies with this organism are needed for confirmation.

These observations indicate that *H. vermiformis*, the predominating protozoan host for *Legionella* spp., is ubiquitous in water supplies and other engineered water installations. This organism can grow at temperatures ranging from less than 20 to 42°C. Proliferation is limited at active biomass concentrations $<$ 1 ng ATP liter^{-1} in combination with NOM concentrations $<$ 0.5 mg C liter^{-1}. However, production of drinking water with such low concentrations of NOM and ATP is not achievable in most cases. Therefore, a combination of measures in water treatment (reducing the NOM concentration) and distribution (prevention of sediment accumulation, disinfection) is needed to reduce the potential for growth of free-living protozoa in water supplies and in water installations.

Contents

Chapter 1

General introduction

1.1 Background of research

Free-living protozoa are ubiquitous in natural freshwater environments, but also multiply in engineered water systems, including wastewater treatment plants, drinking water treatment systems, drinking water distribution systems and tap water installations inside buildings [175, 217, 256, 274]. Free-living protozoa are unicellular heterotrophic eukaryotes, which feed mainly on bacteria and other small biomass particles that are suspended in the water, or are present in biofilms and sediments in natural and engineered freshwater environments [196]. Consequently, free-living protozoa have a considerable impact on the microbial communities in aquatic environments. Many protozoa are selective consumers that can recognize various traits of their potential prey [55, 113]. They play an important role as decomposers and nutrient regenerators, and their metabolization of organic molecules leads to the release of phosphate, ammonia, nitrate and amino acids into the environment [130, 201]. Furthermore, free-living protozoa are an important food source for invertebrates present in aquatic environments [274].

Certain free-living protozoa can be human pathogens and can cause serious inflammations of the brain and eye [132, 287]. Also a wide range of bacteria, including opportunistic human pathogens such as *Legionella* spp, *Burkholderia* spp. and *Mycobacterium* spp., use free-living protozoa as hosts and proliferate within these organisms [257]. Usually, free-living protozoa digest the prey organisms, which are taken up during grazing, but some microorganisms are able to survive or even replicate inside the protozoa [85, 242]. The host environment provides nutrients for growth of these protozoa-resisting bacteria, which are not directly available in drinking water distribution systems and installations [86]. Furthermore, bacteria within the protozoa are protected against ultraviolet radiation and disinfectants used in drinking water treatment [140, 249]. Certain bacteria are able to multiply within protozoa and may subsequently lyse their host, and large numbers of bacteria are released into the aquatic environment or may be expelled from the protozoan in pellets [17, 218]. Host protozoa for pathogenic bacteria and/or human pathogens, such as *Acanthamoeba* spp. and *Hartmannella* spp. [218, 219], and also pathogenic protozoa, e.g., *Naegleria fowleri*, have been isolated from drinking water supplies and warm water installations [175, 204, 217, 274].

Growth of free-living protozoa depends on the diversity and abundance of prey organisms in the biofilm, in the sediments and in the planktonic phase. Water quality is a critical factor for growth of microorganisms including prey of free-living protozoa and therefore will affect the abundance and diversity of free-living protozoa in aquatic systems [267, 278]. Despite the fact that free-living protozoa play a central role in the microbial quality of drinking water in distribution systems and in tap water installations inside buildings, information about their occurrence and conditions that support the growth of these organisms in relation to the water quality is limited [43].

1.2 Free-living protozoa

Description and ecology

Protozoa were described for the first time by the Dutch scientist Antonie van Leeuwenhoek (1632–1723), who had examined various ecosystems with his own developed microscopes. These eukaryotic microorganisms received their name from the Greek words "proton" which means "first" and "zoa" which means "animal". Protozoa and also fungi, metazoa (multicellular animals), viridiplantae and algae belong to the domain of the eukaryotes. Biologists have over the centuries identified about 30,000 species of free-living protozoa [187]. In addition, an equal number of fossil species has been obtained from habitats which range from ocean water to fluid of insects [187]. The cell size of protozoa ranges from 2 to 3000 μm and replication occurs by cell division where one cell splits into new individuals. A variety of protozoa reproduces also sexually, whereby DNA is exchanged between two cells. The majority of protozoa are aerobic, but some are anaerobic and are present, for example, in anaerobic sewage plants or live in symbiosis with other organisms in the rumen. Most protozoa live free in aquatic and wet soil environments, but sporozoa occur as parasites within other organisms. These sporozoa, such as *Cryptosporidium*, *Giardia* and *Entamoeba*, do not belong to the free-living protozoa and are therefore not further described in the present thesis. However, also certain free-living protozoa, which can grow in freshwater, are human pathogens. *Acanthamoeba* spp. [61, 132], *Balamuthia mandrillaris* [277] and *Naegleria fowleri* [287] can cause meningoencephalitis, an infection of the brain and *Acanthamoeba* spp. can also cause keratitis, an infection of the cornea of the eye [132].

Most free-living protozoa are heterotrophic organisms and feed on other organisms including bacteria, algae, fungi, and protozoa, or on organic detritus, to obtain energy and nutrients [196]. However, several protozoa are not exclusively heterotrophic, but combine heterotrophic and autotrophic processes mediated through the presence of functional chloroplasts or symbiotic algae [187]. Protozoa themselves can serve as food for other organisms such as invertebrates in freshwater systems [274] (Fig. 1.1). During grazing, free-living protozoa can take up some microorganisms, including human pathogens such as *Legionella* spp., *Burkholderia* spp. and *Mycobacterium* spp., which cannot be digested by several protozoan species [86, 242, 257]. These bacteria are able to survive or even replicate inside the protozoan host.

Prey of free-living protozoa lives suspended in the water (planktonic) or together with other organisms in sediments or in biofilms attached to living or abiotic surfaces, such as a pipe in a drinking water distribution system. In freshwater systems, biofilms represent the major proportion of bacterial biomass and activity [14, 38]. Biofilms offer a gradient of nutrients and protection towards diverse physical and chemical stress factors including UV exposure [82] and antibiotics [169]. Based on their feeding behavior and their interactions with biofilm-carrying surfaces, free-living protozoa can be divided into four groups [196]. First, the transient group contains predominantly free-swimming organisms which feed on suspended prey. The second group of free-living protozoa, which are attached to a surface, but feed on suspended prey is called the sessile group. The third group consists of amoebae, which

are browsing over a surface and feed on attached prey. The last group is defined as browsers that can do both; swimming freely and feeding on suspended prey, and browsing over a surface and feeding on attached prey.

Grazing by free-living protozoa is an important shaping force for structure and diversity within microbial communities [135, 229, 235]. Free-living protozoa can remove between 30 to 100% of the bacterial production per day [229] and maintain their prey in a "physiological state of youth" [130]. Free-living protozoa graze on bacteria with rates between 0.2 and 1465 bacteria per protozoan per hour, and in general flagellates have the lowest ingestion rates within the free-living protozoa [196]. Many protozoa graze selectively on bacteria and this selection is influenced by various features such as the size of bacterial prey, its motility and the properties of the cell [55, 113]. Therefore, the richness and abundance of bacteria in the planktonic phase, in the biofilm, and in the sediment affects the composition of protozoan communities [113, 196, 231]. However, the bacterial communities are affected by the water quality, for example high concentrations of natural organic matter (NOM) enhance bacterial growth and biofilm formation in drinking water distribution systems [79, 268]. Therefore, the abundance and nature of free-living protozoa in these systems are also affected by the water quality [267, 278]. The quality of the water depends on the composition of the raw water, treatment processes, and the conditions in the distribution system like hydraulics, materials, and residence time. Due to their rapid response to environmental changes, free-living protozoa have been used as water quality indicators [144, 237].

Free-living protozoa are wide-spread all over the world and have been detected in natural habitats including surface water [190], hot mud pools in volcanic areas [236], freshwater lakes in Antarctica [117], desert soil [214] and marine environments [43]. These organisms have been observed in hot springs at temperatures of 56°C [263] and 68°C [76], demonstrating the ability of these organisms to grow or survive under various and extreme conditions. Concentrations of free-living protozoa in drinking water supplies, determined using cultivation methods and microscopy, range from < 1 to 7×10^5 cells liter^{-1} [233, 274]. During harsh conditions such as high temperatures, presence of harmful chemicals, or absence of nutrients or oxygen, free-living protozoa transform to the cyst form to survive. Consequently, bacteria inside the cysts are also protected against most of these harsh conditions [140, 249]. When environmental conditions become more favorable, free-living protozoa transform to the active trophozoite form and feed and reproduce again.

Classification of free-living protozoa

Free-living protozoa are an extremely diverse group of organisms. Consequently, their classification is complex and various taxonomic systems have been developed over the past decades. The distinction between free-living protozoa and algae is often vague, because of the existence of mixotrophic organisms, which feed on other organisms and can also use energy to produce complex organic compounds from inorganic molecules like algae. Until the 1980s, the classification systems were based

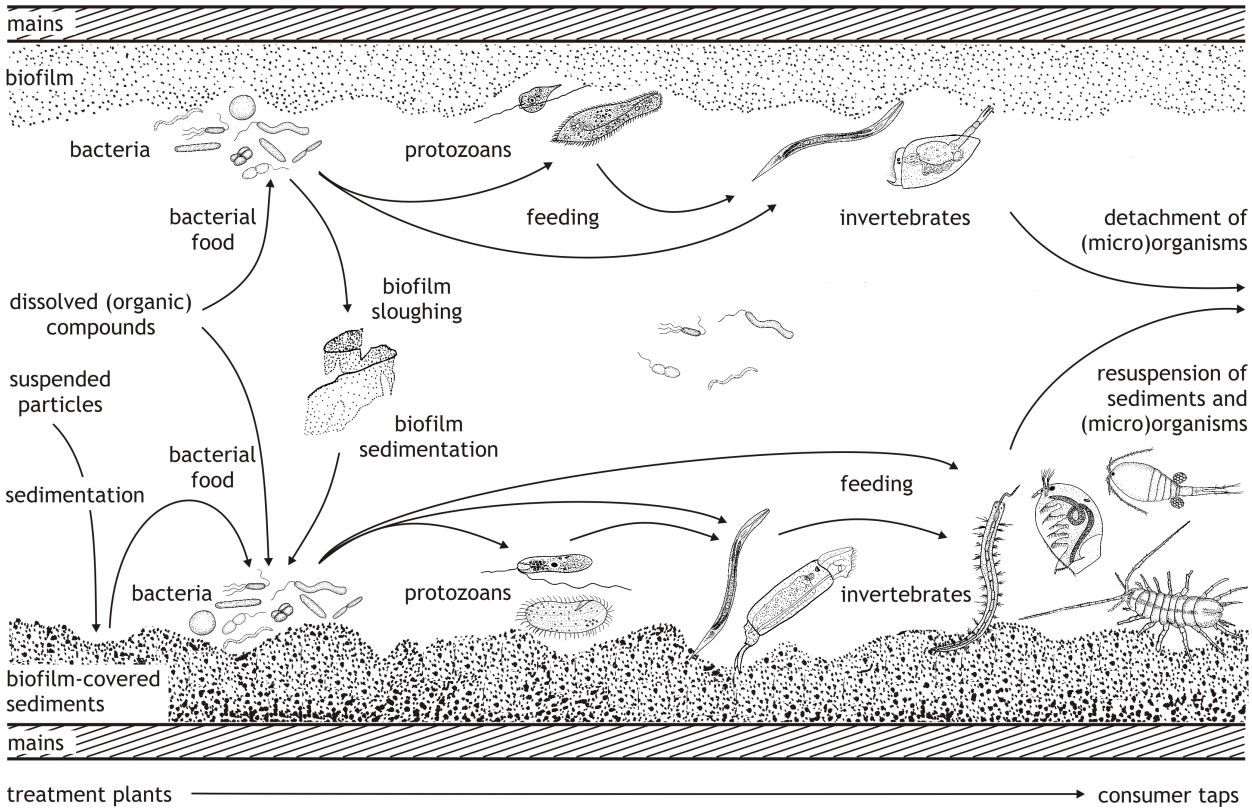

Figure 1.1: Schematic depiction of food supply and feeding in drinking water distribution systems. Source: J. H. M. van Lieverloo et al. 2002 [274]. Reprinted with permission from John Wiley & Sons.

on morphological and biochemical traits of mainly cultured protozoa. However, molecular analyses, which are cultivation-independent, revealed an enormous richness within the free-living protozoa and still yet-undescribed types are discovered. Therefore, classification systems are continuously subjected to changes. Most studies until now have identified free-living protozoa with cultivation methods and microscopy, and classified the free-living protozoa based on morphologic characteristics (traits) into flagellates, ciliates and amoebae. This classification system is first described. Then, a brief overview of the current state of the art of classification of free-living protozoa using molecular techniques is given.

Flagellates are named after the presence of one or more flagella (Latin for whip), which are tail-like projections that have a function in swimming and feeding (Fig. 1.2A). The flagellates are the smallest organisms within the free-living protozoa and their cell size varies between 2 and 20 μm. Flagellates graze mainly on small bacteria ($<$ 1.6 μm) which are suspended in the water [55, 104]. With the flagellum, they draw water containing prey to the base of the flagellum, where the prey is drawn into an oral groove or ingested via pseudopodia. These pseudopodia or “fake feeds”, are temporal bulges filled with cytoplasm, which can be used for feeding and locomotion. Flagellates, however, use these structures only for feeding. Several flagellates are filter feeders; they have a collar of tentacles at the

base of the flagellum that allows only the smallest of prey particles to pass for ingestion. Many flagellates swim freely in the water and are attached temporarily to a surface by means of a stalk. However, they still feed preferentially on suspended prey [220, 232]. Some flagellates graze mainly on attached bacteria and browse over the surface for food [42, 232, 295]. Currently, none of the free-living flagellates have been described as host for pathogenic bacteria or as a human pathogen.

Amoebae are named after the ancient Greek word for "change", because of their irregular and changing cell shape (Fig. 1.2B). Naked amoebae use pseudopodia for crawling over a surface, searching for prey [196]. Prey particles are enclosed by pseudopodia and subsequently digested in food vacuoles. Several amoebae are housed in shells (testate amoebae) and the prey is caught by cytoplasma which reaches through holes in the shell. Afterwards, the prey is brought to the cell body and digested. In general amoebae are more associated with biofilms than with planktonic samples and feed more effectively on attached than on suspended prey [216]. Naked amoebae feed mainly on bacteria while larger species of the testate amoebae feed preferentially on larger ciliates, rotifers and smaller other testate amoebae [102].

Naked and testate amoebae are common inhabitants of freshwater [175, 274] and have been studied extensively, because several species are (opportunistic) human pathogens. *Achanthamoeba* spp. [61], *Balamuthia mandrillaris* [277] and *Naegleria fowleri* [287] can cause meningoencephalitis and *Acanthamoeba* spp. have also been associated with keratitis in persons wearing contact lenses [132]. Furthermore, certain species belonging to the amoebae genera *Acanthamoeba*, *Echinamoeba*, *Hartmannella*, *Naegleria* and *Vahlkampfia* have been described as hosts for *L. pneumophila* (Table 1.1). In addition, also other pathogenic bacteria, like *Bulkholderia* spp., *Mycobacterium* spp., and *Vibrio cholerae*, grow or survive within free-living amoebae [107].

Ciliates are characterized by the hair-like organelles called cilia, which are present in at least one life-cycle of the ciliates (Fig. 1.2C). The cilia are similar in structure to the flagella, but cilia are shorter and present in much larger numbers than flagella. Ciliates used these cilia for locomotion, attachment, feeding and sensation [196]. Ciliates are common inhabitants of natural and engineered freshwater systems. In these environments, ciliates swim freely in the water, browse on a surface or are attached with a stalk to the surface. Ciliates are the most diverse group with regard to their feeding behavior and due to their larger cell size compared to flagellates, ciliates feed on a larger variety of prey such as larger bacteria, algae, fungi and other protozoa [18, 81, 230]. Suspended and attached prey both serves as food for ciliates [3, 81]. Ciliates belonging to the genus *Tetrahymena* have been described as hosts for pathogenic bacteria such as *L. pneumophila* and *Francisella tularensis* [36], but none of the free-living ciliates have been described as human pathogen (Table 1.1).

In recent years, only a few ecological studies have investigated the diversity and identity of free-living protozoan communities in freshwater systems using molecular techniques, which are described in more detail later in this introduction [157, 202, 239]. In the studies described in this thesis, free-living protozoa were identified with cultivation-independent molecular techniques, based on the 18S rRNA gene,

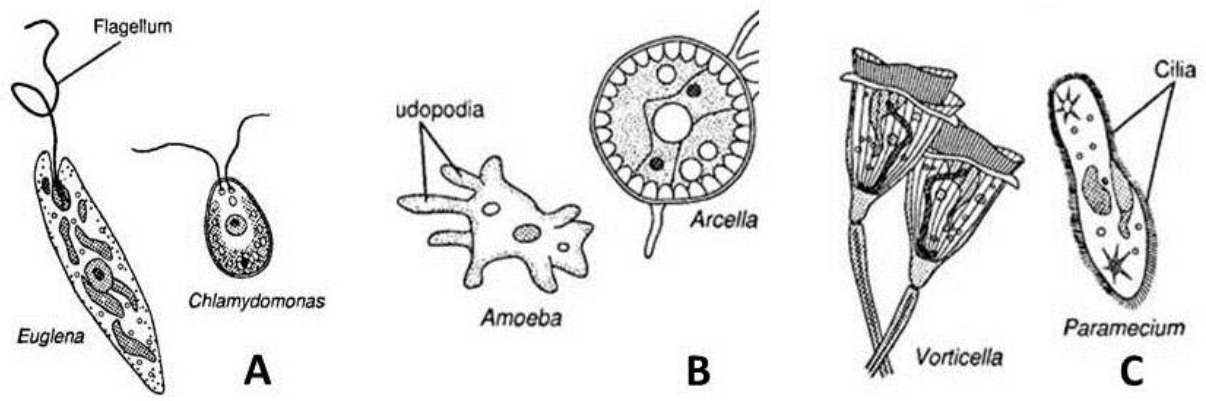

Figure 1.2: Classification of free-living protozoa based on morphological traits. (A) Flagellates (B) Amoebae (C) Ciliates. Reprinted with permission from: http://www.cliffsnotes.com (Classification of protozoa).

and divided into 11 phyla, which clustered within four infrakingdoms (Table 1.1). This classification is based on studies of the taxonomist Cavalier-Smith [46, 48] and the structure in the used database programs [165, 203]. Cavalier-Smith has classified eukaryotes, including protozoa, based on (i) morphological traits, such as the structure of cell organelles, and (ii) molecular phylogenetic research involving ribosomal RNA genes and nuclear protein-coding genes [45].

The **Amoebozoa** phylum is a major group of amoeboid protozoa which vary greatly in size. Many are only 10 to 20 μm in size, but they also include the larger protozoa with a size of about 800 μm. Amoebozoa are common in soils and aquatic habitats. The Amoebozoa phylum contains species which are described as host for pathogenic bacteria (Table 1.1). The Amoebozoa also include the slime molds. The slime mold *Dictyostelium discoideum* has been described as host for *L. pneumophila* [110] and other pathogenic bacteria such as *Aeromonas* spp. and *Pseudomonas aeruginosa* [59, 98]. The **Choanozoa** phylum received its name from the Greek word "choanos" which means "funnel". This group is more closely related to animals than to fungi and they are therefore of great interest to biologists studying animal origins. Choanozoa are either free-swimming in the water column or sessile organisms and a number of species form simple colonies.

The phylum **Apusozoa** comprises several genera of flagellate protozoa [50]. The name Apusozoa derives from the Greek word for footless animals. Apusozoa are usually around 5 to 20 μm in size, and occur in soils and aquatic habitats, where they feed on bacteria. The **Cercozoa** phylum includes many amoebae and flagellates which feed by using pseudopodia [49]. Cercozoan types are commonly abundant and are widespread in soil and freshwater [136], but have never been isolated from marine environments [13]. Amoebae belonging to the **Heliozoa** phylum have a characteristic sun-like appearance for which they are named. They may be found in both freshwater and marine environments.

Table 1.1: Classification of aerobic free-living protozoa in 11 phyla based on molecular techniques.

Infrakingdom	**Phylum**	**Morphological group(s)**	**Species described as host for *L. pneumophila***
Sarcomastigota	Amoebozoa	Amoebae	*Acanthamoeba castellanii* [218]
			Acanthamoeba culbertsoni [177]
			Acanthamoeba palestinensis [6]
			Acanthamoeba polyphaga [218]
			Acanthamoeba royreba [260]
			Balamuthia mandrillaris [227]
			Echinamoeba exundans [88]
			Hartmannella cantabrigiensis [219]
			Hartmannella vermiformis [219]
			Dictyostelium discoideum[a] [110]
	Choanozoa	Flagellates	-[b]
Rhizaria	Apusozoa	Flagellates	-
	Cercozoa	Amoebae,	-
		Flagellates	-
	Heliozoa	Amoebae	-
Excavate	Euglenozoa	Flagellates	-
	Loukozoa	Flagellates	-
	Percolozoa	Amoebae,	*Naegleria fowleri* [186]
		Flagellates	*Naegleria gruberi* [218]
			Naegleria jadini [218]
			Naegleria lovaniensis [260]
			Vahlkampfia jugosa [219]
Alveolata	Myzozoa	Flagellates	-
	Ciliophora	Ciliates	*Tetrahymena pyriformis* [89]
			Tetrahymena thermophila [138]
	Stramenopiles	Flagellates	-

[a] *D. discoideum* is commonly referred to as a slime mold.

[b] - No hosts described.

The **Euglenozoa** phylum includes flagellated protozoa with a cell size between 15 and 40 μm, although some euglenids have larger cells and are about 500 μm long. Some other Euglenozoa feed on bacteria and other small organisms and many euglenids contain chloroplasts to obtain energy through photosynthesis [44]. The **Loukozoa** phylum [48] received its name from the Greek word "loukos" which means "groove". This groove is used for feeding, but little ecological information is available about this group of organisms. The **Percolozoa** phylum is a group of colorless protozoa, including species

that can transform between amoeboid, flagellate and encysted stages. The cell size in the amoeboid stage is typically around 20-40 μm in length, but the flagellate stage is slightly smaller, with two or four flagella anterior to the feeding groove. Usually the amoeboid form prevails when food is plentiful, and the flagellate form is used for rapid locomotion. However, not all members can appear in both forms. Most Percolozoa feed on bacteria and live in soil and freshwater, but some live in marine environments. Species within the Percolozoa are described as hosts for pathogenic bacteria (Table 1.1). Moreover, *Naegleria fowleri* is a human pathogen and infections are often fatal [287].

No ecological information is available about the **Myzozoa** phylum [47]. The **Ciliophora** contains all ciliated protozoa and the name of this phylum refers to the presence of cilia in at least one life-stage. About 8,000 species of these ciliates have been described ranging in size from about 10 to 3,000 μm.

The very diverse group of **Stramenopiles**, also classified as heterokonts include organisms such as algae, fungus-like cells, and parasitic and free-living flagellates which are common in marine environments [156, 197]. More than 100,000 species belonging to this phylum are described. Many are unicellular flagellates and most others produce flagellated cells at some point in their life-cycle. Not all species within the Stramenopiles phylum belong to the free-living protozoa, but a significant fraction of the heterotrophic flagellates feed on other organisms [173].

Detection of free-living protozoa by analyzing the 18S rRNA gene

Free-living protozoa in freshwater habitats were and still are investigated with microscopic techniques and cultivation methods based on enrichment [195]. The cultivated protozoa can be classified based on their morphological characteristics and by using biochemical, immunological and currently also, molecular methods [252]. Cultivation methods are time consuming and selective for certain species, but have the advantage that free-living protozoa become available for further experiments. Over the last two decades, application of molecular methods, mainly based on small subunit ribosomal RNA genes (SSU rRNA), has made it possible to study microbial diversity independently of morphological identification and cultivation [4]. Sequence analysis of the ribosomal RNA (rRNA) genes is currently the method of choice for phylogenetic reconstruction, nucleic acid based detection and quantification of microbial richness. Such genes, which are present in every eukaryotic and prokaryotic cell, contain a conserved DNA-sequence, are relatively straightforward to analyze and contain much information. The classification system of free-living protozoa that is used for the studies in this thesis, is partly based on 18S rRNA gene sequence analysis [45]. Molecular ecology methods based on 18S rRNA amplification and sequencing has revealed an enormous richness of microbial eukaryotes in every environment sampled so far [80]. Each survey yields new protozoan species and genera. Also the identification of free-living protozoa based on the 18S rRNA gene, in a variety of freshwater samples, revealed new sequences.

1.3 Relationship between free-living protozoa and pathogenic bacteria in freshwater systems

Host protozoa for pathogenic bacteria

Bacteria belonging to the genus *Legionella* are the most intensively studied opportunistic pathogens that proliferate within eukaryotic hosts in drinking water systems, warm tap installations, and other engineered water installations. Also other pathogenic bacteria such as *Bulkholderia* spp. and *Mycobacterium* spp. were shown to proliferate within free-living amoebae such as *Acanthamoeba* spp. [242], but less information about the interaction with the protozoan hosts is available. *L. pneumophila* requires a unique combination of nutrients for growth, and can be cultured on or in a medium containing amino acids supplemented with cystein [78]. In 1980, Rowbotham was the first to report growth of *Legionella pneumophila* within the free-living protozoa of the genera *Naegleria* and *Acanthamoeba* using laboratory *in vitro* studies [218]. In 1988, Wadowsky demonstrated that *L. pneumophila* needs free-living protozoa to proliferate in freshwater environments [283]. No growth of *L. pneumophila* was observed in water or biofilms, when protozoa were not added or were removed by a filtration step. However, *Legionella* spp. can survive in water outside the host [151, 181, 283]. Under specific laboratory conditions, with high concentrations of cyanobacteria, growth of *L. pneumophila* has been observed in the absence of protozoan hosts [253, 259, 285]. Microscopic (FISH) analyses demonstrated the presence of *L. pneumophila* and *L. anisa* within amoebae obtained from freshwater systems (Fig. 1.3) [16, 67, 151]. *In vitro* studies with cocultures have revealed that 14 species of amoebae and two species of the ciliated genus *Tetrahymena* can serve as hosts for *L. pneumophila* (Table 1.1). A few *in vivo* studies have demonstrated that *Hartmannella* spp., and *E. exundans*, serve as hosts for *L. pneumophila* in aquatic environments [88, 151, 283]. However, it is unclear whether other eukaryotes that can serve as hosts in *in vitro* studies also serve as hosts for *L. pneumophila* in freshwater environments. The large outbreak of Legionnaires' disease among visitors of a flower show in the Netherlands in 1991 [69] and the presence of *Legionella* spp. in water installations initiated more research on these bacteria and host protozoa in drinking water supplies in the Netherlands [289]. *Hartmannella vermiformis*, a protozoan host for *L. pneumophila*, appeared to be commonly present in natural and man-made freshwater environments in the Netherlands [149].

Growth of *L. pneumophila* in freshwater environments is associated with biofilms and sediments, environments with much food for the protozoan hosts [149]. During grazing, *L. pneumophila* can enter the protozoan cell by phagocytosis. The prey, including *Legionella* spp., are then engulfed by the cell membrane and an internal phagosome is formed by the protozoan. Phagocytosed prey are generally killed in lysosomes, where an acidic pH and lysosomal enzymes digest the bacteria. Afterwards, nutrients are released and can be used by the protozoan hosts. *L. pneumophila* and some other bacteria species have evolved strategies to prevent lysosome-mediated destruction and persist in a vacuole within several protozoan hosts. However, most free-living protozoa are resistant towards this bacterium [71]. In the protected compartment the bacteria multiply and finally many bacteria are released in the water, while the protozoan host is killed (Fig. 1.3). *L. pneumophila* uses similar strategies to establish infection in

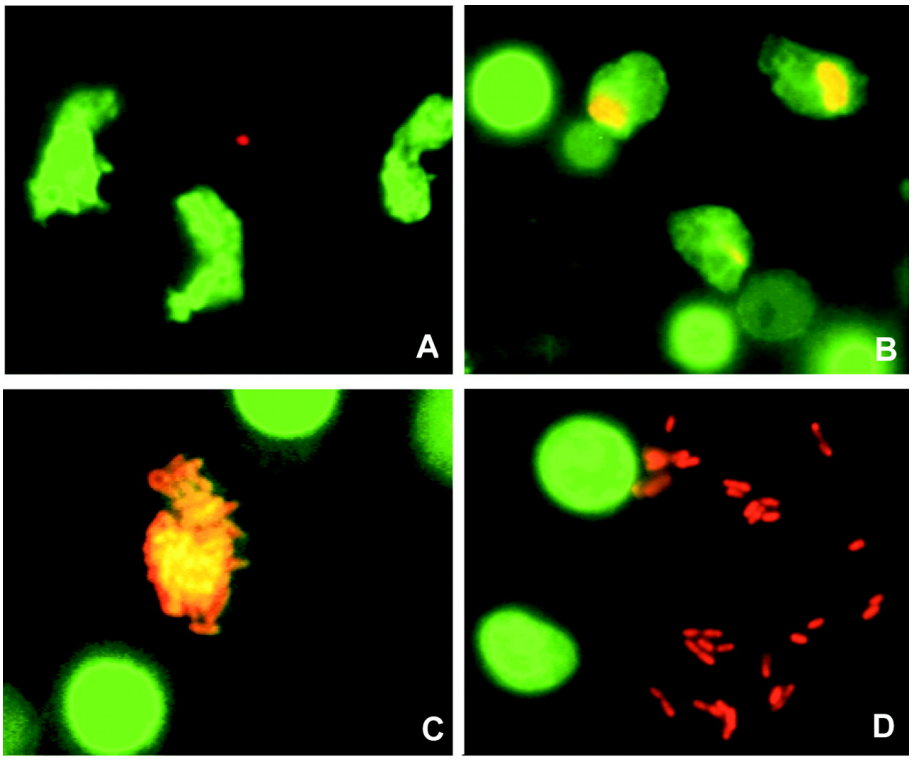

Figure 1.3: Different stages of intracellular proliferation of *L. pneumophila* within amoebae. (A) Amoebae in trophozoite stadium; (B) amoebae just infected with *L. pneumophila*; (C) heavily infected amoeba; (D) *L. pneumophila* just released from a lysed amoeba in the water. Source: Kuiper et al. 2004 [151]. Reprinted with permission from American Society for Microbiology.

both free-living protozoa and human cells [101]. Free-living protozoa do not only provide nutrients for the intracellular *L. pneumophila*, but also provide a shelter when environmental conditions become unfavorable [219]. Inside cysts, *L. pneumophila* is better able to survive disinfection procedures and drying out. After intracellular replication within protozoa, *L. pneumophila* exhibits a higher resistance to chemical disinfection and biocides than *in vitro* grown bacteria [1]. Furthermore, inside the protozoan, *Legionella* bacteria are packaged into pellets, which may enhance a wide distribution of *L. pneumophila* in aquatic environments and in aerosols and thereby favor contact with host protozoa and humans [17, 27].

Legionnaires' disease

Annually, about 6000 cases of the Legionnaires' disease have been reported in Europe in the period from 2005 to 2008 [133]. The first recognized outbreak of pneumonia (inflammation of the lungs), caused by the bacterium *Legionella pneumophila*, occurred among participants of an American Legion in Philadelphia (US) in July 1976 [97]. In total, 180 participants became ill and 29 of them died

due to the pneumonia. The ethological agent was isolated and identified from the air-conditioning system in the lobby of the hotel, and the disease became known as the Legionnaires' disease [174]. Subsequently, three years later the genus *Legionella* was established [33, 97]. Currently, this genus contains about 53 described species, which include 74 different serogroups. The number of species and serogroups within the genus *Legionella* will probably keep increasing like it has done in the recent years [152, 164]. Approximately half of the identified *Legionella* spp., including *L. pneumophila*, *L. bozemanii*, *L. longbeachae*, and *L. dumoffii* have been associated with cases of the disease [86, 180]. In about 95% of the cases of Legionnaires' disease in Europe in 2007 and 2008, *L. pneumophila* was identified as the etiological agent and about 85% of these cases were caused by serogroup 1. This demonstrates that this serogroup is more infectious than the other 14 serogroups of *L. pneumophila*. Currently, with sequence-based typing, using seven genes, hundreds of different types within the *L. pneumophila* species have been distinguished, including patient isolates, isolates from the environment or both [99, 206].

Occurrence of *Legionella* spp. in freshwater

L. pneumophila is a common inhabitant of natural and engineered freshwater systems like hot springs, treated sewage, cooling towers, whirlpools, and (warm) tap water installations [40, 93, 153, 208]. The bacterium has been isolated from freshwater environments at temperatures below 10°C up to 60°C, but growth of *L. pneumophila* is restricted to temperatures between 25°C and about 42°C in aquatic environments [284, 292]. Many studies have investigated the presence of mainly *L. pneumophila* in freshwater systems with a standardized cultivation method on a complex solid medium that contains amino acids, cystein, and antibiotics [78, 184]. However, recent studies using molecular techniques, demonstrated that uncultured yet-unidentified *Legionella* spp. are ubiquitously present in freshwater systems [40, 66, 289]. In drinking water systems in temperate regions, with water temperatures below 20°C, concentrations of uncultured *Legionella* spp., range from 1×10^2 to 8×10^5 cells liter^{-1} [288, 289] and *L. pneumophila* is sporadically present at low concentrations. In warm tap water installations in temperate regions, *L. anisa* is more frequently observed than *L. pneumophila* [75, 291], but the number of reported cases of infection caused by *L. anisa* is extremely low. Also proliferation of *L. anisa* takes place within free-living protozoa in aquatic environments [85, 245]. Probably many species or maybe all species within the *Legionella* genus need free-living protozoa to multiply in water systems. Cultivation of *Legionella* spp. obtained from drinking water with temperatures $<$ 20°C failed. This also indicates that special growth conditions, which prevail within free-living protozoa, are needed for growth [288].

1.4 Aim and outline of the thesis

Free-living protozoa are ubiquitous in natural and engineered freshwater systems, and host protozoa for *L. pneumophila* and other pathogenic bacteria are common inhabitants as well in these systems.

The water quality and temperature affects the abundance and richness of free-living protozoa. However, information about the presence and identity of free-living protozoa, including human pathogens and/or hosts for pathogenic bacteria, in freshwater environments in relation to the water quality is limited. This may be attributed to the limitations of microscopic techniques and cultivation methods for detection and identification of these organisms. Therefore, molecular methods were applied to investigate the communities of free-living protozoa in two drinking water supplies in the Netherlands and in three supplies in the Caribbean region. These water types varied in temperature and in concentrations of natural organic matter (NOM) which are important factors effecting heterotrophic microbial growth in freshwater. In addition, biofilm batch tests with various freshwater types were applied to identify predominating, known, and yet-undescribed potential hosts for *L. pneumophila* and to determine the effect of temperature on the indigenous protozoan communities grown in biofilms.

Concentrations of two frequently observed protozoan hosts for *L. pneumophila*, viz., *Hartmannella vermiformis* and *Acanthamoeba* spp., were determined with specific quantitative PCR methods [149, 205]. No primers were available to amplify all described free-living protozoa; therefore eukaryotic primers, which amplify approximately 550 base pairs of the 18S rRNA gene, were used. Fingerprints of eukaryotic communities were generated using terminal restriction fragment length polymorphism (T-RFLP) analysis [159]. These fingerprints give an overview of the eukaryotic richness and can be used to compare eukaryotic communities present in different water types, but the eukaryotes cannot be identified using this technique. To identify free-living protozoa and other small eukaryotes present in freshwater samples, DNA sequences, obtained with PCR and cloning methods, were compared with sequences in databases. On the basis of the number of analyzed DNA sequences and the similarity between the obtained DNA sequences, the richness within eukaryotic and protozoan communities in freshwater types was estimated. Moreover, free-living protozoa, as well as fungi, metazoa, algae and viridiplantae could be identified using these methods. The identification of fungi yielded information about the abundance of potential pathogenic fungi and metazoa in the examined water types.

The objectives of the research described in the present thesis were:

(i) to elucidate the identities and richness of free-living protozoa predominating in various drinking water supplies;
(ii) to identify known and yet-undescribed protozoan hosts for *L. pneumophila* and other *Legionella* spp. in freshwater environments;
(iii) to identify conditions, favoring the growth of free-living protozoa in drinking water distribution systems.

To achieve these objectives, a number of studies were conducted that are described in the following Chapters:

Chapter 2 describes efforts aiming at determining the richness and identity of free-living protozoa and other small eukaryotes predominant in two drinking water supplies in the Netherlands that both use groundwater as their source. Samples of treated water, distributed water and distribution system

biofilms were collected from a supply with a low concentration of natural organic matter (0.5 mg of C liter^{-1}) and from a supply with a high concentration of organic matter (7.9 mg C liter^{-1}). The eukaryotic communities were studied using terminal restriction fragment length polymorphism, clone library analyses of partial 18S rRNA gene fragments and a *H. vermiformis*-specific qPCR.

In **Chapter 3**, experiments conducted for elucidation of the identity of protozoan hosts for *L. pneumophila* under conditions resembling those in engineered water systems are described. For this purpose a biofilm batch test (BBT) was used. Samples of 600 ml collected from 21 engineered freshwater systems, with added polyethylene cylinders to promote biofilm formation, were inoculated with *L. pneumophila* and subsequently incubated at 37°C for 20 days. During incubation, growth of *L. pneumophila* was determined in the BBT flasks and subsequently predominating known and yet-undescribed hosts for *L. pneumophila* were identified with molecular techniques based on the 18S rRNA gene.

Water temperature has a distinct impact on the protozoan community in water supplies. **Chapter 4** focuses on the richness and identity of free-living protozoa and other small eukaryotes in three drinking water supplies in the Caribbean region with different water treatment and distribution systems. Cultivable *Legionella* spp. are ubiquitously present in this drinking water, which is produced from sea water and has a temperature of about 30°C. The concentrations of *Acanthamoeba* spp. and *H. vermiformis* were quantified with qPCR methods. In addition, predominant free-living protozoa and other hosts for *L. pneumophila* in the three supplies were identified. The conditions favoring growth of free-living protozoa were elucidated by comparing characteristics of the water quality and treatment in the three supplies.

To assess the effects of water temperature, various water types were incubated in the BBT system at 20, 30, 37 and 42°C **(Chapter 5)**. Water samples of 1800 ml of the river Rhine, a cooling tower and two tap water installations were inoculated with *L. pneumophila*, to enable detection and identification of protozoan hosts for *L. pneumophila*. Growth of the host protozoa *Acanthamoeba* spp. and *H. vermiformis* was quantified with qPCR methods. In addition, different predominant free-living protozoa were identified in the BBT at the four incubation temperatures.

Chapter 6 summarizes and discusses the contribution of the present work to the current knowledge about growing conditions of free-living protozoa and especially host protozoa for *L. pneumophila* in drinking water supplies. In addition, some research needs related to the presence of free-living protozoa in water supplies are identified.

Chapter 2

Free-living protozoa in two unchlorinated drinking water supplies, identified by phylogenic analysis of 18S rRNA gene sequences

Abstract

Free-living protozoan communities in water supplies may include hosts for *Legionella pneumophila* and other undesired bacteria and also pathogens. This study aimed at identifying free-living protozoa in two unchlorinated groundwater supplies, using cultivation-independent molecular approaches. For this purpose, samples ($< 20^{\circ}$C) of treated water, distributed water and distribution system biofilms were collected from supply A, with a low concentration of natural organic matter (NOM) ($<$ 0.5 ppm of C), and from supply B with a high NOM concentration (7.9 ppm of C). Eukaryotic communities were studied using terminal restriction fragment length polymorphism and clone library analyses of partial 18S rRNA gene fragments and a *Hartmannella vermiformis*-specific quantitative PCR (qPCR). In both supplies, highly diverse eukaryotic communities were observed, including free-living protozoa, fungi, and metazoa. Sequences of protozoa clustered with Amoebozoa (10 operational taxonomic units [OTUs]), Cercozoa (39 OTUs), Choanozoa (26 OTUs), Ciliophora (29 OTUs), Euglenozoa (13 OTUs) Myzozoa (5 OTUs) and Stramenopiles (5 OTUs). A large variety of protozoa were present in both supplies, but the estimated values for protozoan richness did not differ significantly. *H. vermiformis* was observed in both supplies but was not a predominant protozoan. One OTU with the highest similarity to *Acanthamoeba polyphaga*, an opportunistic human pathogen and a host for undesired bacteria, was observed in supply A. The high level of NOM in supply B corresponded with an elevated level of active biomass and with elevated concentrations of *H. vermiformis* in distributed water. Hence, application of qPCR may be promising in elucidating the relationship between drinking water quality and the presence of specific protozoa.

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2.1 Introduction

Free-living protozoa are ubiquitous in natural freshwater environments [22, 149, 198, 264] but also proliferate in engineered water systems, including water treatment systems [5, 175, 256], distribution systems [21, 274], and tap water installations inside buildings [217, 255]. Concentrations of protozoa, determined using cultivation methods and microscopy, range from < 1 to 10^4 cells liter^{-1} in treated water [5, 175, 256, 274] and from < 1 to 7 x 10^5 cells liter^{-1} in distribution systems [21, 226, 233, 274]. Genera of free-living protozoa commonly observed in these systems and in tap water installations include *Acanthamoeba*, *Echinamoeba*, *Hartmannella*, *Platyamoeba*, *Vahlkampfia*, and *Vannella* [175, 221, 255, 256]. In warm water systems, certain free-living protozoa, e.g., *Acanthamoeba* spp. [219], *Balamuthia mandrillaris* [227], *Echinamoeba exundans* [84], *Hartmannella* spp. [151, 219], *Naegleria* spp. [186, 218], *Tetrahymena* spp. [89, 138], and *Vahlkampfia jugosa* [219], serve as hosts for *Legionella pneumophila*, the etiologic agent of Legionnaires' disease. High concentrations of *L. pneumophila* are generally associated with the proliferation of host protozoa in biofilms [149, 215]. In addition, other amoeba-resistant, potentially pathogenic bacteria, e.g., *Burkholderia* spp. [125] and *Mycobacterium* spp. [147], have been observed in man-made aquatic environments [107]. Free-living protozoa may enhance the multiplication of bacteria, serve as a transmission vector, or serve as a shelter against unfavorable environmental conditions, such as the presence of disinfectants. Furthermore, certain free-living protozoa are human pathogens, e.g., *Naegleria fowleri* [287], *Balamuthia mandrillaris* [277], and *Acanthamoeba* spp. [61] can cause encephalitis. *Acanthamoeba* spp. have also been associated with keratitis in persons wearing contact lenses [132].

Free-living protozoa feed on bacteria, algae, fungi, other protozoa, and organic detritus in biofilms or in the planktonic phase, thereby affecting the structure of microbial communities. In turn, the community of free-living protozoa depends on the richness and abundance of bacteria in the biofilm and in the planktonic phase [113, 196, 198, 211, 231, 234]. Water quality is a critical factor for biofilm formation in distribution systems and tap water installations and therefore will affect the abundance and richness of free-living protozoa in these systems [267, 278]. However, information about the presence and identity of free-living protozoa in water supplies in relation to the quality of treated water is scarce, which may be attributed to the limitations of microscopic techniques and cultivation methods for detection and identification of these organisms, e.g., low detection limits and selectivity for specific groups [95].

In this study, we applied a variety of cultivation-independent techniques, viz., quantitative PCR (qPCR), terminal restriction fragment length polymorphism (T-RFLP) analysis, and cloning and sequencing of eukaryotic 18S rRNA gene fragments, for the detection and identification of free-living protozoa predominating in two unchlorinated groundwater supplies. The concentrations of dissolved natural organic matter (NOM) in treated water at the plant were < 0.5 mg C liter^{-1} and 7.9 mg C liter^{-1}, covering the entire range of NOM concentrations in drinking water in the Netherlands. The objectives of the study were (i) to elucidate the identities of and richness in the free-living protozoa predominating in these two different water supplies and (ii) to trace the presence of host protozoa for *L. pneumophila*

and pathogenic free-living protozoa. The study revealed that treated water and biofilms in the distribution systems of both water supplies contained a large variety of free-living protozoa, including protozoan hosts for *Legionella* spp.

2.2 Materials and methods

Selected water supplies

Two groundwater supplies in the Netherlands, distributing drinking water with different NOM concentrations, were selected (Table 2.1). In supply A, with an annual production of 5.6 x 10^6 m^3 and a supply area of ca. 40 km^2 without service reservoirs, aerobic groundwater abstracted from a sand aquifer is aerated to remove CO_2, followed by limestone filtration to increase the pH and hardness of the water (see Fig. A.1 in Appendix A for details). The treated water of supply A (TW-A) contains a low concentration of NOM ($<$ 0.5 mg C $liter^{-1}$), measured as nonpurgable organic carbon (NPOC). In supply B, with an average annual production of 2.5 x 10^7 m^3 and a supply area of ca. 1,000 km^2 with several service reservoirs, anaerobic groundwater abstracted from below a peat layer is treated by intensive aeration, rapid sand filtration, caustic dosage followed by pellet softening, aeration, and a second stage of rapid sand filtration (see Fig. A.1 for details). The two stages of rapid sand filtration remove ammonia, iron, and manganese. The NOM concentration in the treated water of supply B (TW-B) is 7.9 mg C $liter^{-1}$. Both water types are treated and distributed without chemical disinfection [267].

Sample collection

During all seasons of the year 2005, samples of treated water were collected at both plants. In September and October 2005, samples of the biofilms in pipe segments of both distribution systems were taken. Distances between sample locations and the treatment plant ranged from 0.4 to 6.0 km for supply A and from 17.1 km to 35 km for supply B. In July and November 2007, treated water and water from both distribution systems were collected. The samples of the distributed water were taken at the same locations where biofilms were collected. The numbers and letters in sample names indicate the locations of samples in the distribution systems, e.g., BF-A1 and DW-A1 indicate that the biofilm sample and the distributed water sample, respectively, were collected at location 1 in supply A (see Fig. A.2 for details). The water samples were stored at 4°C in sterile glass containers and processed within 24 h. At seven locations in the distribution system of supply A and at eight locations in the distribution system of supply B, segments (30 cm) of unplasticized polyvinyl chloride pipes (diameter, 110 mm) were removed, after thorough cleaning of the outer surface, and subsequently placed in plastic cylinders containing water from the distribution system. The samples were stored at 4°C and processed within 24 h. The attached biomass at the inside surface was collected by swabbing $\pm$ 20 cm^2 with three sterile cotton swabs (Copan Innovation, Italy). These swabs were placed in 10 ml phosphate-buffered saline, and the biomass was removed from the swabs by four 2-min sonication steps in a water bath at

Table 2.1: Quality characteristics treated water at the treatment plants of supply A and supply B. Mean, minimum, and maximum values are shown, based on routine monitoring over a period of one year.

Parameter	TW-A value			TW-B value		
	Mean	Min.	Max.	Mean	Min.	Max.
Temperature (°C)	10.0	9.5	11.5	11.5	10.0	13.5
pH	7.8	7.2	8.2	7.6	7.4	8.1
O_2 concn (mg liter^{-1})	6.4	5.6	7.8	5.9	3.9	8.3
HCO_3 concn (mg liter^{-1})	98	92	124	282	273	308
Cl concn (mg liter^{-1})	13	11	14	28	27	31
Ca concn (mg liter^{-1})	35.4	32.9	39.6	32.7	25.7	52.8
Mg concn (mg liter^{-1})	2.37	2.07	2.73	9.72	8.36	10.9
Total hardness (mmol liter^{-1})	0.98	0.90	1.09	1.22	1.04	1.68
Fe concn (μg liter^{-1})	<20	<20	<20	25	<20	73
Mn concn (μg liter^{-1})	<10	<10	<10	<10	<10	<10
SO_4 concn (mg liter^{-1})	16	13	19	<10	<10	<10
NH_4 concn (mg liter^{-1})	<0.05	<0.05	<0.05	<0.05	<.05	<0.05
NPOC concn (mg C liter^{-1})	0.33	<0.3	0.49	7.9	7.6	8.3

a frequency of 40 kHz and an average power input of 0.015 W/ml [168]. Total ATP concentrations in biofilms and the planktonic phase, representing the active biomass, were determined by ATP analysis as described by Magic-Knezev and van der Kooij [168].

Water filtration and DNA extraction

Samples of 1.25 to 3 liters of treated water and 0.5 liter of distributed water were filtered through a 1.2-μm-pore-size and 55-mm-diameter RTTP Isopore membrane (Millipore, Molsheim, France). Samples of biomass suspended in phosphate-buffered saline were filtered through a 1.2-μm-pore-size and 25-mm-diameter RTTP Isopore membrane (Millipore). DNA was isolated and purified using a Fast DNA spin kit for soil (Bio 101, Carlsbad, CA) following the instructions of the manufacturer, with the exception that 2-ml tubes containing lysing matrix E, sodium phosphate, MT buffer, and filter with sample were processed in a FastPrep instrument (Bio 101) two times for 30 s each at speed setting 5.5. The isolated DNA was eluted in 200 μl DNase- and pyrogen-free water. Distilled water (DNase and RNase free) was used as a negative control in each experiment to check for possible DNA contamination during filtration, DNA extraction, and PCR amplification. In addition, all samples were spiked with *Hartmannella vermiformis* ATCC 50237 to check for the presence of inhibitors in the samples. DNA was subsequently used for the characterization of eukaryotic community composition and for quantification of *H. vermiformis* populations.

Detection of *H. vermiformis* by quantitative PCR

Quantitative PCR assays were performed in 96-well plates, using an iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Veenendaal, the Netherlands) as described by Kuiper et al. [149]. Experiments were performed in duplicate, using undiluted and 10-fold diluted DNA extracts as templates. Quantification was based on a calibration curve for a suspension with a known number of *H. vermiformis* cells that was analyzed in different DNA dilutions with each series of samples. The detection limit was one *H. vermiformis* cell per reaction.

PCR for T-RFLP and cloning

PCR was performed with a GeneAmp PCR 9700 system (Applied Biosystems, Nieuwerkerk aan de IJsel, the Netherlands), using a reaction mixture (50 μl) with 10 μl template DNA. PCR was performed with 5% and 6.7% of the total DNA extracted from the treated water and biofilms, respectively. Fragments of the 18S rRNA gene were amplified with the eukaryotic primers (labeled at the 3' end with 6-carboxyfluorescein) Euk1a-f [243] and Euk516-r [4]. Amplification conditions were as follows: preheating at 94°C for 130 s, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 130 s, and a terminal extension at 72°C for 7 min.

T-RFLP analysis

Fluorescently labeled PCR products (45 μl) were purified by using a DNA Clean & Concentrator-5 kit (BaseClear, Leiden, the Netherlands) and redissolved in 20 μl of distilled water. The digestion reaction mixture (20 μl) contained 5 U of HhaI (Promega), 2 μl of buffer C (Promega), 12.5 μl of distilled water, and 5 μl of the PCR product and was incubated at 37°C for 6 h. The mixture was cleaned as described above and redissolved in 15 μl distilled water. The restriction digestion product (5 μl) was mixed with 15 μl loading buffer (Hi-Di formamide [Applied Biosystems] and GS-500 ROX [Applied Biosystems], 15:1 [vol/vol], as an internal standard). The injection time was 5 s for analysis of terminal restriction fragments, and the run time was 35 min. The fluorescently labeled terminal restriction fragments were analyzed by electrophoresis on an ABI Prism 310 genetic analyzer (Applied Biosystems) in Genescan mode. Electropherograms were imported into a genomic fingerprint analysis program, Bionumerics v. 4.6 (Applied Maths, Sint-Martens-Latem, Belgium), and fragment sizes were calculated. Banding patterns were compared using a densitometric curve-based method that evaluates the positions and intensities of bands to generate pairwise similarity scores (Pearson coefficients), and these were subsequently used for cluster analysis.

Cloning and sequencing of PCR product

The identities of the predominant eukaryotes in the treated water at the plant and in the biofilm of the distribution system were determined by cloning and sequence analysis of approximately 550-bp 18S rRNA gene fragments amplified with the primers Euk1a-f and Euk516-r. The PCR products were cloned using the pGEM-T Easy II vector system. The DNA inserts of randomly selected positive clones were sequenced using the Euk1a-f and Euk516-r primers (BaseClear). One hundred thirty-four and 136 clones of the treated water samples and 43 to 50 clones of the biofilm samples were analyzed.

Phylogenetic analysis of partial sequences

Operational taxonomic units (OTUs) were defined as 18S rRNA gene sequences that shared $\geq$ 99% sequence similarity. The obtained sequences of approximately 550 bp were compared to sequences in the National Center for Biotechnology (NCBI) database by BLAST searches and were also imported and aligned into the SILVA 94 SSU Ref database [203], released in April 2008, using the ARB software package [165]. A distance matrix (no filter and no corrections) was calculated for all clones. This distance matrix was used as an input file in the DOTUR program [223]. OTUs for the purpose of community analysis were defined by a 1% difference in nucleic acid sequences, as determined using the furthest neighbor algorithm in DOTUR. Similarity percentages were determined for complete and partial 18S rRNA gene sequences in the SILVA 94 SSU Ref database for genera and species closely related to the obtained OTUs. The partial sequences used correspond to the fragments amplified with the primers Euk1a-f and Euk516-r. The OTU richness was estimated by the Chao1 estimator [51] and was calculated from randomized data as described by Hughes et al. [122].

The similarity of each OTU to 18S rRNA gene sequences in the SILVA 94 SSU Ref database was analyzed by adding one representative sequence of each OTU to the main phylogenic tree by using parsimony criteria without changing the overall tree topology. The POS_VAR_Eukarya_94 filter (excludes highly variable positions 1 to 7) was used. The obtained sequences were divided into taxa based on the classification system of Cavalier-Smith [46] and the structure in the SILVA 94 SSU Ref database [203]. Sequences with similarities to described species of $<$ 75% were excluded from further analysis.

Statistical analysis

The *F* test, with log transformation of the concentrations, was used to determine the difference between the concentrations of *H. vermiformis* in the distributed water in the summer and the autumn.

Nucleotide sequence accession numbers

All partial 18S rRNA gene sequences determined in this study have been deposited in GenBank under accession numbers EU860442 to EU860974.

2.3 Results

Active biomass (ATP) and water temperature

ATP concentrations in treated water and in distributed water at supply A generally were below 1.0 ng liter^{-1}, and the average was 123.4 $\pm$ 87.7 pg ATP cm^{-2} in the biofilm in the pipes. The concentration of active biomass in treated water of supply B was 10.6 $\pm$ 4.9 ng ATP liter^{-1}, and that in the distribution system was 4.7 $\pm$ 1.2 ng ATP liter^{-1}, with a biofilm concentration of 334.7 $\pm$ 226.1 pg ATP cm^{-2}. The temperature of the treated water at both plants was close to 10°C and showed little variation during the seasons (Table 2.1). For supply A, the average temperature of the water samples collected from the distribution system was 14.8 $\pm$ 2.3°C in July and 11.1 $\pm$ 0.6°C in November. The average temperature of the distributed water in supply B was 13.6 $\pm$ 1.5°C in July and 12.5 $\pm$ 0.8°C in November. Hence, the temperature of the water in the distribution system increased during the summer. In summary, the concentration of active biomass in supply B was higher than that in supply A, and both water types were characterized by relatively low temperatures.

T-RFLP analysis of eukaryotic communities

T-RFLP analyses using 18S rRNA gene primers revealed complex eukaryotic communities in the water samples of both supplies (Fig. 2.1). The fingerprints of the samples of each supply clustered together, indicating that the water type affected the eukaryotic community. Fingerprints of TW-A analyzed in duplicate showed a minimum similarity of 90%, and those obtained in different seasons showed similarities between 61.8% and 78.7%. Duplicate fingerprints of each TW-B sample showed a minimum similarity of 87%, and the fingerprints of different samples showed similarities between 70.5% and 81.3%. Hence, the eukaryotic communities in treated water showed some variation, but more variation was observed between the fingerprints of the biofilm samples within each supply.

Table 2.2: Classification of clones of eukaryotes retrieved from treated water and biofilms of supply A and supply B.

Kingdom or subkingdom	Water supply A[a]				Water supply B[a]				All analyzed samples	
	TW-A1		BF-A		TW-B1		BF-B			
	No.(%) OTUs	% in library	No.[b](%) OTUs	% in library	No.(%) OTUs	% in library	No.[b](%) OTUs	% in library	No.[b](%) OTUs[b]	% in library
Free-living protozoa	26(48.1)	33.4	29(43.3)	64.5	43(67.2)	52.2	31(56.4)	36.6	127(58.0)	253(46.4)
Fungi	7(13.5)	8.9	12(17.9)	20.3	4(6.3)	2.9	6(10.9)	5.2	27(12.3)	51(9.4)
Metazoa	4(7.7)	24.4	3(4.4)	4.3	10(15.6)	23.5	14(25.5)	47.8	28(12.8)	134(24.6)
Protophyta and plants	3(5.8)	4.4	2(3.0)	1.5	0.0(0.0)	0.0	1.0(1.8)	0.7	5(2.3)	9(1.7)
Organisms with $<$ 75% similarity	12(25.0)	28.9	10(14.9)	9.4	7(10.9)	21.4	3(5.5)	9.7	32(14.6)	98(17.9)
Total	52(100)	100	56(100)	100	64(100)	100	55(100)	100	219(100)	545(100)

[a] TW, treated water obtained directly from plant; BF, biofilm. The biofilm data are totals for the three analyzed biofilm samples.

[b] The total number of OTUs includes OTUs which were obtained from more than one sample type.

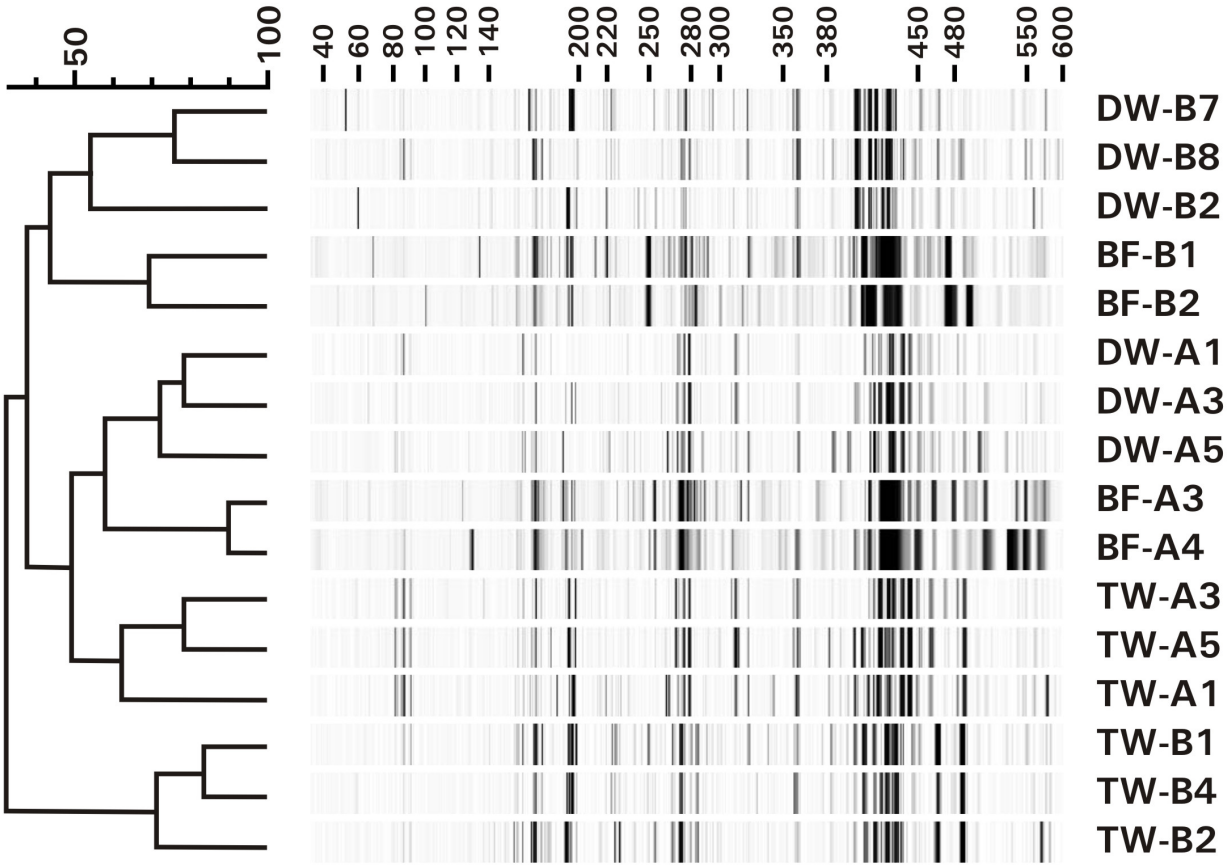

Figure 2.1: Dendogram, created by unweighted-pair group method using average linkages, of T-RFLP fingerprints of treated water (TW), biofilm (BF) from the distribution system and distributed water (DW) of supply A and supply B. Samples of distributed water were taken from different locations in July, and biofilm samples from different locations were taken in September and October.

Richness of eukaryotic communities in treated water and in biofilms

Clone libraries were constructed for one treated water sample and three biofilm samples from each supply. In total, 545 partial 18S rRNA gene sequences of 550 bp were analyzed (Table 2.2). The results for the three biofilm samples were combined for comparison with the results for the treated water samples. All sequences showed the highest similarity to 18S rRNA gene sequences in the NCBI and the SILVA databases (release 94; April 2008), confirming the specificity of the primers used for eukaryotic sequences.

A total of 219 different OTUs (sequence similarity of $\geq$ 99%) were distinguished (Table 2.2). Eight OTUs were observed in more than one sample type, and therefore the sums of the OTUs in Tables 2.2 and 2.3 give excess values. The other 211 OTUs were unique for specific samples, demonstrating the

Table 2.3: Richness of organisms in clone libraries for treated water at the plant and biofilms in the distribution systems of supply A and supply B.

Source and type of organism	No. of clones	No. of OTUs identified	Coverage index[a]	Total OTU richness (Chao1 estimation)[b]		
				Mean	Min.	Max.
Eukaryotes in clone libraries						
Supply A	272	107	39.7	159	136	204
Supply B	273	115	42.1	145	91	277
Total	545	219[c]	40.2	390	328	487
Free living protozoa in clone libraries						
Supply A	133	55[c]	40.6	113	81	187
TW-A	44	26	56.8	34	28	55
BF-A[d]	89	29	33.7	158	70	444
Supply B	120	72[c]	60.0	163	112	274
TW-B	71	43	60.6	134	75	297
BF-B[d]	49	31	63.3	45	32	88
Total	253	127[c]	50.2	281	212	407

[a] Number of OTUs/number of sequences x 100% [105].

[b] The Chao index [51] was calculated with DOTUR [223].

[c] The total number of OTUs excludes OTUs which were obtained from more than one sample type.

[d] The data are totals for the three analyzed biofilm samples.

high level of richness in the eukaryotic communities in the two supplies. Table 2.3 shows that the coverage of the clone libraries for both supplies was similar and also that the estimated total OTU richness values were not significantly different between the two supplies. In treated water of both supplies, the free-living protozoa constituted the largest proportion ($>$ 48%) of the obtained OTUs, with fungi having the second largest number in supply A and metazoa having the second largest number in supply B (Table 2.2). In addition, protophyta and plants were represented. Thirty-two (14.6%) of the obtained OTUs, one of which was detected in TW-A and in BF-A, had similarity percentages below 75% for described sequences in the SILVA database and remained unidentified (Table 2.2).

A total of 27 OTUs (12.3%) showed highest similarity to phyla within the fungi, viz., Chytridiomycota (3 OTUs), Zygomycota (2 OTUs), Ascomycota (20 OTUs), and Basidiomycota (2 OTUs). Two of these OTUs, which showed the highest similarity to *Triparticalar arcticum* and an uncultured Banisveld eukaryote, were retrieved from both supplies. A total of 28 OTUs (12.8%) showed highest similarity to metazoan phyla, viz., Porifera (5 OTUs), Cnidaria (8 OTUs), Platyhelminthes (1 OTU), Rotifera (2 OTUs), Gastrotricha (4 OTUs), Nematoda (2 OTUs), Annelida (2 OTU), and Arthropoda (4 OTUs).

Two of these OTUs were retrieved from both supplies and showed the highest similarity to *Lepadella patella* and *Rhabdolaimus terrestris*. The clone libraries of BF-B5 and BF-B6 are predominated by an OTU with highest similarity (99%) to the metazoan freshwater jellyfish *Craspedacusta sowerbyi*.

Five OTUs (2.3%) clustered with protophyta or plants, and one of these OTUs was obtained from BF-A and BF-B. The other four OTUs were obtained only from supply A (Table 2.2). Four OTUs (1.8%) clustering with the protophyta showed highest similarity with species within the phylum Cryptophyta, viz., *Plagioselmis prolonga* (75.8% similarity), *Chlorella* sp. (76.5% similarity), *Staurastrum polymorphum* (82.7% similarity), and *Goniomonas pacifica* (91.3% similarity). One OTU clustered within the family Poaceae (grasses).

Identity and richness of free-living protozoa in treated water and in biofilms

A total of 253 sequences (46.4%) and more than half of the obtained OTUs (127 OTUs) showed highest similarity to free-living protozoa (Table 2.2). The coverage of the clone libraries for supply A was lower than that for supply B, but the estimated total OTU richness values for these supplies were not significantly different (Table 2.3). The obtained OTUs had similarities of 57% to 100% with eukaryotic sequences in the SILVA database (release 94; April 2008). Similarity threshold percentages for eukaryotic genera and species at the 18S rRNA gene level have not yet been established. Therefore, similarity percentages for 18S rRNA gene sequences most closely related to the same genera and species included in the SILVA database were derived. Data for nine different genera of free-living protozoa revealed that the minimum similarities ranged from 75% to 92%. For sequences in the SILVA database most closely related to the cluster of *Hartmannella* (n = 19), *Acanthamoeba* (n = 211), and *Vorticella* (n = 7) species, minimum similarities of 75.2%, 78.1%, and 91.9%, respectively, were obtained. Minimum similarities ranging from 86.6% (*Bodo saltans*; n = 23) to 99.7% (*H. vermiformis*; n = 15) were calculated for sequences of seven protozoan species (not all data shown) most closely related to those collected in this study. A total of 98 sequences (32 OTUs) had $\leq$ 75% similarity to sequences in the database and thus were considered unidentifiable (Table 2.2). These unidentified OTUs showed a minimum similarity of 44.4% and a maximum similarity of 98.2% to each other. Eleven of these OTUs, including nine OTUs from supply A and two OTUs from supply B, clustered with each other with more than 95% similarity.

In the clone libraries for both supplies, we observed sequences clustering with seven protozoan phyla (Fig. 2.2 and see Table A.1 for more details). The results show that a few protozoan phyla predominated in the different clone libraries and that the diversity within each phylum varied between the different sample locations. None of the 127 OTUs with highest similarity to free-living protozoa were observed in both supplies, demonstrating that there are highly diverse protozoan communities in each supply (see Table A.1 for details).

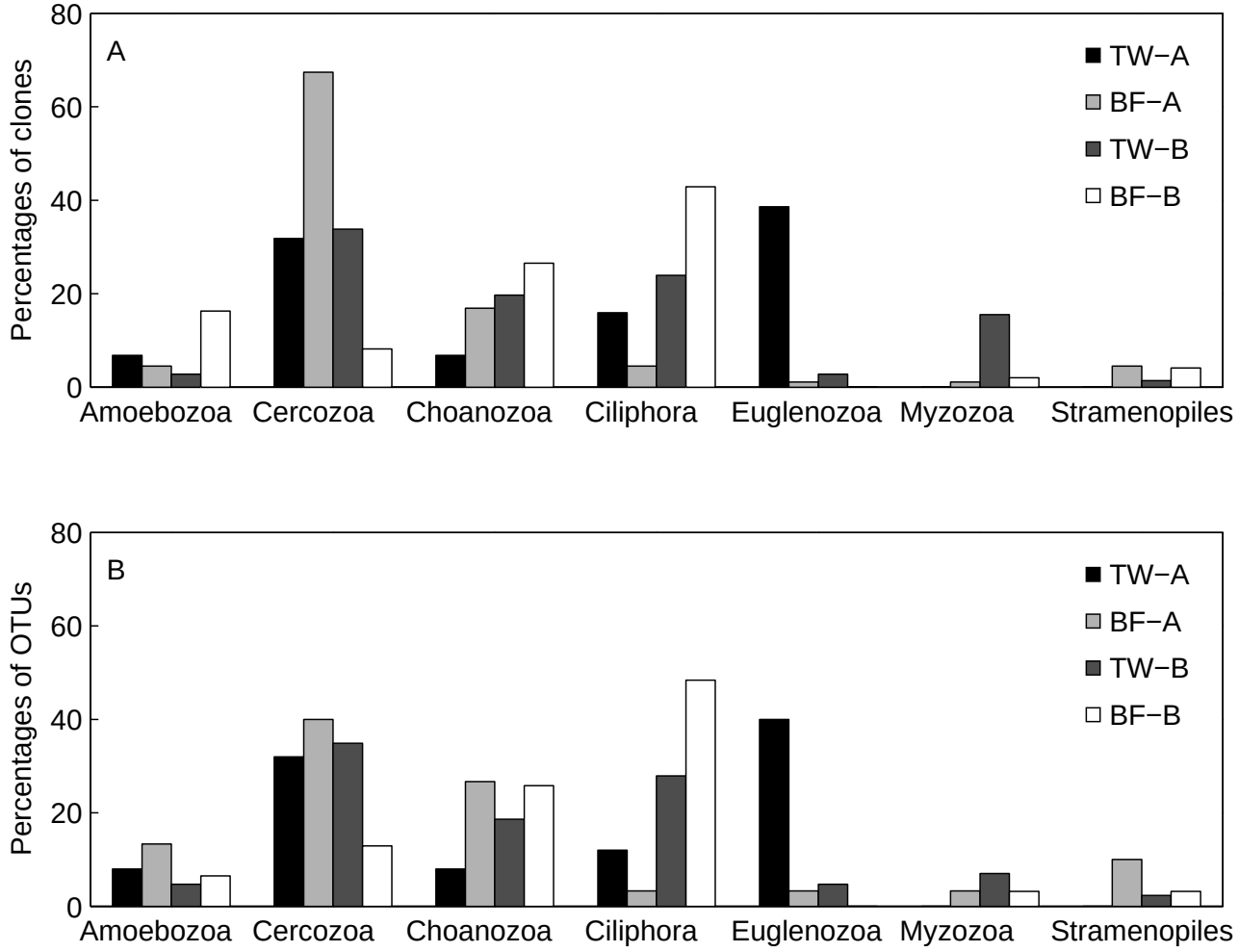

Figure 2.2: (A) Taxonomic distribution of free-living protozoa based on 18S rRNA gene clones retrieved from treated water (TW) and biofilms (BF) of supply A and supply B. (B) Taxonomic distribution of the OTUs with the highest similarity to a free-living protozoan retrieved from treated water (TW) and from biofilms (BF) of supply A and supply B.

Occurrence of protozoan hosts and pathogenic free-living protozoa in treated water and biofilms

All samples of treated water, water from distribution systems, and biofilms for both supplies were analyzed for the presence of the *L. pneumophila* host *Hartmannella vermiformis*, using qPCR [149]. Inhibition of PCR amplification was not observed in any of the samples. *H. vermiformis* was detected in four of the seven samples of TW-A, at concentrations between 0.49 and 29.3 cells liter^{-1} (median, 1 cell liter^{-1}), but was not detected in any of the DW-A samples or in the BF-A samples. Two of the seven samples of TW-B were positive for *H. vermiformis*, both at a concentration of 1.5 cells liter^{-1}, and one (of eight) biofilm sample was positive for *H. vermiformis*, at a concentration of 4.3 cells per

10 cm^2. The organism was detected in all DW-B samples, at concentrations between 2.3 and 815 cells $liter^{-1}$, and concentrations in July (median, 70 cells $liter^{-1}$) were significantly ($p < 0.05$) higher than the concentrations in November (median, 4 cells $liter^{-1}$). *H. vermiformis* was not detected in the clone libraries. These observations demonstrated that this organism was commonly present but constituted a minor fraction of the protozoan community. One OTU of the clone library of BF-B2 showed the highest similarity (77.9%) to a sequence belonging to the family of Hartmannellidae (see Table A.1 for details). A total of 2.2% of the sequences representing free-living protozoa obtained from supply A showed highest similarity (85.9% to 89.3%) to species within the genus *Acanthamoeba*. Several *Acanthamoeba* spp. can serve as hosts for *L. pneumophila* and other undesired bacteria [107]. One OTU (0.8%) obtained from TW-B showed the highest similarity to *Echinamoeba thermarum* (85.7%), a potential host for *L. pneumophila*. One OTU had the highest similarity to *Acanthamoeba polyphaga*, a potential pathogen (see Table A.1 for details).

2.4 Discussion

Analytical procedures

To our knowledge, primers for the amplification of all free-living protozoa included in public databases are not available. Therefore, we selected 18S rRNA gene primers amplifying most, but not all, eukaryotic organisms represented in public databases. Two genera serving as hosts for *L. pneumophila*, viz., *Naegleria* spp. and *Vahlkampfia* spp., were not amplified with these primers. Recently, a primer set for vahlkampfiid amoeba has been developed for direct detection of *Acanthamoeba* spp., *Naegleria* spp., and *Vahlkampfia* spp. [65].

The variation in the T-RFLP fingerprints of treated water and biofilms exceeded the reproducibility of the T-RFLP method, demonstrating differences in the involved eukaryotic communities. However, a limitation of the T-RFLP method is that similar fragment lengths may represent different sequences, implying that the richness in the sample may be higher than the number of observed fragments. The use of clone libraries to study the richness in eukaryotic communities and the estimation of richness with the Chao1 index [51] also have a few limitations. In most eukaryotes, 18S rRNA genes are organized in tandem repeat units [160], and the copy number differs significantly by genus, e.g., *H. vermiformis* has about 1,330 copies per cell [149] and *Acanthamoeba* spp. have about 600 copies per cell [39]. The clone libraries from the treated water and biofilm samples were constructed using a fraction (5% to 6.7%) of the isolated DNA, and therefore only organisms with more than 15 to 20 copies of the 18S rRNA gene per cell could be represented in the clone libraries. Hence, the composition of the clone libraries does not exactly reflect the composition of the involved eukaryotic communities. The effect of copy number is most pronounced with multicellular eukaryotes, containing more DNA (copies) than unicellular eukaryotes, as demonstrated in the clone libraries of BF-B5 and BF-B6, with an OTU with $>$50% of the clone sequences representing metazoa (Table 2.2). Furthermore, only the predominating sequences were analyzed (Table 2.3).

Identification of obtained partial 18S rRNA gene sequences

Similarity percentages at the 18S rRNA gene level have not been published for members of eukaryotic genera and species. For the genera of free-living protozoa most closely related to those observed in this study, similarities between 76% and 92% were derived from the 18S rRNA gene sequences in the SILVA database. On the species level, similarities between 86.6% and 99.7% were calculated for sequences of a number of protozoan species most closely related to those collected in this study. Morphologically well-defined ciliated species vary highly at the small-subunit rRNA sequence level [131, 239]. Hence, genera and species of many free-living protozoa may show relatively high levels of sequence diversity in the 18S rRNA gene. Therefore, with the division of sequences into OTUs with 99% similarity, almost all different species can be distinguished. The large proportion of sequences with relatively low similarity percentages to sequences included in the databases further indicates that many eukaryotic organisms in freshwater and marine environments are not yet described [12, 80, 172, 239, 256, 279]. A total of 32 OTUs showed the highest similarity to a specific eukaryote but clustered in the phylogenic tree with another group of eukaryotic organisms. We used the information on highest similarity (by BLAST search) for these OTUs for identification. These observations demonstrate that identification of freshwater protozoa is limited by the currently available database, but the large variety of sequences retrieved in the present study will facilitate further investigations of free-living protozoan communities in water supplies.

Host protozoa for *Legionella* spp. and pathogenic free-living protozoa

H. vermiformis, a commonly observed protozoan host for *L. pneumophila* [88, 151, 283], was detected in both supplies. This protozoan has also been observed in treated groundwater in Germany [143, 175], using culture methods, in drinking water supplies [175, 256], in warm water supplies [217, 255], and in surface water [149, 175, 256], demonstrating its ubiquitous presence in the freshwater environment. However, *H. vermiformis* was not a predominant protozoan in the eukaryotic communities in any of the samples in this study for which clone libraries were prepared. In the distributed water, *H. vermiformis* was detected only in supply B, with higher concentrations in the summer than in the autumn. The presence of *H. vermiformis* in supply B is associated with an elevated level of active biomass and a high level of NOM.

A total of 2.3% of the protozoan sequences retrieved from supply A and 6.7% of the protozoan sequences of supply B had highest similarities to genera with one or more protozoan species described as hosts for *L. pneumophila*. Water temperatures in supplies A and B were below 20°C and thus were too low for growth of *L. pneumophila* [137], but uncultured *Legionella* spp., including *Legionella*-like amoeba pathogens [289], can multiply in this temperature range in water supplies. A number of the detected free-living protozoa can probably serve as hosts for these uncultured *Legionella* spp. At elevated temperatures in warm water installations, *H. vermiformis* and *Acanthamoeba* spp. are available to promote the growth of *L. pneumophila* and other undesired bacteria [107, 219, 283]. *Acanthamoeba* spp. [61, 132, 183] have also been identified as opportunistic human pathogens, but it is unclear

whether the sequences related to such species represent organisms with pathogenic characteristics.

Fungi, metazoa, protophyta, and plants

Fungi, metazoa, protophyta, and plants were detected in the clone libraries of nearly all samples (Table 2.2). Fungi [111] and metazoa [274] can multiply in water treatment and distribution systems [9]. Some of the OTUs had highest similarities to fungi, e.g., *Aspergillus* spp., *Fusarium* spp., and *Cladosporium* spp., which have also been observed in drinking water in Slovakia [96], Norway [111], and Germany [106]. A few OTUs had highest similarities ($>$ 99%) to pathogenic fungi, e.g., *Candida albicans* [73], but it is not possible to determine whether the obtained partial sequences represent pathogenic organisms.

Metazoa such as nematodes and cnidarians (e.g., freshwater jellyfish [272]) are common inhabitants of treated water in distribution systems and play a role in the food chain [9, 171, 172, 239, 274]. None of the sequences obtained in the present study were related to pathogenic metazoa.

Four OTUs (1.8%) clustered with the Cryptophyta, which contains a large number of mixotrophic species [198], but identification of these protophyta is limited by the currently available database. The OTU clustering with the family of grasses probably originated from a contamination with pollen via the air during sampling or sample treatment, although it was not observed in the negative control.

Eukaryotic richness in supply A and supply B

The concentration of NOM in treated water of supply A ($<$ 0.5 ppm of C) was much lower than that in supply B (7.9 ppm of C) (Table 2.1). This difference is reflected in the concentrations of active biomass measured as ATP in these water types, viz., $<$ 1 ng liter^{-1} for TW-A and 10.6 g liter^{-1} for TW-B, confirming the ultraoligotrophic nature of water type A. PCR-based identification methods can detect more variation at a low DNA concentration than at a high concentration [224]. Indeed, the fingerprints of TW-A showed more variation than the fingerprints of TW-B (Fig. 2.1), but overall, the total number of OTUs observed in the clone libraries of supply A was not significantly different from the number observed in the clone libraries of supply B (Table 2.3).

The coverage index of the clone libraries for all eukaryotes was 40% based on 99% similarity between the sequences within one OTU and 37% when OTUs were based on 97% similarity (Table 2.3 and see Table A.1 for more details). These coverage indexes are low in comparison with the values derived for the communities of small eukaryotes in an anaerobic aquifer (66%) [29] and in a mesotrophic lake (91%) [157] but higher than the value (22%) reported for eukaryotes in a suboxic and an oxid lake in France [239]. Only eight OTUs were observed in more than one biofilm sample from supply A, and only two OTUs were obtained from more than one biofilm sample from supply B. Obviously, differences

in environmental conditions for biofilms sampled at different locations within the distribution system promoted the growth of different types of eukaryotes. Still, the T-RFLP fingerprints of the communities of eukaryotes clustered within each supply (Fig. 2.1).

Richness of free-living protozoa in supplies A and B

Free-living protozoa feed on bacteria, other protozoa, and detritus and play an important role in the transfer of energy through the trophic levels [22, 196, 276]. Due to their rapid response to environmental changes, free-living protozoa have been used as water quality indicators, and the diversity in free-living protozoa generally increases with improved water quality [128, 143, 144, 145, 171]. Consequently, differences in the protozoan communities in the two supplies can be attributed to differences in raw water composition, treatment processes, and conditions in the distribution system (hydraulics, materials, and residence time). However, the estimated richness in the free-living protozoa was not significantly different between the two supplies (Table 2.3).

Based on morphological studies, free-living protozoa have been divided into flagellates, ciliates, and amoebae [196, 276]. Table A.1 in Appendix A shows that representatives of these groups were identified in the different samples by molecular techniques. Microscopic studies have shown that sand filters operating under similar conditions in water treatment systems harbor different numbers and types of ciliates and amoebae [167, 256]. Microscopic analysis also showed that flagellates predominated (93%) in drinking water in an experimental distribution system with pipes of concrete and polyvinyl chloride supplied for 4 months with treated water with a dissolved organic carbon concentration of 2.3 mg C liter^{-1} [233]. However, in the biofilm, no flagellates were detected, but ciliates (52%) and amoebae (48%) were observed. In the present study, many OTUs observed in the biofilms had highest similarities to flagellates, including *Cercomonas* spp., *Bodo saltans*, and *Rhynchomans nasuta* [see Table A.1 for details; 198].

Twelve OTUs of the clone libraries for supply A and 26 OTUs of the clone libraries for supply B had highest similarities to genera that have been used as indicator organisms in the saprobic index for organic pollution [94, 237]. A total of 87% of these organisms belong to genera that indicate moderate pollution at a high dissolved oxygen content, e.g., *Hemiophrys*, *Rhynchomonas*, and *Vorticella* (see Table A.1 for details). However, elucidation of the relationship between environmental conditions in water treatment and distribution systems, e.g., water composition, and the occurrence of free-living protozoa is not possible because (i) the communities are highly diverse, (ii) species and genus boundaries of eukaryotes are still unclear, (iii) little information is available about the growth conditions of free-living protozoa, and (iv) the richness in the clone libraries is not proportional to the richness in the protozoa in the samples.

In conclusion, in two groundwater supplies with a large difference in the concentration of NOM, highly diverse communities of free-living protozoa were observed. These communities differed between lo-

cations within the distribution system. Hence, a large variety of microhabitats, defined by as yet unknown environmental conditions, exist within water supplies and affect the eukaryotic composition. Furthermore, high levels of NOM and active biomass in treated water corresponded with elevated concentrations of *H. vermiformis*. Consequently, quantitative detection of selected protozoa by molecular techniques may be promising in elucidating the relationship between drinking water quality and the presence of specific organisms.

Acknowledgments

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Chapter 3

Detection of protozoan hosts for *Legionella pneumophila* in engineered water systems by using a biofilm batch test

Abstract

Legionella pneumophila proliferates in aquatic habitats within free-living protozoa, 17 species of which have been identified as hosts by using *in vitro* experiments. The present study aimed at identifying protozoan hosts for *L. pneumophila* by using a biofilm batch test (BBT). Samples (600 ml) collected from 21 engineered freshwater systems, with added polyethylene cylinders to promote biofilm formation, were inoculated with *L. pneumophila* and subsequently incubated at 37°C for 20 days. Growth of *L. pneumophila* was observed in 16 of 18 water types when the host protozoan *Hartmannella vermiformis* was added. Twelve of the tested water types supported growth of *L. pneumophila* or indigenous *Legionella anisa* without added *H. vermiformis*. In 12 of 19 BBT flasks *H. vermiformis* was indicated as a host, based on the ratio between maximum concentrations of *L. pneumophila* and *H. vermiformis*, determined with quantitative PCR (qPCR), and the composition of clone libraries of partial 18S rRNA gene fragments. Analyses of 609 eukaryotic clones from the BBTs revealed that 68 operational taxonomic units (OTUs) showed the highest similarity to free-living protozoa. Forty percent of the sequences clustering with protozoa showed $\geq$ 99.5% similarity to *H. vermiformis*. None of the other protozoa serving as hosts in *in vitro* studies were detected in the BBTs. In several tests with growth of *L. pneumophila*, the protozoa *Diphylleia rotans*, *Echinamoeba thermarum*, and *Neoparamoeba* sp. were identified as candidate hosts. *In vitro* studies are needed to confirm their role as hosts for *L. pneumophila*. Unidentified protozoa were implicated as hosts for uncultured *Legionella* spp. grown in BBT flasks at 15°C.

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3.1 Introduction

Legionella pneumophila, the causative agent of Legionnaires' disease, is a common inhabitant of natural freshwater environments and human-made water systems, including cooling towers, whirlpools, air-conditioning systems, and installations for warm tap water [86]. In the aquatic environment *L. pneumophila* proliferates within certain free-living protozoa, which serve as its hosts [88, 151, 283]. Environmental factors favoring the growth and survival of *L. pneumophila* in freshwater systems include a water temperature between 20 and 45°C [215, 284] and the presence of biofilms and sediments on which the protozoan hosts can graze [151, 215, 271].

Rowbotham [218] was the first to report the growth of *L. pneumophila* within free-living amoebae, which belonged to the genera *Acanthamoeba* and *Naegleria*. *In vitro* studies with cocultures have revealed that 14 species of amoebae, viz., *Acanthamoeba* spp. [6, 177, 218, 260], *Balamuthia mandrillaris* [227], *Echinamoeba exundans* [88], *Hartmannella* spp. [219], *Naegleria* spp. [186, 218, 260], and *Vahlkampfia jugosa* [219]; the slime mold *Dictyostelium discoideum* [110, 244]; and two species of the ciliate genus *Tetrahymena* [88, 138] can serve as hosts for *L. pneumophila*. Recently, it has been reported that *L. pneumophila* can also replicate within the intestinal tract of the microbiovorous nematode *Caenorhabditis elegans* [30].

A number of the free-living protozoa mentioned above and others, e.g., *Vannella* spp. and *Saccamoeba* spp., have been observed in aquatic environments from which *L. pneumophila* was cultivated or in which it was detected with PCR [32, 215, 255, 256]. However, it remains unknown which of these protozoa actually serve as hosts for *L. pneumophila* in the aquatic environment, including human-made water systems. Moreover, it cannot be excluded that free-living protozoa other than those tested *in vitro* can serve as hosts for *L. pneumophila* as well. Information is also lacking about protozoan hosts for *Legionella anisa* [85, 245], which is frequently present in water installations in temperate regions [75, 291]. Furthermore, it is unknown which free-living protozoa serve as hosts for uncultured *Legionella* spp. that can grow at temperatures of about 15°C [288, 289].

L. pneumophila can proliferate in samples of surface water, effluent of wastewater treatment plants, potable water, and water from cooling towers incubated at 25, 35, or 37°C [149, 221, 271]. Consequently, incubation of freshwater samples can be used to amplify protozoan hosts for *L. pneumophila* and other *Legionella* spp. In this study, different engineered water types were investigated using a biofilm batch test (BBT) system to (i) amplify and subsequently identify predominating, known, and yet-undescribed hosts for *L. pneumophila* and (ii) identify potential protozoan hosts for *Legionella* spp. that can grow at 15°C.

3.2 Materials and methods

Experimental setup of the BBT system

A biofilm batch test (BBT) was used to amplify and subsequently identify protozoan hosts for *L. pneumophila*. The BBT consists of well-cleaned, heat-sterilized (4 h at 150°C) Pyrex glass Schott flasks (1 liter) with 600 ml of test water and polyethylene cylinders that were incubated to develop a biofilm of indigenous and inoculated microorganisms. Initial tests were incubated with 12 cylinders of cross-linked polyethylene (PE-Xa) (diameter, 16 mm; each with surface area of about 9 cm^2). In subsequent tests each BBT flask contained six gamma-irradiated (Isotron, Ede, the Netherlands) polyethylene (PE-80) cylinders (diameter, 20 mm; each with a surface area of about 10 cm^2). Nitrate and phosphate were added to each BBT flask at final concentrations of 72.5 μM and 13.5 μM, respectively, to prevent growth limitation by these nutrients.

L. pneumophila was inoculated at a concentration between 4.2×10^3 and 5.0×10^4 *mip* gene copies $liter^{-1}$ [56] to ensure the presence of this organism. Each test was carried out in duplicate flasks. The BBT flasks were incubated at 37°C ($\pm$ 1°C) in the dark for at least 20 days without shaking. The concentration and richness of *Legionella* and eukaryotic communities in the planktonic phase and biofilm were monitored during the incubation period. Control BBTs in which flasks were inoculated with *L. pneumophila* and the protozoan host *Hartmannella vermiformis* were performed to verify if growth of *L. pneumophila* occurs under the test conditions in the investigated water type. *H. vermiformis* was inoculated at a concentration between 3.3×10^5 and 4.8×10^5 cells $liter^{-1}$. Control tests were done in single flasks.

A number of BBT flasks were additionally incubated at 15°C with tap water and with filtered tap water (3.0-μm-pore-size and 47-mm-diameter TSTP Isopore membrane [Millipore, Molsheim, France]) from groundwater supply C to test if growth of indigenous *Legionella* spp. occurred in the presence and the absence of free-living protozoa (Table 3.1). Furthermore, BBT flasks inoculated with biomass from the filter beds of groundwater supplies A and B were incubated at 15°C to determine whether growth of indigenous *Legionella* spp. occurred in the presence and the absence of inoculated *H. vermiformis*.

Preparation of inoculum for *L. pneumophila* and *H. vermiformis*

A suspension of *L. pneumophila* serogroup 1, grown for 7 days on buffered charcoal-yeast extract (BCYE) agar plates [78] at 37°C, was diluted in autoclaved tap water and used for inoculation (600 μl per flask). *H. vermiformis* (ATCC 50237) was axenically cultivated in modified PYNFH medium (ATCC 1034) for 2 weeks at 30°C [87] and was used as inoculum (600 μl per flask). Total direct cell counts of the inoculum suspensions of *L. pneumophila* and *H. vermiformis* and suspensions of protozoa for calibration curves were determined using acridine orange and epifluorescence microscopy [118].

Table 3.1: Characteristics of the water types tested and results of the controls of the biofilm batch test (BBT) incubated at 37°C, where Hv indicates *H. vermiformis* and Lp *L. pneumophila*.

Origin of sample	Temp (°C)[a]	Conc. of ATP (ng l^{-1})[a]	Conc. of Hv (cells l^{-1})[a]	Lp growth in control[b]	Lp/Hv-ratio control[c]
Drinking water supplies					
Groundwater supply A					
Treated water	11	<1	<0.5	N.D.[f]	N.A.[g]
Biomass from filter bed (limestone)	11	4.6±0.04[d,e]	<2[e]	+	2.1; 2.5
Installation A1	39	1.9±0.3	51±4[d]	+	3.6
Installation A2	37	3.3±0.2	8.2±2.2	+	2.9
Groundwater supply B					
Treated water	11	6.7±0.3	<0.5	N.D.	N.A.
Biomass from filter bed (sand)	11	52.6±0.7[e]	11.4±7.6[e]	+	2.4; 3.5
Installation B1	35	4.1±0.2	6.8±2.5	+	2.4
Installation B2	37	7.8±0.9	1530±290	+	3.3
Flushed tap water	12	16.0±3.3	805±74	+	3.0
Groundwater supply C; tap water	18	3.3±0.4	<4	+	3.1
Surface water supply D					
Biomass from filter bed (granular activated carbon)	5	22.1±1.3[e]	5.0±1.8[e]	+	2.6
Biomass from filter bed (sand)	5	55.5±4.3[e]	12.3±1.7[e]	+	2.0
Surface water					
Storage reservoir for surface water supply D	5	14.2±0.5	<6.7	+	3.5
Water of river Rhine (autumn)	10	83.2±5.4	<10	N.D.	N.A.
Water of river Rhine (winter)	4	61.1±2.4	<10	+	3.0
Treated sewage	15	705.8±4.9	28.0±10.8	+	1.9
Water from cooling tower					
Cooling tower 1 (pH 7.3)	27	101.8±1.5	<6.6	+	2.6
Cooling tower 2 (pH 8.5)	9	35.7±0.3	<2	+	3.2
Cooling tower 3 (pH 7.8)	24	19.0±0.1	<2	−	-
Cooling tower 4 (pH 6.6)	19	14.7±0.6	<2	−	-
Cooling tower 5 (pH 8.5)	47	55.8±4.9	19±1.6	+	2.9

[a] At time of sampling

[b] +, significant growth ($p<0.025$); −, no growth.

[c] Log-transformed ratio between the maximum concentrations of *L. pneumophila* (Lp) and *H. vermiformis* (Hv) in control flasks of BBT.

[d] Standard deviation of analysis.

[e] Concentration per gram (wet-weight) of filter bed material.

[f] N.D., not determined.

[g] N.A., not applicable.

Sample collection and preparation

Samples included biomass from four filter beds and treated water from three different drinking water supplies (A, B, and D), six tap water types from three supply areas (A, B, and C), two surface water types, the effluent of a wastewater treatment plant, and five cooling tower samples (Table 3.1; see Fig. B.1 in Appendix B for details of the treatment systems for the four drinking water supplies). The samples were collected in containers of sterile glass or polyethylene, stored at 4°C, and processed within 24 h. In supply A, aerobic groundwater is aerated to remove CO_2, followed by limestone filtration to increase the pH and the hardness of the water as described in Chapter 2 (see Fig. B.1 for details). In supply B, anaerobic groundwater is treated by intensive aeration, rapid sand filtration, caustic dosage followed by pellet softening, aeration, and a second stage of rapid sand filtration. In supply C, anaerobic groundwater is aerated, followed by rapid sand filtration. In supply D, seepage water is treated with iron(III) chloride, storage in a lake, rapid sand filtration, ozonation, pellet softening, granular activated carbon filtration, and slow sand filtration. All four drinking water types are treated and distributed without chemical disinfection [269].

The biomass from the filter beds of supply A (limestone filter), supply B (slow sand filter), and supply D (granular activated carbon filter and slow sand filter) was collected by adding 45 g (wet weight) of filter bed material to 900 ml of the associated treated water or autoclaved tap water, followed by low-energy sonication as previously described [168]. Subsequently, the biomass suspension was diluted to obtain an initial ATP concentration in the BBT flask of 10 times the ATP concentration in the related treated water. Biomass in samples of warm tap water (about 40 liters) was concentrated to about 5 liters with ultrafiltration using the hemoflow method [275]. The effluent from a sewage plant was diluted 5-fold with autoclaved tap water, and the sample of flush water was diluted 3-fold before incubation in the BBT flask. All other samples were directly incubated in the BBT flask.

Microbiological analyses

The attached microorganisms were removed from the polyethylene cylinders and suspended in autoclaved tap water by using low-energy sonication as described elsewhere [151]. Total ATP concentrations in the planktonic phase and in the biomass suspension, representing the active biomass, were determined as previously described [168]. Direct plating on BCYE medium and incubation at 37°C for detection of culturable *Legionella* spp. [78, 184] were used in the initial BBT flasks inoculated with biomass from the filter beds of supplies A and B. For DNA isolation, volumes of 50 to 200 ml of the planktonic samples and 50 to 100 ml of biofilm suspensions were filtered through a 0.22-μm-pore-size and 55-mm-diameter polycarbonate Track-Etch membrane (Sortorius, Goettingen, Germany) to isolate the microorganisms. Subsequently, DNA was isolated according to procedures as described in Chapter 2. *L. pneumophila* bacteria were quantified at days 0, 3, 5, 10, and 20 of the incubation by quantitative PCR (qPCR) by applying the primers LpneuF and LpneuR and the specific TaqMan probe LpneuP as described earlier [288]. *Legionella* spp. were quantified with qPCR on the same sample days as was *L. pneumophila* with the primers LEG-225 and LEG-858 as described earlier [178]. *L. anisa* was detected

with the primers LaF (5'-CAATGTCTACTGTAATGGCAGC-3') and LaR (5'-AACCGCTTGGAGTACCGT-3') and the specifc TaqMan probe LaP (5'-AGACGGAATGTCTGGTGCCCAATTGA-3') targeting the *mip* gene. The thermal cycling conditions were similar to the conditions of qPCR for *L. pneumophila*. All primers and probes were produced at Biolegio (Malden, the Netherlands), and all qPCR assays were performed in 96-well plates in an I-cycler real-time PCR detection system (Bio-Rad, Veenendaal, the Netherlands). Quantification was based on plasmid-based calibration curves. The concentrations of *Legionella* spp. are expressed in genome units (GU) liter^{-1} or GU cm^{-2}. The concentrations of *L. pneumophila* and *L. anisa* are measured in *mip* gene copies liter^{-1} or *mip* gene copies cm^{-2}.

qPCR assays for detection of *Acanthamoeba* spp. were performed with the primers AcantF900 and AcantR1100 [205] at days 0 and 10 after incubation and for *H. vermiformis* were performed with the primers Hv1227F and Hv1728R at all sample days [149]. The quantification of *Acanthamoeba* spp. and *H. vermiformis* was based on calibration curves which were constructed by preparing 10-fold dilutions of DNA extracted from suspensions with known numbers of cells of *Acanthamoeba castellanii* (CCAP 1501) and *H. vermiformis* (ATCC 50237).

The richness and composition of the eukaryotic communities in the planktonic and biofilm samples were determined by terminal restriction fragment length polymorphism (T-RFLP) analysis and sequence analysis of 18S rRNA gene fragments ($\pm$ 550 bp) as described earlier in Chapter 2. T-RFLP analysis was done for all BBT flasks at day 0 and after 20 days of incubation in planktonic samples of 50 to 200 ml and/or in biofilm suspensions of 50 to 100 ml. For each water type, this analysis was performed on the same day to minimize the experimental variation. From the BBT flasks incubated at 37 and 15°C, a total of 820 eukaryotic clones, varying from 5 to 59 clones per library depending on the complexity of the band pattern of T-RFLP fingerprints, were analyzed. All 18S rRNA gene sequences obtained were grouped in operational taxonomic units (OTUs) with 99% similarity [223]. The composition of the *Legionella* community in the biofilm of the BBT flasks incubated at 15°C was determined by sequence analysis of genus-specific 16S rRNA gene fragments ($\pm$ 650 bp) of clones retrieved as described earlier [289]. All 16S rRNA gene sequences obtained were grouped in OTUs with 97% similarity. From each BBT, about 45 16S RNA gene sequences were analyzed.

The sequences obtained were compared to sequences in the National Center for Biotechnology (NCBI) database by BLAST search and imported and aligned into the SSU Ref SILVA94 database released in April 2008 by using the ARB software package as described in Chapter 2 [165, 203]. The eukaryotic sequences obtained were organized into taxa based on the classification system of Cavalier-Smith [46] and the structure in the SILVA database [203].

Statistical analysis

The analysis of variance (ANOVA) *F* test was used for the comparison of the concentrations of *L. pneumophila* and *Legionella* spp. measured with qPCR with the concentrations of cultivated *Legionella* spp.

in the BBT flasks with biomasses of supplies A and B. The statistical significance of the growth of *L. pneumophila*, *L. anisa*, and *H. vermiformis* populations in the BBT flasks between day 0 and day 10, and between day 0 and day 20, was determined using log-transformed concentrations. First the *F* test was used (with 95% confidence) to test on equality of variance of two populations. If the *F* test rejected the equality of variance, the adjusted *t* test was applied, whereas a regular *t* test was used when the *F* test did not reject equality of variance. The (adjusted) *t* test was applied with 95% confidence (one-sided testing). For each separate sample, the Bonferroni-correction for multiple comparisons was used as two tests were applied for each sample. The Shapiro-Wilk normality test was applied to the log10-transformed ratios of the maximum concentrations of *L. pneumophila* and *H. vermiformis* (*L. pneumophila/H. vermiformis* ratio) observed in the BBT flasks. From the normally distributed values for the *L. pneumophila/H. vermiformis* ratio of the control group results, the upper limit (tolerance limit) of the one-sided 95% confidence interval of the 99th percentiles was calculated.

Nucleotide sequence accession numbers

The 18S rRNA gene sequences determined in this study have been deposited in GenBank under accession numbers GU970094 to GU970913. The sequences of the *Legionella* 16S rRNA gene have been deposited in GenBank under accession numbers GU970914 to GU971083.

3.3 Results

Development of the biofilm batch test (BBT) system

Despite the presence of *H. vermiformis*, no growth of *L. pneumophila* was observed in initial BBT flasks incubated at 37°C with biomass from the filter beds of supply A (initial concentration of 7.9 ng ATP liter^{-1}) and supply B (131.7 ng ATP liter^{-1}), without inoculation with *L. pneumophila* (Fig. 3.1). Furthermore, inoculated *L. pneumophila* did not multiply in the BBT flasks with two types of treated water without exogenous addition of *H. vermiformis*. In contrast, significant growth ($p < 0.025$) of *L. pneumophila* was observed in both BBT flasks with water from cooling tower 1 in which *H. vermiformis* was not detected at day 0 (< 20 cells liter^{-1}; Fig. 3.2). Within a few days of incubation, a significant growth ($p < 0.025$) of *H. vermiformis* also was observed in this water, as was growth of *L. pneumophila*, which reached its maximum level of growth after about 10 days. Based on these observations, *L. pneumophila* was added to all BBT flasks to ensure the presence of this organism in the test, and control flasks with *L. pneumophila* and *H. vermiformis* were included in each test to verify if the water under investigation supports its growth in the presence of a host protozoan.

Significant growth ($p < 0.025$) of *L. pneumophila* was observed in 16 of 18 control BBT flasks (Table 3.1). In these flasks, at least a 2-log-unit increase of the *L. pneumophila* concentration was observed in the planktonic phase and in the biofilm within 10 days of incubation. Maximum concentrations of

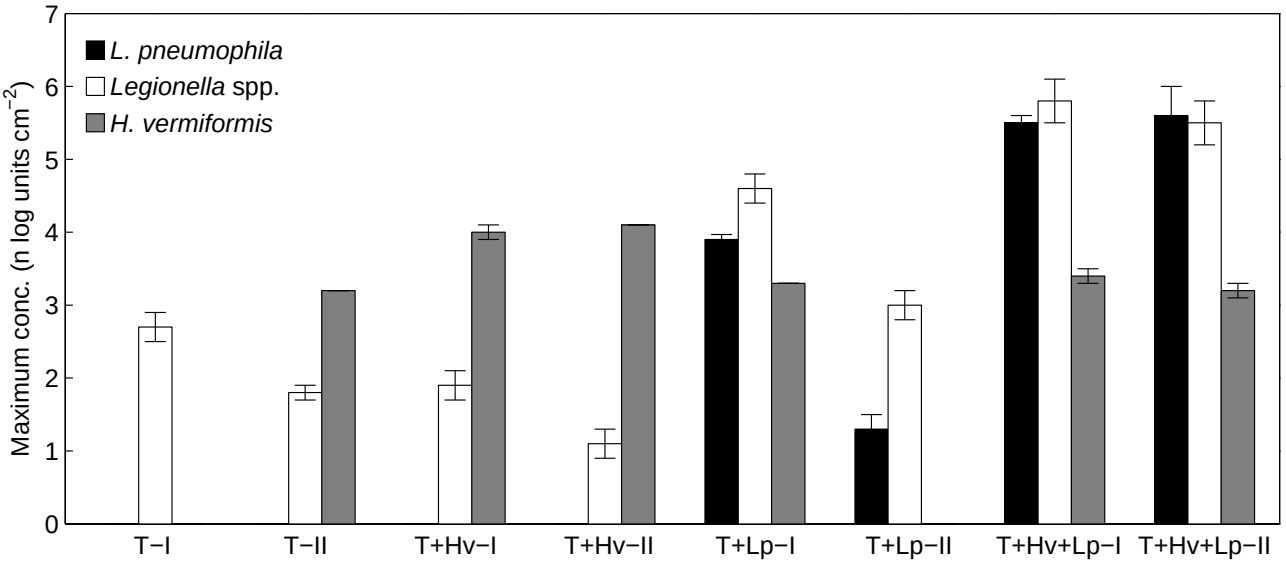

Figure 3.1: Maximum concentrations of *L. pneumophila* (*mip* gene copies cm^{-2}), *Legionella* spp. (GU cm^{-2}) and *H. vermiformis* (cells cm^{-2}) in the biofilm on PEX-a in the BBT flasks with biomass from a limestone filter bed in treated water of supply A incubated at 37°C for 20 days. Initial concentrations of *L. pneumophila* (about 2 log units of *mip* gene copies cm^{-2}) and *H. vermiformis* (about 3 log units of cells cm^{-2}) are converted from units liter^{-1} to units cm^{-2}. Abbreviations: T-I and T-II, blank test flasks (no inoculation); T+Hv-I and T+Hv-II, duplicate test flasks inoculated with *H. vermiformis*; T+Lp-I and T+Lp-II, duplicate test flasks inoculated with *L. pneumophila*; T+Lp+Hv-I and T+Lp+Hv-II, duplicate test flasks inoculated with *L. pneumophila* and *H. vermiformis* (controls). Error bars indicate standard deviations of the analysis.

colony-forming cells of *Legionella* and maximum concentrations of *Legionella* spp. and *L. pneumophila* as measured by qPCR were not significantly different ($p < 0.05$) in the BBT flasks with biomass from the filter beds of supplies A and B incubated at 37°C. Therefore, qPCR was used in subsequent tests to quantify growth of *L. pneumophila* and *Legionella* spp. The log-transformed *L. pneumophila/H. vermiformis* ratios for the controls ranged from 1.9 to 3.6 (average, 2.8 ± 0.5) and were normally distributed according to the Shapiro-Wilk test ($p = 0.577$). From these data an upper tolerance limit of 4.5 was derived, above which it is most likely that protozoan hosts for growth of *L. pneumophila* other than *H. vermiformis* are present in the involved BBT flask. *L. pneumophila* did not proliferate in the control BBT flasks with water from cooling towers 3 and 4 (Table 3.1). Probably, a biocide residual inhibited biofilm development and/or inactivated *L. pneumophila* because the ATP concentration decreased from 40 ng ATP liter^{-1} to 12 ng ATP liter^{-1} during the incubation in the flask with water from cooling tower 3. In the other controls, the maximum ATP concentrations in the water varied between 9.3×10^2 and 3.7×10^3 ng ATP liter^{-1}. In the tests, the maximum ATP concentration in water varied between 5.4×10^1 and 5.1×10^2 ng ATP liter^{-1} and the maximum concentration of the biofilm on the surface of the PE-80 cylinders ranged from 1.3 to 9.3 ng ATP cm^{-2}.

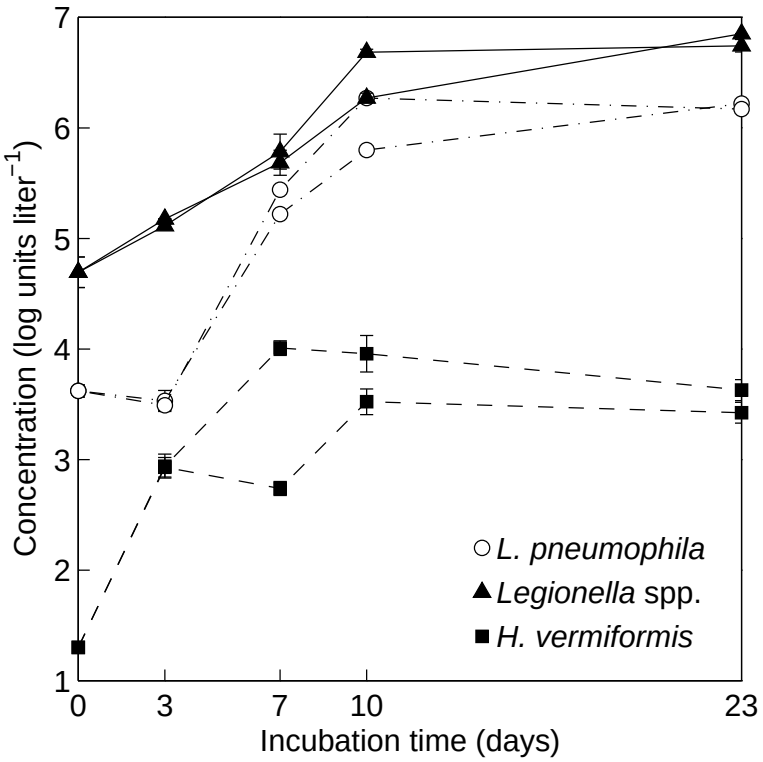

Figure 3.2: Growth of inoculated *L. pneumophila* in two biofilm batch test flasks with water from cooling tower 1 during incubation at 37°C for 23 days. *L. pneumophila* is expressed as *mip* gene copies liter^{-1}), *Legionella* spp. in genome units (GU) liter^{-1}), and *H. vermiformis* as cell liter^{-1}. The detection limit for *H. vermiformis* is 20 cells liter^{-1}. Error bars indicate standard deviations of the analysis.

Growth of *Legionella* spp. in the BBT flasks at 37°C

The concentration of *L. pneumophila* increased significantly ($p < 0.025$) by 1 to 3 log units in one or both BBT flasks with 11 of 21 water types inoculated with this organism (Table 3.2; see also Tables B.1 to B.3 for details). The maximum concentration of *L. pneumophila* ranged from 5.3×10^3 to 5.7×10^7 *mip* gene copies liter^{-1} and from 4.9×10^2 to 5×10^5 *mip* gene copies cm^{-2} in the biofilm depending on the water type. In these BBT flasks the maximum concentration of *Legionella* spp. was similar to the maximum concentration of *L. pneumophila*, indicating that the *Legionella* community was dominated by *L. pneumophila*. Growth of *Legionella* spp. was observed, but *L. pneumophila* did not multiply in two of four BBT flasks with water originating from two tap water installations in the distribution system of supply B. In these water types significant growth ($p < 0.025$) of indigenous *L. anisa* to maximum concentrations ranging from 2.1×10^6 to 2.6×10^7 *mip* gene copies liter^{-1} was observed (Table 3.2). No growth of *Legionella* spp. was observed in 9 of 21 water types.

Table 3.2: Predominant 18S rRNA gene sequences clustering with free-living protozoa obtained from the biofilm in BBT flasks with growth of *L. pneumophila* and/or *L. anisa* after ≥ 20 days of incubation at 37°C.

Water type	BBT flask	Growth[a]		Lp/Hv[b] ratio	% of clones clustering with:		Predominant OTU clustering with protozoa		
		Lp	Hv		Protozoa	Hv	Organism with highest similarity (accession no.)	Similarity (%)	% of clones
Biomass from limestone filter bed (supply A)	I	+	+	1.1	100	100	Hv (AY680840)	99.5	100
Installation A2 (supply A)	II	+	+	3.0	100	93	Hv (AY680840)	100	93
Biomass from sand filter bed (supply B)	I	+	−	>6.8[e]	91	<2	*Rhinosporidium sp.* (AY477020)	89.3	73
	II	+	+	2.0	97	93	Hv (AY680840)	99.8	97
	III	+	−	>6.6[e]	16	<3	Uncultured cercozoan clone (AY620304)	>97.7	80
Installation B1 (supply B)	II	−[c]	+	1.9[f]	100	27	*Sphaeroeca volvox* (Z34900)	83.6	73
Installation B2 (supply B)	I	+[c]	+	2.0(2.3[f])	100	100	Hv (AY680840)	100	100
	II	−[c]	+	2.9[f]	100	100	Hv (AY680840)	100	100
Biomass from granular activated carbon filter bed (supply D)	I	+	+	3.4	100	<13	*Nuclearia simplex* (AF484687)	100	100
Biomass from sand filter bed (supply D)	II	+	+	1.6	92	92	Hv (AY680840)	100	100
Water of river Rhine (autumn)	I	+	−	>5.6[e]	15	<5	*Neoparamoeba sp.* (AF371972)	95.6	67
	II	+	−	>5.7[e]	87	<4	Uncultured eukaryote from treated water of groundwater supply B (EU860860)	93.9	100
Water of river Rhine (winter)	I	+	+	5.5[e]	<3	<3	No protozoan observed	-	-
	II	+	+	2.1	61	52	Hv (AY680840)	99.8	86
Treated sewage	II	+	−	>4.6[e]	52	<4	*Diphylleia rotans* (AF420478)	99.3	100
Cooling tower 1	I	+	+	2.7	42	21	Hv (AF426157)	100	50
	II	+	+	2.1	50	17	Hv (AF426157)	99.8	33
Cooling tower 5	I	+	−	>4.5[e]	64	<4	*Echinamoeba thermarum* (AJ489262-68)[g]	96.2-98.7	79
	II	+	−[d]	3.6	89	7	*E. thermarum* (AJ489262-67-68)[g]	96.2-98.9	42

[a] +: significant growth ($p < 0.025$) of *H. vermiformis* (Hv) or *L. pneumophila* (Lp), −: no growth. [b] Log-transformed ratio between the maximum concentration of Lp and the maximum concentration of Hv. [c] Significant growth ($p < 0.025$) of *L. anisa* in the BBT. [d] *H. vermiformis* was detected, but no significant growth was observed. [e] Log-transformed Lp/Hv ratio exceeds the tolerance limit of Lp/Hv ratio for the control group (4.5). [f] Log-transformed ratio between the maximum concentration of *L. anisa* and the maximum concentration of *H. vermiformis*. [g] More than one OTU with highest similarity to *E. thermarum*.

Growth of *H. vermiformis* and *Acanthamoeba* spp. in the BBT flasks at 37°C

H. vermiformis was detected at day 0 in 10 of 21 water types at concentrations ranging from 6.8 to 1,530 cells liter^{-1} and from 6.2 to 12.3 cells g (wet weight)$^{-1}$ of filter bed material (Table 3.1). Significant growth of indigenous *H. vermiformis* in the BBT flasks was observed in 11 of 21 water types tested, with significant growth of *L. pneumophila* or *L. anisa* in 9 of these 11 water types (Table 3.2). The concentration of *H. vermiformis* increased by 0.6 to 4 log units in the planktonic phase and in the biofilm depending on the water type and the initial concentration. In four water types, *H. vermiformis* was not observed at day 0 but appeared within a few days of incubation. *Acanthamoeba* spp. were detected in water collected from tap water installation A2 and in water from cooling tower 4 at concentrations of 0.3 cells liter^{-1} and 1.7 cells liter^{-1}, respectively. However, in none of the BBT flasks growth of *Acanthamoeba* spp. was observed.

The *L. pneumophila/H. vermiformis* ratios in 12 of 19 BBT flasks with growth of *L. pneumophila* did not exceed the derived tolerance limit, and in 11 of these 12 BBT flasks significant growth of *H. vermiformis* was observed (Table 3.2). In BBT flask II with cooling water 5, no significant growth of *H. vermiformis* was observed, but this protozoan was detected during the incubation and the *L. pneumophila/H. vermiformis* ratio did not exceed the derived tolerance limit. These observations indicate that *H. vermiformis* served as a host for *L. pneumophila* in these 12 BBT flasks. In seven BBT flasks with growth of *L. pneumophila*, the *L. pneumophila/H. vermiformis* ratio did exceed the derived tolerance limit and in six of these seven BBT flasks no significant growth of *H. vermiformis* or *Acanthamoeba* spp. was observed (detection limit, $<$ 20 cells liter^{-1}; Table 3.2). The absence of *H. vermiformis* and/or the high *L. pneumophila/H. vermiformis* ratio indicates that free-living protozoa other than *H. vermiformis* served as hosts for *L. pneumophila* in these seven BBT flasks.

Identity and richness of eukaryotes and free-living protozoa predominating in the BBT flasks at 37°C

T-RFLP fingerprints revealed complex eukaryotic communities in the water types directly after sampling. During incubation, the eukaryotic richness in the planktonic phase decreased and different eukaryotic communities developed in duplicate BBT flasks (Fig. 3.3). Free-living protozoa constituted the largest proportion of the OTUs ($>$ 40%) and clones ($>$ 56%) in the BBT flasks with water samples from drinking water supplies and cooling towers, but OTUs (64%) and sequences (61%) with the highest similarity to fungi predominated in the BBT flasks with surface water (Table 3.3). A total of 68 OTUs representing 58% of the clones showed the highest similarity to free-living protozoa (Table 3.3; see also Tables B.1 to B.3 for details). Almost 40% of these clones represent one OTU, which showed 99.5% similarity to *H. vermiformis*, and were obtained from all three water types. Only three other OTUs were obtained from two water types, and all 64 of the other OTUs were observed only once in a single BBT flask.

Table 3.3: Classification of eukaryotic clones retrieved from the biofilm in the BBT flasks after $\geq$ 20 days of incubation at 37°C with samples from drinking water supplies, surface water and cooling towers inoculated with *L. pneumophila*.

Kingdom or subkingdom	**Drinking water supplies**		**Surface water**		**Cooling towers**		**All analyzed samples**	
	No.[a] of OTUs (%)	% of clone libraries	No.[a] of OTUs (%)	% of clone libraries	No.[a] of OTUs (%)	% of clone libraries	No.[a] of OTUs (%)	% of clone libraries
Free-living protozoa	35(40.4)	66.4	7(25.0)	34.8	30(56.6)	57.4	68(41.2)	57.7
Amoebozoa	8(22.2)	58.2	4(57.1)	57.4	21(70.0)	66.7	29(42.6)	59.7
Cercozoa	8(22.2)	8.4	3(42.9)	42.6	5(16.7)	15.2	17(25)	14.2
Choanozoa	11(30.6)	25.9	0(0.0)	0	3(10.0)	16.7	14(20.6)	20.7
Euglenozoa	2(5.6)	5.0	0(0.0)	0	0(0.0)	0.0	2(2.9)	3.4
Myzozoa	5(13.9)	2.1	0(0.0)	0	0(0.0)	0.0	5(7.4)	1.4
Stramenopiles	1(2.8)	0.4	0(0.0)	0	1(3.3)	1.5	2(2.9)	0.6
Fungi	25(28.1)	15.8	18(64.3)	60.7	17(32.1)	31.3	60(36.4)	28.7
Metazoa	14(15.7)	10.8	0(0.0)	0	2(3.8)	4.3	14(8.5)	7.2
Protophyta and plants	3(3.4)	1.1	3(10.7)	4.4	2(3.8)	3.5	8(4.8)	2.3
Organisms with <75% similarity	12(13.5)	5.8	0(0.0)	0	2(3.8)	3.5	15(9.1)	4.1
Total	89(100)	100	28(100)	100	53(100)	100	165(100)	100

[a] OTUs obtained from more than one sample type are included only once.

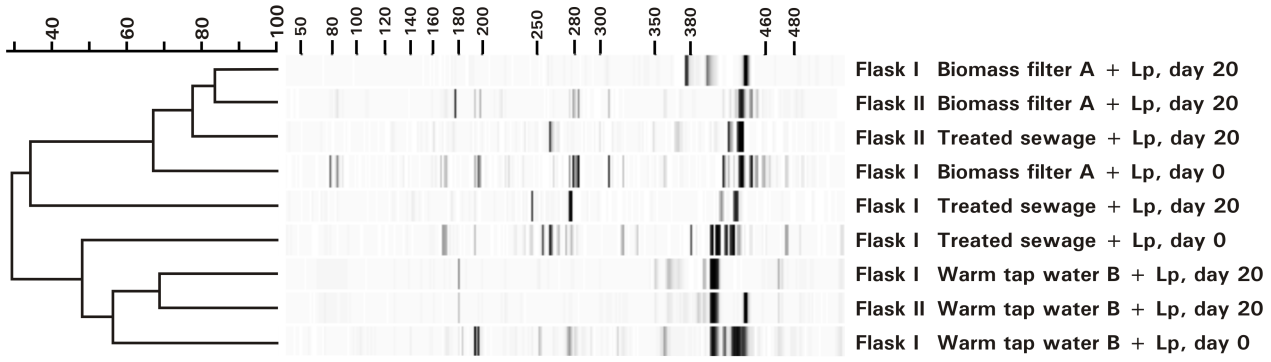

Figure 3.3: UPGMA (unweighted-pair group method using average linkages) dendogram of T-RFLP fingerprints of the BBT flasks with (i) biomass from a limestone filter bed of supply A (biomass filter A), (ii) treated sewage, and (iii) water from warm tap water installation B1 in distribution area of supply B (warm tap water B) at day 0 and after 20 days of incubation at 37°C with *L. pneumophila* (+ Lp). Sample volumes, 50 to 200 ml of the planktonic sample and 50 to 100 ml of the biofilm suspensions. The numbers above the dendrogram represent percent similarity, and those above the fingerprints represent fragment length (number of nucleotides).

Sequences clustering with free-living protozoa predominated in 16 of 19 clone libraries of the BBT flasks with growth of *L. pneumophila* and/or *L. anisa* (Table 3.2; see also Tables B.1 to B.3 for details). *H. vermiformis* constituted more than 50% of the clones clustering with free-living protozoa in 8 of the 19 clone libraries of BBT flasks with growth of *L. pneumophila* and/or *L. anisa*. The *L. pneumophila/H. vermiformis* ratios in these BBT flasks varied between 1.1 and 3.0 and did not exceed the derived tolerance limit (Table 3.2). The *L. pneumophila/H. vermiformis* ratio also did not exceed the tolerance limit in four other BBT flasks, viz., with water from tap water installation B1, with biomass from the granular activated carbon filter bed of supply D, and with water from cooling towers 1 and 5. Obviously, *H. vermiformis* served as host for *L. pneumophila* without predominating in these clone libraries.

The predominating OTUs in the clone libraries of the seven BBT flasks with *L. pneumophila/H. vermiformis* ratios exceeding the tolerance limit showed the highest similarity to *Diphylleia rotans* (99.3%), an uncultured cercozoan clone (97.7%), *Echinamoeba thermarum* (>96%), *Neoparamoeba* sp. (95.6%), an uncultured eukaryote from treated water of supply B (93.9%), and *Rhinosporidium* sp. (89.3%) (Table 3.2). These free-living protozoa may have served as hosts for *L. pneumophila* in these BBT flasks. No sequences related to free-living protozoa were obtained from the BBT flask with water from the Rhine River (winter) in which growth of *L. pneumophila* was observed.

Sequences related to *Sphaeroeca volvox* (>83.6% similarity) predominated in the clone libraries of two BBT flasks with water from tap water installation B1, in one of which growth of *L. anisa* and *H. vermiformis* was observed (Table 3.2; also see Table S2.1 for details). This observation suggests that the free-living protozoan related to *S. volvox* did not serve as a host for *L. anisa* or for *L. pneumophila*. Most

clone libraries of the BBT flasks without growth of *L. pneumophila* and *Legionella* spp. were dominated by OTUs clustering with fungi and metazoa. Predominating OTUs of the protozoan community in these BBT flasks had the highest similarities to *Ichthyophonus irregularis* (89.2%), *Platyamoeba stenopodia* (93.9%), an uncultured cercozoan (>98.9%), and an uncultured eukaryote retrieved from treated water from supply A in Chapter 2 (see Tables B.1 to B.3 for details). Obviously, these free-living protozoa did not serve as hosts for *L. pneumophila* under the test conditions.

Transfer of one PE cylinder with biofilm and 60 ml of the planktonic phase from BBT flasks with the different water types which had been incubated for at least 50 days to freshly prepared BBT flasks with autoclaved tap water and five PE cylinders, followed by incubation at 37°C, did not induce growth of *L. pneumophila* in most flasks. Growth of *L. pneumophila* in freshly prepared BBT flasks was observed only in the presence of *H. vermiformis*, e.g., in the flasks inoculated from the BBT flask with treated sewage and with biomass from the granular activated carbon filter bed of supply D. The *L. pneumophila/H. vermiformis* ratios in these BBT flasks indicated that *H. vermiformis* served as a host for *L. pneumophila*.

Growth of *Legionella* spp. and eukaryotes in the BBT system at 15°C

Growth of indigenous *Legionella* spp. was observed in one of the BBT flasks with tap water of supply C and in the BBT flask inoculated with *H. vermiformis* but not in the membrane-filtered (3.0-μm) sample. At least a 2-log-unit increase of the *Legionella* community was observed in all flasks inoculated with biomass from filter beds of supplies A and B, but no colonies were observed on the BCYE medium. The inoculated *L. pneumophila* did not multiply in the BBT flasks during incubation at 15°C. The *Legionella* community (maximum concentration, 3.1×10^5 GU cm^{-2}) grown in the BBT flasks with biomass from the limestone filter bed of supply A with and without addition of *H. vermiformis* was dominated (>92% of the clone library) by OTUs clustering with sequences obtained from treated water and raw water from supply A in an earlier study [288]. These sequences differ from the species described (data not shown). The *Legionella* community (maximum concentration, 3.0×10^4 GU cm^{-2}) in the BBT flasks with biomass from a sand filter bed of supply B was dominated by one OTU (84% of the clone libraries), which differs from reported sequences.

Acanthamoeba spp. were not observed in the BBT flasks incubated at 15°C, and *H. vermiformis* was observed only in the BBT flasks inoculated with this protozoan. Changes in the T-RFLP fingerprints of the eukaryotic communities in the biofilm during incubation at 15°C indicated that growth of indigenous eukaryotes occurred (results not shown). Sequences clustering with free-living protozoa were obtained from all four eukaryotic clone libraries of the BBT flasks with biomass from filter beds of supplies A and B incubated at 15°C. A total of 51 OTUs showed the highest similarity to protozoan phyla, viz., Amoebozoa (7 OTUs), Cercozoa (16 OTUs), Choanozoa (17 OTUs), Ciliophora (1 OTU), Euglenozoa (7 OTUs), Myzozoa (2 OTUs), and Stramenopiles (1 OTU). More than 80% of the clones clustering with free-living protozoa that were retrieved from the BBT flasks inoculated with biomass from the limestone filter bed of supply A showed the highest similarity with the Cercozoa phylum (see

Tables B.4 and B.5 for details). Sequences clustering with Choanozoa and Euglenozoa predominated in the clone libraries of the BBT flasks with biomass from a sand filter bed of supply B. Most (57%) of the OTUs that clustered with free-living protozoa showed the highest similarity to sequences which differ from already-described species retrieved in an earlier study from treated water or distribution system biofilms of supplies A and B [Chapter 2]. These observations indicate that yet-undescribed protozoa serve as hosts for yet-uncultured *Legionella* spp.

3.4 Discussion

Performance of the biofilm batch test (BBT) system

Only a few *in vivo* studies have shown that certain free-living protozoa serve as hosts for *L. pneumophila* [88, 151, 283], but a large number of free-living protozoa have been identified as hosts for *L. pneumophila* by using *in vitro* studies [89, 138, 177, 186, 218, 219, 227, 260]. Random *in vitro* testing of free-living protozoa to determine whether they serve as hosts for *L. pneumophila* is time-consuming, and only a minor fraction of the free-living protozoa is available in culture collections. Furthermore, it is unknown if free-living protozoa which serve as hosts when tested *in vitro* with a pure culture of *L. pneumophila* serve as hosts in aquatic environments. Therefore, a batch test with biofilm growth in combination with molecular techniques for detection and identification was used to identify potential protozoan hosts for *L. pneumophila* in different freshwater types (Table 3.1).

The present study shows that *Legionella* spp. and free-living protozoa multiplied in the BBT flasks at 37°C and also at 15°C. Therefore, this approach can be used to amplify and subsequently identify free-living protozoa that can serve as hosts for *L. pneumophila* and other *Legionella* spp. in freshwater types of different origins. The maximum concentrations of active biomass on the surface of the PE-80 cylinders varied between 1.3 and 9.3 ng ATP cm^{-2} and are in the same range as biofilm concentrations measured in tap water installations [270]. Therefore, the BBT design represents the environment in water installations. *L. pneumophila* serogroup 1 was added to ensure the presence of this organism in the BBT flasks (Fig. 3.1). Growth of indigenous *L. anisa* was observed in two water types despite the inoculation with a relatively high number of *L. pneumophila*. *L. anisa* is a common inhabitant of engineered water systems [75, 291] and also needs free-living protozoa for growth in aquatic environments [88, 245].

Growth of *H. vermiformis* in the BBT flasks was followed by growth of *L. pneumophila* (Fig. 3.2). The *L. pneumophila/H. vermiformis* ratio in the test in combination with the tolerance limit for this ratio derived from the controls can be used as an indicator for the role of *H. vermiformis* as protozoan host in the BBT flasks. The obtained *L. pneumophila/H. vermiformis* ratios, which ranged from 1.9 to 3.6 log units, are consistent with the average ratios of 2.5 to 2.6 log units obtained in a biofilm batch model system with autoclaved tap water inoculated with pure cultures of *L. pneumophila* and *H. vermiformis* [149] (Table 3.1). The *Legionella*-to-host ratio may be different for other protozoan hosts, e.g., *Acan-*

thamoeba spp., depending on their cell size. In more than 50% of the tested water types, growth of *L. pneumophil*a or *L. anisa* was observed only in one of two BBT flasks incubated at 37°C. Different protozoan communities can develop in duplicate samples (Fig. 3.3 and Table 3.2), indicating that slight differences in environmental conditions result in predominance of other free-living protozoa. This observation is consistent with the observation of highly diverse free-living protozoan communities at different locations within drinking water distribution systems as described in Chapter 2.

The procedure for the identification of the free-living protozoa in clone libraries, as applied in this study, has some limitations. First of all, 18S rRNA gene sequences of a few genera within the phyla Amoebozoa, Euglenozoa, and Percolozoa were not amplified with the primers used. These organisms include *Naegleria* spp. [186, 218] and *Vahlkampfia jugosa* [219], which have been identified as hosts for *L. pneumophila* by using *in vitro* tests. Furthermore, the composition of the clone libraries does not exactly reflect the composition of the involved eukaryotic communities, because 18S rRNA genes are present in different copy numbers in each eukaryotic species [160]. Moreover, although one OTU related to free-living protozoa predominated in the clone libraries of most BBT flasks with growth of *L. pneumophila* (Table 3.2), it cannot be excluded that more than one protozoan type served as hosts for *L. pneumophila* in one BBT flask. Despite these limitations, a number of candidate hosts for *L. pneumophila* were detected.

Identity of protozoan hosts for *L. pneumophila* and *L. anisa*

The observation of *H. vermiformis* in 14 of the 21 water types tested (before or during incubation) is consistent with the ubiquitous presence of this organism in drinking water supplies [see Chapter 2 and 175, 217, 255, 256], cooling towers [149], and surface water [149, 175, 256] (Tables 3.1 and 3.2). In 12 of the 19 flasks with growth of *L. pneumophila* or *L. anisa*, growth of *H. vermiformis* also was observed. The *L. pneumophila/H. vermiformis* ratios in 11 of these 12 BBT flasks did not exceed the derived tolerance limit. OTUs with the highest similarity to *H. vermiformis* predominated in 8 of the associated clone libraries, thus confirming that *H. vermiformis* served as host for *L. pneumophila* or *L. anisa* in these BBT flasks. Significant growth of *H. vermiformis* was observed in two water types without growth of *L. pneumophila* and other *Legionella* spp., and also no growth of *L. pneumophila* was observed in the control for one of these water types. All sequences with the highest similarity to *H. vermiformis* showed a minimum of 99.5% similarity to each other and to the *H. vermiformis* strain (ATCC 50237) used in the controls. The absence of growth of *Legionella* spp. in the presence of *H. vermiformis* remains unexplained.

Acanthamoeba spp. were observed at low initial concentrations in 2 of the 21 water types, indicating that this organism is much less common in freshwater in temperate regions than is *H. vermiformis*. Still, *Acanthamoeba* spp. are frequently used to study proliferation and growth of *L. pneumophila* within free-living protozoa and numbers between $>1.0 \times 10^2$ and 4.5×10^4 CFU of *L. pneumophila* per amoeba or vesicle have been reported [107, 120, 123, 218, 219]. Growth of *Acanthamoeba* spp.

was not observed in the BBT flasks, although most species of this genus can multiply at 37°C [63, 108]. Of the free-living protozoa, the slime mold, and the metazoan which have been identified as hosts for *L. pneumophila* by using *in vitro* studies, only *H. vermiformis* was identified as a host for *L. pneumophila* or *L. anisa* in the present study. The prominent position of *H. vermiformis* as a host for *L. pneumophila* in aquatic environments is consistent with results of other *in vivo* experiments [88, 151, 283]. However, the potential of the other listed organisms to serve as hosts for *L. pneumophila* in the aquatic environment needs confirmation using *in vivo* tests.

L. pneumophila did not proliferate in the absence of *H. vermiformis* in freshly prepared BBT flasks inoculated with the microbiota grown in BBT flasks with selected water types, and no attempts were made to culture free-living protozoa from these BBT flasks. Consequently, not yet-recognized protozoan hosts for *L. pneumophila* were not isolated in this study. The composition of a number of eukaryotic clone libraries and the values of the *L. pneumophila/H. vermiformis* ratio suggest that testing of certain protozoa in coculture with *L. pneumophila* may lead to the identification of novel protozoan hosts for *L. pneumophila* (Table 3.2). The involved organisms include (i) *Diphylleia rotans* (99.3% similarity), an algivorous heterotrophic flagellate that feeds on cyanobacteria [141]; (ii) *Echinamoeba thermarum* (>96%), an extremely thermophilic protozoan [15] related to *E. exundans*, which is described as a host for *L. pneumophila* [88]; and (iii) *Neoparamoeba* sp. (95.6% similarity) of the class Flabelinea with the genera *Platyamoeba* and *Vannella*, which are affiliated with *Legionella* spp. [246]. These candidate hosts were observed in BBT flasks with surface water, water from a cooling tower, and biomass from a sand filter bed of a groundwater supply, but not from tap water (Table 3.2).

Free-living protozoa growing at 37°C were dominated by OTUs clustering within Amoebozoa, while the clone libraries of the BBT flasks at 15°C were dominated by OTUs with the highest similarity to Cercozoan and Choanozoan types. Also in drinking water supplies (< 20°C) described in Chapter 2, only a minor fraction of OTUs of free-living protozoa clustered with Amoebozoa. OTUs related to free-living protozoa with pathogenic properties, viz., *Acanthamoeba* spp. [61, 132] and *Balamuthia mandrillaris* [277], were observed in several water types (see Tables B.1 to B.3 for details). However, it is unclear whether these sequences represent pathogenic organisms. In most BBT flasks without growth of *Legionella* spp., OTUs that showed the highest similarity to fungi and metazoa predominated. The free-living protozoa related to *Sphaeroeca volvox*, *Ichthyophonus irregularis*, and *Platyamoeba stenopodia* and an uncultured cercozoan did not serve as hosts for *L. pneumophila* under the test conditions.

Identity and richness of *Legionella* spp. and free-living protozoa in the BBT system at 15°C

Legionella spp., including several yet-uncultured species, are ubiquitously present in freshwater [66, 72, 288, 289]. Growth of *Legionella* spp. at 15°C was observed in the BBT system but not with membrane-filtered water (3.0 μm), confirming that these bacteria require free-living protozoa for growth. The yet-undescribed *Legionella* (sequence) types predominating in the BBT flasks with biomass from a

limestone filter bed of supply A clustered with several sequence types previously obtained from raw and treated water of the associated supply [288]. The sequences of the protozoa predominating in the BBT flasks with *Legionella* spp. growing at 15°C clustered with sequences related to Cercozoan types retrieved from the treated water of supplies A and B as described in Chapter 2. However, inoculated *L. pneumophila* did not grow at 15°C, nor did it grow in one of the BBT flasks incubated at 37°C with biomass from a limestone filter bed of supply A, despite the predominance of Cercozoan types (see Tables B.4 and B.5 for details). Growth of unidentified *Legionella* spp. at 15°C in the presence of free-living protozoa related to Choanozoan and Euglenozoan types in the BBT flasks with biomass from a sand filter bed of supply B suggests that also these protozoa can serve as hosts. One choanozoan type (*Rhinosporidium* sp.) also predominated in the clone library of a BBT flask with growth of *L. pneumophila* at 37°C (*L. pneumophila/H. vermiformis* ratio, >6.8), suggesting that this organism also served as a host for *L. pneumophila* (Table 3.2). Most (57%) OTUs of free-living protozoa in the BBT flasks inoculated with biomass from filter beds and incubated at 15°C showed the highest similarity to sequences obtained from treated water or distribution system biofilms of supplies A and B as described in Chapter 2. Obviously, either the many free-living protozoa present in the treated water and biofilms in the distribution systems have their origin in the filter beds and/or the environmental conditions in the filter bed resemble those in the biofilm in the distribution systems.

In conclusion, our observations confirm the prominent position of *H. vermiformis* as a host for *L. pneumophila*, whereas none of the other protozoa serving as hosts in *in vitro* studies were observed in the BBT system. A few protozoa, e.g., *Diphylleia rotans*, *Echinamoeba thermarum*, *Neoparamoeba* sp., and *Rhinosporidium* sp., were identified as candidate hosts for *L. pneumophila*, but *in vitro* studies with these organisms are needed for confirmation.

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Chapter 4

Relationships between free-living protozoa, cultivable *Legionella* spp. and water quality parameters in three drinking water supplies in the Caribbean

Abstract

The present study aimed at detecting potential protozoan hosts for cultivable *Legionella* spp. and identifying conditions favoring the growth of free-living protozoa in drinking water supplies in a tropical region. Treated and distributed water ($\pm$ 30°C) were sampled from the water supplies of three Caribbean islands and were investigated with molecular techniques, based on the 18S rRNA gene. Cultivable *Legionella pneumophila* and the protozoan host *Hartmannella vermiformis* were observed in all three supplies. Operational taxonomic units (OTUs) with the highest similarity to the potential or candidate hosts *Acanthamoeba* spp., *Echinamoeba exundans*, *Echinamoeba thermarum* and *Neoparamoeba* sp. were detected as well. In total, 59 OTUs of free-living protozoa were identified, but the estimated protozoan richness did not differ significantly between the three supplies. In supply CA-1, the concentration of *H. vermiformis* correlated with the concentration of *Legionella* spp. and clones related to Amoebozoa predominated (82%) the protozoan community. These observations, the low turbidity ($<$ 0.2 NTU) and the varying ATP concentrations (1-12 ng liter^{-1}) suggest that biofilms promoted microbial growth. Ciliophora represented 25% of the protozoan OTUs in supply CA-2 with elevated ATP concentrations (max. 55 ng liter^{-1}) correlating with turbidity (max. 62 NTU) caused by corroding iron pipes. Cercozoan types predominated (70% of protozoan clones) in supply CA-3 with ATP concentrations $<$ 1 ng liter^{-1} and turbidity $<$ 0.5 NTU in most samples of distributed water. The absence of *H. vermiformis* in most samples from supply CA-3 suggests that growth of this protozoan is limited at ATP concentrations $<$ 1 ng liter^{-1}.

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4.1 Introduction

In tropical regions, the water temperature in drinking water distribution systems is permanently about 30°C [26]. In these regions, *Legionella pneumophila*, the main etiologic agent of Legionnaires' disease [86] that proliferates at temperatures about 25°C [284], is commonly present in freshwater environments e.g., surface water, waste water, cooling towers and drinking water [103, 193, 222]. Also free-living protozoa, serving as hosts for *L. pneumophila*, including, *Acanthamoeba* spp. [6, 218], *Hartmannella* spp. [219] and *Naegleria* spp. [218, 260] have been observed in these environments [25, 41, 124, 248]. Furthermore, certain free-living protozoa with pathogenic properties, viz., *Acanthamoeba* spp. [61, 132], *Balamuthia mandrillaris* [277] and *Naegleria fowleri* [287] can grow in drinking water related biofilms at elevated temperatures [213]. Free-living protozoa in aquatic environments feed on bacteria, fungi, other protozoa and organic detritus in biofilms, sediments or in the planktonic phase [196]. The abundance of prey organisms and detritus depends on the water composition and the hydraulic conditions in distribution systems, which therefore also affect both the free-living protozoa abundance and community composition [267, 278]. Most information on community composition and abundance of free-living protozoa in freshwater environments has been obtained in the past by using cultivation methods and microscopy. Recently, however the presence and identities of such organisms in drinking water supplies in temperate regions have been studied by using molecular methods for detection and identification [202, Chapter 2]. In groundwater supplies in the Netherlands a total of 127 operational taxonomic units (OTUs) of free-living protozoa were identified based on their 18S rRNA gene sequences. Free-living protozoa, mostly pathogens, have been characterized in only a few studies in tropical regions [25, 41, 124].

Cases of Legionnaires' disease have been reported in relation to the presence of *L. pneumophila* in drinking water supplies in the Caribbean [60, 222, 225], but information about water quality parameters is not provided. Therefore, in this study, the occurrence and identity of free-living protozoa and other small eukaryotes in treated and distributed water of drinking water supplies of three islands in the Caribbean region were investigated with molecular techniques. In these supplies, drinking water is produced from seawater by using distillation and/or reverse osmosis (RO) for desalination. The objectives of this study were: (i) to determine concentrations of the protozoa *Acanthamoeba* spp. and *H. vermiformis* and cultivable *Legionella* spp. in treated and distributed water of three different water supplies, (ii) to identify the predominant free-living protozoa in these supplies, and (iii) to identify conditions favoring the growth of free-living protozoa and *Legionella* spp. by comparing the characteristics of water quality and distribution systems.

4.2 Materials and methods

Drinking water supplies

Water treatment plant CA-1 has a daily production of 4.4×10^4 m^3, 18% of which is produced by RO and 82% by distillation. Post treatment with dolomite filtration to increase the hardness of the water and addition of corrosion inhibitors (pyrophosphate: 1.5 ppm and zinc orthophosphate: 2.5 ppm) is followed by storage in steel tanks and UV disinfection (38-171 mJ cm^{-2}) prior to distribution. Mains of copper (42%) and cement-lined cast iron (39%) lead the treated water to seven service reservoirs from steel in the supply area. Supply CA-2 includes two treatment facilities with a total daily production of 5.8×10^4 m^3 drinking water. The distribution systems of both plants are interconnected. Desalination at plant CA-2a is done with RO and at plant CA-2b with RO (80%) and distillation (20%). Post treatment of RO filtrate and distillate includes calcium hypochlorite dosage (0.3 mg $liter^{-1}$), addition of carbon dioxide, limestone and granular activated carbon (GAC) filtration, addition of fluoride (0.3-0.7 mg $liter^{-1}$) and disinfection with UV radiation (CA-2a: 135 J s^{-1} m^{-2}; CA-2b: 10 J s^{-1} m^{-2}). Pipes of high density polyethylene, copper and galvanized iron comprise about 70% of the distribution system with seven service reservoirs. The main pipes (26%) and the transportation pipes consist of cast iron, with and without cement lining. In supply CA-3, with a daily production of 3.8×10^3 m^3 and five service reservoirs, seawater is treated with RO, followed by limestone filtration, GAC filtration, storage in steel tanks and addition of chlorine (residual $<$ 0.2 mg $liter^{-1}$) prior to distribution. The distribution system with five cast iron reservoirs consists of pipes of cast iron (63%), polyethylene (11%) and polyvinyl chloride (9%). Maximum residence times, in all three supplies, in the distribution systems of the supplies, including the storage tanks, range from 48 h to about 96 h.

Sample collection

From supply CA-1, two sample series were collected, one in November 2007 and one in November 2009. Both series included 4 samples at different treatment stages and 7 samples from the distribution system after each reservoir (6-12 km from the plant). From supply CA-2, samples were collected in May 2008 and in January 2009. Both series included one sample before UV from both treatments plant. In addition, 15 samples were collected from the distribution system (5-15 km from the plant) in 2008 and 7 samples in 2009. Treated water of supply CA-3 was sampled before UV at the plant and 13 samples from the distribution system were collected (5-10 km from plant). All samples, contained in sterile 1-liter PE flasks, were stored on ice and processed within 24 to 72 hours. The flasks for the samples of supply CA-3 contained 1 ml of sterile sodium thiosulfate (0.12 M) to neutralize the chlorine residual.

Analytical methods

Total concentrations of adenosine triphosphate (ATP), representing active biomass, were measured in all water samples as described earlier [168]. Buffered charcoal-yeast extract (BCYE) agar, incubated at

37°C for 7 days, was used to detect cultivable cells of *Legionella* spp. in the water samples [78, 184]. Subsequently, the fraction of colonies related to *L. pneumophila* was determined by an agglutination test (*Legionella* Latex Test; Oxoid, UK).

Duplicate water samples of 500 ml were filtered using an RTTP Isopore membrane (Millipore, Molsheim, France) with 1.2-μm-pore-size and 55-mm-diameter. Of the samples taken from supply CA-1 in 2007, volumes of 1.75 liter were filtered. Subsequently, DNA of the organisms retained on the membrane filter was isolated as described in Chapter 2. Concentrations of *H. vermiformis* and *Acanthamoeba* spp. in the water samples were determined using quantitative PCR (qPCR) as described earlier [149, 205]. In brief, quantification of *H. vermiformis* and *Acanthamoeba* spp. was based on calibration curves which were constructed by preparing 10-fold dilutions of DNA extracted from suspensions with counted numbers of cells of *H. vermiformis* (ATCC 50237) and *Acanthamoeba castellanii* (CCAP 1501) [149]. All primers were produced at Biolegio (Malden, the Netherlands). All qPCR assays were performed in duplicate, using undiluted and 10-fold diluted DNA extracts as templates in 96-well plates in a C1000^{TM} Thermal Cycler (Biorad, Veenendaal, the Netherlands).

The richness and composition of the eukaryotic communities in the water samples were determined by T-RFLP analyses, and cloning followed by sequence analyses of 18S rRNA gene fragments ($\pm$ 550 bp) as described earlier [Chapter 2]. Clone libraries were constructed of two treated water samples from plant CA-1 and five, ten and six samples of distributed water from supplies CA-1, CA-2 and CA-3, respectively. These samples were selected based high concentrations of cultivable *Legionella* spp., low concentration of *H. vermiformis*, and preferably from the periphery of the distribution systems. Approximately 45 clones per sample were analyzed, resulting in a total of 991 partial 18S rRNA gene sequences.

The obtained 18S rRNA gene sequences were divided in operational taxonomic units (OTUs) using a threshold of 99% sequence similarity. Subsequently, these sequences were compared to sequences in the National Center for Biotechnology (NCBI)-database by BLAST search and imported and aligned into the SSU Ref SILVA98 database released in March 2009 using the ARB software package [165, 203], as described in Chapter 2. The estimated OTU richness was determined with the ChaoI estimator from randomized data [122]. The obtained eukaryotic sequences were divided into higher taxa based on the classification system of Cavalier-Smith [46] and the structure in the SILVA database [203].

Table 4.1: Average values of chemical and physical water quality characteristics of treated water of supplies CA-1, CA-2 and CA-3[a].

Quality characteristic	Treated water CA-1	Treated water CA-2a	Treated water CA-2b	Treated water CA-3
Temp. (°C)	30	28	31.6	28.7
pH	9.3	8.2	8.3	8.5
Conductivity (μS cm^{-1})	25	132	125	420
Total hardness (CaCO3 mg liter^{-1})	9.9	48.2	42.2	66.0
Turbidity (NTU[b])	0.16	0.20	0.30	0.26
Cl concn. (mg liter^{-1})	0.6	6.7	10.2	74
Cu concn. (mg liter^{-1})	0.01	5	15	0.01
Fe concn. (mg liter^{-1})	0.01	0.01	0.01	0.04
NPOC[c] concn. (mg C liter^{-1})	$<$0.05	$<$0.05	$<$0.05	$<$0.05

[a] Average values, based on routine monitoring over a period of one year.
[b] NTU: nephelometric turbidity units.
[c] NPOC: non-purgeable organic carbon.

Statistical analyses

The Kruskal-Wallis test was applied to determine differences in the concentrations of selected water quality parameters between the three supplies. In case of a statistical significant test result for a parameter, subsequent pairwise comparisons were used to determine which supplies differed. All tests were performed with 95% confidence and for the multiple comparisons the Bonferroni correction was applied. Linear regression analysis using log transformed concentrations was used to assess possible relationships between physical-chemical and microbiological parameters in the distributed water of the three supplies.

Nucleotide sequence accession numbers

All partial 18S rRNA gene sequences determined in this study have been deposited in GenBank under accession numbers HQ998878 to HQ999868.

4.3 Results

Quality characteristics of treated and distributed drinking water

The water temperature at the treatment facilities and in the distribution systems of the three supplies ranged from about 28 to 34°C (Tables 4.1 and 4.2). The turbidity of treated water was low ($\leq$ 0.3

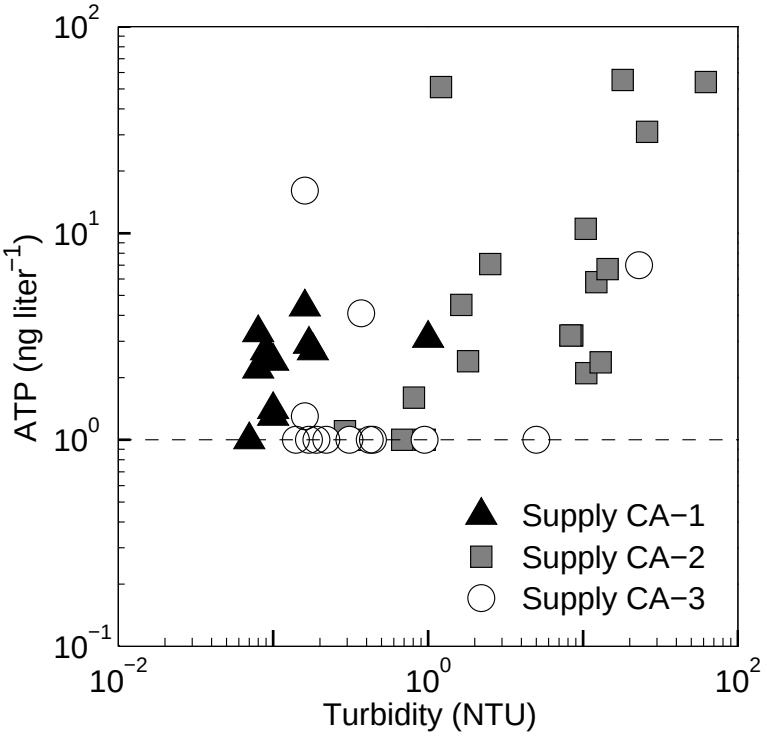

Figure 4.1: Concentrations of turbidity and active biomass, measured as ATP, in the distributed water of supplies CA-1 (n = 14), CA-2 (n = 17) and CA-3 (n = 13). Detection limit for active biomass was 1 ng ATP liter^{-1}.

NTU) at all facilities and remained low in the distribution system of supply CA-1. Turbidity exceeded 5 NTU at ten locations in supply CA-2 and at two locations in supply CA-3 (Fig. 4.1). Similarly, the concentration of iron in treated water was low ($\leq$ 0.04 mg liter^{-1}) and remained low ($<$ 0.01 mg Fe liter^{-1}) in the distribution system of supply CA-1, but locally exceeded 1 mg Fe liter^{-1} in supply CA-2 (nine samples) and in supply CA-3 (one sample). The turbidity of the water in distribution systems CA-2 and CA-3 correlated significantly with the iron concentration indicating that iron is the main cause of turbidity in these supplies (Table 4.3). These relationships yielded average iron to turbidity ratios of 0.24 $\pm$ 0.12 mg Fe liter^{-1}/NTU^{-1} (supply CA-2) and 0.22 $\pm$ 0.13 mg Fe liter^{-1}/NTU^{-1} (supply CA-3).

In treated water of supply CA-1 a higher concentration of active biomass (3.7 ng ATP liter^{-1}) was observed than in treated water of supplies CA-2 and CA-3, where the concentration was below the detection limit ($<$ 1 ng ATP liter^{-1}). The elevated ATP concentration in treated water of supply CA-1 is due to an increase from $<$ 1 to 9 ng ATP liter^{-1} in the storage tanks at the plant. The ATP concentration did not increase during distribution in supply CA-1. ATP concentrations in distributed water of supply CA-2 (median, 3.2 ng ATP liter^{-1}) were significantly higher than in supply CA-3 (median, $<$ 1 ng ATP liter^{-1}) and correlated with turbidity (Fig. 4.1 and Table 4.3).

H. vermiformis, Acanthamoeba spp. and cultivable *Legionella* spp. in treated and distributed water

H. vermiformis (about 3 cells liter^{-1}) and cultivable cells of *Legionella* spp. (1.0×10^2 CFU liter^{-1}) were detected in the treated water of supply CA-1 after UV treatment. These microorganisms had grown in the storage tanks at the treatment plant where the concentration of *H. vermiformis* increased from $<$ 2 to 18 cells liter^{-1} and the concentration of *Legionella* spp. increased from $< 1\times10^2$ to 1.5×10^4 CFU liter^{-1}. *H. vermiformis* was detected in all 14 samples collected from the distribution system of supply CA-1 (median, 18 cells liter^{-1}), in 12 of 20 samples of supply CA-2 (median, 4 cells liter^{-1}) and in 4 of the 13 samples from supply CA-3 at concentrations of about 3 cells liter^{-1} (Fig. 4.2 and Table 4.2). The concentrations of *H. vermiformis* in the distributed water of supply CA-1 were significantly higher than in the distributed water of supplies CA-2 and CA-3 (Table 4.3). *Acanthamoeba* spp. were observed in five samples of distributed water of supply CA-2 at concentrations ranging from 2 to 56 cells per liter^{-1} (median, 8.0 cells liter^{-1}; Table 4.2). In these samples also *H. vermiformis* was detected.

Legionella spp. were cultured from 41 of the 49 samples of distributed water and 28 of these 41 samples containing *H. vermiformis* (Fig. 4.2). In supplies CA-1 and CA-2, *L. pneumophila* represented 80 to 100% of the cultured *Legionella* colonies and 40 to 100% in supply CA-3. The colony counts of *Legionella* spp. in the distributed water of supplies CA-1 and CA-2 were significantly higher than those in supply CA-3 (Table 4.3). The concentration of *Legionella* spp. correlated significantly with turbidity in the distributed water of supplies CA-2 and CA-3, with the concentrations of ATP in supply CA-2 and with concentrations of *H. vermiformis* in supplies CA-1 and CA-2. In 15 of 41 distributed water samples with cultivated *Legionella* spp. the concentrations of *H. vermiformis* and *Acanthamoeba* spp. were below the detection limit of 2 cells liter^{-1}.

Richness and identity of free-living protozoa

T-RFLP analyses using 18S rRNA gene-targeting primers revealed that the eukaryotic richness in the distributed water was higher than in the treated water at the four plants (data not shown). A total of 225 (25%) of the 908 partial 18S rRNA gene sequences in the clone libraries of the three types of distributed water clustered within free-living protozoa and represented 59 (30%) of the 195 OTUs (sequence similarity of $\geq$ 99% sequence similarity) (Table 4.4). Up to eleven OTUs of free-living protozoa were obtained from 0.5 liter of distributed water of supplies CA-1, CA-2 and CA-3. The highest protozoan richness was observed in supply CA-2, but the estimated total OTU richness for free-living protozoa did not differ significantly between the three supplies (Table 4.5).

OTUs of Amoebozoa represented a large proportion of the clones retrieved from the distributed water of supply CA-1 (Fig. 4.3 and see Table C.1 in Appendix C for more details). Eukaryotic clone libraries were also constructed from the two samples of treated water before UV treatment at plant CA-1, because *Legionella* spp. were detected in the water before and after UV treatment. OTUs which clustered with Amoebozoa also predominated ($>$ 90%) in these clone libraries (see Table C.2 for details). A

Table 4.2: Quality characteristics of water sampled from the distributions systems of supplies CA-1, CA-2 and CA-3.

Parameter	**Supply CA-1**			**Supply CA-2**			**Supply CA-3**		
	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.
Temp. (°C)	32.7	31.5	34.4	31.9	27.4	32.9	29.1	27.8	31.4
Turbidity (NTU)	0.1	0.07	0.2	5.4	0.2	62.1	0.3	0.1	23.0
Fe concn (mg l^{-1})	<0.01	<0.01	<0.01	0.7	0.03	9.5	0.03	<0.01	1.5
ATP concn (ng l^{-1})	2.6	1.0	12.2	3.2	<1.0	55.2	<1.0	<1.0	16.1
H. vermiformis concn (cells l^{-1})	18	3	245	4	<2	1670	<2	<2	4
Acanthamoeba spp. concn (cells $liter^{-1}$)	<2	<2	<2	<2	<2	56	<2	<2	<2
Cultivated *Legionella* spp. concn (CFU $liter^{-1}$)	1.3×10^4	3.0×10^2	2.5×10^5	7.1×10^3	$<2.5\times10^2$	1.0×10^5	7.5×10^2	$<2.5\times10^2$	6.5×10^4

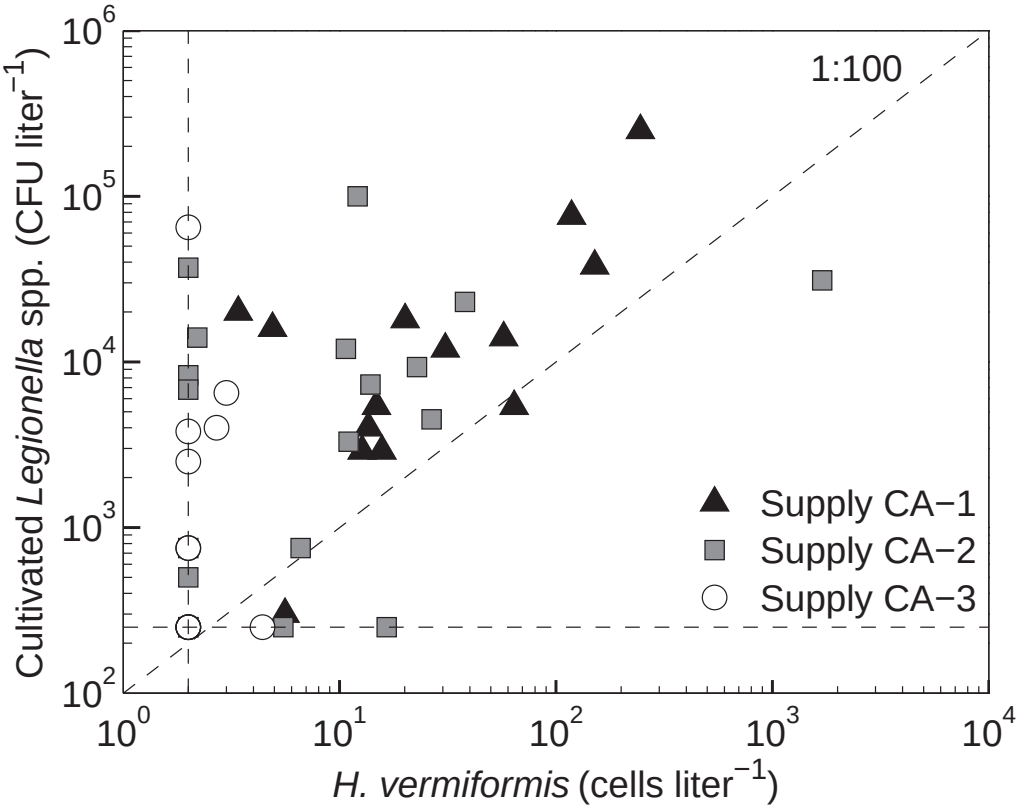

Figure 4.2: Concentrations of *H. vermiformis* and cultivated *Legionella* spp. in distributed water of supplies CA-1, CA-2 and CA-3. Detection limits for *H. vermiformis* (2 cells liter^{-1}) and for cultivated *Legionella* spp. (250 CFU liter^{-1}) are shown as dotted lines.

large proportion (39%) of the protozoan-related OTUs in the clone libraries of distributed water of supply CA-3 clustered within the phylum of the Cercozoa, whereas a large evenness of Amoebozoa (33%), Ciliophora (25%) and Cercozoa (25%) was observed in the clone libraries of distributed water of supply CA-2. A few OTUs clustering within the Choanozoa phylum were obtained from supplies CA-1 and CA-2. Two (3%) of the 59 OTUs of free-living protozoa, which showed the highest similarity to *H. vermiformis* and *Hemiophrys procera*, were obtained from all three supplies (see Table C.1 for details). Nine (15%) of these 59 OTUs were obtained from the clone libraries of two supplies. Approximately 30% of the OTUs of supply CA-1 were observed in more than one sample and about 20% of the OTUs in supplies CA-2 and CA-3 were observed at more than one location in the distribution system. Hence, the protozoan communities in the three supplies differed from each other and between locations within one supply. *H. vermiformis* predominated in the clone libraries of distributed water in four of five samples from supply CA-1, in two of nine samples from supply CA-2 and in one of six samples from supply CA-3. OTUs with the highest similarity to *Acanthamoeba* spp. and to candidate hosts for *L. pneumophila*, viz., *Echinamoeba exundans*, *Echinamoeba thermarum* and *Neoparamoeba* spp. were observed in supplies CA-1 and CA-2.

Table 4.3: Statistical analyses of differences in median concentrations (log-transformed values) of selected water quality parameters of three drinking water supplies and correlations between the parameters in these supplies. Only results of parameters which are significantly different ($p < 0.05$) between two or three supplies are presented.

Comparison between supplies			
Parameters	**Supply CA-1**	**Supply CA-2**	**Supply CA-3**
Turbidity (NTU)	<CA-2	>CA-3	<CA-2
Iron concn (mg l^{-1})	<CA-2	>CA-1,>CA-3	<CA-2
ATP concn (ng l^{-1})	N.S.[a]	>CA-3	<CA-2
H. vermiformis (cells l^{-1})	>CA-2,>CA-3	<CA-1	< CA-1
Legionella spp. (CFU l^{-1})	>CA-3	>CA-3	<CA-1, <CA-2
Correlation between parameters			
Parameters	**Supply CA-1**	**Supply CA-2**	**Supply CA-3**
Turbidity vs. iron concn	N.S.	R=0.94 ($p=4.2\times10^{-10}$)	R=0.87 ($p=2.0\times10^{-4}$)
Turbidity vs. ATP concn	N.S.	R=0.71 ($p=5.2\times10^{-4}$)	N.S.
Turbidity vs. *Legionella* spp.	N.S.	R=0.82 ($p=9.1\times10^{-6}$)	R=0.83 ($p=7.4\times10^{-4}$)
ATP vs. *Legionella* spp.	N.S.	R=0.76 ($p=4.9\times10^{-6}$)	N.S.
ATP vs. *H. vermiformis*	N.S.	N.S.	N.S.
H. vermiformis vs. *Legionella* spp.	R=0.64 (p=0.014)	R=0.48 (p=0.024)	N.S.

[a] N.S.: not significant, $p > 0.05$.

Fungi and other small eukaryotes

A total of 83 OTUs (43%) showed the highest similarity to fungi, 46 of which clustered within the Ascomycota, the predominating fungus ($\geq$ 49%) in all three supplies (see Table C.3 for details). In supply CA-1, most eukaryotic clones (53%) and OTUs (52%) showed the highest similarity to fungi (Table 4.4 and see Table C.3 for details). Nine of the 83 OTUs, which clustered within fungi, were obtained from the distributed water of two supplies, while the other 74 OTUs were retrieved from one supply. One OTU with $>$ 99% similarity to the potential pathogen *Mucor racemosus* was obtained from treated water of supply CA-1 and two OTUs with $>$ 99% similarity to *M. racemosus* and the potential pathogen *Malassezia restricta* were obtained from distributed water of supply CA-2.

The metazoa were represented by 29 OTUs, 19 (66%) of which showed the highest similarity to species of nematodes (see Table C.4 for details). One of these 19 OTUs showed 99% similarity to *Rhabdolaimus cf. terrestris* and included 140 (51%) of the 274 clones which clustered with metazoa obtained from water supplies CA-2 and CA-3. From supply CA-1 only one metazoan OTU, which clustered with Ro-

Table 4.4: Classification of eukaryotic clones, with >75% similarity to sequences in the SSU Ref SILVA98 database, obtained from distributed water of supplies CA-1, CA-2 and CA-3.

Kingdom or subkingdom	**Distributed water CA-1**[a]			**Distributed water CA-2**[a]		
	No.[b] of OTUs[c]	% of OTUs	% of clones in libraries	No.[b] of OTUs[c]	% of OTUs	% of clones in libraries
Free-living protozoa	23	36.5	36.0	36	33.6	25.5
Fungi	33	52.4	53.3	41	38.3	35.1
Metazoa	1	1.6	3.3	22	20.6	34.2
Cryptophyta and Viridiplantae	2	3.2	2.3	7	6.5	4.7
Sequences with < 75% similarity	4	6.3	5.1	1	0.9	0.5
Total	**63**	**100**	**100**	**107**	**100**	**100**
Kingdom or subkingdom	**Distributed water CA-3**[a]			**All analyzed samples**		
	No.[b] of OTUs[c]	% of OTUs	% of clones in libraries	No.[b] of OTUs[c]	% of OTUs	% of clones in libraries
Free-living protozoa	13	25.0	14.6	59	30.3	24.8
Fungi	17	32.7	15.0	83	42.6	33.5
Metazoa	9	17.3	47.9	29	14.9	30.9
Cryptophyta and Viridiplantae	12	23.1	21.7	18	9.2	9.1
Sequences with < 75% similarity	1	1.9	0.7	6	3.1	1.7
Total	**52**	**100**	**100**	**195**	**100**	**100**

[a] Data are totals for all analyzed samples of distributed water of the indicated supply.

[b] OTUs obtained from more than one sample are included only once.

[c] Each OTU contain 18S rRNA gene sequences with a minimum of 99% similarity.

tifera, was retrieved and the estimated average OTU richness for metazoa in this supply (one OTU) was significantly lower than in the supplies CA-2 (40 OTUs) and CA-3 (34 OTUs). Ten OTUs, obtained from all three supplies, clustered within the Cryptophyta phylum and one of these OTUs, with 93% similarity to *Chroomonas* sp., was obtained from all three supplies (see Table C.5 for details). A total of eight OTUs, obtained from all three distributed water types, showed the highest similarity to viridiplantae. One of these eight OTUs, obtained from supply CA-1, clustered within the Chlorophyta and showed 98.5% similarity to *Chlorella luteoviridis*. Six (3%) of the 195 obtained OTUs had similarities below 75% for described sequences in the SILVA database and remained unidentified as described before [Chapter 2]. These observations show that the eukaryotic communities and the concentrations of cultivable *Legionella* spp. differed in the investigated supplies all using seawater as source.

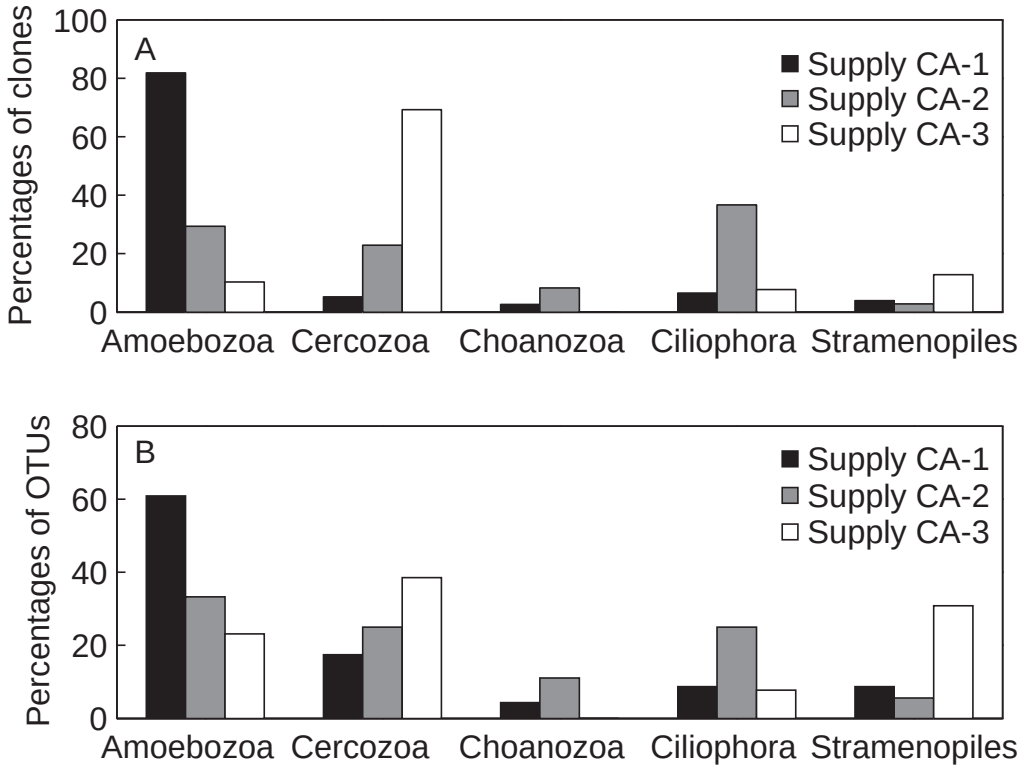

Figure 4.3: (A) Taxonomic distribution of free-living protozoa, based on 18S rRNA gene clones retrieved from distributed water of supplies CA-1, CA-2 and CA-3. (B) Taxonomic distribution of OTUs with highest similarity to free-living protozoa retrieved from distributed water of supplies CA-1, CA-2 and CA-3. Data are totals for all analyzed samples of distributed water of the indicated supply.

4.4 Discussion

Detection of free-living protozoa with PCR-based methods

In the present study, molecular techniques targeting the 18S rRNA gene were instrumental for the detection and identification of a large variety of small eukaryotes, including potential protozoan hosts for *Legionella* spp. in the three drinking water supplies. The relative abundances of different sequences in the clone libraries may not represent the community composition because different eukaryotic species can largely differ in 18S rRNA gene copy numbers, in particular in metazoa [160]. For obtaining quantitative information, specific qPCR assays were used for the detection of two groups of free-living protozoa serving as environmental hosts for *L. pneumophila*, viz., *H. vermiformis* and *Acanthamoeba* spp.

H. vermiformis was observed with the specific qPCR [149] in all samples from which clones with $\geq$ 99% similarity to this organism were retrieved. However, such clones were not obtained from a few samples of supplies CA-2 and CA-3 which were positive with the qPCR for *H. vermiformis*. Obviously, the

Table 4.5: Numbers of retrieved clones, OTUs and estimated richness of OTUs (sequence similarity of $\geq$ 99%) in distributed water of supplies CA-1, CA-2 and CA-3.

Source and type of organism	No. of clones	No.[a] of OTUs identified	Coverage index[b]	Total estimated OTU richness (Chao1[c])		
				Mean	Min.	Max.
Eukaryotes in clone libraries						
Distributed water CA-1[d]	214	63	30	129	89	236
Distributed water CA-2[d]	427	107	25	222	165	337
Distributed water CA-3[d]	267	52	19	118	81	209
Total[e]	**908**	**195**	**21**	**452**	**347**	**633**
Free-living protozoa in clone libraries						
Distributed water CA-1[d]	77	23	30	42	27	95
Distributed water CA-2[d]	109	36	33	59	43	110
Distributed water CA-3[d]	39	13	33	22	15	58
Total[e]	**225**	**59**	**26**	**111**	**79**	**190**

[a] OTUs obtained from more than one sample are included only once, therefore the sum of the OTUs in the three supplies gives excess values.

[b] Number of OTUs/number of sequences $\times$ 100%.

[c] The Chao1 index [51] was calculated with DOTUR [223].

[d] Data are totals for all analyzed samples of distributed water of the indicated water supply.

[e] Chao1 estimation is based on the total of the clones, therefore the sums of the estimated values per supply give other values.

eukaryotic communities in these samples were predominated by other organisms. Clones with 82-86% similarity to *Acanthamoeba* spp. were retrieved from several samples of supplies CA-2 and CA-3, but the specific qPCR for *Acanthamoeba* spp., [205] was negative in these samples. These 18S rRNA genes were not amplified with the *Acanthamoeba* genus-specific primers, suggesting that these sequences did not represent *Acanthamoeba* spp. These observations confirm the utility of qPCR methods for detecting specific free-living protozoa.

Conditions affecting microbial growth and protozoan richness in the three supplies

Water quality of the three supplies, using seawater as source, is influenced by the treatment processes, e.g., type of desalination, filtration processes, softening, addition of corrosion inhibitors or chlorine, and the conditions in the distribution system, e.g., pipe materials, hydraulics and residence time. Treated water of the examined treatment plants, all using seawater as source, contained a very low concentration of NOM ($<$ 0.1 mg C liter^{-1}) and a low turbidity (Table 4.1). Still, the three supplies differed in concentrations of ATP, colony-forming *Legionella* spp., *H. vermiformis* and compositions of

communities of free-living protozoa and other eukaryotes in the distributed water (Table 4.3 and 4.5). These parameters also varied between the different locations within one supply area, demonstrating the complexity of the interactions with environmental conditions. Comparison of the observations in the three Caribbean supplies with those in a similar study of two water supplies in the Netherlands [Chapter 2] and the typical behavior of certain identified organisms enable the identification of several conditions affecting the microbial communities.

The varying ATP concentrations combined with the low turbidity and the low iron concentration in distributed water of supply CA-1, suggests that microbial growth mainly occurs in biofilms on the walls of reservoirs and pipes of this supply (Table 4.2). The relatively high concentrations of *H. vermiformis* and the large proportion and high richness of Amoebozoa in this supply supports this suggestion, because amoebae feed much more effectively on attached microorganisms in a biofilm than on suspended prey [196]. The absence of metazoa in most samples of supply CA-1 also indicates that sediments, which are needed for their growth, were insufficiently available [274].

The high turbidities in supply CA-2 correlate with iron concentrations, indicating that sediments originate from corroding cast iron pipes. The correlations between turbidity and ATP and between turbidity and cultivable *Legionella* spp. demonstrate that the sediments support microbial growth. In comparison with supplies CA-1 and CA-3, sequences related to Ciliophora constituted a relatively high proportion (25%) of OTUs of the free-living protozoa in supply CA-2 (Fig. 4.3). Ciliates feed effectively on suspended bacteria, and their relatively large cell size enables these organisms to consume a large variety of prey types, such as algae, flagellates and other ciliates [196]. In addition, a large number of metazoan OTUs, namely 22, were observed in supply CA-2. Ciliates and metazoa also constituted significant proportions of the eukaryotic community in the distribution system of a groundwater supply in the Netherlands, with elevated concentrations of ATP (10 ng liter^{-1}) and NOM (8 mg C liter^{-1}) [Chapter 2]. Obviously, these conditions and accumulation of sediments promote growth of metazoa, ciliates and also cultivable *Legionella* spp.

At most locations in supply CA-3, low turbidities ($<$ 0.5 NTU) and low concentrations of iron ($<$ 0.05 mg liter^{-1}), ATP ($<$ 1 ng liter^{-1}), *H. vermiformis* ($<$ 2 cells liter^{-1}) and cultivable *Legionella* spp. ($<$ 1×10^3 CFU liter^{-1}) were observed (Table 4.2 and 4.3). Elevated ATP concentrations ($>$ 4 ng liter^{-1}) at three locations indicating local accumulation of biomass, did not all correspond with elevated turbidity. Small flagellated Cercozoan types, mainly *Cercomonas* spp., predominated (69% of clones) the free-living protozoan communities in the clone libraries of supply CA-3 (Fig. 4.3). These flagellates can produce pseudopodia which attach to surfaces, but preferentially feed on suspended prey [182, 196]. In an experimental distribution system, flagellates predominated in the drinking water, but were absent in the related biofilm [233]. Cercozoan types also predominated in the biofilm in a groundwater supply in the Netherlands with low concentrations of ATP ($<$ 1 ng liter^{-1}) and NOM ($<$ 0.5 mg C liter^{-1}), but were a minor fraction in a groundwater supply with elevated concentrations of ATP and NOM [Chapter 2]. No significant correlation was observed between the concentrations of ATP and *H. vermiformis* in the water samples collected from the three supplies. In supply CA-3, the *H. ver-*

miformis concentration was below the detection level (< 2 cell liter^{-1}) in all but one of the samples with a low ATP concentration (< 1 ng ATP liter^{-1}). Also, this protozoan was not detected in water and biofilms in the distribution system of the groundwater supply in the Netherlands with ATP concentrations < 1 ng liter^{-1} and low biofilm concentrations [Chapter 2]. *H. vermiformis* was observed at concentrations up to 815 cells liter^{-1} in the summer in the Netherlands in distributed water with elevated concentrations of NOM and ATP. These observations suggest that growth of *H. vermiformis* in drinking water distribution systems is limited at ATP concentration < 1 ng liter^{-1}. The proportion of eukaryotic clones and OTUs which clustered within the Amoebozoa, retrieved from supplies CA-2 and CA-3, were similar to the proportions in treated water and distribution system biofilms in the Netherlands at temperatures that were 15 to 20°C below those in the three Caribbean supplies. Thus, the proportion of free-living amoebae in the eukaryotic community in drinking water may not depend on water temperature [163, 256].

Both, in temperate and tropical regions, clones clustered within Amoebozoa, Cercozoa, Choanozoa, Ciliophora and Stramenopiles, but sequences related to Euglenozoa and Myzozoa were only observed in the temperate region. Probably, these organisms grow only in drinking water with temperatures below 20°C, although several Euglenozoan types were obtained from a volcanic area at temperature above 30°C [236]. Many stramenopiles types are observed in marine environments, but the obtained Stramenopiles OTUs clustered with flagellates and algae earlier obtained from freshwater or soils [24, 212].

Host protozoa and pathogenic free-living protozoa

The detection of *H. vermiformis*, *Acanthamoeba* spp. and *L. pneumophila* in the investigated supplies is consistent with other studies on drinking water systems in tropical regions [25, 41, 193, 222, 248]. The present study confirmed that *H. vermiformis* is a much more common amoeba in drinking water than *Acanthamoeba* spp. [Chapter 3]. This difference may in part be explained by the much higher yield of *H. vermiformis* as compared to *Acanthamoeba* spp. when feeding on prey bacteria [286].

The colony counts of *Legionella* spp. in supplies CA-1 and CA-2 correlated significantly with the concentration of *H. vermiformis* (Fig. 4.2 and Table 4.3). The log value of the ratios between the concentrations of *Legionella* spp. and *H. vermiformis* in distributed water samples containing both organisms, ranged from 1.2 to 3.9. These values are below the upper tolerance limit of 4.5 as determined in biofilm batch tests using different types of freshwater inoculated with *L. pneumophila* and *H. vermiformis* and incubated at 37°C [Chapter 3]. Above this upper tolerance limit, protozoan hosts for growth of *L. pneumophila*, other than *H. vermiformis* were observed in these tests. The observations of the present study thus confirm the prominent position of *H. vermiformis* as host for *L. pneumophila* in freshwater environments [88, 151, 283, Chapter 3]. In all three supplies OTUs related to the described hosts *E. exundans* [88] and *Acanthamoeba* spp. [6, 218] were detected in samples with *H. vermiformis*, indicating that more than one protozoan species may have served as host for *Legionella* spp. at these locations. Also the candidate hosts *Neoparamoeba* sp. and *E. thermarum* [Chapter 3] were observed in the present

study, but none of the other protozoa identified as hosts by using *in vitro* experiments [88, 138, 227]. However, certain protists belonging to the genera *Naegleria* and *Vahlkampfia* which include hosts for *L. pneumophila* [219, 260] and/or human pathogens [287], were not amplified with the primers used. *Acanthamoeba* spp. have been identified as opportunistic human pathogens [61, 132], but it is unclear whether the sequences related to such species represent organisms with pathogenic characteristics.

Fungi, metazoa and other eukaryotes

Fungi are relatively common in drinking water distribution systems [108, 111, 112, Chapter 2]. Fungi predominated in the libraries of clones retrieved from treated and distributed water of supply CA-1 despite the high pH (Table 4.1). Clones with $>$ 99% similarity to the pathogenic fungi *Mucor racemosus* and *Malassezia restricta* [115, 250] were obtained from supplies CA-1 and CA-2. OTUs clustering with the genera *Basidiobolus, Candida*, *Pichia* and *Penicillium*, which include pathogenic species [31, 70, 191, 294], were also obtained from the distributed water of all three supplies. Fungi are commonly present in water supplies, also in the Netherlands, but the public health significance of the presence of fungi related to pathogenic species is not clear [111, Chapter 2].

Sequences related to metazoa predominated in the clone libraries of supplies CA-2 and CA-3. Clones related to metazoa also predominated in the distribution system biofilms of the groundwater supply in the Netherlands with a high concentration of NOM [Chapter 2]. Certain metazoa are common inhabitants of drinking water systems and grow in these systems at elevated concentrations of biofilm and sediments [111, 274].

The observation of DNA sequences related to viridiplantae, some of which probably originating from pollen, and Chryptophyta, is consistent with observations on drinking water in Europe [Chapter 2] and in the USA [129]. Various detected algae are mixotrophic and some algae may have grown at locations where the water is disinfected with UV radiation [290].

In conclusion, highly diverse communities of free-living protozoa and other small eukaryotes were observed in the three investigated supplies. The growth of these organisms and *Legionella* spp. is enhanced by biofilms and corrosion-related sediments. An ATP concentration $<$ 1 ng liter^{-1} in drinking water indicates growth-limiting conditions for *H. vermiformis*. Limiting the multiplication of *Legionella* spp. therefore implies reduction of the growth potential of the water and prevention of sediment accumulation.

Acknowledgments

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Chapter 5

Effect of temperature on the communities of free-living protozoa in four freshwater types in a biofilm batch system

Abstract

Temperature affects the grazing and metabolic activities of free-living protozoa, including hosts for *Legionella pneumophila*, in freshwater environments. The present study aimed at determining the effect of temperature on indigenous communities of free-living protozoa by incubating duplicate water samples (1.8 liter) from four freshwater environments at 20, 30, 37 and 42°C for 19 days in a biofilm batch test (BBT). *L. pneumophila* serogroup 1 sequence type 1 was added to enable identification of host protozoa for this bacterium. Incubation at the four temperatures caused shifts in the composition of free-living protozoan communities, present on PVC-P tubing segments added to promote biofilm formation. Only two of the 53 operational taxonomic units (OTUs) related to free-living protozoa obtained from the four freshwater types at day 0 were also detected after incubation. Indigenous *Acanthamoeba* spp., detected with qPCR, multiplied at 20 and 30°C, and at 37°C in one flask with cooling tower water. *Hartmannella vermiformis* proliferated at these temperatures and at 42°C in one flask with river Rhine water. Hence, this protozoan can multiply over the entire temperature range at which *L. pneumophila* can grow. Growth of organisms related to Amoebozoa and Stramenopiles was observed at all four temperatures, whereas Cercozoan and Euglenozoan types predominated in the BBT system incubated at 30 and/or 37°C. Yet-undefined sequence types of *L. pneumophila* multiplied in river Rhine water and in cooling tower water incubated at 42°C. The Amoebozoan *Arachnula* sp. was identified as a candidate host for *L. pneumophila* at 42°C, but *in vitro* studies with this organism are needed for confirmation.

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5.1 Introduction

Free-living protozoa are ubiquitous in natural and engineered freshwater systems and have been detected in freshwater environments at temperatures ranging from 0.5 to 68°C [108, 217, 255, 256, Chapter 2]. Obviously, free-living protozoa proliferate or survive at a wide temperature range. Temperature affects the grazing activity, cell size and the metabolic activity of these organisms and therefore will have an impact on the protozoan community composition [126, 213, 258]. The temperature range or the optimal temperature for growth of selected free-living protozoa has been investigated in various *in vitro* or animal studies. Several of the protozoa studied, e.g., *Hartmannella vermiformis*, *Acanthamoeba* spp. and *Tetrahymena* spp. [89, 219, 218] can serve as hosts for pathogenic bacteria including *Legionella pneumophila* [86] and/or are human pathogens, e.g., *Acanthamoeba* spp. and *Naeglaeria* spp. [61, 132, 287]. The highest rate of cell reproduction of *Tetrahymena pyriformis* was observed between 17.5 and 33.5°C with an optimal growth temperature between 27.5 and 29°C [258]. However, the maximum growth temperature can differ between species of one genus; of 19 tested *Acanthamoeba* spp. grown at 30°C on non-nutrient agar, only a few, e.g., *A. culbertsoni* and *A. royreba*, proliferated at 40°C [63].

The ability of strains of the genera *Naegleria* and *Acanthamoeba* to grow at elevated temperatures seems directly related to virulence and nonvirulent strains were unable to grow at normal or elevated human body temperatures [108]. Temperature is also an important condition for the growth of these free-living protozoa in aquatic environments, such as *Acanthamoeba castellanii* and *Acanthamoeba palestinensis*, as they do not multiply at temperatures above 37°C [63]. Certain *Naegleria* spp. are thermotolerant and have been observed in aquatic environments at elevated temperatures up to 64°C [37, 199], but *in vitro* growth of these protozoa has been demonstrated only at temperatures up to 46°C [108]. Growth of *H. vermiformis* has been observed at 55°C using *in vitro* cultures [148, 217] and this organism has been described as one of the most thermotolerant host protozoa for *L. pneumophila* [217].

Information about the effects of temperature on free-living protozoan communities in freshwater environments is still rather limited [43], despite the important role of these organisms in affecting bacterial communities and public health concerns about pathogenic free-living protozoa. Assessment of the effect of temperature on free-living protozoan communities in engineered water systems is complicated, because of fluctuations in temperature and limitations in detection and identification of free-living protozoa with microscopy and cultivation methods. Therefore, a biofilm batch test (BBT) in combination with molecular methods [Chapter 3], based on the 18S rRNA gene for detection and identification of free-living protozoa, was used for determining the effects of temperature on protozoan communities in freshwater. Incubation temperatures were selected based on the following considerations: (i) 20°C is the temperature of drinking water during distribution in summer in temperate regions [202, Chapter 2]; (ii) 30°C is the water temperature in tropical regions and in 'cold' pipes of warm tap water [26, Chapter 4]; (iii) 37°C is the optimal growth temperature of *L. pneumophila* and other human pathogens [137], and (iv), 42°C is close to the maximum temperature at which growth of *L. pneu-*

Table 5.1: Characteristics of the examined water types.

Parameter	Cooling tower water	River Rhine water	Tap water I	Tap water II
Temperature (°C)	24.4	9.8	12.2	9.6
Active biomass (ng ATP liter^{-1})	148	150	2.7	2.7
NPOC[a] (mg C liter^{-1})	21	2.7	7.1	4.5
Acanthamoeba spp. (cells liter^{-1})	1.3×10^{2}	1.5×10^{1}	<2	8.2×10^{0}
H. vermiformis (cells liter^{-1})	6.8×10^{2}	3.6×10^{1}	7.9×10^{1}	9.0×10^{1}
Cultivated *Legionella* spp.(CFU liter^{-1})	nd[b]	nd[b]	200	<100
L. pneumophila (mip genes liter^{-1})	1.6×10^{6}	6.0×10^{4}	<100	<100
Legionella spp. (GU liter^{-1})	5.6×10^{6}	1.3×10^{7}	4.7×10^{6}	3.4×10^{6}

[a]: NPOC: non-purgeable organic carbon.

[b]: nd: not detectable, due to growth of other bacteria on plates.

mophila has been observed in tap water [192, 284, 292]. In the present study, water from the river Rhine, a cooling tower and two tap water installations were incubated in the BBT system at the temperatures mentioned above. The objectives of the study were (i) to determine at which temperatures indigenous *H. vermiformis* and *Acanthamoeba* spp. can proliferate in the test system; (ii) to identify which free-living protozoa predominate at the selected temperatures and (iii) to identify potential protozoan hosts for *L. pneumophila* at these temperatures.

5.2 Materials and methods

Experimental setup

Four freshwater types were incubated in a biofilm batch test (BBT) to determine the growth of indigenous free-living protozoa at selected temperatures [Chapter 3]. The BBT setup consisted of thoroughly-cleaned heat-sterilized (4 hrs at 150°C) Pyrex glass Schott flasks with a volume of 2 liter containing 1.8 liter of test water and six segments of plasticized polyvinylchloride (PVC-P) tubing (internal diameter: 15 mm, length 15 mm, total surface area about 12.5 cm^2), which had been heat-treated in tap water (30 min at 100°C). Nitrate and phosphate were added to each flask at final concentrations of 72.5 μM and 13.5 μM respectively, to prevent growth limitation by these nutrients. Also *L. pneumophila* (serogroup 1, sequence type 1) was added at a concentration of about 4×10^{4} *mip* gene copies liter^{-1} to ensure the presence of this bacterium as described in Chapter 3. Duplicate flasks were incubated at 20, 30, 37 and 42°C ($\pm$ 1°C) in the dark during 19 days. The concentrations of *Acanthamoeba* spp., *H. vermiformis* and *Legionella* spp. and the richness of eukaryotic communities in the planktonic phase and biofilm were monitored at day 0 and after 10 and 19 days of incubation.

Four freshwater types, with different eukaryotic communities, viz., cooling tower water, river Rhine water and water from two tap water installations were incubated in the BBT system (Table 5.1). Tap water I was collected from an installation in the distribution area of a groundwater supply with a high concentration of NOM (7.9 mg C liter^{-1})[Chapter 2]. Tap water II was collected from an installation in the distribution area of a surface water supply using lake water as source [Chapter 3]. Both drinking water types are distributed without a chemical disinfectant. Samples of about 20 liter were collected in containers of polyethylene, stored at 4°C and processed within 24 h.

Microbiological methods

The microorganisms grown on the surface of PVC-P segments were removed and suspended in 60 ml of water from the involved BBT flask using low energy sonication as described elsewhere [151]. Total concentrations of adenosine triphosphate (ATP) in the planktonic phase and in the biomass suspension were determined as previously described [168]. Total direct cell counts of the suspension of *L. pneumophila* serogroup 1 used for inoculation, and of suspensions of *Acanthamoeba castellanii* (CCAP 1501), and *H. vermiformis* (ATCC 50237) used for the calibration curves, were determined using acridine orange and epifluoresecnce microscopy (Leica DMRXA, the Netherlands) [118]. Direct plating on BCYE medium and incubation at 37°C was used for detection of culturable *Legionella* spp. at days 0 and 10 [78, 184]. For characterization of the isolated colonies, five colonies per sample were analyzed by sequence-based typing (SBT), a molecular typing tool for *L. pneumophila*. This method uses digitized allelic profiles of seven predetermined genes [99, 100, 206]. The SBT database is available through the website of the European Working Group for *Legionella* Infections (EWGLI; www.ewgli.org).

Volumes of 200 ml of the planktonic samples at day 0 and 50 ml of the biofilm suspensions were filtered through a 0.22-μm-pore-size 55-mm-diameter polycarbonate Track-Etch Membrane (Sortorius, Goettingen, Germany) to isolate the microbiota. In addition, 500 ml of the planktonic samples collected at day 0 were filtered through a 1.2-μm pore-size 55-mm-diameter polycarbonate Track-Etch Membrane (Sortorius, Goettingen, Germany) for collection of the eukaryotes in these water types. Subsequently, DNA of the collected microorganisms was isolated following previously described procedures in Chapter 2.

Concentrations of *Acanthamoeba* spp., *H. vermiformis, L. pneumophila* and *Legionella* spp. were determined by quantitative PCR (qPCR) at days 0, 10 and 19 as described earlier [149, 178, 205, 288]. Quantification of *Acanthamoeba* spp. and *H. vermiformis* was based on calibration curves which were constructed by preparing 10-fold dilutions of DNA extracted from suspensions with known numbers of cells of *A. castellanii* and *H. vermiformis*. The detection limit was one cell of *Acanthamoeba* spp. and *H. vermiformis* per filtered volume. The diversity of *H. vermiformis* in the four water types detected before and after incubation was determined by sequencing directly the PCR amplicons (464 bp) obtained with the qPCR for 18S rRNA gene-targeted *H. vermiformis* [149]. Quantification of *L. pneumophila* and *Legionella* spp. was done using a plasmid-based calibration curve [288, Chapter 3]. The concentrations

of *L. pneumophila* are measured as *mip* gene copies liter^{-1} or cm^{-2}. The concentration of *Legionella* spp. is expressed in genome units (GU) liter^{-1} or cm^{-2}. All primers and probes were produced at Biolegio (Malden, the Netherlands) and all qPCR assays were performed in 96-wells plates in an I-cycler real-time PCR detection system (Biorad, Veenendaal, the Netherlands).

The richness and composition of the eukaryotic communities in the planktonic and biofilm samples were assessed by terminal restriction fragment length polymorphism (T-RFLP), and cloning and sequence analysis of 18S rRNA gene fragments (about 550 bp) as described in Chapter 2. T-RFLP analysis was done for all BBT flasks at day 0 in the water samples of 500 ml and in the suspensions of attached biomass (50 ml) collected after 10 and 19 days of incubation. All T-RFLP analyses were performed on a single day to minimize the experimental variation. Forty-five clones of each water type at day 0 were analyzed and also 23 or 45 clones retrieved from the suspensions of biomass removed from the PVC-P segments at day 10, depending on the complexity of the band pattern of the T-RFLP fingerprints. All obtained 18S rRNA gene sequences were grouped into operational taxonomic units (OTUs) with $\geq$ 99% similarity.

The obtained sequences were compared to sequences in the National Center for Biotechnology (NCBI)-database by BLAST search and imported and aligned into the SSU Ref SILVA94 database released in February 2010 using the ARB software package as previously described [165, 203, Chapter 2]. The obtained eukaryotic sequences were organized into taxa based on the classification system of Cavalier-Smith [46] and the structure in the SILVA database [203].

Statistical analysis

The statistical significance of the growth of *Acanthamoeba* spp., *H. vermiformis*, *L. pneumophila* and *Legionella* spp. in the BBT flasks between day 0 and day 10, and between day 0 and day 19, was determined using log-transformed concentrations. First the *F* test was used (with 95% confidence) to test on equality of variances of the two populations. If the *F* test rejected the equality of variances, the adjusted *t* test was applied, whereas the regular *t* test was used when the *F* test did not reject the equality of variances. The (adjusted) *t* test was applied with 95% confidence (one-sided testing). For each separate sample the Bonferroni correction for multiple comparisons was used, as two tests were applied for each sample.

Nucleotide sequence accession numbers

The 18S rRNA gene sequences determined in this study have been deposited in GenBank under accession numbers JF774827 to JF775360.

5.3 Results

Growth of *Acanthamoeba* spp., *H. vermiformis*, and *Legionella* spp. at 20, 30, 37 and 42°C

ATP concentrations increased in the water and on the surface of the PVC-P segments during incubation demonstrating that microbial growth occurred in all BBT flasks, except for one flask with tap water I incubated at 42°C (see Table D.1 in Appendix D for details). The maximum ATP concentrations observed in the BBT flasks with cooling tower water, river Rhine water and tap water I ranged from 1.1 to 13.8 ng ATP cm^{-2}, whereas the maximum concentrations in the BBT flasks with tap water II ranged from 20.1 to 31.3 ng ATP cm^{-2} (except for one flask incubated at 42°C with a concentration of 15.0 ng ATP cm^{-2}).

Acanthamoeba spp. were observed at day 0 in three of the four tested water types, and *H. vermiformis* was present in all four water types at day 0 (Table 5.1). The Amoebozoa phylum, however, did not predominate in the corresponding eukaryotic clone libraries (Fig. 5.1 and see Table D.6 for more details). A significant increase ($\geq$ 1 log unit, $p < 0.025$) of the concentration of *Acanthamoeba* spp. was observed in three of the eight BBT flasks incubated at 20°C, in five flasks incubated at 30°C and in one flasks incubated at 37°C (Fig. 5.2 and see Tables D.2 to D.5 for more details). *Acanthamoeba* spp. were also detected with qPCR in the BBT flasks with cooling tower water incubated at 42°C, but did not multiply in the flasks incubated at this temperature. Multiplication of *H. vermiformis* was observed at 20, 30 and 37°C in all four tested water types. At 42°C, growth ($>$ 1 log units $p < 0.025$) of *H. vermiformis* was detected in one of the two BBT flasks with river Rhine water, but the protozoan was also observed at 42°C in the BBT flasks with cooling tower water. All 18S rRNA gene sequences (464 bp), amplified with the specific *H. vermiformis* primers [149] retrieved from the tested water types before and after incubation at 20, 30 and 37°C, showed $\geq$ 99% similarity to each other. Clones related to *Acanthamoeba* spp. and *H. vermiformis* predominated in the libraries of tap water II after incubation.

Indigenous *L. pneumophila* was observed in cooling tower water and in river Rhine water at day 0 (Table 5.1). A strain of *L. pneumophila* (ST 1) was inoculated into each BBT flask to ensure the presence of viable *L. pneumophila* in all water types. The bacterium did not multiply in the BBT flasks incubated at 20°C, but significant growth ($p < 0.025$) of *L. pneumophila* was detected in 17 of the 24 BBT flasks incubated at 30, 37 and 42°C (Fig. 5.2 and see Tables D.2 to D.5 for more details). Colony counts increased to values of 4.5×10^2 CFU cm^{-2} to 1.9×10^6 CFU cm^{-2}, confirming that multiplication had occurred in the involved samples. In these 17 BBT flasks also *H. vermiformis* was observed. The maximum concentrations of *L. pneumophila* were 1.2 to 5.1 log units higher than the maximum concentrations of *H. vermiformis*. *Acanthamoeba* spp. were detected in ten of these 17 BBT flasks with concentrations below those of *H. vermiformis* in seven of these ten flasks (Fig. 5.2).

Most colonies of *L. pneumophila* grown at 30 and 37°C were identified as ST 1, identical to the inoculated strain. In the BBT flasks with river Rhine water incubated at 42°C also ST 93 and an unknown

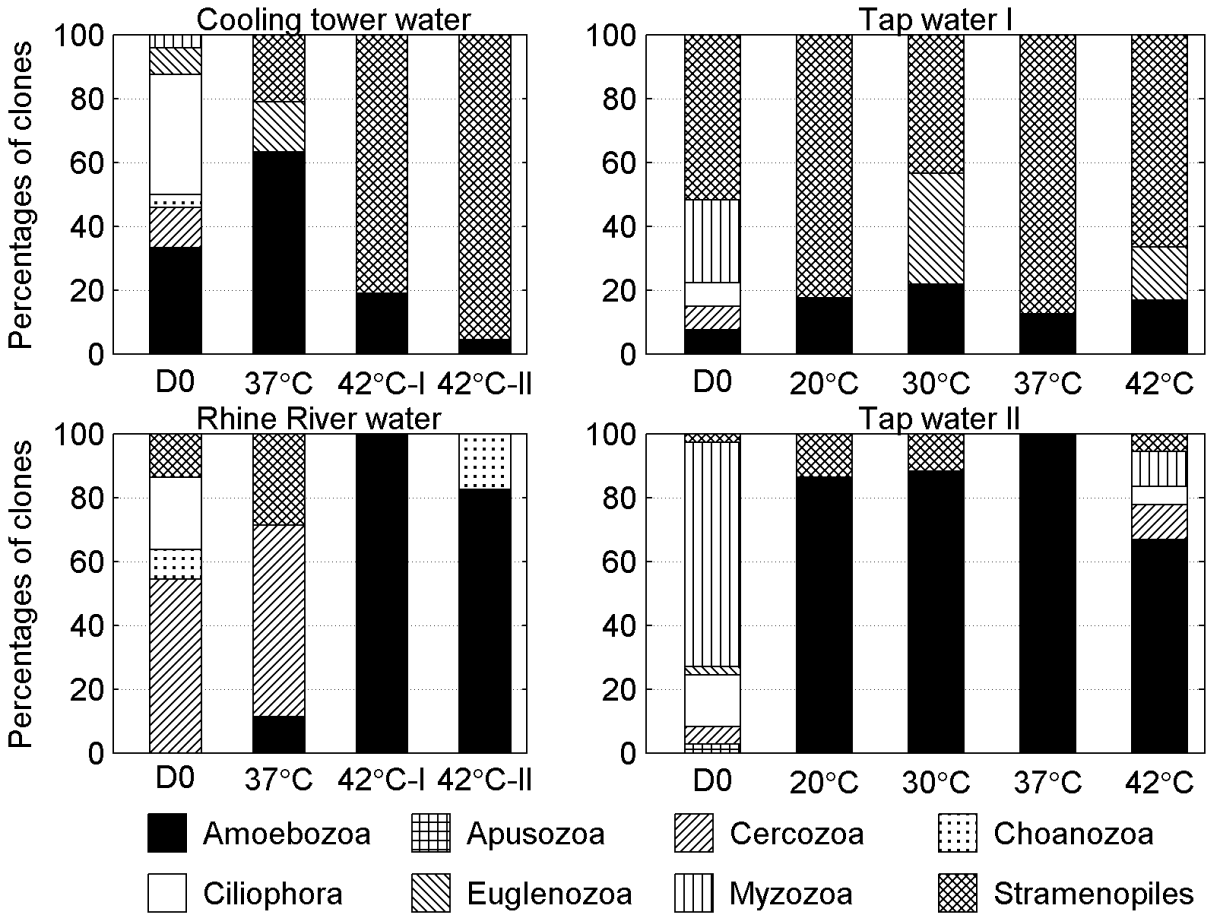

Figure 5.1: Taxonomic distribution of free-living protozoa, based on 18S rRNA gene sequences retrieved before incubation (D0) and after incubation at 20, 30, 37, and 42°C from the BBT flasks inoculated with cooling tower water, river Rhine water and tap water I and II.

sequence type were found. Cooling tower water incubated at 42°C yielded five yet undefined sequence types. *L. pneumophila* did not grow in 15 of the 32 BBT flasks, eight of which were incubated at 20°C, although growth of *Acanthamoeba* spp. and/or *H. vermiformis* was observed in 10 of these flasks. The concentration of *L. pneumophila* decreased with more than 1 log unit in 6 of the 8 BBT flasks incubated at 20°C. In two BBT flasks incubated at 20°C the concentration of *Legionella* spp. increased significantly ($p < 0.025$) with 1 log unit. No growth of *Acanthamoeba* spp., *H. vermiformis* and *Legionella* spp. was observed in the BBT flasks with river Rhine water (one flask) and tap water types I and II (duplicate flasks) incubated at 42°C.

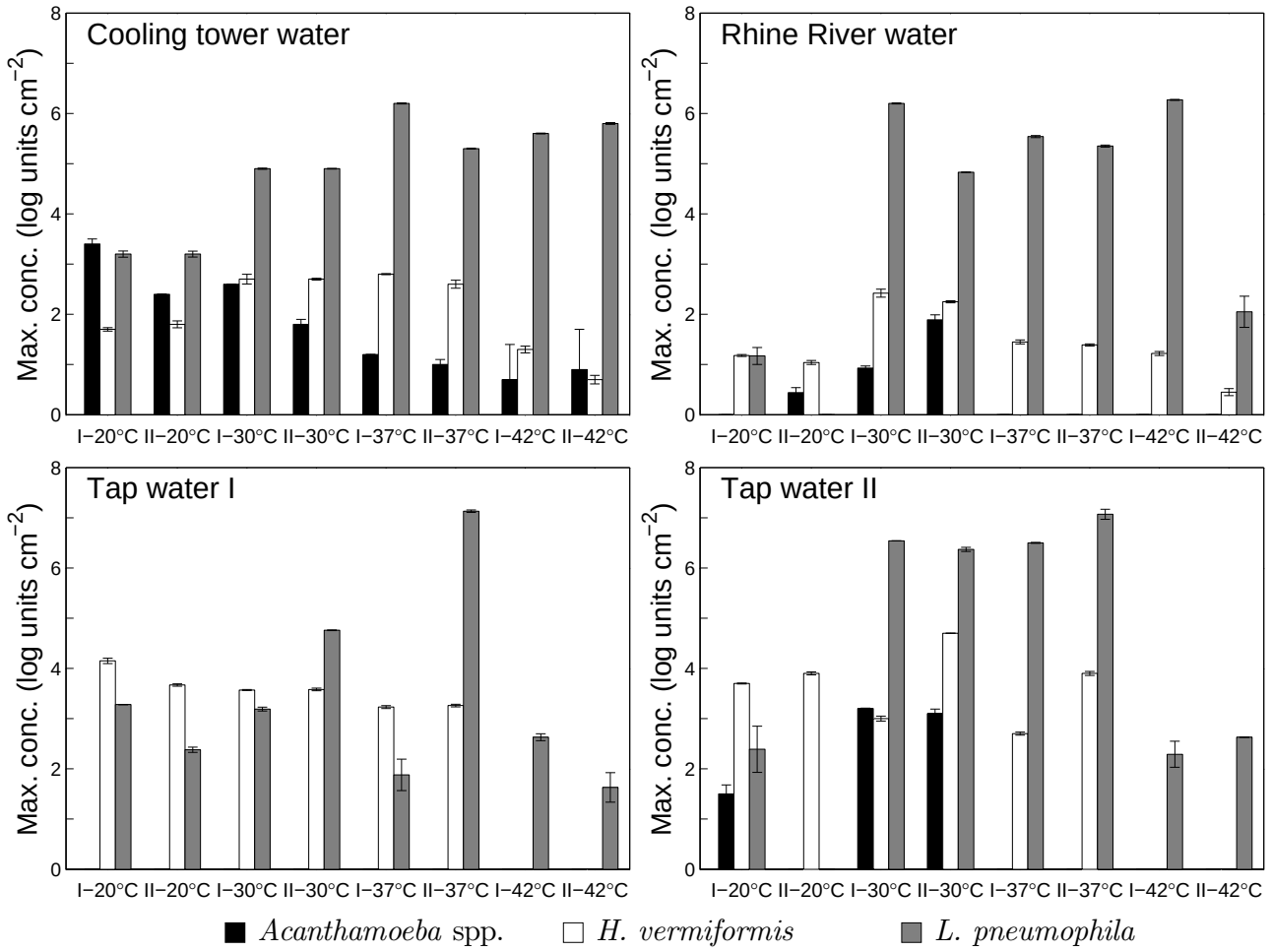

Figure 5.2: Observed maximum concentrations of *Acanthamoeba* spp. (log cells cm^{-2}), *H. vermiformis* (log cells cm^{-2}) and *L. pneumophila* (*mip* gene copies cm^{-2}) in the biofilm on PVC-P in duplicate BBT flasks incubated at 20, 30, 37 and 42°C for 19 days. Abbreviations: CTW: cooling tower water, RRW: river Rhine water, TWI: tap water I, and TWII: Tap water II. Initial concentrations of *Acanthamoeba* spp. (CTW: 0.5, RRW:-0.5, TWI: < -1.3, TWII: -0.7), *H. vermiformis* (CTW: 1.2, RRW: -0.07, TWI: 0.26, TWII: 0.3) and *L. pneumophila* (CTW: 4.6, RRW: 3.3, TWI: 3.0, TWII: 3.0) have been converted from log units liter^{-1} to log units cm^{-2} using the surface/volume ratio. Error bars indicate standard deviations of the analyses.

Richness and identity of free-living protozoa

Clones related to free-living protozoa predominated in the libraries of the four water types at day 0 (Table 5.2). A total of 29 OTUs related to free-living protozoa were obtained from the cooling tower water at day 0 and after incubation at 37 and 42°C, and 31 from river Rhine water. From tap water I and II at day 0 and all four incubation temperatures, respectively 28 and 34 OTUs related to free-living protozoa were obtained. Only one of the 53 OTUs, related to free-living protozoa, retrieved from the

four water types at day 0, was observed in more than one water type. This OTU, which was obtained from both tap water types, clustered with Ciliophora (see Tables D.6 to D.9 for details). Obviously, different free-living protozoa predominated in the four water types at day 0.

T-RFLP fingerprints of the eukaryotic communities in the biofilms in the BBT flasks after incubation generally showed less bands than the fingerprints retrieved from the water at day 0 (data not shown). Also the number of detected phyla of free-living protozoa at day 0 was higher than in the biofilm samples collected after incubation (Fig. 5.1 and see Tables D.10 to D.13 for more details). Furthermore, only two OTUs, related to Myzozoa and Stramenopiles, were obtained from both tap water types before and after incubation in the BBT system. These observations confirmed that incubation caused shifts in the composition of eukaryotic communities in the four water types.

Clones related to Amoebozoa predominated ($>$ 60%) in seven of the 14 libraries after incubation, whereas these amoebae constituted less than 33% of the libraries of the same water type at day 0 (Fig. 5.1). Nine OTUs related to the Amoebozoan *Korotnevella stella* were observed in cooling tower water at day 0 and after incubation at 37 and 42°C. In cooling tower water incubated at 37°C, one Amoebozoan-related OTU showed 99.8% similarity to a clone retrieved from another cooling tower water incubated at the same temperature in Chapter 3. The two libraries of river Rhine water incubated at 42°C were predominated by Amoebozoan-related OTUs with 98.9% similarity to *Arachnula* sp. and with 98.1% similarity to a clone which had been obtained earlier from cooling tower water incubated at 37°C [Chapter 3]. The libraries of tap water II incubated at 20 and 37°C were predominated by one OTU with 100% similarity to *H. vermiformis*. This OTU was also obtained from the BBT flasks with tap water I incubated at 20, 30 and 37°C and from cooling tower water incubated at 37°C. Clones with $>$ 96.4% similarity to an *Acanthamoeba* sp. predominated after incubation at 30 and 42°C in tap water II. Furthermore, one Amoebozoan-related OTU with 90.5% similarity to a *Lobosea* sp. was obtained from the libraries after incubation at 20, 30 and 42°C of tap water II.

Stramenopiles-related clones predominated ($>$ 43%) in six of the 14 clone libraries of water after incubation. In cooling tower water incubated at 42°C, 95% of the clones showed 98.7% similarity to a 'Spumella-like' flagellate which clustered with Stramenopiles. This OTU predominated also in the BBT flasks with tap water I at day 0 and after incubation at all four temperatures and was also observed in drinking water of the Caribbean [Chapter 4].

In river Rhine water, clones related to Cercozoan types predominated ($>$ 54%) in the libraries at day 0 and after incubation at 37°C. A higher OTU richness within the Cercozoa phylum was observed after incubation at 37°C (10 OTUs) than before incubation (2 OTUs). After incubation of tap water I at 30°C, a prominent part (35%) of the clones clustered with Euglenozoa and showed 99.8% similarity to *Bodonidae* sp., while this phylum was not detected at day 0.

Two OTUs with $>$93% similarity to *Tetrahymena* spp., which include protozoan hosts for *L. pneumophila* [89, 138], were observed in the libraries of cooling tower water and tap water II at day 0. OTUs re-

Table 5.2: Classification of clones (%) clustering with eukaryotes obtained from four different water at day 0 (D 0) and after incubation at 20, 30, 37, and 42°C. Numbers in the table are given as percentage of total number of clones per sample.

Type of organism	**Cooling tower water**			**River Rhine water**			**Tap water I**					**Tap water II**				
	D 0	37	42	D 0	37	42	D 0	20	30	37	42	D 0	20	30	37	42
Free-living protozoa	57	39	100	59	73	99	68	100	100	100	70	88	100	77	100	86
Fungi	41	61	0	3	27	1	5	0	0	0	15	10	0	23	0	14
Metazoa	0	0	0	35	0	0	28	0	0	0	0	2	0	0	0	0
Viridiplantae	0	0	0	3	0	0	0	0	0	0	15	0	0	0	0	0
Unknown	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total number of clones	**42**	**46**	**44**	**37**	**48**	**69**	**40**	**17**	**23**	**24**	**13**	**42**	**22**	**22**	**24**	**21**

lated to the candidate hosts *Diphylleia rotans*, *Neoparamoeba* sp. and *Rhinosporidium seeberi* [Chapter 3] were observed in the BBT flasks containing river Rhine water with growth of *L. pneumophila* incubated at 37 and 42°C.

These observations demonstrate that a large variety of free-living protozoa were present in the water types tested at day 0 and after incubation at the four temperatures. *Acanthamoeba* spp. and *H. vermiformis* multiplied in these flasks at temperatures from 20 to 37°C. *H. vermiformis* even proliferated in river Rhine water at 42°C and at this temperature also growth of *L. pneumophila* was observed in this water.

Richness and identity of fungi and other small eukaryotes

Fungi were observed in the clone libraries of all four water types at day 0 (Table 5.2 and see Table D.7 for more details). Fungi-related OTUs obtained from cooling tower water at day 0 and after incubation at 37°C, showed the highest similarity to Ascomycota (see Tables D.7 and D.14 for details). Also, one OTU from tap water I incubated at 42°C showed the highest similarity to Ascomycota, but clones related to unidentified fungi were obtained at day 0. River Rhine water clones related to Chytridiomycota were observed at day 0, but clones which clustered with unidentified fungi were observed after incubation at 37 and 42°C. None of the four fungi-related OTUs predominated in the library of tap water II at day 0, but after incubation at 30°C clones clustering with Ascomycota predominated and clones related to Basidimycota and unidentified fungi were observed after incubation at 42°C. None of the obtained OTUs were related to known fungal pathogens.

All OTUs related to metazoa obtained from river Rhine water at day 0 clustered with Mollusca and clones related to Nematoda were retrieved from both tap water types at day 0. Moreover, clones from tap water I clustered with Cnidaria and Rotifera. OTUs related to metazoa were not observed after incubation of the four water types, suggesting that the BBT system did not promote growth of these organisms within the applied incubation period.

Two OTUs related to viridiplantae were obtained after incubation of tap water I, but viridiplantae were not observed at day 0. One OTU which clustered with viridiplantae was obtained from river Rhine water at day 0. One OTU from cooling tower water at day 0 remained unidentified.

5.4 Discussion

Experimental setup of the BBT system

Incubation of indigenous organisms in batch tests with biofilm formation, simulating *in vivo* conditions, has yielded information about conditions promoting growth of protozoa in engineered water systems

[151, 283, Chapter 3]. Free-living protozoa in stock cultures at laboratories may become adapted to growth at higher or lower temperatures as a result of maintenance of the culture before the actual experiment [258]. Therefore, in the present study the effect of the temperature was investigated on free-living protozoa in the indiginous eukaryotic communtities in four different water types (Table 5.1 and Fig. 5.2). With molecular techniques targeting the 18S rRNA gene, a large variety of small eukaryotes, including potential protozoan hosts for *L. pneumophila*, were detected and identified before and during incubation at 20, 30, 37 and 42°C. The incubation period of 19 days results in a young biofilm, favoring the growth of free-living protozoa (Table 5.2). Duplicate BBT flasks did not always show similar results indicating that growth of these organisms depends on complex environmental conditions, or possibly the number of certain free-living protozoa was low at day 0 [Chapter 3].

The relative abundances of different OTUs in the clone libraries may not represent the community composition because different eukaryotic species differ in 18S rRNA gene copy numbers, in particular in metazoa [160]. *Acanthamoeba* spp. and *H. vermiformis* were observed with the specific qPCR in all samples from which clones with $\geq$ 99% similarity to these organisms were retrieved, except for one flask that was incubated with tap water II at 42°C. However, such clones were not included in 8 of the 18 libraries of BBT flasks, which were positive with qPCR for *Acanthamoeba* spp. and/or *H. vermiformis*. These observations confirm the superior sensitivity of qPCR methods for detection of specific free-living protozoa.

Effect of temperature on growth of *Acanthamoeba* spp. and *H vermiformis*

Growth of *H. vermiformis* was observed at 20, 30 and 37°C in all four water types. In a previous study with BBTs no growth of this protozoan was observed at 15°C, but *H. vermiformis* proliferated at 37°C in the water types tested [Chapter 3]. Growth of *H. vermiformis* at 53°C has been reported [217], but in the present study significant growth of this amoeba at 42°C was observed only in river Rhine water. Hence, strains of this species with different maximum growth temperatures may be present in the aquatic environment. Most strains of *Acanthamoeba polyphaga*, *A. castellani* and *A palestinensis*, which have been described as host protozoa for *L. pneumophila* [6, 218], did not grow at temperatures above 37°C [63, 108]. Strains of the *Acanthamoeba* genus, which are able to grow at temperatures to 43°C, e.g., *A. culbertsoni* [108], have rarely been found in hot water systems [217]. The absence of growth of *Acanthamoeba* spp. in the BBT system at 42°C and the limited growth at 37°C is consistent with these reports (Fig. 5.2). Identification of *Acanthamoeba* spp. grown at different temperatures in the present study was not possible, because analysis of the 180 bp amplified by the specific qPCR for *Acanthamoeba* spp. [205] does not allow for unambiguous identification beyond the genus level. Growth of *Acanthamoeba* spp. was detected in nine of the 19 BBT flasks with multiplication of *H. vermiformis*, in water in which both genera were observed at day 0 (Table 5.1 and Fig. 5.2). The concentration of *Acanthamoeba* spp. was higher than the concentration of *H. vermiformis* in two of these 19 BBT flasks. These two BBT flasks had been incubated at 20 and 30°C, indicating that stronger growth of *Acanthamoeba* spp. occurred only at a temperature $<$ 37°C. The present study confirmed that *H. vermi-*

formis is frequently present at higher concentrations than *Acanthamoeba* spp. in freshwater [Chapter 3]. The high yield of *H. vermiformis* feeding on prey bacteria [286] may enhance its competition with *Acanthamoeba* spp.

Effect of temperature on growth of other free-living protozoa

The obtained results confirm that Amoebozoan types can proliferate in the aquatic environment at temperatures ranging from 20 to 42°C (Figs. 5.1 and 5.2). Amoebae feed more effectively on attached than on suspended bacteria [196]. Therefore, the biofilms in the BBT system will favor the growth of this protozoan type. Still, seven of the 14 libraries of water incubated in the BBT system were dominated by clones related to other protozoan phyla than Amoebozoa.

'Spumella-like' flagellates, belonging to the Stramenophiles, which predominated at day 0 and after incubation at all four temperatures in tap water I and in cooling tower water after incubation at 37 and 42°C, have also been isolated from freshwater and soil habitats around the world [24]. However, temperatures of these sources were not reported. In addition, heterotrophic flagellates such as *Spumella* spp. and *Paraphysomonas* spp. have been isolated from drinking water supplies [241, Chapters 2 and 4]. Growth after incubation at different temperatures and the widespread presence suggest that representatives of the Stramenopiles proliferate or survive at a broad temperature range.

Different Cercozoan types predominated in river Rhine water at day 0, but were not observed after incubation at 42°C. Species of the *Cercomonas* genus are common inhabitants of drinking water systems in temperate (< 20°C) and tropical regions (about 30°C), and growth of these Cercozoan types has also been observed at 5°C [62, Chapters 2 and 4]. Thus, Cercozoan types grow or survive at temperatures between 5 and 37°C, but have not been observed at more elevated temperatures.

Euglenozoan types were not observed in drinking water in a tropical region (about 30°C; Chapter 4). However, in the present study Euglenozoan types were obtained from tap water I after incubation at 30 and 42°C, in which they were not detected at day 0. Growth and survival of Euglenozoan types at 42°C in the present study is consistent with observations of these organisms in a volcanic area with temperatures up to 40°C [236].

Clones related to Ciliophoran, Myzozoan and Choanozoan types were only obtained after incubation at 42°C, whereas these free-living protozoa were observed in at least two of the four water types at day 0 (Fig. 5.1). These organisms were unable to compete with other free-living protozoa in the BBT system at 20, 30 and 37°C and probably also did not grow at 42°C, but remained present at 42°C. In addition, Apusozoan types also did grow in the BBT system. Ciliates were also not retrieved in a previous study using the BBT system [Chapter 3]. Ciliophora constituted significant proportions of the free-living protozoan community in the distribution system of a groundwater supply, with elevated concentrations of ATP (10 ng liter^{-1}) and NOM (8 mg C liter^{-1}) and in a drinking water supply in the Caribbean, with

elevated levels of ATP (max. 55 ng liter^{-1}) and turbidity (max. 62 NTU; Chapters 2 and 4). These observations and the feeding characteristics of ciliates suggest that elevated concentrations of biomass or sediments may promote their growth in freshwater systems.

Identity of protozoan hosts for *L. pneumophila*

The presence of *Acanthamoeba* spp. and/or *H. vermiformis* in the four examined water types confirmed that protozoan hosts for *L. pneumophila* are ubiquitous in freshwater [143, 175, 217, 256] (Table 5.1). Two OTUs related to the *Tetrahymena* genus were obtained at day 0, but were not observed after incubation despite their ability to grow at 20 and 30°C [245, 258]. *H. vermiformis* was observed in all 17 BBT flasks with growth of *L. pneumophila*. In two of these 17 BBT flasks, one with river Rhine water and the other with cooling tower water, both incubated at 42°C, the log value of the ratio between the maximum concentrations of *L. pneumophila* and *H. vermiformis* exceeded the upper tolerance limit of 4.5 determined in a previous study with BBT flasks incubated at 37°C [Chapter 3]. This observation may indicate that not *H. vermiformis*, but other protozoa had served as hosts for *L. pneumophila* in these two BBT flasks. Clones related to the Amoebozoan *Arachnula* sp. predominated in the library of one of these flasks. This large naked amoeba (up to 1 mm), which has been observed in soils irrigated with treated sewage effluent [109], feeds on a large variety of prey including bacteria, fungi, algae and small metazoa. Consequently, amoebae belonging to the *Arachnula* genera may have served as host for *L. pneumophila*. The protozoan community in the other flask, with cooling water, was predominated by a Stramenopiles-related OTU with 99% similarity to the 'Spumella-like' flagellate. However, this OTU also predominated in two other libraries of flasks without growth of *L. pneumophila* and no information is available indicating that flagellates can serve as hosts for *Legionella* spp. Therefore, the identity of the protozoan host in one of the flasks with cooling tower water incubated at 42°C remains unknown. In a number of BBT flasks, *Acanthamoeba* spp. and *H. vermiformis* may both have served as hosts for *L. pneumophila*, e.g., in cooling tower water and tap water II. However, information about the ratio between the maximum concentrations of *L. pneumophila* and *Acanthamoeba* spp. is not available for verification. OTUs related to the candidate hosts *Diphylleia rotans*, *Neoparamoeba* sp. and *Rhinosporidium seeberi* [Chapter 3] were observed in BBT flasks with growth of *L. pneumophila* in river Rhine water incubated at 37 and 42°C. However, certain protists belonging to the genera *Naegleria* and *Vahlkampfia* also include hosts for *L. pneumophila* [219, 260] and/or human pathogens [287], but these organisms were not amplified with the primers used.

The concentration of *L. pneumophila* decreased in six of the eight BBT flasks incubated at 20°C, despite the growth of *Acanthamoeba* spp. and/or *H. vermiformis* in these flasks. This observation is consistent with reports about digestion of *L. pneumophila* by amoebae at temperatures below 20°C [192] and the minimum growth temperature of 25°C for *L. pneumophila* in aquatic environments [284]. In a previous study growth of unidentified *Legionella* types was observed in BBT flasks at 15°C [Chapter 3]. In the present study growth of *Legionella* spp. was only observed in two of the eight BBT flasks incubated at 20°C and which all contained *Acanthamoeba* spp. and/or *H. vermiformis*. Information about the

physiology of yet-uncultured *Legionella* spp. growing at 20°C is limited and the absence of growth of these bacteria at this temperature remains unexplained. Also, the absence of growth of *L. pneumophila* in two BBT flasks with tap water I incubated at 30 and 37°C in the presence of *H. vermiformis* remains unexplained.

The absence of growth of *Acanthamoeba* spp., *H. vermiformis* and *Legionella* spp. in five of the BBT flasks incubated at 42°C indicated that the host protozoa present in these water types were unable to multiply at this temperature. Also the inoculated *L. pneumophila* strain (ST 1) probably was unable to multiply at 42°C, because sequence types differing from the inoculated type were obtained from three BBT cultures with river Rhine water and cooling tower water. In the flasks in which no growth of *L. pneumophila* occurred, either no indigenous *L. pneumophila* or protozoan host with the ability to grow at this temperature were present. Previous studies demonstrated that the maximum growth temperature, e.g., 37°C [284], 42°C [292] and 45°C [259] of natural occurring *L. pneumophila* varied between different strains and various test conditions.

Fungi and other small eukaryotes

The BBT system also promoted growth of fungi (Table 5.2), which obviously can grow in freshwater and related biofilms [74, 111, Chapter 2]. Clones related to pathogenic fungi species were neither obtained at day 0 (see Tables D.7 and D.14 for details), nor from water incubated at four temperatures, although pathogenic fungi such as *Candida bombi* and *Pichia ohmeri* are thermotolerant and can grow at temperatures up to 40 to 45°C [185, 262].

No growth of metazoa was observed in the BBT system, although these organisms proliferate in biofilms and sediments in drinking water distribution systems at temperatures between 10 and 30°C [Chapters 2 and 4]. Also in a previous study with BBT systems incubated at 15 and 37°C, metazoan-related OTUs were not dominant in the clone libraries [Chapter 3], suggesting that the BBT system with the (young) biofilms and no sediments do not promote growth of metazoa. The nematode *Caenorhabditis elegans*, described as host for *L. pneumophila* [30], was not observed in the water samples at day 0. Viridiplantae did not grow in the BBT system, because the flasks were incubated in the dark. Hence, the OTUs of viridiplantae obtained after incubation of tap water I at 42°C were not observed at day 0, because other eukaryotes predominated in the clone library of this sample.

In conclusion, growth of indigenous strains of *Acanthamoeba* spp. and *H. vermiformis* was observed at 20, 30, and 37°C, but growth of *Acanthamoeba* spp. was only observed in one of the eight flasks incubated at 37°C. At 42°C only growth of *H. vermiformis* was observed in one flask. Thus, the host *H. vermiformis* can multiply over the entire temperature range at which *L. pneumophila* can grow. Free-living protozoa related to Amoebozoa and Stramenopiles multiplied at temperatures ranging from 20 to 42°C. However, no growth at any temperature of organisms related to Apusozoa, Choanozoa, Ciliophora, and Myzozoa was observed in the BBT system, while these organisms were present at day

0. The Amoebozoan *Arachnula* sp. was identified as candidate host for *L. pneumophila*, but *in vitro* studies with this organism are needed for confirmation.

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Chapter 6

General Discussion

6.1 Study objectives

Free-living protozoa are ubiquitous in freshwater environments, including engineered water systems. As described in the Chapters 2 to 5, these organisms can affect the water quality in drinking water supplies and in (warm) tap water installations. Furthermore, physicochemical and biological water quality characteristics and other environmental conditions determine the abundance and diversities of free-living protozoa in these systems. In the studies described in this thesis molecular methods were applied to study the free-living protozoan communities in drinking water supplies. Other small eukaryotes, mainly fungi and metazoa, were also observed using the applied methods and subsequently have been identified, but this discussion will focus on the identify and abundance of free-living protozoa in drinking water supplies. The selected water supplies varied in concentrations of natural organic matter (NOM), active biomass (ATP) and water temperature. The key objectives of the study described in the present thesis were:

(i) to elucidate the identities and richness of free-living protozoa predominating in drinking water supplies;
(ii) to identify known and yet-undescribed host protozoa for *L. pneumophila* and other *Legionella* spp. in drinking water and other freshwater environments;
(iii) to identify conditions favoring the growth of free-living protozoa in drinking water distribution systems.

Selection of examined freshwater types

The identity of predominant free-living protozoa in treated water at the plant and in water in the distribution system was investigated in two groundwater supplies in the Netherlands and in three supplies in the Caribbean [Chapters 2 and 4]. The concentration of NOM was low ($<$ 0.5 mg of C liter^{-1}) in the treated water of the supplies in the Caribbean and in one of the supplies (supply A) in the Netherlands. In contrast, the other groundwater plant (supply B) produces drinking water with the highest concentration of NOM (about 8 mg of C liter^{-1}) found in the Netherlands. The water types selected for the studies described in this thesis cover the entire range of NOM concentrations in treated water in the Netherlands and in most European countries [251, 202]. The water temperature in the two groundwater supplies in the Netherlands was below 18°C, well within the range of temperatures between 10 and 20°C that are common in temperate regions. It should be noted, however, that the temperature of distributed water produced from surface water can increase up to 25°C in the summer in the Netherlands. The maximum temperature of treated water worldwide, including tropical regions, is about 30°C, but in distribution systems the temperature can increase up to 45°C [57]. Microorganisms present in the distributed water mainly grow in biofilms, and also in other engineered freshwater systems biofilms represent the major proportion of microbial biomass and activity [14, 38]. Therefore distribution system biofilms in the two supplies in the Netherlands were analyzed to obtain information about the occurrence and growth of free-living protozoa in drinking water distribution systems.

To identify protozoa that can serve as hosts for *Legionella* spp. a variety of freshwater types was incubated in a biofilm batch test (BBT) and multiplication of free-living protozoa and *Legionella* spp. was followed during the incubation [Chapters 3 and 5]. Assessment of the effect of temperature on free-living protozoan communities in engineered water systems is complicated and therefore the BBT system was also incubated at temperatures ranging from 15 to 42°C. At 15°C, growth of uncultured *Legionella* spp. occur, whereas 42°C is close to the maximum temperature at which growth of *L. pneumophila* in aquatic systems has been observed [288, 292]. By analyzing a variety of freshwater types in the BBT system, including drinking water, (warm) tap water, surface water and water from cooling towers, growth conditions of different indigenous free-living protozoa obtained from various habitats could be studied. Finally, it is well documented that cooling towers are commonly colonized by *L. pneumophila* and it has been shown that such systems are a source of outbreaks of Legionnaires' disease [68, 179]. Therefore, investigating the communities of free-living protozoa in cooling tower water will also contribute to elucidate the growth conditions of *L. pneumophila* and free-living protozoa.

6.2 Detection and identification of free-living protozoa based on the 18S rRNA gene in freshwater environments

Natural richness of eukaryotes and free-living protozoa

Sequencing the ribosomal RNA (rRNA) gene is currently the method of choice for phylogenetic analyses, nucleic acid based detection and quantification of microbial richness. Molecular ecology methods based on 18S rRNA gene amplification and sequencing has revealed an enormous richness of microbial eukaryotes in every water-related environment sampled so far [80]. Each survey yielded yet-undescribed protozoan species and genera. Consequently, public databases with 18S rRNA gene sequences increased exponentially in the last decade. Also in the investigations described in this thesis, the identification of free-living protozoa based on the 18S rRNA gene, in a variety of freshwater samples, revealed yet-unknown sequences. The newest SSU ref SILVA database release 104, used for processing the data obtained from the BBT system with water samples incubated at four temperatures [Chapter 5], contains more than 50,000 aligned 18S rRNA gene sequences, a number that has doubled since the first release in February 2007 [165, 203]. The 18S rRNA gene is mainly used to quantify concentrations of free-living protozoan species [35, 205] and to identify protozoan communities in aquatic environments [202, 239, 247]. Only a few ecological studies have been conducted on the protozoan communities in drinking water supplies. Currently, amoebae cultivated from freshwater environments are identified based on their 18S rRNA gene [256]. The classification system of Cavalier-Smith of free-living protozoa, used in the investigations described in this thesis, is partly based on 18S rRNA gene sequences [46, and Chapter 1].

About 80% of the obtained OTUs related to free-living protozoa, analyzed in Chapter 5, showed $<$ 99% similarity to OTUs described in the Chapters 2, 3 and 4, confirming that each survey still yields

Table 6.1: Quality characteristics of five examined water supplies and numbers of retrieved clones related to free-living protozoa, OTUs and estimated richness of OTUs (sequence similarity of $\geq$ 99%).

Supply	**Characteristic of the supply**			**No. of clones**	**No.[b] of OTUs**	**Cover-age index[c]**	**Total estimated OTU richness (Chao1 [d])**		
Supplies in the Netherlands[d]	NOM (mg C l^{-1})	ATP (ng l^{-1})	Temp. (°C)[a]				Mean	Min.	Max.
Supply A [Chapter 2]	<0.5	< 1	10	133	54	41	113	81	187
Supply B [Chapter 2]	8	10	10	120	72	60	163	112	274
Total in the Netherlands	**-**	**-**	**-**	**253**	**127**	**50**	**281**	**212**	**407**
Supplies in the Caribbean[e]									
Supply CA-1 [Chapter 4]	<0.5	3.5	30	77	23	30	42	27	95
Supply CA-2 [Chapter 4]	<0.5	< 1	30	109	36	33	59	43	110
Supply CA-3 [Chapter 4]	<0.5	< 1	29	39	13	33	22	15	58
Total in the Caribbean	**-**	**-**	**-**	**225**	**59**	**26**	**111**	**79**	**190**

[a] Temperature of the treated water at the treatment plants.
[b] OTUs obtained from more than one sample are included only once.
[c] Number of OTUs/number of sequences 100%.
[d] The Chao1 index [51] was calculated with DOTUR [223].
[e] Data are totals for all analyzed samples of distributed water of the indicated water supply.

new protozoan species and genera. A number of OTUs related to a few protozoan genera, such as *Hartmannella*, *Acanthamoeba*, *Hemiophrys* and *Cercomonas*, were repeatedly observed in the supplies in the Netherlands and in the supplies in the Caribbean. Several of these free-living protozoa have also frequently been isolated from other engineered freshwater systems [149, 175, 217, 256]. This is consistent with observations of Esteban et al. [83], which indicated that local and global abundances of protozoa in soils are correlated. Species that are locally rare tend to be globally rare, and those that are locally abundant tend to be globally abundant. Finlay and Esteban [90] also postulated that protozoan species are globally ubiquitous and that all species of freshwater protozoa could eventually be discovered in one small pond.

Table 6.2: Numbers of retrieved clones related to eukaryotes, OTUs and estimated richness of OTUs (sequence similarity of $\geq$ 99%).

Supply	**No. of clones**	**No.**[a] **of OTUs**	**Coverage index**[b]	**Total estimated OTU richness (Chao1** [c]**)**		
				Mean	Min.	Max.
Supplies in the Netherlands[d]						
Supply A [Chapter 2]	272	108	40	159	136	204
Supply B [Chapter 2]	273	115	42	145	91	277
Total in the Netherlands	**545**	**219**	**40**	**390**	**328**	**487**
Supplies in the Caribbean[d]						
Supply CA-1 [Chapter 4]	214	63	30	129	89	236
Supply CA-2 [Chapter 4]	427	107	25	222	165	337
Supply CA-3 [Chapter 4]	267	52	19	118	81	209
Total in the Caribbean	**908**	**195**	**21**	**452**	**347**	**633**

[a] OTUs obtained from more than one sample are included only once.

[b] Number of OTUs/number of sequences $\times$ 100%.

[c] The Chao1 index [51] was calculated with DOTUR [223].

[d] Data are totals for all analyzed samples of distributed water of the indicated water supply.

Esteban et al. [83] recorded 365 protozoan species, including flagellates, testate and naked amoebae and ciliates, in the soil of 1 hectare of pasture land. This protozoan species richness is higher than the estimated mean OTU richness observed in the five examined drinking water supplies (Table 6.1). In a study with forest soil, using a comparable primer set as in the investigations described in this thesis, 27% of the sequences were related to protists and types clustering within Cercozoa predominated [11]. The eukaryotic richness was estimated to exceed 180 species, but no standard error was reported. This estimated eukaryotic species richness is low in relation to the estimated mean OTU richness of the supplies in the Netherlands (390 OTUs) and the Caribbean (452 OTUs). The coverage index of the clone libraries, for the study in forest soil, was 46%, based on 99% similarity between the sequences within one OTU. In the marine environment of the North Atlantic and North Pacific Oceans, the coverage index of the clone libraries with partial 18S rRNA gene sequences was 43%, based on OTUs also containing sequences with $>$ 99% similarity [43]. These two coverage indexes are comparable with those for all eukaryotes observed in the drinking water supplies in the Netherlands (Table 6.2). Estimations, with standard errors, of the richness of protozoa in forest soils and marine environments have not been reported; therefore the obtained protozoan richness in the drinking water supplies can not be compared with the protozoan richness in those environments.

Species definition in free-living protozoa

Species of microorganisms are not clearly defined and not all environments have been exhaustively studied, therefore the number of free-living protozoan species globally is unknown and a matter of debate [91, 95]. Genera and species of many free-living protozoa show a relatively high level of sequence divergence of their 18S rRNA gene [131, 239]. Therefore, defining species can be resolved by considering rRNA gene sequences based on operational taxonomic units (OTUs). For the studies described in this thesis, OTUs were defined by a 1% difference in nucleic acid sequences. Also several other studies, dealing with the identification of eukaryotes, divided clone sequences in OTUs with $\geq$ 99% similarity [43, 202], and therefore the protozoan richness can be compared. As described in Chapter 2, similarities between 86.6% and 99.7% were calculated for 18S rRNA gene sequences of a number of protozoan species, which is consistent with the reported percentages of similarity observed between species in another study [43]. However, the investigated species were identified based on morphological traits and the classification of these organisms can be incorrect. Consequently, similarities determined between strains included in the same species either were too low or too high. With the division of sequences into OTUs with 99% similarity, almost all different protozoan species can be distinguished. For analyzing the OTUs as described in Chapter 2 the identification of the free-living protozoa was limited by the available database, which contained approximately 26,000 aligned 18S rRNA gene sequences [203]. In the course of the studies described in this thesis, the identification of sequences from drinking water supplies became progressively more reliable, as related sequences from previous studies, described in this thesis, were available. Still, a few OTUs described in Chapter 5 showed $<$ 75% similarity to sequences in the database. These OTUs could not be further identified, because a number of selected free-living protozoan genera showed a minimal similarity of 76% [Chapter 2]. In view of the ever increasing speed with which new sequencing technologies are currently being developed [166], it can be expected that these OTUs can be identified in the near future when the DNA databases cover a larger proportion of the true diversity of such organisms.

Limitations of the used molecular techniques

The main focus of the research described in this thesis was to identify free-living protozoa in drinking water supplies, but to the best of our knowledge primers for the amplification of all free-living protozoa included in public databases are not available. Therefore, 18S rRNA gene primers, which amplify most eukaryotic organisms represented in public databases, were used for the detection of these organisms. Also other studies, which mainly focus on the identification of communities of free-living protozoa, have identified eukaryotic communities based on the 18S rRNA gene [202, 239]. Consequently, also fungi, metazoa and viridiplantae were observed in the studies described in the previous chapters. Many of these organisms are multicellular and contain more DNA (copies) than unicellular eukaryotes. Furthermore, the copy number of 18S rRNA genes differ significantly between different genera [160]. Therefore, the composition of the clone libraries does not exactly reflect the composition of the involved eukaryotic communities. Partial 18S rRNA gene sequences (about 550 bp) were used for T-RFLP analyses and the eukaryotic clone libraries. These partial sequences demonstrated the

presence of highly diverse eukaryotic communities in the drinking water supplies but did not provide sufficient information for in depth phylogenetic analyses of certain eukaryotic groups [80].

Many of the obtained sequences showed high percentages of similarity (>95%) to described species in the database. The molecular approaches used here, especially the library construction and analyses, remain relatively labor intensive. Nevertheless, the clone libraries described in previous chapters, provide important information about the richness of free-living protozoa in freshwater systems. The application of next generation sequencing approaches, such as 454 pyrosequencing of rRNA gene amplicons, will further improve the knowledge of the eukaryotic richness in various habitats, including that of free-living protozoa [28, 54]. However, collecting information about more gene sequences of yet-unindentified organisms or about organisms with yet-unknown properties and which are small fractions of the protozoan communities, may not be productive. Application of qPCR methods for organisms selected on the basis of their presence and role in protozoan communities in the selected aquatic environment may be a more effective approach aiming at elucidating the interactions between environmental conditions and the occurrence of specific protozoan groups in these environments, e.g., drinking water supplies.

The applied DNA-based approaches do not differentiate between nonviable or even fragmented organisms and living organisms. Two molecular approaches have been described that can add a viability component to PCR methods. The first method is based on the amplification of RNA instead of DNA [20], but isolation of mRNA from complex environmental samples is technically challenging because of the instability of mRNA and the ubiquitous presence of RNAses [146]. Secondly, samples can be treated with DNA-modifying dyes, such as prodidium monoazide and ethidium monoazide bromide, which leads to irreversible modification of the DNA in cells with compromised membranes, prior to molecular analysis [188, 189]. Such DNA-modifying dyes have so far been mostly applied for the assessment of viability of prokaryotes, including *Legionella pneumophila* [52], but also for yeasts [8, 207] and *Cryptosporidium* spp. [34]. Interpretation of these data, however, needs more attention, because of the risk of false-positive results.

Nevertheless, various studies, based on cultivation and microscopic methods, demonstrated intact and viable free-living protozoa, fungi, metazoa and algae in drinking water systems [111, 129, 202, 217, 274]. The examined groundwater supplies did not apply chemical disinfection in water treatment and distribution and also in the Caribbean supplies microbial growth was observed in the distribution systems. Hence, in these supplies, in most cases, the observed microorganisms were viable. Furthermore, during incubation in the BBT system, the concentrations of *Acanthamoeba* spp. and *H. vermiformis* increased and also shifts in eukaryotic communities were observed. This strongly suggests that most of the organisms detected with the molecular analyses represented viable organisms in the examined drinking water systems in the present thesis. The DNA sequences related to viridiplantae probably originated from pollen, which were present in the water sample or were a contamination via air during sampling or sample treatment [Chapters 2 and 4]. However, DNA sequences related to these plants were not observed in the negative controls. Furthermore, local intrusion of plant roots into reservoirs

or pipes in the distribution system has been observed (W. Hoogenboezem, personal communication, December 2010). This may be a source of the obtained sequences related to viridiplantae.

6.3 Free-living protozoa serving as host for pathogenic bacteria and pathogenic free-living protozoa

Occurrence of protozoan hosts for pathogenic bacteria and pathogenic protozoa

Several free-living protozoa, such as species belonging to the genera *Acanthamoeba*, *Echinamoeba*, *Balamuthia*, *Hartmannella*, *Neagleria*, *Vahlkampfia*, and *Tetrahymena* can serve as hosts for pathogenic bacteria, e.g., *Legionella* spp., *Burkholderia* spp., and *Mycobacterium* spp. [Chapter 1]. A few of these protozoa have also been recognized as human pathogens, viz., *Acanthamoeba* spp. [61], *Balamuthia mandrillaris* [277] and *Naegleria fowleri* [287]. One of the objectives of the studies described in this thesis was to identify protozoan hosts for *L. pneumophila*. However, information is lacking about the presence of pathogenic free-living protozoa in drinking water supplies and (warm) tap water installations in the Netherlands and therefore, when possible, these organisms were also identified.

Free-living protozoa related to pathogenic species and/or hosts for *L. pneumophila* such as *Acanthamoeba* spp. and *H. vermiformis*, were observed in drinking water types in the Netherlands and the Caribbean, which is consistent with the abundance of these amoebae in drinking water supplies worldwide [254]. Water treatment plants play a role in reducing the number of these amoebae in water, but several protozoan hosts such as *Hartmannella* and *Echinamoeba*, can proliferate in water treatment processes, e.g., filter beds with granular activated carbon, sand or limestone [58, 256]. In addition, amoebae have the capacity to encyst and are therefore very resistant against disinfection processes like chlorination and ozonation [161]. Furthermore, higher concentrations of *H. vermiformis* and *Acanthamoeba* spp. were observed in the distributed water than in the related treated water at plants [Chapters 2 and 4]. Amoebae related to hosts for pathogenic bacteria and potential human pathogens belonging to the *Acanthamoeba* genus can thus grow in distribution systems. Therefore, it is also essential to take measures to minimize the growth of free-living amoebae in drinking water distribution systems and (warm) tap water installations inside buildings.

H. vermiformis was the most commonly observed protozoan host for *L. pneumophila* in the Netherlands and in the Caribbean [Chapters 2 to 5]. This is consistent with the ubiquitous presence of this organism in other engineered freshwater systems [149, 175, 217, 256]. *Acanthamoeba* spp. were also detected, but the present study demonstrated that *H. vermiformis* is frequently present in freshwater at higher concentrations than *Acanthamoeba* spp. [Chapter 3, 4 and 5]. The high yield of *H. vermiformis* when feeding on prey bacteria may enhance its competition with *Acanthamoeba* spp. [286]. This is probably related to the smaller cell size of *H. vermiformis* compared to *Acanthamoeba* spp. OTUs related to *Balamuthia mandrillaris* and *Tetrahymena* spp. were only incidentally observed in the examined water

samples, but *Echinamoeba* spp. were more often detected in samples from drinking water systems. This is consistent with the common presence of *Echinamoeba* spp. in drinking water supplies and in (warm) tap water installations, but limited information is available about the presence of *Balamuthia* spp. and *Tetrahymena* spp. in these systems [175, 217, 256]. It should also be noted that *Vahlkampfia* spp. and *Naegleria* spp. could not be detected in the studies described here, because the used 18S rRNA gene primers could not amplify the sequences of these genera. The main objective of the studies described in this thesis was to identify predominant free-living protozoa in drinking water supplies and detection of specific pathogenic protozoa was beyond the scope of our study.

Identity of protozoan hosts for *L. pneumophila*

Interactions between free-living protozoa and pathogenic bacteria are generally species specific [257], but *in vitro* studies have demonstrated that *L. pneumophila* can proliferate inside 14 species of amoebae, two species of ciliates, a slime mold and also in a nematode [Chapter 1]. Several *in vivo* studies have shown that certain protozoa, viz., *Acanthamoeba castellanii*, *Hartmannella* spp. and *Echinamoeba exundans* serve as hosts for *L. pneumophila* [67, 88, 151, 283]. In addition, *Legionella* spp. have been isolated from amoebae belonging to genera such as *Acanthamoeba*, *Echinamoeba*, *Hartmannella* and *Naegleria* obtained from drinking water supplies [254]. This demonstrates that these amoebae do have the ability to serve as host for *Legionella* spp. in drinking water supplies.

The BBT system was applied to identify potential protozoan hosts for *L. pneumophila* in various engineered freshwater types [Chapters 3 and 5]. This approach has yielded the following candidate hosts for *L. pneumophila*: *Diphylleia rotans*, an uncultured cercozoan clone, *Echinamoeba thermarum*, *Neoparamoeba* sp., an uncultured eukaryote obtained from treated water in the Netherlands, *Rhinosporidium* sp., and an *Arachnula* sp. Several of these candidate hosts were also observed in other freshwater environments, including the drinking water supplies in the Caribbean. However, *in vitro* studies are needed to confirm the role of these organisms as hosts for *L. pneumophila*.

OTUs related to *Tetrahymena* spp. were not observed in the BBT system which probably did not promote growth of ciliates. Therefore, it remains unknown if ciliates can serve as host for *L. pneumophila* in aquatic environments. This is also the case for *B. mandrillaris*, the presence of which is limited in engineered freshwater systems, including the systems described in this thesis [175, 217, 256]. In addition, the slime mold *Dictyostelium discoideum* and the nematode *Caenorhabditis elegans* have been identified as hosts for *L. pneumophila* with *in vitro* studies [30, 110]. No information is available about proliferation of *L. pneumophila* within these organisms in aquatic systems and these organisms were not detected in the studies described in present thesis. These observations suggest that proliferation of *L. pneumophila* in aquatic systems occurs mainly in *H. vermiformis*, *Echinamoeba* spp. and *Acanthamoeba* spp.

Pathogenic free-living protozoa in relation to drinking water supply

In the Netherlands, meningoencephalitis infections caused by *Acanthamoeba* spp. [61, 132], *B. mandrillaris* [277] and *N. fowleri* [287] have not been reported. However, amoebic keratitis, caused by *Acanthamoeba* spp. is reported in the Netherlands in persons wearing soft contact lenses [53]. In other countries, cases of Amoebic keratitis have been related to the presence of *Acanthamoeba* spp. in drinking water [127, 139]. *Acanthamoeba* spp. were also detected in distributed water in the Netherlands and the Caribbean [Chapters 4 and 5]. Still, it is unclear whether the detected *Acanthamoeba* spp. represent organisms with pathogenic characteristics. Most of the reported meningoencephalitis infections related to *Acanthamoeba* spp., and *Naegleria fowleri* occurred in Australia, the southern states of the USA and in the north of South America [23, 176, 293]. Information about an epidemiological relation between meningoencephalitis infections caused by *Acanthamoeba* spp. and *B. mandrillaris* and the presence of these organisms in drinking water does not seem available. A few cases of meningoencephalitis infections caused by *N. fowleri* in Asia, Australia and the USA were related to drinking water [7, 170, 228], but most cases were related to infected surface water or soils [114, 293]. In Belgium, children died due to a *N. fowleri* infection after swimming in surface water [265].

6.4 Effects of environmental conditions on the communities of free-living protozoa in freshwater

Environmental conditions in water supplies

Water quality is a critical factor for growth and abundance of (micro)organisms and also affects the composition of microbial communities in drinking water systems. In turn, the quality of drinking water depends on the raw water source, the applied water treatment processes, e.g., desalination, filtration processes, softening, disinfection and the conditions in the distribution system, e.g., pipe materials, concentration of particles/sediment, hydraulics and residence time [154, 270, 273]. Microbial growth in drinking water distribution systems in the Netherlands is controlled by producing biologically stable drinking water instead of maintaining a disinfectant residual during distribution [267]. Biologically stable drinking water has a low concentration of assimilable organic carbon (AOC) and a low potential for biofilm formation [209, 268]. The concentrations of AOC and NOM are reduced as much as possible during water treatment [10], because (i) NOM can give color, taste and odor problems, (ii) NOM affects the performance of water treatment process and disinfection in a negative way [154, 194], and last but not least (iii) it can enhance biofilm formation in water distribution systems [79, 268]. The biodegradable fraction of NOM is a critical factor for biofilm formation in distribution systems and tap water installations and will therefore affect also the abundance and diversity of free-living protozoa in these systems [267, 278]. Technical problems such as corrosion of pipe materials may also be related to growth of microorganisms [155].

Water temperature also affects the composition of free-living protozoan communities in drinking water supplies and (warm) tap water installations. Seasonal temperature variations in distributed water can affect the concentration and the composition in communities of free-living amoebae [119]. The variation between the T-RFLP fingerprints of the treated water within each of the two groundwater supplies in the Netherlands is small because the quality of the raw water is not affected by seasonal changes [Chapter 2]. Free-living protozoa respond rapidly to environmental changes, and with improved water quality the protozoan abundance decreases whereas the protozoan richness increases [128, 143, 171]. Therefore, species of free-living protozoa have been used as water quality indicators in the saprobic index for organic pollution [94, 237]. Still, information about the abundance and identity of free-living protozoa in drinking water supplies in relation to conditions in drinking water supplies is very limited.

Effects of NOM and active biomass (ATP) on the richness of free-living protozoa

The availability of biodegradable NOM is one of the most important environmental factors that affects heterotrophic growth in most ecosystems [79]. In unchlorinated drinking water, higher NOM concentrations generally corresponded with increased concentrations of active biomass (ATP), which demonstrate growth of microorganisms (Table 6.1)[273, Chapter 2]. However, a major proportion of the NOM concentration does not serve as energy source for bacteria, because it is not easily biodegradable [266]. In drinking water in distribution systems of supplies in the Caribbean variable concentrations of ATP were observed, although the NOM concentrations in the treated water types were low ($<$ 0.5 mg liter^{-1}). In one of these systems, sediments were present, most likely related to corrosion of the iron pipes [Chapter 4]. Therefore, the growth of microorganisms in distribution systems also depends on conditions which affect sediment accumulation [154].

In the two supplies in the Netherlands with different concentrations of NOM and ATP in the treated water the protozoan communities were predominated by different OTUs, but the estimated values for protozoan richness did not differ significantly. However, the concentration of *H. vermiformis* in the supply with the high concentration of NOM and ATP was higher than in the supply with the low NOM concentration. These observations suggest that the abundance of free-living protozoa is affected by the concentrations of NOM and ATP, but that these parameters do not necessarily affect the richness in free-living protozoan communities in drinking water supplies. A study of the concentration and identities of *Legionella* spp. in the groundwater supplies A and B revealed that the concentration of *Legionella* spp. was the lowest in the supply with the low concentrations of NOM and ATP. In this supply, however, a higher richness of *Legionella* spp. was observed than in the supply with the high NOM and ATP concentrations [288].

In line with observations in the different groundwater supplies in the Netherlands, the composition of protozoan communities varied between the three supplies in the Caribbean, whereas the estimated OTU richness in free-living protozoan communities also did not differ significantly [Chapter 4]. Supply CA-3 in the Caribbean and supply A in the Netherlands both had low concentrations of NOM and active

biomass in the treated and distributed water (Table 6.1). However, the estimated mean OTU richness for free-living protozoa in supply CA-3 (22 OTUs) was significantly lower than in supply A (113 OTUs). A major difference between supply CA-3 and supply A is the use of aerobic groundwater in supply A, whereas CA-3 uses reverse osmosis (RO) treated seawater as source. The free-living protozoa observed in supply A originate from the groundwater, whereas free-living protozoa present in seawater were all removed by RO-treatment or the distillation process. RO-treated or distilled water passes one or two filter beds, but due to the prior water treatment, it is unlikely that these filter beds contain high concentrations of biomass. These circumstances may lead to the lower estimated protozoan richness in the supplies in the Caribbean than in the supplies in the Netherlands (Table 6.1).

Information about relationships between concentrations of NOM and ATP concentrations and the abundance and richness of free-living protozoan communities in drinking water supplies is limited, because only a few studies in various drinking water supplies are available for comparison. In general the protozoan richness increases with improved water quality [128, 143, 171], but it is unknown if this is also the case for oligotrophic habitats such as drinking water supplies. Concentrations of free-living amoebae show a positive correlation with NOM and ATP concentrations in water at treatment plants and in distribution systems [162, and Chapter 2]. Consequently, quantitative detection of selected protozoan species or groups by molecular techniques may be promising in elucidating the relationships between NOM and ATP concentrations and the presence of specific organisms in drinking water supplies. It should, however, be noted that elucidation of the impact of environmental conditions, including NOM and ATP concentrations, on the richness and composition of microbial communities, requires the analysis of large numbers of samples from different drinking water supplies.

Effect of temperature on the communities of free-living protozoa

Free-living protozoa proliferate and/or survive at a wide range of temperatures and have been isolated from drinking water supplies and (warm) tap water installations at temperatures ranging from 0.5 to 68°C [217, 119]. Seasonal temperature variations in distributed water can affect the concentration and the composition in communities of free-living amoebae. For example, *Naegleria* spp. could only be isolated from drinking water supplies in Germany during summer, whereas *Acanthamoeba* spp. have been isolated throughout the year [119]. Shifts in the composition of protozoan communities were also observed during incubation of freshwater types at various temperatures [Chapter 5].

Protozoa related to the flagellated Euglenozoa predominated in river Rhine water incubated at 37°C, but after incubation at 42°C, Amoebozoan types predominated. Only very limited growth of *Acanthamoeba* spp. was observed at 37°C, and at this temperature *H. vermiformis* predominated in the BBT system. Furthermore, no growth of *Acanthamoeba* spp. was observed at 42°C, and at this temperature growth of *H. vermiformis* was observed. These observations demonstrate that in addition to NOM and ATP concentrations, also the water temperature affects the composition in free-living protozoan communities. In contrast to the limited information about the effects of the temperature on the protozoan

communities in drinking water supplies, some more information is available about the effect of the temperature on specific protozoan groups.

Growth of indigenous *H. vermiformis* was observed in the BBT system at temperatures between 20 and 42°C [Chapters 3 and 5]. However, no growth of this protozoan was observed at 15°C, although viable cells of *H. vermiformis* were present, because growth of this organism was observed at 37°C in the same water types [Chapter 3]. Furthermore, concentrations of *H. vermiformis* were higher in the summer (temperature varied between 12.3 and 16.8°C), than in the autumn (temperature varied between 11.5 and 13.5°C) in the distributed water of supply B in the Netherlands. In addition, *H. vermiformis* is commonly observed in treated water leaving the treatment facility at temperatures between 10 and 15°C [175, 256]. Obviously, *H. vermiformis* can multiply at temperatures around 15°C. Moreover, *H. vermiformis* has been described as one of the most thermoresistant amoebae in hot water systems [217]. Growth of *H. vermiformis* strains, isolated from hot tap water systems with temperatures between 46.5 and 55.7°C, was observed at 53°C on NN-A medium. *H. vermiformis* was also isolated from a hospital network with a temperature of 60°C [255]. Growth ($>$ 1 log unit) at 42°C of *H. vermiformis* was only observed in one of the four tested water types, although viable organisms were present in all water types [Chapter 5]. Altogether, the observations made in the framework of this study and by others indicate that this species contains various strains with different maximum growth temperatures. *H. vermiformis* has also been divided in different groups based on partial 18S rRNA gene sequences [149]. However, no distinction could be made in this study, neither based on the partial 18S rRNA gene sequences amplified with the specific *H. vermiformis* primers nor the sequences obtained with eukaryotic primers from the Netherlands and the Caribbean, because the sequences showed $\geq$ 99% similarity to each other. Therefore, genetic characterization of cultured strains is needed to confirm the existence of thermotolerant strains within *H. vemiformis*.

Growth of indigenous *Acanthamoeba* spp. was observed at temperatures between 20 and 37°C, but not at 15 and 42°C [Chapters 3, 4 and 5]. Growth of *Acanthamoeba* spp. in these BBT flasks incubated at 37°C was very limited. Furthermore, no growth of *Acanthamoeba* spp. was observed in a series of BBT flasks with 21 different water types, incubated at 37°C and no growth of this organism was observed in BBT flask incubated at 15°C [Chapter 3]. In addition, in hot water systems, strains of the *Acanthamoeba* genus, which are able to grow at temperatures up to 45°C, have only rarely been obtained [217]. Strains isolated from patients with keratitis throughout Europe all grew at 37°C, but only a few multiplied at 40°C [64]. De Jonkheere [64] suggested that cases of keratitis caused by *Acanthamoeba* spp. are more frequently observed in Europe than meningoencephalitis infections caused by this genus, since *Acanthamoeba* strains in the environment grow at relatively low temperatures. The cornea is at a lower temperature than the rest of the body and *Acanthamoeba* strains that infect the eye do not require temperatures as high as those required by strains that cause infections in other parts of the body. De Jonkheere [64] also suggeseted that the number of *Acanthamoeba* spp. responsible for keratitis infections may be limited. A study with *in vitro* and animal experiments indicated that the ability of strains of the genera *Naegleria* and *Acanthamoeba* to grow at elevated temperatures is related to virulence, because nonvirulent strains were unable to grow at normal or elevated body temperatures [108]. In

addition, most strains which have been described as host protozoa for *L. pneumophila* [6, 218], e.g., *Acanthamoeba polyphaga*, *A. castellani* and *A. palestinensis*, are unable to grow at temperatures above 37°C [63, 108]. Hence, at temperatures $\geq$ 37°C other protozoa than *Acanthamoeba* spp. including *H. vermiformis* serve as host for *L. pneumophila* [217]. *Acanthamoeba* spp. and *H. vermiformis* can support growth of naturally occurring *L. pneumophila* in aquatic environments at the minimum growth temperature of 25°C [284]. At about 20°C, *L. pneumophila* is digested by the amoebae [192], which was also observed in the BBT system incubated at this temperature [Chapter 5]. *Acanthamoeba* spp. have been observed in river Rhine water [Chapter 5] and in a drinking water production plant [175], both with temperatures of about 10°C, but the minimum growth temperature of these amoebae in the environment is still unknown.

Properties of free-living protozoa

Based on morphological studies, free-living protozoa have been divided into flagellates, ciliates and amoebae [Chapter 1]. Each of these groups has their own characteristics in the interactions with their prey. Therefore, these three groups are compared with the growth conditions in relation to the environmental conditions in the drinking water supplies studied.

Flagellates

Many species of flagellates attach to surfaces temporarily by means of a stalk, but they still preferentially feed on suspended prey [196]. Flagellates generally are smaller than ciliates and amoebae and feed on smaller prey than the other protozoa [90]. In addition, flagellates have also a lower food ingestion rate (2.5 to 11.8 prey cell^{-1} h^{-1}) than ciliates (37 to 421 prey cell $^{-1}$ h^{-1}) [196]. In one supply from the Netherlands and in one supply from the Caribbean, both with low concentrations of NOM and ATP, the predominating clones were related to small flagellates, e.g., *Cercomonas* spp. and *Neobodo* sp., belonging to the phyla Euglenozoa and/or Cercozoa [Chapters 2 and 4]. Experiments with the BBT system indicated that organisms related to an uncultured cercozoan clone can serve as hosts for *L. pneumophila*, but no other flagellates have been described as host for pathogenic bacteria [Chapters 1, 3 and 5]. Cercozoan types predominated also in the biofilm in the groundwater supply in the Netherlands at low concentrations of ATP and NOM [Chapter 2]. Microscopic analyses revealed that flagellates predominated (93%) in the drinking water in an experimental distribution system, but were not observed in the biofilm [233]. In several rivers, flagellates were the most abundant type of free-living protozoa in the planktonic phase, and elevated concentrations of flagellates were also observed on surfaces of water plants and in sediments [240]. In drinking water (TOC: 4 mg C liter^{-1}) in the Netherlands, relatively large numbers (2-500 cells ml^{-1}) of heterotrophic nanoflagellates were determined using microscopy. Drinking water prepared from surface water contained the highest numbers of these flagellates and drinking water produced from groundwater contained lower concentrations [121]. Hence, small flagellates can live in oligotrophic freshwater environments, which are present in drinking water supplies with low concentrations of NOM and ATP, but also in rivers and lakes. Growth of flagellates belonging to the Cercozoa phylum has been observed at 5°C [62] and these organisms

were mainly observed in freshwater types with temperatures between 10 and 37°C [Chapter 2 to 5]. Heterotrophic flagellates belonging to the phyla Euglenozoa and Stramenopiles have been observed in drinking water samples with temperatures between 10 and 42°C, indicating growth over a broad temperature range.

Amoebae

Amoebae are know as browsers, which move over a surface and graze on attached prey, whereas they feed less effectively on suspended prey [196]. Most of the free-living protozoa, which can serve as hosts for undesired bacteria and/or are human pathogens, belong to the free-living amoebae. Therefore, in studies of water quality the free-living amoebae attracted more attention than the ciliates and flagellates [162, 254]. The young biofilm in the BBT system seems to favor the growth of amoebae, which is consistent with the notion that growth of amoebae mainly occurs in biofilms [Chapters 3, 4 and 5]. Also in an experimental drinking water distribution system, amoebae were not observed in the planktonic phase, but predominated the protozoan community in the biofilm [233]. Concentrations of free-living amoebae show a positive correlation with NOM concentrations in water at treatment plants and surface water sources, which is used for drinking water production [162, 163]. ATP concentrations and heterotrophic plate counts (both indicators for higher nutrient availability) in cooling tower water correlated also positively with *H. vermiformis* [149]. Similarly, elevated levels of NOM and active biomass corresponded with elevated concentrations of *H. vermiformis* in the summer, demonstrating that growth of this amoeba increased at rising concentrations of active biomass and at higher temperatures in drinking water supplies [Chapters 2 and 4]. Seasonal temperature variations in distributed water thus can affect both the abundance and the composition of communities of free-living amoebae. However, Loret et al. [163] did not find a correlation between water temperature and concentrations of these organisms in surface water sources, in a range of temperature of 7 to 28.5°C, used for drinking water production. Concentrations of thermophilic amoebae such as the pathogenic species *Naegleria fowleri* had been shown to increase with increasing water temperatures [261]. Together, these observations indicate that water temperature and the presence of high concentrations of NOM and biofilms affect the richness and abundance of free-living amoebae in freshwater environments. Species belonging to the Amoebozoa phylum, including *Acanthamoeba* spp. and *H. vermiformis* do have the ability to grow at temperatures between 11 and 53°C, but this varied between species and also between strains of one species [116, 217].

The investigations reported in this thesis confirmed that *H. vermiformis* is the most frequently observed free-living amoebae of the list of protozoa which can serve as host for undesired bacteria. The high yield of *H. vermiformis* when feeding on prey bacteria may enhance its competition with *Acanthamoeba* spp. and probably other amoebae [286]. Information about threshold values for growth of *H. vermiformis*, including the minimum concentrations of NOM and ATP and the minimum and maximum growth temperature, is needed to define measures for limiting the growth of this amoeba in drinking water supplies and (warm) tap water installations. In an *in vitro* study using solid medium, *H. vermiformis* exhibited the maximum growth rate at concentrations greater than 1×10^7 prey cells cm^{-2} [200] which corresponds to an active biomass concentration of about 1000 pg ATP cm^{-2} (concentra-

tion of ATP in one bacterial cell in biofilm is about 7.4×10^{-5} pg ATP [268]). This ATP concentration is higher than the biofilm concentrations in distribution systems (about 100 and 350 pg ATP cm^{-2}) in the two groundwater supplies in the Netherlands [Chapter 2] and similar to biofilm concentrations in a model warm water system supporting the growth of *L. pneumophila* [270]. A biofilm batch model system with pure cultures of *L. pneumophila*, *H. vermiformis* and *Acidovorax* sp. demonstrated a minimal concentration of prey bacteria of about 4×10^4 cells cm^{-2} ($<$ 5 pg ATP cm^{-2}) or about 3×10^6 cells ml^{-1} (ca. 200 pg ATP ml^{-1}) for growth of *H. vermiformis* [149]. From the observations in supply A and supply CA-3 can be derived that growth of *H. vermiformis* is limited in drinking water distribution systems at ATP concentrations $<$ 1 ng liter^{-1} in temperate and tropical regions [Chapters 2 and 4].

Ciliates

Ciliates are common inhabitants of biofilms in natural and engineered freshwater systems, but less abundant in the planktonic phase [233, 240]. None of the free-living ciliates is described as a human pathogen and only a few species within the *Tetrahymena* genus have been described as hosts for *Legionella* spp. [Chapter 1]. These organisms probably browse over a surface and feed on suspended and attached prey organisms [196]. Species within the ciliates can grow at a broad range of NOM concentrations, but different species are abundant at different concentrations of NOM [94, 237]. Protozoa related to Ciliophora have been obtained from hot springs in Iceland with temperatures between 15 and 47°C [2]. The highest rate of cell reproduction of *Tetrahymena pyriformis* in *in vitro* experiments was observed between 17.5 and 33.5°C with an optimal growth temperature between 27.5 and 29°C [258]. In liquid proteose peptone medium, growth of *Tetrahymena pyriformis* was observed at 39.5°C [142].

In one of the three supplies in the Caribbean ciliates (Ciliophora) were a prominent fraction in the protozoan communities. Ciliates related to *Colopoda cucullus* and *Lembadion bullinum* were predominant and *Tetrahymena* spp. were not observed despite the optimal water temperature of about 30°C [Chapter 4]. The elevated ATP concentrations in the involved supply correlated with turbidity and iron, indicating the presence of corrosion-related sediments in the distributions system. In aquatic environments, ciliates are usually the dominant free-living protozoa and probably the most important protozoan grazers in sediments [90]. Ciliates were also a predominant group in the treated water and in the biofilm in the distribution system of groundwater supply B in the Netherlands with concentrations of NOM of 8 mg C liter^{-1} and 10 ng ATP liter^{-1} in the treated water. In both regions, ciliates related to *Hemiophrys* spp., which feed by engulfing large "particles" such as flagellates and other ciliates [77], were observed in supply B [Chapter 2] and in supplies in the Caribbean [Chapter 4].

Classification of water types using free-living protozoa as bioindicators

The saprobic index is used to classify water types with different organic pollution levels and is based on empirical studies involving the abundance of certain aquatic organisms, including free-living protozoa [94, 237]. For this index, mainly ciliates (Ciliophora) are used to categorize water types from

xenosaprobic (with low concentrations of organic material and high concentrations of oxygen), to metasaprobic (with high concentrations of organic material and low concentrations of oxygen). Only a few other protozoan genera belonging to the phyla Amoebozoa, Cercozoa and Euglenozoa are included in the list of indicator organisms [210, 238]. This system has been and is still used to investigate the water quality of surface water and mainly river water [134, 280]. In the investigations described in this thesis, the free-living protozoan communities were investigated in three samples of the river Rhine with a NOM concentration of 3 mg C liter^{-1} and an active biomass concentration varying between 60 and 120 ng ATP liter^{-1} [Chapters 3 and 5]. However, only a few of the observed OTUs, before and after incubation in the BBT system, were related to a protozoan genus (*Cercomonas*), which is described as indicator organism [94]. In addition, only a few of the obtained free-living protozoan genera in the two groundwater supplies in the Netherlands and the supplies in the Caribbean are included in the list of indicator organisms, e.g., *Brusaria*, *Hemiophrys*, *Cercomonas Rhynchomonas*, and *Vorticella* [94, 210]. The presence of these organism indicates moderate organic pollution at a high dissolved oxygen content [94, 210]. However, the saprobic index can only be used if the biological indictor species are present in sufficient numbers. The 18S rRNA studies revealed the presence of many yet-undescribed protozoan genera and species, which are not (yet) included in the saprobic system. To classify drinking water types according to the saprobic index, based on the identification of free-living protozoa with molecular techniques, specific qPCR methods for protozoan groups can be applied. To classify drinking water types, however, other parameters can also be determined.

Based on the information described above it can be concluded that amoebae are indicative for the presence of biofilms, and that ciliates indicate the presence of sediments. Flagellates are present in rivers and in oligotrophic environments. In the absence of biofilms and sediments, flagellates become predominant, but these organisms as such are not indicative for oligotrophy. Ciliates may be used as an indicator for sediments, but the presented studies do not yet reveal which organism(s) can serve as indicator(s). A separate qPCR for detection of several genera of ciliates may be useful. However, ATP is a simple indicator for bioactivity and techniques are available for rapid and accurate assessment of this parameter in water supplies [273]. Furthermore, turbidity can be used for quantifying the presence of sediments in distribution systems [281, 282]. Quantitative data about the amoeba *H. vermiformis* can be used as an indication of biofilm formation. From a public health perspective, amoebae are the most important free-living protozoa, because various species serve as hosts for pathogenic bacteria, including *L. pneumophila*, and several species have been identified as human pathogens. Limiting the growth potential for *H. vermiformis*, the most commonly observed amoeba, will very likely also limit the growth of other amoebae.

6.5 Concluding remarks and future perspectives

Free-living protozoa are ubiquitous in drinking water supplies and in (warm) tap water installations and proliferate in these systems. Each survey on free-living protozoa in drinking water supplies yielded

yet-undescribed species. Concentrations of *H. vermiformis* in the supply with the high concentrations of NOM and ATP were higher than in the supply with the low NOM and ATP concentrations. These observations suggest that the abundance of free-living protozoa is affected by the concentrations of NOM and ATP. *H. vermiformis* was the most commonly observed protozoan host for *L. pneumophila* in the drinking water supplies. This amoeba has the ability to growth at higher temperatures than *Acanthamoeba* spp., which growth is limited at temperatures ≥ 37°C. However, information about the conditions favoring the growth of specific free-living protozoa in drinking water distribution systems is in its infancy. This can be attributed to the following reasons:

(i) the free-living protozoan communities are highly diverse;
(ii) free-living protozoan communities have been identified in only a few drinking water supplies;
(iii) a large variety of environmental conditions ("niches") occurs in water supplies and identification of the physicochemical parameters in these niches is complicated;
(iv) the database for free-living protozoa based on the available 18S rRNA gene sequences database is still limited;
(v) information about the growth conditions of the observed free-living protozoa is very scarce;
(vi) the richness in the clone libraries is not proportional to the richness in the protozoa in the samples due to large differences in the number of rRNA gene clusters in different protozoa.

Investigations of a wide range of different drinking water supplies are needed to obtain detailed information about the abundance and richness of free-living protozoa in relation to the water quality. Although analyzing the 18S rRNA gene clone libraries provided a large number of new DNA sequences, this method is labor intensive and only the predominant sequences were analyzed. The used qPCR methods for the detection and quantification of specific groups of free-living protozoa, like indicator organisms, hosts for undesired bacteria and potential pathogenic free-living protozoa in drinking water supplies may be an attractive alternative approach.

H. vermiformis was the most commonly observed host for *L. pneumophila* in drinking water supplies in the Netherlands and in the Caribbean. This amoeba was also found in (warm) tap water installations, in other engineered water systems and in surface water in the Netherlands [149, 150]. Measures for limiting growth of *H. vermiformis* in engineered freshwater systems, will very likely also limit the growth of other amoebae, which have pathogenic traits and/or can serve as host for pathogenic bacteria. To verify if *H. vermiformis* is the most common present amoeba, also the abundance of *Echinamoeba* spp. and *Acanthamoeba* spp. should be determined, with qPCR methods, in different drinking water supplies and (warm) tap water installations. These two genera were often observed in freshwater environments and species related to these genera can serve as hosts for *L. pneumophila* [88, 218]. In addition, several *Acanthamoeba* spp. can be human pathogens [61].

The role of *Naegleria* spp. as hosts for pathogenic bacteria is unknown, and was also not addressed in the present study. A specific qPCR for *Naegleria* spp. will enable the elucidation of the presence and role of species of this genus in water supplies. Although, identifying pathogenic fungi was out of the scope

of the studies described in this thesis, several clones with $>$ 99% similarity to potential pathogenic fungi were observed in the drinking water supplies. Therefore, further research is needed to assess the abundance and growth of potential pathogenic fungi in drinking water supplies and (warm) water installations.

Growth of *H. vermiformis* is limited at active biomass concentrations $<$ 1 ng ATP liter^{-1} in combination with NOM concentrations $<$ 0.5 mg C liter^{-1}. However, these values are based on a limited number of ecological studies; therefore, controlled batch experiments with different concentrations of biofilm and NOM should be conducted to determine in more detail the threshold values of these parameters for growth of *H. vermiformis*. Growth of *H. vermiformis* was observed in the BBT system at temperatures between 20 and 42°C. *H. vermiformis* can probably multiply at temperatures below 20°C and above 42°C. Assessment of the minimum and maximum growth temperature of *H. vermiformis* requires additional testing. Different strains of *H. vermiformis* have probably various minimum and maximum growth temperatures. Therefore, genetic characterization of cultured strains is needed to confirm the existence of thermotolerant strains within *H. vermiformis*.

Production of drinking water with low concentrations of NOM ($<$ 0.5 mg C liter^{-1}) and ATP ($<$ 1 ng liter^{-1}) is not achievable in most cases. Furthermore, several pipe materials may promote biofilm formation, and/or sediment formation in the distribution systems [270]. A combination of measures in water treatment and distribution may be most effective in reducing the potential for growth of free-living protozoa in water supplies and water installations. In addition, specific measures, such as hot-water flushing or copper-silver ionization, can be used in warm tap water installations, to prevent the proliferation of free-living amoebae in these systems [19, 92, 158]. Measuring the concentrations of selected species of free-living protozoa with qPCR can be used to verify the effects of these measures.

Appendix A

Supplemental material to Chapter 2

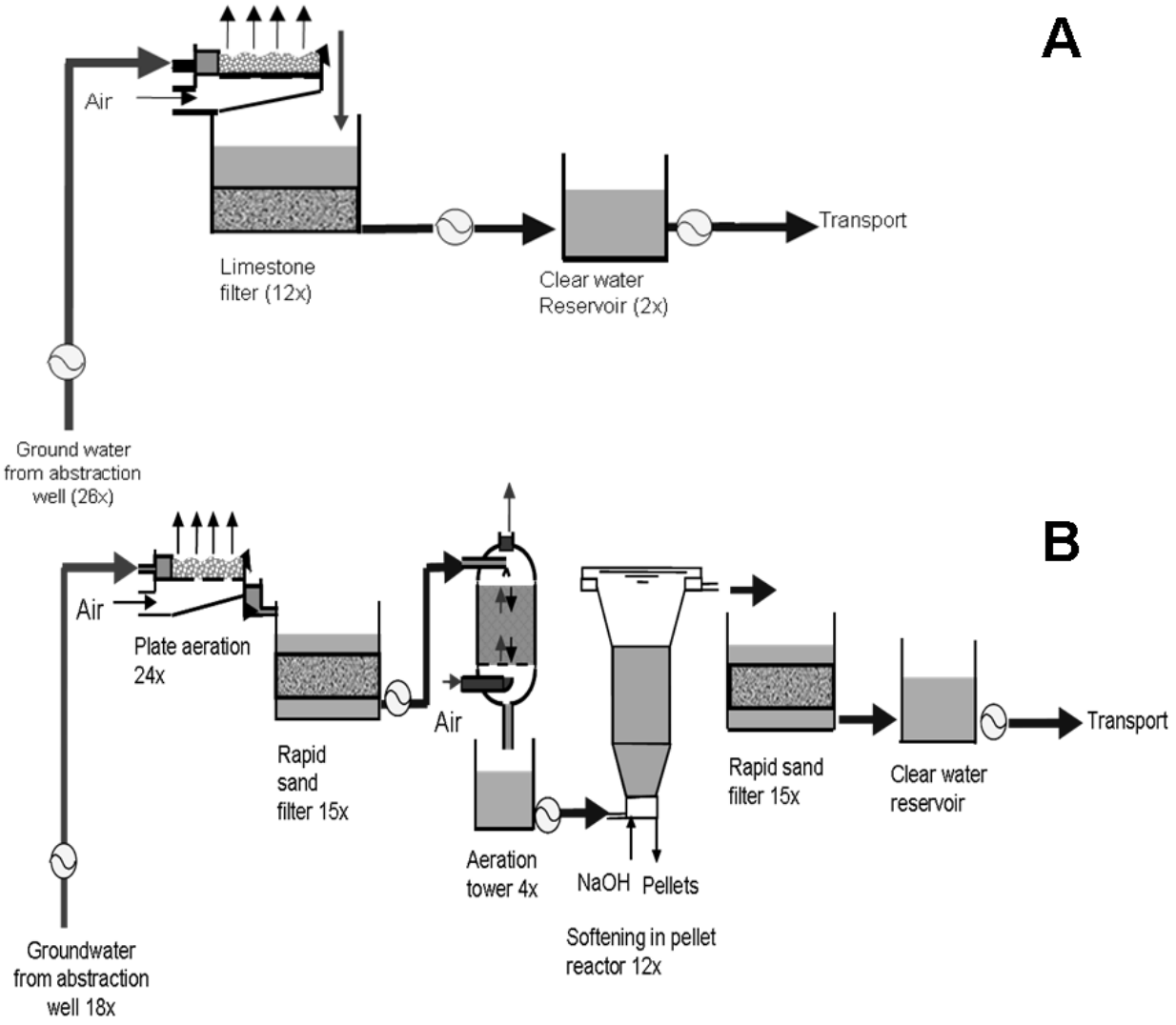

Figure A.1: Flowcharts of treatment systems at plants A (top) and B (bottom).

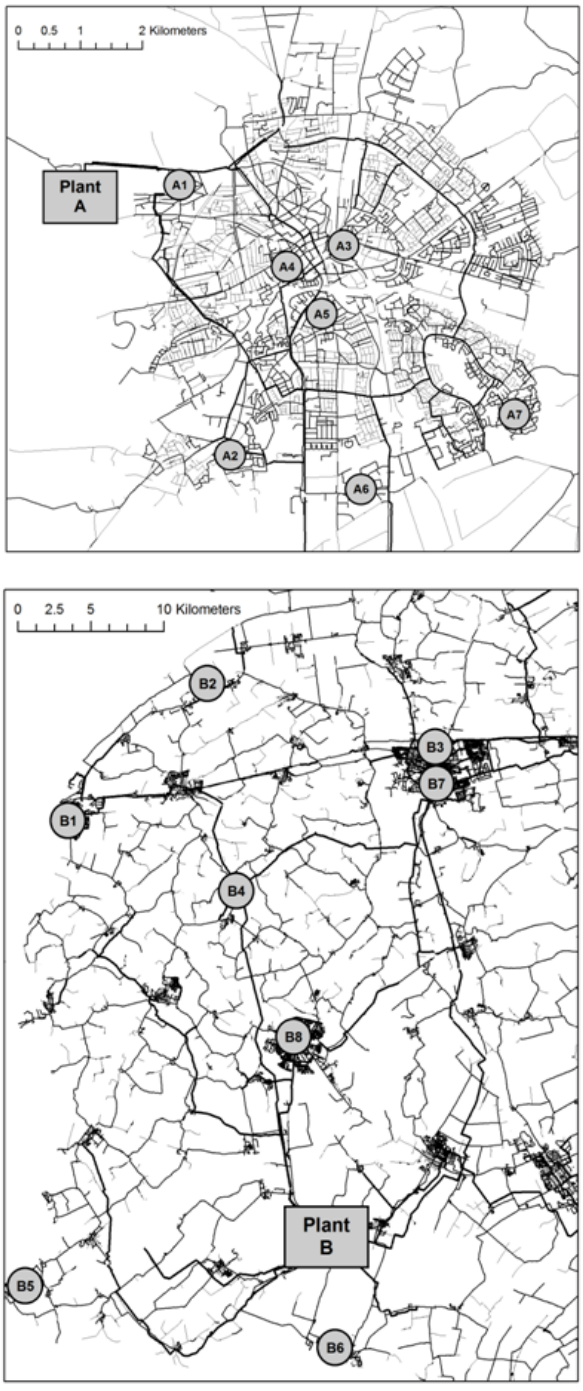

Figure A.2: Sample locations of the distributed water and the biofilms in distribution systems A (top) and B (bottom).

Table A.1: Classification of OTUs clustering with free-living protozoa obtained from treated water and from biofilms in the distribution system of water supply A and water supply B[a].

Organism(s) with highest similarity (GenBank accession no.)[b]	Similarity (%)	No. of OTUs	No. of clones			
			TW-A	BF-A[c]	TW-B	BF-B[c]
Amoebozoa		10	3	4	2	8
Acanthamoeba polyphaga (AF260725)	89.3	1	1	-	-	-
Echinamoeba thermarum (AJ489264)	85.7	1	-	-	1	-
Eimeriidae environmental sample clone (EF024503)/ *Acanthamoeba* sp. (AY173000)	96.8/85.9	1	2	-	-	-
Uncultured endolithic amoeba (AB257667)/ *Hartmannellidae* sp. LO57N/1 (AY145442)	79.2/77.9	1	-	-	-	7
Neoparamoeba aestuarina (AY121851)	89.3	1	-	-	1	-
Pterocystis tropica (AY749612)	93.4	1	-	1	-	-
Raineriophrys erinaceoides (AY749633)	94.7, 93.3	2	-	2	-	-
Uncultured eukaryote clone (AY749523)/ *Raineriophrys* sp. (AY749606)	89.0/88.4	1	-	1	-	-
Uncultured Sarcosomataceae clone (EF023269)/ *Amastigomonas mutalitis* (AY050182)	78.3/77.1	1	-	-	-	1
Cercozoa	-	39	14	60	24	4
Athalamea environmental sample clone (EF024169)/ Soil flagellate AND25 (AY965868)	91.1/88.5	1	-	-	2	-
Athalamea environmental sample clone (EF024169)/ *Hedriocystis reticulata* (AY305010)	90.3/85.0	1	-	-	1	-
Athalamea environmental sample clone (EF024169)/ *Exuviaella pusilla* (DQ388459)	81.4/81.0	1	-	1	-	-
Cercomonas longicauda (AY496047, AF411270, AY496047)	96.3, 94.6, 91.9	3	4	-	1	-
Cercomonas metabolicus (DQ211597)	97.5, 95.6, 94.8	3	6	-	-	-
Cercomonas sp. (AF534712)	95.2, 95.0, 80.6	3	-	3	5	-
Cercomonadida environmental sample clone (EF024293, EF024163)/ Soil Flagellate AND 24 (AY965867)	98.1, 93.4/ 94.1, 92.6	2	-	1	1	-
Cercomonadidae environmental sample clone (EF024692)/ *Cercomonas* sp. (AF411266)	95.1/95.0	1	-	-	-	1
Cercomonadida environmental sample clone (EF024163)/ *Masisteria marina* strain DFS1 (AF174371)	87.4/84.6	1	-	1	-	-
Dimorpha-like sp. ATCC 50522 (AF411283)	93.4	1	-	10	-	-
Dodomorpha sp. HFCC57 (DQ211596)	99.4	1	-	24	-	-
Ebria triparrtita (DQ303922)	88.2	1	-	3	-	-

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Table A.1 – continued from previous page

Organism(s) with highest similarity (GenBank accession no.)[b]	Similarity (%)	No. of OTUs	No. of clones			
			TW-A	BF-A[c]	TW-B	BF-B[c]
Pseudodifflugia cf. gracilis (AJ418794)	88.7	1	-	-	-	1
Trachelocorythion pulchellum (AJ418789)	76.5	1	-	13	-	-
Uncultured Banisveld eukaryote clone (EU091827)/ Soil flagellate AND21 (AY965866)	99.8/99.1	1	-	1	-	-
Uncultured cercozoan sample clone (EF023523)/ Cercomonadida environmental sample clone (EF024163)	92.6/91.9	1	-	-	2	-
Uncultured cercozoan clone (AY620301)/ *Cercomonas* sp. (AF411266)	98.0/91.4	1	-	-	1	-
Uncultured cercozoan clone (AY620269)/ *Massisteria marina* (AF174373)	89.1/87.4	1	-	-	1	-
Uncultured cercozoan clone (AY821946)/ *Pseudodifflugia cf. gracilis* (AJ418794)	90.0/87.5	1	-	-	2	-
Uncultured cercozoan partial 18S rRNA gene (AM114807)/ Soil Flagellate AND 24 (AY965867)	99.0/93.0	1	-	-	3	-
Uncultured cercozoan clone (AY620268)/ *Cercomonas* sp. (AF411266)	97.9/89.1	1	-	-	1	-
Uncultured eukaryote clone (AY082981)/ *Ebria tripartita* (DQ303922)	96.1/88.0	1	-	1	-	-
Uncultured eukaryote clone (EF024996)/ *Protaspis grandis* (DQ303924)	96.1/92.6	1	-	-	1	-
Uncultured eukaryote clone (AY082993)/ Uncultured freshwater Cercozoan (DQ243993)	95.1/94.0	1	-	1	-	-
Uncultured eukaryote clone (EF023764)/ *Protaspis grandis* (DQ303924)	99.4/94.0	1	-	-	-	1
Uncultured freshwater cercozoan clone (DQ244000)/ Cercomonadidae environmental sample clone (EF024294)	83.3/81.4	1	1	-	-	-
Uncultured freshwater cercozoan clone (DQ243992, DQ243993)/ *Cercomonas* sp. (AF411271, AF411266)	92.6, 89.0/91.4, 88.5	2	2	-	-	1
Uncultured freshwater cercozoan clone (DQ243993)/ Rigidomastix-like sp. (AF411279)	93.5/92.9	1	-	1	-	-
Uncultured rhizosphere cercozoan (AJ506007)/ Soil Flagellate AND 21 (AY905866)	78.9/74.5	1	1	-	-	-
Unidentified eukaryote (AJ130856)/ *Lecythium* sp. (AJ514867)	97.0, 95.9/95.9, 94.4	2	-	-	3	-

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Table A.1 – continued from previous page

Organism(s) with highest similarity (GenBank accession no.)[b]	Similarity (%)	No. of OTUs	No. of clones			
			TW-A	BF-A[c]	TW-B	BF-B[c]
Choanozoa	-	26	3	15	14	13
Amoebidium parasiticum strain ATCC 32708 (Y19155)	90.5	1	-	-	1	-
Codonosigidae evironmental sample clone (EF024012)/ *Monosiga ovata* (AF271999)	97.1/93.5	1	-	1	-	-
Corallochytrium limacisporum (L42528)	79.5	1	-	-	1	-
Diaphanoeca grandis (DQ103820, AY753614, AF084234)	92.9, 82.8, 75.7	3	3	-	-	3
Eimeriidae environmental sample clone (EF024885)/ *Diaphanoeca grandis* (AF084234)	92.3/90.1	1	-	-	-	1
Eimeriidae environmental sample clone (EF024885)/ *Endochytrium* sp. (AY635844)	91.9/90.2	1	-	1	-	-
Eimeriidae environmental sample clone (EF023936)/ *Monosiga ovata* (AF084230)	94.9, 94.1, 90.2/ 92.1, 91.4, 88.9	3	-	2	-	1
Eimeriidae environmental sample clone (EF024885)/ *Stephanoeca diplocostata* (AF084235, AY149899)	91.5, 93.5/ 90.2, 92.8	2	-	7	1	-
Ichthyophonus irregularis (AF232303, AF232303)	92.4, 79.4	2	-	2	-	1
Nuclearia moebiusi (AF484686)	91.8	1	-	-	-	1
Rhinosporidium seeberi (AF118851)	90.1	1	-	1	-	-
Uncultured eukaryote (AB275066)/ *Diaphanoeca grandis* (AF684234)	90.7/89.6	1	-	-	1	-
Uncultured eukaryotic picoplankton clone (AY642728)/ *Monosiga ovata* (AF084230)	94.8, 98.5, 90.7/94.4, 90.4	3	-	-	5	1
Uncultured eukaryotic picoplankton clone (AY642707)/ *Stephanoeca diplocostata* (AY149899)	92.2/92.0	1	-	-	1	-
Uncultured marine eukaryote clone (EF526879)/ *Corallochytrium limacisporum* (L42528)	90.4/89.9	1	-	-	-	2
Uncultured marine eukaryote clone (EF526803, DQ103820)/ *Diaphanoeca grandis* (AF084234, L10824)	94.0, 88.7/92.4, 88.4	2	-	1	4	-
Uncultered marine eukaryote clone (EF526803)/ *Stephanoeca diplocostata* (AY149899)	93.4/92.0	1	-	-	-	3
Ciliophora	-	29	7	4	17	21
Dexitrichides pangi (AY212805)	91.2	1	2	-	-	-
Heliophrya erhardi (AY007445)	86.3, 85.6	2	-	-	-	5
Hemiophrys macrostoma (AY102173)	82.6	1	-	-	1	-
Hemiophrys procera (AY102175)	98.1, 96.8	2	-	-	2	-
Holosticha diademata (DQ059583)	98.7, 96.7	2	-	-	2	-
Oxytrichidae environmental sample clone (EF024903)/	97.5/96.9	1	-	-	1	-

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Table A.1 – continued from previous page

Organism(s) with highest similarity (GenBank accession no.)[b]	Similarity (%)	No. of OTUs	No. of clones			
			TW-A	BF-A[c]	TW-B	BF-B[c]
Gonostomum namibiense (AY498655)						
Parabirojimia similis (DQ503584)	97.3	1	-	-	1	-
Tokophrya lemnarum clone (AY332720)	87.6	1	-	4	-	-
Unidentified eukaryote (AJ130855)/ *Carchesium polypinum* (AF401522)	90.4/90.2	1	-	-	-	1
Uncultured eukaryote clone (EF024996)/ *Dexitrichides pangi* (AY212805)	98.9, 87.1	1	-	-	1	-
Unidentified eukaryote (AJ130851)/ *Ophrydium versatile* (AF401526)	85.9, 85.3	1	-	-	-	1
Unidentified eukaryote (AJ130851)/ *Vorticella campanula* (AF335518, DQ662849)	94.8/92.4, 94.6, 92.1	2	-	-	-	4
Uncultured hypotrichid ciliate clone (AY821937)/ *Aspidisca steini* (AF305625)	88.9/87.7	1	-	-	2	-
Uncultured marine eukaryote clone (DQ103847)/ Uncultured cilate (AM114813)	77.0/76.2	1	3	-	-	-
Uncultured marine eukaryote clone (EF526916)/ *Plagiopyliella pacifica* (AY541685)	94.5/94.5	1	2	-	-	-
Uroleptus gallina (AF164130)	87.6	1	-	-	1	-
Vorticella campanula (DQ662849, AF335518)	99.2, 98.4, 93.6, 95.2, 93.4, 91.6	6	-	-	1	7
Vorticella fusca (DQ190468)	99.3	1	-	-	-	1
Vorticella sp. JCC-2006-4 (DQ868349)	96.6	1	-	-	5	1
Zoothamnium niveum (DQ868350)	94.8	1	-	-	-	1
Euglenozoa	-	13	17	1	2	0
Bodo saltans (DQ207571, AY490229)	95.6, 77.2	2	2	-	-	-
Neobodo designis (AY753616, AY753616, DQ207583)	97.4, 94.3, 93.0, 91.1, 88.3, 87.7	6	10	-	1	-
Petalomonas cantuscygni CCAP 1259/1 (AF386635)	85.8	1	-	1	-	-
Rhynchomonas nasuta (DQ207595, AY425023)	98.1, 85.4	2	4	-	-	-
Uncultured eukaryote clone (AY753980)/ *Bodo saltans* (AY490232)	94.5/93.6	1	1	-	-	-
Uncultured eukaryote clone (EF100316)/ *Petalomonas cantuscygni* (U84731)	80.5/78.5	1	-	-	1	-
Myzozoa	-	5	0	1	11	1
Pseudoperkinsus tapetis (AB300505)	86.8	1	-	1	-	-
Uncultured alveolate clone (AF372776)/	87.8/78.9	1	-	-	1	-

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Table A.1 – continued from previous page

Organism(s) with highest similarity (GenBank accession no.)[b]	Similarity (%)	No. of OTUs	No. of clones			
			TW-A	BF-A[c]	TW-B	BF-B[c]
Corallochytrium limacisporum (L42528)						
Uncultured eukaryote clone (EF100258)/ Uncultured alveolate clone (AF372776)	97.3/91.0	1	-	-	9	-
Uncultured eukaryote clone (EF100258)/ *Colpodella pontica* (AY078092)	98.1/77.3	1	-	-	1	-
Uncultured marine eukaryote clone (DQ103862)/ *Peridinium wierzejskii* (AY443018)	97.1/96.7	1	-	-	-	1
Stramenopiles	-	5	0	4	1	2
Aphanomyces invadans (DQ403202, AF396684)	98.7, 98.4	2	-	3	-	-
Hyphochytrium catenoides (AF163294)	97.3	1	-	-	-	2
Paraphysomonas foraminifera (AB022864)	98.5	1	-	-	1	-
Rhizidiomyces apophysatus (AF163295)	98.3	1	-	1	-	-
Total	-	127	44	89	71	49

[a] OTUs were compared with sequences in the ARB database [46, 149]

[b] Most closely related sequence/most closely related genus or species. More than one accession number indicates that more than one OTU had the highest similarity to the same genus or species, but to different sequence types.

[c] Data are totals for the three analyzed biofilm samples.

Appendix B

Supplemental material to Chapter 3

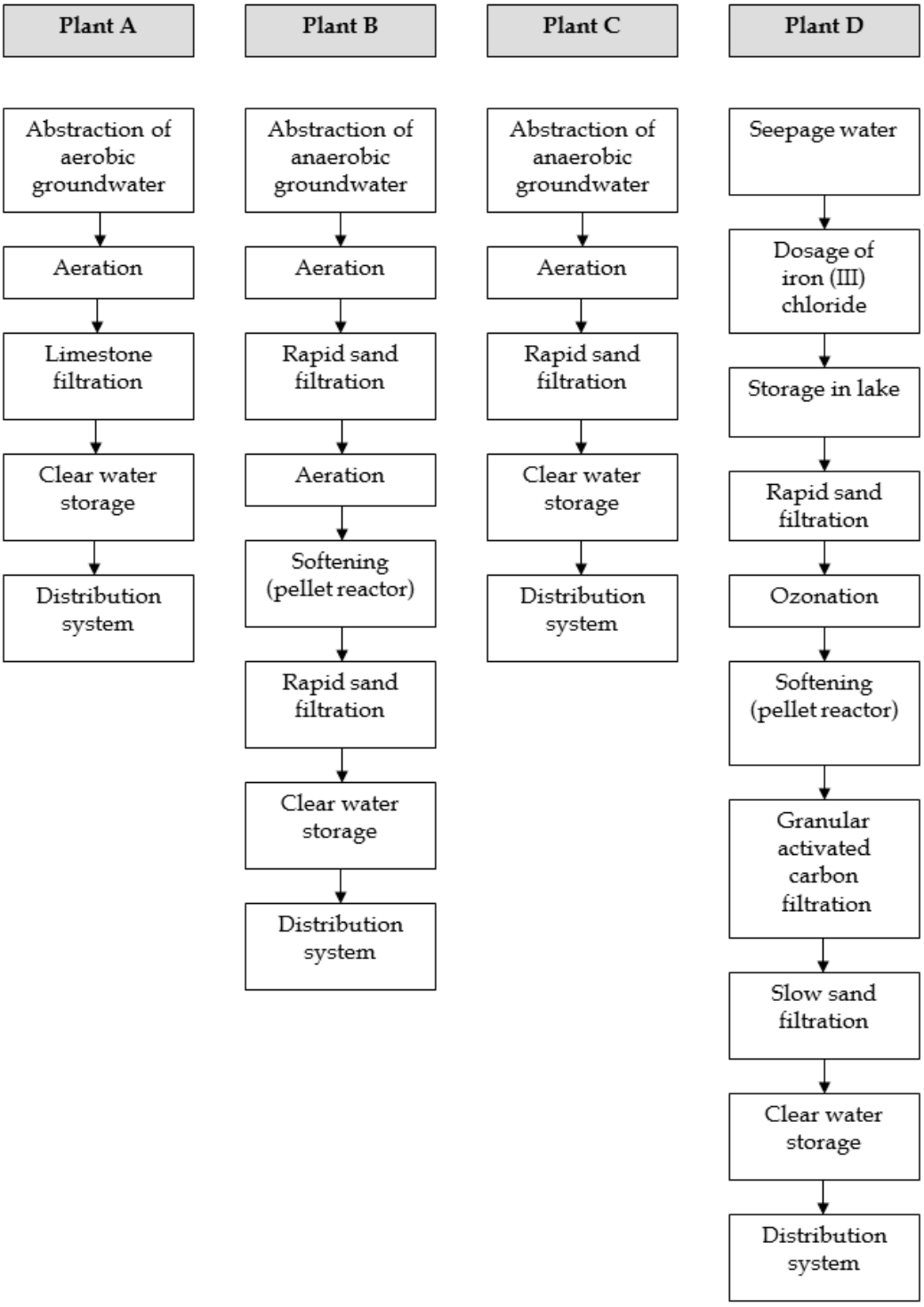

Figure B.1: Schematic representation of water treatment plants A, B, C and D.

Table B.1: Classification of OTUs clustering with free-living protozoa obtained from BBTs after ≥ 20 days of incubation at 37°C with samples related to drinking water supplies.

Water type	BBT flask	Growth in flask[a]		Organism(s) with highest similarity (access. no.)	Similarity (%)	No. of OTUs	No. of seq.	% of OTU(s) clustering with protozoa
		Lp	Hv					
Supply A (treated water)	I	−	−	Not determined	-	-	-	-
	II	−	−	Not determined	-	-	-	-
Supply A (biomass from limestone filter bed)	I	+	+	*H. vermiformis* (AY680840)	99.5	1	29	100
	II	−	−	Uncultured eukaryote from treated water of supply A (EU860610)	99.3	1	9	37.5
				Uncultured Banisveld eukaryote (EU091848	99.4	1	5	20.8
				Cercomonas metabolicus (DQ211597)	98.1	1	4	16.7
				Uncultured eukaryote (AY082993)/ Uncultured eukaryote from biofilm of supply A (EU860465)	95.5/94.4	1	2	8.3
				Korotnevella stella (AY686573)	97.4	1	1	4.2
				H. vermiformis (AF426157)	99.1	1	1	4.2
				Uncultured eukaryote from biofilm of supply A (EU860465)	99.8	1	1	4.2
				Uncultured eukaryotic picoplankton (AY642694)/ Soil flagellate AND 27 (AY965869)	97.7/96.8	1	1	4.2
Supply A (installation A1)	I	−	+	*H. vermiformis*(AY680840)	99.8	1	8	100
	II	−	+	*Platyamoeba stenopodia* (AY294144)	93.9	1	8	100
Supply A (installation A2)	I	−	−	Not determined	-	-	-	-
	II	+	+	*H. vermiformis* (AY680840)	100	1	13	92.9
				Uncultured marine eukaryote (EF526997)/*H. vermiformis*(AY502960)	95.6/93.7	1	1	7.1
Supply B (treated water)	I	−	−	Not determined	-	-	-	-
	II	−	−	Not determined	-	-	-	-
Supply B (biomass from sand filter bed)	I	+	−	*Rhinosporidium cygnus* (AF399715)	87.8	1	29	72.5
				Rhinosporidium seebri (AF118851/AF158369)	78.9-89.8	4	4	10
				Uncultured eukaryote (EF100316)/ *Petalomonas cantiscygni* (U84731)	85.0/77.0	1	3	7.5
				Balamuthia mandrillaris (AF477020)	87.8	1	1	2.5
				Uncultured eukaryote from treated	99.6	1	1	2.5

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Table B.1 – continued from previous page

Water type	BBT flask	Growth in flask[a]		Organism(s) with highest similarity (access. no.)	Similarity (%)	No. of OTUs	No. of seq.	% of OTU(s) clustering with protozoa
		Lp	Hv					
				water of supply A (EU860731)				
				Aphanomyces invadans (DQ403202)	100	1	1	2.5
				Neoparamoeba pemaqiodensis (AF371972)	91.3	1	1	2.5
	II	+	+	*H. vermiformis* (AY680840)	99.8	1	28	96.6
				Uncultured freshwater cercozoan (DQ243994)	97.0	1	1	3.4
	III	+	−	Uncultured Cercozoan clone (AY620304)/ Soil flagellate AND 11 (AY962866)	97.7/89.5	1	2	40
				Uncultured cercozoan clone (AY620304)/ *Cercomonas* sp. (AF411266)	98.1/92.4	1	2	40
				Cyanophora paradoxa (X68483)	87.8	1	1	20
Supply B (installation B1)	I	−	−	*Sphaeroeca volvox* (Z34900)	79.4-85.0	3	7	100
	II	−[b]	+	*Sphaeroeca volvox* (Z34900)	83.6	1	12	73.3
				H. vermiformis (AY680840)	100	1	4	26.7
Supply B (installation B2)	I	+[b]	+	*H. vermiformis* (AY680840)	100	1	16	100
	II	−[b]	+	*H. vermiformis* (AY680840)	100	1	15	100
Supply B (flushed tap water)	I	−	−	None of the sequences are related to free-living protozoa	-	-	-	-
	II	−	−	Not determined	-	-	-	-
Supply C (tap water)	I	−	−	Not determined	-	-	-	-
	II	−	−	Not determined	-	-	-	-
Supply D (biomass from granular activated carbon filter bed)	I	+	+	*Nuclearia simplex* (AF484687)	100	1	8	100
	II	−	−	Uncultured eukaryote of treated water of supply A (EU860696, EU860731, EU860636)	95.3-99.5	3	3	75
				Uncultured eukaryote from biofilm of supply B (EU860576)	95.3	1	1	25
Supply D (biomass from sand filter bed)	I	−	−	*Ichthyophonus irregularis* (AF232303)	89.2	1	2	40
				Bodomorpha sp. (DQ211596)	100	1	1	20
				Rhinosporidium sp. (AY372365)	84.7	1	1	20
				Uncultured eukaryote from treated water of supply B (EU860791)	99.4	1	1	20
	II	+	+	*H. vermiformis* (AY680840)	100	1	11	100

[a] +: significant growth ($p<0.025$) of *L. pneumophila* (Lp) and *H. vermiformis* (Hv); −: no growth.
[b], growth of *L. anisa*.

Table B.2: Classification of OTUs clustering with free-living protozoa obtained from BBTs after $\geq$ 20 days of incubation at 37°C with samples of surface water.

Water type	BBT flask	Growth in flask[a]		Organism(s) with highest similarity (access. no.)	Similarity (%)	No. of OTUs	No. of seq.	% of OTU(s) clustering with protozoa
		Lp	Hv					
Storage reservoir for	I	−	−	Not determined	-	-	-	-
surface water plant D	II	−	−	Not determined	-	-	-	-
Water of river Rhine	I	+	−	*Neoparamoeba* sp. (AF371972)	95.6	1	2	66.7
(autumn)				Uncultured eukaryote (AB275097)/ *Protaspis grandis* (DQ303924)	98.0/94.0	1	1	33.3
	II	+	−	Uncultured eukaryote from treated water of supply B (EU860860)/ Uncultured cercozoa (AY620269)	93.9/88.6	1	20	100
Water of river Rhine	I	+	+	Clone library contains only sequences related to fungi	-	-	-	-
(winter)	II	+	+	*H. vermiformis* (AY680840)	99.8	1	12	85.8
				Stenamoeba sp. (EU377587)	90.6	1	1	7.1
				Uncultured eukaryote from treated water of supply B (EU860773)	95.0	1	1	7.1
Treated sewage	I	−	−	Clone library contains only sequences related to fungi	-	-	-	-
	II	+	−	*Diphylleia rotans* (AF420478)	99.3	1	12	100

[a] +: significant growth ($p<0.025$) of *L. pneumophila* (Lp) and *H. vermiformis* (Hv); −: no growth.

Table B.3: Classification of OTUs clustering with free-living protozoa obtained from BBTs after ≥ 20 days of incubation at 37°C with water of cooling towers.

Water type	BBT flask	Growth in flask[a]		Organism(s) with highest similarity (access. no.)	Similarity (%)	No. of OTU	No. of seq.	% of OTU(s) clustering with protozoa
		Lp	Hv					
Cooling tower 1	I	+	+	*H. vermiformis* (EU137741/AF426157)	93.5/100	2	4	50.0
				Uncultured eukaryote from treated water of supply B (EU860803)/ Uncultured cercozoan (AY620271)	91.0, 91.4/ 89.8, 90.1	2	2	25.0
				Corallochytrium limacisporum (L42528)	92.2	1	1	12.5
				Chromonas sp.(EF165144)	98.8	1	1	12.5
	II	+	+	Uncultured eukaryote from biofilm of supply A (EU860525)/*Cercomonadida* environmental sample clone (EF024515)	90.4/87.0	1	4	44.5
				H. vermiformis (AF426157)	99.82	1	3	33.3
				Uncultured eukaryote of TW-A (EU860633)	83.8	1	1	11.1
				Stenamoeba sp. (EU377587)	90	1	1	11.1
Cooling tower 2	I	−	−	Uncultured freshwater cercozoan (DQ243994)	99.1	1	2	100
	II	−	−	Uncultured freshwater cercozoan (DQ243994)	98.9	1	2	100
Cooling tower 3	I	−	−	Not determined	-	-	-	-
	II	−	−	Not determined	-	-	-	-
Cooling tower 4	I	−	+	*H. vermiformis* (AF426157)	100	1	5	100
	II	−	−	Not determined	-	-	-	-
Cooling tower 5	I	+	−	*Echinamoeba thermarum* (AJ489267, AJ489268)	96.6-97.9	4	10	62.4
				Stenamoeba sp. (EU377587)	90.0	2	4	25
				Pavlova gyrans (AF106055)	78.0	1	1	6.3
				Rhinosporidium seeberi (AF158369)	87.2	1	1	6.3
	II	+	−	*Echinamoeba thermarum* (AJ489268)	85.1-99.1	5	13	54.2
				Rhinosporidium seebri (AF118851)	87.9	1	9	37.5
				H. vermiformis (AY502960)	99.8	1	2	8.3

[a] +: significant growth ($p<0.025$) of *L. pneumophila* (Lp) and *H. vermiformis* (Hv); −: no growth.

Table B.4: Classification of OTUs clustering with free-living protozoa obtained from the BBTs incubated at 15°C with biomass from limestone filter bed of groundwater supply A inoculated with and without *Hartmannella vermiformis* (+Hv).

Water type	Organism(s) with highest similarity	Similarity (%)	No. of OTU(s)	No. of seq.	% of OTU(s) clustering with protozoa
Supply A (biomass from limestone filter bed)	Uncultured eukaryote from treated water of supply A (EU860670)/ *Cercomonas metabolicus* (DQ211597)	97.2-9.1/ 95.4-98.3	4	28	84.9
	Uncultured eukaryote from treated water of supply A (EU860661)/ *Neobodo designis* (AY753616)	99.8/88.2	1	4	12.1
	Uncultured freshwater cercozoan (DQ243993)/ *Gymnophrys cometa* (AF411284)	94.0/91.0	1	1	3.0
Supply A (biomass from limestone filter bed + Hv)	Uncultured eukaryote (AJ130856)/ Uncultured eukaryote from treated water of supply B (EU860779)	98.9/96.4	1	14	64
	Cercomonas metabolicus (DQ211597)	98.3	1	1	4.5
	Hartmannella vermiformis (DQ084363)	99.8	1	1	4.5
	Rhynchomonas nasuta (DQ207598)	92.1	1	1	4.5
	Spumella-like flagellate (DQ388549)	99.1	1	1	4.5
	Vanella miroides (AY183888)	91.7	1	1	4.5
	Uncultured marine eukaryote (EF526997)/ *Hartmannella vermiformis* (DQ084363)	97.7/97.0	1	1	4.5
	Uncultured eukaryote from treated water of supply A (EU860684)/ *Cercomonas metabolicus* (DQ211597)	97.9/93.8	1	1	4.5
	Uncultured Cercozoa (AY620280))/Uncultured eukaryote from treated water of supply B (EU860779)	97.1/95.5	1	1	4.5

Table B.5: Classification of OTUs clustering with free-living protozoa obtained from the BBTs incubated at 15°C with biomass from sand filter bed of groundwater supply B.

Water type	Organism(s) with highest similarity	Similarity (%)	No. of OTUs	No. of seq.	% of OTU(s) clustering with protozoa
Supply B (biomass from sand filter bed)	*Corallochytrium limacisporum* (L42528)	81.3/81.5	2	15	35.7
	Uncultured eukaryote from treated water of supply B (EU860879)/ Uncultured sphenomonad euglenozoan (AY821957)	99.4/68.5	1	9	21.4
	Uncultured eukaryote from treated water of supply B (EU860809)/*Sphaeroeca volvox* (234900)	99.8/80.2	1	5	11.9
	Uncultured cercozoan (AY620280)/Uncultured eukaryote from treated water of supply B (EU860779)	98.9, 100/ 96.8, 98.9	2	2	4.8
	Eimeriidae environmental sample (EF024467)	79.56	1	1	2.4
	Uncultured endolithic amoeba (AB257667)/Uncultured eukaryote from treated water of supply B (EU860945)	80.8/80.4	1	1	2.4
	Uncultured eukaryote from treated water of supply B (EU860849)/ *Ephydatia fluviatilis* (DQ167158)	79.1/76.4	1	1	2.4
	Uncultured eukaryote from treated water of supply A (EU860670)/ *Cercomonas metabolicus* (DQ211597)	97.8/97.7	1	1	2.4
	Uncultured eukaryote from treated water of supply B (EU860779)/*Lecytium* sp. (AJ514867)	93.4/91.7	1	1	2.4
	Uncultured eukaryote from treated water of supply B (EU860849)	82.2	1	1	2.4
	Uncultured eukaryote from treated of supply B (EU860809)/ *Corallochytrium limacisporum* (L42528)	93.6/78.8	1	1	2.4
	Uncultured eukaryote from treated water of supply B (EU860809)/ *Monosiga ovata* (AF084230)	94.3/81.1	1	1	2.4
	Uncultured haptorid ciliate (AY821926)/*Hemiophrys procera* (AY102175)	99.8/97.3	1	1	2.4
	Uncultured eukaryote from biofilm of supply A (EU860447)/*Gonimonas* sp. (AY360454)	84.3/82.8	1	1	2.4
	Uncultured eukaryote from treated of supply A (EU860737)/ Uncultured freshwater eukaryote (AY919695)	95.3/76.0	1	1	2.4
Supply B (biomass from sand filter bed)	Uncultured eukaryote from treated water of supply B (EU86087 9)/ Uncultured sphenomonad euglenozoan (AY821957)	99.3, 100/ 68.7, 68.3	2	9	20.9
	Uncultured marine eukaryote (EF526728)/ Uncultured eukaryote from biofilm of supply A (EU860463)	79.9/77.5	1	8	18.6
	Uncultured eukaryote from treated water of supply B (EU860879)/Uncultured eukaryote (EF100248)	99.8/69.3	1	7	16.3

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Table B.5 – continued from previous page

Water type	Organism(s) with highest similarity	Similarity (%)	No. of OTUs	No. of seq.	% of OTU(s) clustering with protozoa
	Uncultured eukaryote from biofilm of supply A (EU860456)/*Bodomopha* sp. (DQ211596)	94.8, 99.8/ 94.2, 99.2	2	2	4.7
	Uncultured eukaryote from treated water of supply A (EU860670)/*Codonosiga gracilis* (AY149897)	98.6/80.3	1	2	4.7
	Uncultured eukaryote from treated water of supply A (EU860670)/*Sphaeroeca volvox* (Z234900)	99.4/81.4	1	2	4.7
	Saccamoeba limax (AF293902)	81.8	1	1	2.3
	Rigidomastix-like sp. (AF411279)/*Bodomopha* sp. (DQ211596)	92.2/92.1	1	1	2.3
	Uncultured eukaryote from treated water of supply B (EU860810)/Uncultured eukaryote (EF023281)	86.8/83.1	1	1	2.3
	Uncultured eukaryote from TW-B (EU860779)/ Uncultured eukaryote (AJ130856)	98.3/97.7	1	1	2.3
	Corallochytrium limacisporum (L42528)	80.5	1	1	2.3
	Uncultured eukaryote from biofilm of supply A (EU860468)/*Diaphanoeca grandis* (AF084234)	97.9/93.6	1	1	2.3
	Uncultured eukaryote from biofilm of supply A (EU860521)/*Ichthyophonida* sp. (EU124913)	92.4/90.6	1	1	2.3
	Uncultured eukaryote from from treated water of supply A (EU860670)/*Monosiga ovata* (AF084230)	94.5/80.5	1	1	2.3
	Uncultured eukaryote from treated water of supply B (EU860866)/Codonosigidae environmental sample (EF024012)	93.2/91.8	1	1	2.3
	Uncultured eukaryote from treated water of supply B (EU860670)/*Monosiga brevicollis* (AF100940)	83.3/77.5	1	1	2.3
	Uncultured eukaryote from treated water of supply B (EU860866)/*Monosiga ovata* (AF271999)	98.7/92.4	1	1	2.3
	Uncultured eukaryote from treated water of supply B (EU860843)/*Petalomonas cantuscygni* (U84731)	99.8/62.7	1	1	2.3
	Uncultured haptorid ciliate (AY821926)/*Litonotus paracygnus* (DQ190464)	99.5/97.5	1	1	2.3

Appendix C

Supplemental material to Chapter 4

Table C.1: Classification of OTUs clustering with free-living protozoa obtained from distributed water of supplies CA-1, CA-2 and CA-3.

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones		
			DW-CA-1	DW-CA-2	DW-CA-3
Amoebozoa	**-**	**24**	**63**	**32**	**4**
Andalucia godoyi (AY965870)	98.7	1	-	-	2
Echinamoeba exundans (AF293895, AF293896)	96.2-100	3	7	-	-
Echinamoeba thermarum (AJ489268, AJ489265)	83.7, 90.3	2	4	-	-
Uncultured eukaryote obtained from treated water of groundwater supply A (EU860633)/*Acanthamoeba castellanii* (AF114438)	99.6/85.9	1	1	1	-
Eimeriidae environmental sample (EF024492)/ *Acanthamoeba* sp.(AY176047)	86.9/85.4	1	-	2	-
Gephyramoeba sp. (AF293897)	98.6	1	-	2	-
Hartmannella vermiformis (AY680840, AY502961, DQ084363)	98.3-99.8	6	43	20	1
Hartmannellidae environmental sample (EF023499)	91.0, 96.4	2	2	-	-
Neoparamoeba pemaquidensis (AY714355)	90.8	1	-	1	-
Platyamoeba stenopodia (AY294144)	88.3	1	4	-	-
Uncultured eukaryote (AY082995)/ *Paravahlkampfia* sp.(AJ550994)	79.6/69.1	1	-	1	-
Uncultured eukaryote obtained from BBT with biomass from sand filter bed of groundwater supply B after incubation at 37°C (GU970396)/*Neoparamoeba pemaquidensis* (AF371972)	94.3/91.5	1	-	1	-
Uncultured eukaryote obtained from BBT with biomass from limestone filter bed of groundwater supply A after incubation at 37°C (GU970344)/*Korotnevella stella* (AY686573)	93.6/93.6	1	-	2	-
Uncultured eukaryote from treated water of groundwater supply A (860633)/*Acanthamoeba* sp. (AY176047)	86.0/82.7	1	-	-	1
Uncultured eukaryote from treated water of groundwater supply B (EU860839)/*Echinamoeba thermarum* (AJ489266)	98.9/94.1	1	3	2	-
Cercozoa	**-**	**15**	**4**	**25**	**27**
Bodomorpha minima (AF411276)	99.4	1	-	5	-
Cercomonas metabolicus (DQ211597)	97.5, 95.1	2	-	2	22
Hyphodontia sp. (DQ873632)	95.0	1	1	-	-
Ichthyophonida galbana (AJ246266)	77.8	1	-	1	-
Uncultured cercozoan clone (AY62027)/ Soil Flagellate AND25 (AY965868)	100/92.0	1	1	7	-
Uncultured cercozoan (AY620271)/Uncultured eukaryote from treated water of groundwater supply B (EU860814)	99.2/97.2	1	-	3	-
Uncultured eimeriidae (EF024879)/Uncultured eukaryote from treated water of groundwater supply B (EU860773)	92.1/91.9	1	-	-	1
Uncultured eukaryote from treated water of groundwater supply A (EU860670)/*Cercomonas metabolicus* (EF095190)	95.8/95.9	1	-	-	1
Uncultured eukaryote from treated water of groundwater supply A (EU860636)/Uncultured freshwater cercozoan (DQ243993)	91.7/83.7	1	-	1	3
Uncultured eukaryote from treated water of groundwater supply B (EU860803)/Uncultured eukaryote (AB275050)	91.9/90.4	1	-	5	-
Uncultured eukaryote from treated water of groundwater supply B	95.8/96.4	1	1	-	-

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Table C.1 – continued from previous page

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones		
			DW-CA-1	DW-CA-2	DW-CA-3
(EU860814)/Uncultured cercozoan (AY620271)					
Uncultured freshwater cercozoan (DQ243994)/ *Gymnophrys cometa* (AJ514866)	99.6/91.1	1	1	-	-
Uncultured freshwater cercozoan (DQ243992)/ *Cercomonas* sp.(AF411271)	96.9/95.6	1	-	1	-
Uncultured eukaryote obtained from BBT with surface water after incubation at 37°C (GU970532)/ *Cercomonas* sp. (AF411266)	99.1/86.1	1	-	1	-
Choanozoa	**-**	**4**	**2**	**9**	**0**
Acanthoeca spectabilis (AF084233)	77.7	1	-	1	-
Uncultured eukaryote obtained from BBT with water from tap water installation B1 after incubation at 37°C (GU970716)/*Sphaeroeca volvox* (Z34900)	100/85.0	1	-	1	-
Uncultured freshwater eukaryote (AY919771)/ *Corallochytrium limacisporum* (L42528)	92.6, 91.6/ 88.6, 88.1	2	2	7	-
Ciliophora	**-**	**10**	**5**	**40**	**3**
Bursaria trancatella (EU039889)	95.7	1	-	2	-
Colopoda cucullus (EU039893)	99.0	1	-	12	-
Lembadion bullinum (AF255358)	84.1, 88.4	2	-	10	-
Prorodontidae environmental sample (EF024334)	94.7	1	-	4	-
Pseudoplatyophrya nana (AF060452)	97.5, 97.7	2	-	2	-
Uncultured eukaryote (AJ130862)/ *Hemiophrys procera* (AY162175)	98.3/96.8	1	2	9	3
Uncultured eukaryote (EF032797)/ *Pseudocyrtolophosis alpestris* (EU264564)	99.4/98.5	1	3	-	-
Uncultured marine eukaryote ((EU446414)/ *Cyrtolophosis mucicola* (EU039898)	89.8/88.4 1	-	1	-	-
Stramenopiles	**-**	**6**	**3**	**3**	**5**
Ochromonas sp. (EF165144)	98.8, 99.6	2	-	-	2
Posterioochromonas malhamensis (EF165114)	99.0	1	-	2	-
Spumella-like flagellate (AY651076, DQ388542)	98.4, 99.6	2	2	-	3
Uncultured eukaryote (AJ564770)/ *Floydiella terrestis* (D86498)	87.5/85.7	1	1	1	-
Total		**59**	**77**	**109**	**39**

Table C.2: Classification of OTUs clustering with eukaryotes obtained from treated water from supply CA-1.

Organism(s) with highest similarity (GenBank accession no.)	**Phylum**	**Similarity (%)**	**No. of OTUs**	**No. of clones**
Free-living protozoa	**-**	**-**	**10**	**25**
Echinamoeba exundans (AF293895)	Amoebozoa	94.5-99.3	3	4
Hartmannellidae environmental sample (EF023499)	Amoebozoa	92.3, 96.7	2	3
Hartmannella vermiformis (DQ123623, DQ084363)	Amoebozoa	85.2-99.3	3	14
Malawimonas jakobiformis (AY117420)	Amoebozoa	95.8	1	3
Uncultured eukaryotic picoplankton (AJ130857)	Choanozoa	90.9	1	1
Fungi	**-**	**-**	**17**	**47**
Mucor racemosus (AF113430)	Zygomycota	100	1	1
Cladosporium sp. (EU167574)	Ascomycota	100	1	1
Uncultured Banisveld eukaryote (EU091865)	Ascomycota	82.7, 82.8	2	3
Uncultured eukaryote (AJ564770)	Ascomycota	88.4	1	2
Uncultured eukaryote (AJ130869)/Uncultured freshwater eukaryote (AY919786)	Ascomycota	89.0/73.6	1	1
Uncultured eukaryote obtained from surface water after incubation at 37°C (GU970494)/ Uncultured Banisveld eukaryote (EU091865)	Ascomycota	82.4/80.2	1	1
Cryptococcus bhutanensis (AB032623)	Basidiomycota	100	1	1
Dioszegia buhagiarii (EU517065)	Basidiomycota	100	1	1
Filobidiella depauperata (AJ568017)	Basidiomycota	81.1	1	1
Lycogala flavofuscum (AY187083)	Basidiomycota	99.4	1	2
Uncultured banisveld eykaryote (EU091865)	Unidentified	96.5	1	1
Uncultured eukaryote (AJ130857) Uncultured eukaryote (AJ130857)/	Unidentified	91.6	2	28
Uncultured banisveld eukaryote (EU091838)	Unidentified	91.6, 91.5	2	2
Uncultured nucleariidae (EF024210)/ Uncultured Chytidiomycota (EU162642)	Unidentified	90.0/89.2	1	1
Viridipantae	**-**	**-**	**2**	**2**
Bryum caespiticium (AF023703)	-	100	1	1
Fagus grandifolia (AF206910)	-	99.8	1	1
Sequence with $<$ 75% similarity	**-**	**-**	**4**	**9**
Total	**-**	**-**	**33**	**83**

Table C.3: Classification of OTUs clustering with fungi obtained from distributed water of supplies CA-1, CA-2 and CA-3.

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones		
			DW-CA-1	DW-CA-2	DW-CA-3
Chytridiomycota	**-**	**3**	**0**	**4**	**21**
Entophlyctis sp. (AY635824, AY635828)	83.8	2	-	3	21
Rhizophydium sp. (AY635821)	98.0	1	-	1	-
Zygomycota	**-**	**7**	**3**	**11**	**0**
Basidiobolus microsporus (AF368505)	95.6	1	1	-	-
Batrachochytrium dendrobatidis (AATT01000051)	78.3, 78.5	2	-	2	-
Catenaria anguillulae (EF014365)	99.6	1	2	-	-
Mucor racemosus (AF113430)	99.6	1	-	1	-
Uncultured eukaryote obtained from BBT with cooling water after incubation at 37°C (GU970807)	94.7, 98.5	2	-	8	-
Ascomycota	**-**	**46**	**71**	**68**	**12**
Botryosphaeria rhodina (U42476)	99.6	1	-	1	-
Candida fermenticarens (AB013525)	99.2	1	2	-	-
Capronia epimyces (AJ232938)	97.8	1	-	2	-
Citeromyces matritensis (AB018164)	99.9	1	1	-	-
Cordyceps ochraceostromata (AY245660)	99.0	1	-	1	-
Eurotium herbariorum (AB008402)	100	1	-	-	1
Galactomyces reessii (AB000646)	81.8	1	-	1	-
Hyphodontia paradoxa (AF026612)	99.0	1	1	1	-
Paecilomyces aerugineus (AB023942)	94.1	1	-	1	-
Penicillium purpurogenum (AF245257)	99.8	1	-	7	-
Phaeosphaeria nodorum (AAG101000078)	99.6	1	-	13	-
Pichia jadinii (AB054369)	100	1	-	2	2
Pichia pastoris (EF550392, AB018182)	77.3, 78.0	2	-	2	-
Saccharomyces cerevisiae (DJ324968)	99.6	1	-	-	1
Uncultured Ascomycota (AB074653)/ *Zasmidium cellare* (EF137362)	99.6	1	4	1	-
Uncultured Banisveld eukaryote (EU091865)	77.0-87.6	4	6	5	-
Uncultured Banisveld eukaryote (EU091865)/ Uncultured eukaryote obtained from BBT with surface water after incubation at 37°C (GU970636)	96.2/94.6	1	3	-	1
Uncultured eukaryote (AJ130869)	88.8-90.4	4	6	3	-
Uncultured eukaryote (AJ130869)/ Eimeriidae environmental sample clone (EF023236, EF024865)	86.6-89.6/ 80.2-73.4	4	9	-	-
Uncultured eukaryote (AY082990)/ *Pseudosigmoidea cranei* (DQ104808)	96.2/95.5	1	-	8	-
Uncultured eukaryote (AJ130869)/ *Saccinobaculus doroxostylus* (DQ525704)	91.1/72.8	1	-	-	1
Uncultured eukaryote (AJ130869)/ Uncultured eukaryote obtained from BBT with surface water after incubation at 37°C (GU970495)	99.8/95.0	1	4	-	-
Uncultured eukaryote obtained from biofilm of distribution system from anaerobic groundwater plant (EU860538,	78.7, 79.4/ 78.5, 79.3	2	17	-	-

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Table C.3 – continued from previous page

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones		
			DW-CA-1	DW-CA-2	DW-CA-3
EU860937)/Uncultured Banisveld eukaryote (EU091865)					
Uncultured eukaryote obtained from BBT with cooling water after incubation at 37°C (GU970830)/ Uncultured Banisveld eukaryote (EU091865)	92.6, 93.0/ 82.7	2	13	-	-
Uncultured eukaryote obtained from surface water after incubation at 37°C (GU970494)/ Uncultured Banisveld eukaryote (EU091865)	9 1.7, 93.3/ 85.2, 85.4	2	-	-	4
Uncultured eukaryote obtained from surface water after incubation at 37°C (GU970602)/ Eimeriidae environmental sample clone (EF024865)	98.0/80.5	1	2	-	-
Uncultured eukaryote obtained from treated sewage after incubation at 37°C (GU970554)	92.8-94.0	5	-	20	1
Uncultured fungus (EU162635)	79.4	1	3	-	-
Zopfia rhizophila (L76622)	99.2	1	-	-	1
Basidiomycota	**-**	**10**	**1**	**41**	**2**
Cryptococcus bhatanensis (AB032623)	99.6	1	1	-	-
Eimeriidae environmental sample (EF023382)	98.5	1	-	1	-
Filobasidium floriforme (D13460)	100	1	-	35	-
Hexagonia hirta (AY336759)	98.8	1	-	-	1
Malassezia restricta (AAXK01002636)	99.8	1	-	1	-
Mycocia pinicola (DQ873636)	98.9	1	-	1	-
Psathyrella gracilis (DQ851582)	99.6	1	-	1	-
Rhodotorula benthica (AB126647)	93.6	1	-	1	-
Rhodotorula murilaginosa (EU563925)	99.8	1	-	-	1
Wallemia sebi (AY741379)	99.8	1	-	1	-
Unidentified	**-**	**17**	**39**	**25**	**5**
Uncultured Banisveld eukaryote (EU091865, EU091859)	85.8-96.7	7	24	6	3
Eimeriidae environmental sample clone (EF024492)	82.7	1	-	-	1
Uncultured eukaryote (AY916588, AJ130849, AJ130857)	91.2-98.3	4	1	20	-
Uncultured eukaryote obtained from BBT with surface water after incubation at 37°C (GU970467)/ *Camarops microspora* (DQ471036)	95.0/81.2	1	9	-	-
Uncultured eukaryote obtained from BBT with cooling water after incubation at 37°C (GU970802)	89.0	1	-	-	1
Uncultured freshwater eukaryote (AY919771)	91.5	1	1	-	-
Uncultured fungus (EU162635, DQ244011)	86.1, 97.1	2	4	-	-
Total	**-**	**83**	**114**	**150**	**40**

Table C.4: Classification of OTUs clustering with metazoa obtained from distributed water of supplies CA-1, CA-2 and CA-3.

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones		
			DW-CA-1	DW-CA-2	DW-CA-3
Porifera	**-**	**1**	**0**	**0**	**10**
Uncultured eukaryote obtained from BBT with biomass from sand filter of anaerobic groundwater plant B after incubation at 15°C (GU970303)/ *Baikalospongia fungiformis* (EF095190)	99.6/80.6	1	-	-	10
Platyhelminthes	**-**	**4**	**0**	**48**	**11**
Catenula sp. (AJ012532)	94.2, 89.3	2	-	16	11
Stenostomum sp. (U95947)	99.1	1	-	4	-
Suomina sp. (AJ012532)	99.1	1	-	28	-
Rotifera	**-**	**1**	**7**	**3**	**0**
Lecane leontina (DQ297700)	100/99.6	1	7	3	-
Gastrotricha	**-**	**1**	**0**	**0**	**11**
Chaetonotus sp. (AJ001735)	97.9	1	-	-	11
Nematoda	**-**	**19**	**0**	**87**	**95**
Achromadora terricola (AY593940)	98.2, 98.8	2	-	-	2
Ceratoplectus tusarmatus (AY284706)	98.8	1	-	1	-
Diplolai melloides (EF659919)	86.6	1	-	1	-
Monohystera riemanni (AY593938)	89.2	1	-	1	-
Eimeriidae environmental sample (EF023774)/*Paratriphyla* sp. (AY284737)	87.2/86.7	1	-	1	-
Uncultured Diplolaimelloides (EF659919)/ *Monohystera riemanni* (AY593938)	89.6/89.6	1	-	2	-
Uncultured eukaryote obtained from BBT with biomass from sand filter of plant D after incubation at 37°C (GU970705)/ *Rhabdolaimus terrestris* (AY284710)	99.2/97.5	1	-	1	-
Uncultured eukaryote (EF032796)/ *Rhabdolaimus terrestris* (AY284710)	94.6-99.1/ 93.4-98.7	3	-	56	85
Paratriphyla sp. (AY284737)	84.4, 84.7	2	-	15	-
Phaseoleae environmental sample (EF023598)/ *Prismatolaimus intermedius* (AY284729)	97.7-99.8/ 99.6-97.5	3	-	6	8
Rhabdolaimus terrestris (AY284711, AY284712)	77.5-96.7	3	-	3	-
Annelida	**-**	**1**	**0**	**6**	**1**
Enchytraeus sp. (U95948)	95.4	1	-	6	-
Arthropoda	**-**	**2**	**0**	**2**	**0**
ELiposcelis bostrychophyla (AY630476)	99.5	1	-	1	-
Sinella curviseta (DQ016565)	97.5	1	-	1	-
Total	**-**	**29**	**7**	**146**	**128**

Table C.5: Classification of OTUs clustering with cryptophyta and viridiplantae obtained from distributed water of supplies CA-1, CA-2 and CA-3.

Organism(s) with highest similarity (GenBank accession no.)	**Similarity (%)**	**No. of OTUs**	**No. of clones**		
			DW-CA-1	**DW-CA-2**	**DW-CA-3**
Cryptophyta	**-**	**10**	**4**	**16**	**37**
Chroomonas sp. (AJ007277)	93.1	2	4	10	31
Goniomonas sp. (AY360455)	80.7, 83.0	2	-	2	-
Ostreococcus tauri (AY329635)	88.0	1	-	1	-
Phoma herbarum (AY864822)	91.6	1	-	1	-
Uncultured eukaryote obtained from BBT with biomass from sand filter of anaerobic groundwater supply B (GU970196)/ *Goniomonas* sp. (AY360455)	84.6-85.4/ 79.7-80.2	4	-	2	6
Viridiplantae	**-**	**8**	**1**	**4**	**21**
Albizzia julibrissin (U42536)	94.7-99.1	3	-	-	10
Caprifoliaceae environmental sample (EF023237)/ *Atractylodes japonica* (EU678363)	100/99.6	1	-	4	-
Chlorella luteoviridis (AB006045)	98.5	1	1	-	-
Hordeum jubatum (AF168852)	99.8	1	-	-	5
Laurus nobilis (AF197580)	99.6	1	-	-	2
Nicotiana tabacum (AJ236016)	99.8	1	-	-	4
Total	**-**	**18**	**5**	**20**	**58**

Appendix D

Supplemental material to Chapter 5

Table D.1: Maximum concentrations of active biomass (ng ATP cm^{-2}) on the PVC-P cylinders during the incubation at four temperatures in the BBT flasks with four freshwater types.

BBT flask	Cooling tower water	River Rhine water	Tap water I	Tap water II
I-20°C	8.9	6.6	17.9	31.3
II-20°C	8.2	6.8	14.8	24.6
I-30°C	8.0	5.8	5.2	31.2
II-30°C	7.4	5.5	3.8	26.9
I-37°C	7.3	11.4	3.7	20.4
II-37°C	6.0	6.7	5.3	24.0
I-42°C	7.4	5.6	7.1	21.3
II-42°C	4.3	10.6	1.1	15.0

Table D.2: Growth of microorganisms at four temperatures in the BBT flasks with cooling tower water.

BBT Flask	Growth[a] of			Ratio (log units) between maximum concentrations of	
	Acanthamoeba spp.	*H. vermiformis*	*L. pneumophila*	*L. pneumophila* and *H. vermiformis*	*L. pneumophila* and *Acanthamoeba* spp.
I-20°C	+	+	−	−	−
II-20°C	+	+	−	−	−
I-30°C	+	+	+	2.1	2.2
II-30°C	+	+	+	2.1	3.0
I-37°C	+	+	+	3.4	4.8
II-37°C	−[b]	+	+	2.8	4.4
I-42°C	−[b]	−[b]	+	4.3	4.9
II-42°C	−[b]	−[b]	+	5.1	4.9

[a] +, significant growth ($p<0.025$); −, no growth.
[b] Free-living protozoa were detected, but no significant growth was observed.

Table D.3: Growth of microorganisms at four temperatures in the BBT flasks with river Rhine water.

BBT Flask	Growth[a] of			Ratio (log units) between maximum concentrations of	
	Acanthamoeba spp.	*H. vermiformis*	*L. pneumophila*	*L. pneumophila* and *H. vermiformis*	*L. pneumophila* and *Acanthamoeba* spp.
I-20°C	−	+	−	−	−
II-20°C	−[b]	+	−	−	−
I-30°C	−[b]	+	+	3.8	5.3
II-30°C	+	+	+	2.6	2.9
I-37°C	−	+	+	4.1	−
II-37°C	−	+	+	4.0	−
I-42°C	−	+	+	5.0	−
II-42°C	−	−[b]	−	−	−

[a] +, significant growth ($p<0.025$); −, no growth.
[b] Free-living protozoa were detected, but no significant growth was observed.

Table D.4: Growth of microorganisms at four temperatures in the BBT flasks with tap water I.

BBT Flask	Growth[a] of			Ratio (log units) between maximum concentrations of	
	Acanthamoeba spp.	*H. vermiformis*	*L. pneumophila*	*L. pneumophila* and *H. vermiformis*	*L. pneumophila* and *Acanthamoeba* spp.
I-20°C	−	+	−	−	−
II-20°C	−	+	−	−	−
I-30°C	−	+	−	−	−
II-30°C	−	+	+	1.2	−
I-37°C	−	+	−	−	−
II-37°C	−	+	+	3.9	−
I-42°C	−	−	−	−	−
II-42°C	−	−	−	−	−

[a] +, significant growth ($p<0.025$); −, no growth.

Table D.5: Growth of microorganisms at four temperatures in the BBT flasks with tap water II.

BBT Flask	Growth[a] of			Ratio (log units) between maximum concentrations of	
	Acanthamoeba spp.	*H. vermiformis*	*L. pneumophila*	*L. pneumophila* and *H. vermiformis*	*L. pneumophila* and *Acanthamoeba* spp.
I-20°C	+	+	−	−	−
II-20°C	−	+	−	−	−
I-30°C	+	+	+	3.5	3.3
II-30°C	+	+	+	1.6	3.2
I-37°C	−	+	+	3.8	−
II-37°C	−	+	+	3.2	−
I-42°C	−	−	−	−	−
II-42°C	−	−	−	−	−

[a] +, significant growth ($p<0.05$); −, no growth.

Table D.6: Classification of OTUs clustering with free-living protozoa obtained from cooling tower water (CTW), river Rhine water (RRW), tap water I (TW-I) and tap water II (TW-II) at day 0.

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones			
			CTW	RRW	TW-I	TW-II
Amoebozoa	**-**	**7**	**8**	**0**	**2**	**0**
Acanthamoeba jacobsi (AY262365)	94.5	1	1	-	-	-
Korotnevella stella (AY686573)	88.6	1	1	-	-	-
Uncultured eukaryote obtained from treated water of groundwater supply B (EU860820)/ Uncultured Hartmannellidae (EF023499)	93.3/76.5	1	3	-	-	-
Uncultured eukaryote obtained from BBT with biomass from sand filter bed of groundwater supply B after incubation at 15°C (GU970253)/Uncultured endolithic amoeba (AB257667)	98.9/80.2	1	2	-	-	-
Uncultured eukaryote (AY749481)/ *Pterocystis foliacea* (AY749599)	99.8/99.2	1	1	-	-	-
Uncultured Eimeriidae (EF024503)/ *Acanthamoeba polyphaga* (AF132135)	99.4/96.2	1	-	-	1	-
Uncultured eukaryote obtained from treated water of groundwater supply B (EU860760)/ *Korotnevella hemistylolepis* (AY121850)	99.7/70.9	1	-	-	1	-
Apusozoa	**-**	**1**	**0**	**0**	**0**	**1**
Ancyromonas sigmoides (DQ207565)	91.5	1	-	-	-	1
Cercozoa	**-**	**12**	**3**	**12**	**2**	**2**
Uncultured cercozoan (AY620356)/ *Cercozoa* sp. (FJ824126)	92.9-99.6/ 92.5-98.3	5	-	11	-	-
Uncultured eukaryote obtained from treated water of groundwater supply B (EU860803)/ Uncultured cercozoan clone (AY620300)	86.3/85.4	1	2	-	-	-
Uncultured eukaryote obtained from BBT with cooling water 1 after incubation at 37°C (GU970882)/Uncultured cercozoan clone (AY620301)	94.2/91.4	1	1	-	-	-
Uncultured eukaryote (AB275061)/ *Spongospora subterranean* (AF310899)	87.0/86.9	1	-	-	-	1
Uncultured eukaryote obtained from distribution biofilm of groundwater supply A (EU860489)/ *Dimorpha* sp. (EF455769)	96.1/93.0	1	-	-	1	-
Uncultured eukaryote obtained from treated water of groundwater supply B (EU860798)/ *Allas* sp. (AY268040)	95.7/92.6	1	-	-	1	-
Uncultured eukaryote obtained from treated water of groundwater supply B (EU860803)/ Uncultured cercozoan clone (AY620297)	98.9/87.8	1	-	-	-	1
Uncultured freshwater cercozoan (DQ243991)/ Uncultured marine eukaryote (EF526780)	91.6/89.7	1	-	1	-	-

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Table D.6 – continued from previous page

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones			
			CTW	RRW	TW-I	TW-II
Choanozoa		**2**	**1**	**2**	**0**	**0**
Rhinosporidium seebri (AF118851)	86.9	1	1	-	-	-
Uncultured marine eukaryote (EU446411)/ *Diaphanoeca grandis* (DQ059033)	92.7/92.2	1	-	2	-	-
Ciliophora	-	**12**	**9**	**5**	**2**	**6**
Acineta sp.(AY332717)	90.9	1	-	-	1	-
Colpoda aspera (EU039892)	99.0, 92.5	2	2	-	-	-
Ophryoglena catenula (U17355)	90.7	1	1	-	-	-
Uncultured marine eukaryote (EF527130)/ *Miamiensis avidus* (AY642280)	94.5/93.0	1	3	-	-	-
Tetrahymena bergeri (AF364039)	93.6	1	-	-	-	2
Tetrahymena mobilis (AF364040)	99.6	1	3	-	-	-
Uncultured alveolate (DQ244028)/ *Rimostrombidium lacustris* (DQ986131)	94.6/94.2	1	-	5	-	-
Uncultured eukaryote obtained from treated water of groundwater supply A (EU860686)/ *Anophryoides haemophila* (U51554)	94.7/92.2	1	-	-	-	1
Uncultured eukaryote obtained from treated water of groundwater supply A (EU860732)/ *Miamiensis avidus* (AY550080)	96.1/90.7	1	-	-	-	1
Uncultured spirotrichid ciliate (AY821927)/ *Holosticha diademata* (DQ059583)	99.9/99.2	1	-	-	1	2
Uroleptus retractilis (EF486865)	85.6	1	-	-	-	1
Euglenozoa	-	**3**	**2**	**0**	**0**	**1**
Procryptobia sp. (AY490216)	99.3; 98.0	2	2	-	-	-
Uncultured eukaryote obtained from treated water of groundwater supply A (EU860686)/ *Petalomonas cantuscygni* (AF386635)	99.8/62.7	1	-	-	-	1
Myzozoa	-	**8**	**1**	**0**	**7**	**26**
Cryptosporidium muris (AAZY02000007)	84.7	1	-	-	-	1
Reclinomonas Cityamericana (AY117417)	91.8	1	1	-	-	-
Uncultured freshwater eukaryote (AY919736)/ *Dinophyceae* sp. (DQ116021); (AM08889)	96.7/86.1; 84.0	1	-	-	-	3
Uncultured freshwater eukaryote (AY919736)/ *Gyrodinium aureolum* (AF1272713)	97.5/84.4	1	-	-	-	1
Uncultured freshwater eukaryote (AY919736)/ *Ochromonas* sp. (EF165142)	94.0/87.8	1	-	-	-	1
Uncultured marine eukaryote (EF526851)/ *Takayama cf. pulchellum* (AY800130)	89.5/89.2	1	-	-	-	20
Stramenopiles	-	**8**	**0**	**3**	**14**	**1**
Heterococcus pleurococcoides (AJ5799335)	82.2	1	-	-	2	-
Ochromonas sp. (EF165133)	96.0	1	-	-	1	-
Thalassiosira guillardii (DQ514869)	97.2, 99.8	2	-	2	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999674)/Spumella-like flagellate JBC30 (AY651073)	99.8/98.5	1	-	-	10	-

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Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones			
			CTW	RRW	TW-I	TW-II
Uncultured freshwater eukaryote (AY919789)/ *Chromulina chionophila* (M87332)	97.28/93.7	1	-	1	-	-
Uncultured marine eukaryote (EF527177)/ *Ochomonas* sp. (EF165133)	93.7/93.6	1	-	-	1	-
Uncultured marine eukaryote (AY381186)/ *Pirsonia verrucosa*(AJ561113)	92.8/91.2	1	-	-	-	1
Total	**-**	**53**	**24**	**22**	**27**	**37**

Table D.7: Classification of OTUs clustering with fungi obtained from cooling tower water (CTW), river Rhine water (RRW), tap water I (TW-I) and tap water II (TW-II) at day 0.

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones			
			CTW	RRW	TW-I	TW-II
Chytridiomycota	**-**	**3**	**0**	**1**	**0**	**2**
Uncultured Chytridiomycota(GQ995372)/ *Rhizophydium elyensis* (DQ536479)	91.6/89.4	1	-	1	-	-
Uncultured eukaryote obtained from BBT with biomass from limestone filter bed of groundwater supply A after incubation at 37°C (GU970335)/*Triparticalcar arcticum* (DQ536480)	92.8/90.7	1	-	-	-	1
Uncultured Chytridiomycota (EU162640)/ *Kappamyces laurelenisis* (DQ536478)	97.1/95.0	1	-	-	-	1
Zygomycota	**-**	**1**	**1**	**0**	**0**	**0**
Uncultured alveolate (EU910604)/ *Basidiobolus microsporus* (AF368505)	87.1/86.8	1	1	-	-	-
Ascomycota	**-**	**12**	**14**	**0**	**0**	**1**
Candida bombi (AB013576)	88.6	1	1	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-2 (HQ999245)/*Galactomyces citri-aurantii* (AB000665)	97.5/85.0	1	3	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999654)/Uncultured Banisveld eukaryote (EU091865)	96.2/84.4	1	2	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-2 (HQ999561)/Uncultured Banisveld eukaryote (EU091851)	93.7/79.6	1	1	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-2 (HQ999237)/*Savillea micropora* (EU011928)	99.2/78.0	1	1	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999649)/Uncultured Banisveld eukaryote (EU091865)	98.7/85.8	1	1	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-2 (HQ999223)/*Acanthoeca spectablilis* (AF084233)	97.1/77.7	1	1	-	-	-
Uncultured eukaryote obtained from BBT with of river Rhine after incubation at 37°C (GU970493)/ Uncultured Banisveld eukaryote (EU091865)	98.3/85.4	1	1	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999645)/Uncultured Banisveld eukaryote (EU091865)	95.4/83.8	1	1	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-2 (HQ999230)/Uncultured Chytridiomycota (GQ995289)	97.5/79.2	1	1	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999649)/Uncultured Banisveld eukaryote (EU091865)	96.4/84.4	1	1	-	-	-

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Table D.7 – continued from previous page

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones			
			CTW	RRW	TW-I	TW-II
Strobiloscypha keliae (AF006310)	95.4/96.1	1	-	-	-	1
Unidentified	**-**	**5**	**2**	**0**	**2**	**1**
Uncultured eukaryote (AJ130850)/ *Spizellomyces punctatus* (ACOE01000320)	97.2/88.9	1	-	-	1	-
Uncultured eukaryote obtained from distributed water of supply CA-2 (HQ999233)/Uncultured eukaryote (AJ230849)	99.0/96.1	1	-	-	1	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999690)/Uncultured Banisveld eukaryote (EU091865)	99.2/85.8	1	1	-	-	-
Uncultured fungus (DQ244016)/ Aristolochiaceae environmental sample (EF023617)	83.8/79.6	1	1	-	-	-
Uncultured fungus (EU162635)	99.4	1	-	-	-	1
Total	**-**	**21**	**17**	**1**	**2**	**4**

Table D.8: Classification of OTUs clustering with metazoa obtained from cooling tower water (CTW), river Rhine water (RRW), tap water I (TW-I) and tap water II (TW-II) at day 0.

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones			
			CTW	RRW	TW-I	TW-II
Cnidaria	**-**	**1**	**0**	**0**	**2**	**0**
Plumatella fungosa (DQ221748)	90.5	1	-	-	2	-
Rotifera	**-**	**2**	**0**	**0**	**4**	**0**
Lecane leontina (DQ297700)	99.8	1	-	-	3	-
Uncultured eukaryote obtained from BBT with cooling water 1 after incubation at 37°C (GU970886)/*Lecane leontina* (DQ297700)	97.0/96.0	1	-	-	1	-
Nematoda	**-**	**3**	**0**	**0**	**5**	**1**
Uncultured nematode (EU910601)/ *Eumonhystera cf. simplex* (AY284692)	98.9/97.0	1	-	-	2	-
Paralamyctes environmental sample (EF024168)/ *Eumonhystera cf. simplex* (AY284692)	98.5/97.0	1	-	-	3	-
Uncultured eukaryote obtained from BBT with biomass of sand filter of surface water supply D after incubation at 37°C (GU970682)/ *Rhabdolaimus aquaticus* (FJ969139)	100/99.1	1	-	-	-	1
Mollusca	**-**	**2**	**0**	**13**	**0**	**0**
Dreissena bugensis (AF305703)	99.8, 97.2	2	-	13	-	-
Total	**-**	**8**	**0**	**13**	**11**	**1**

Table D.9: Classification of OTUs clustering with viridiplantae obtained from cooling tower water (CTW), river Rhine water (RRW), tap water I (TW-I) and tap water II (TW-II) at day 0.

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones			
			CTW	RRW	TW-I	TW-II
Viridiplantae	**-**	**1**	**0**	**1**	**0**	**0**
Chlamydomonas sp. (GQ122366)	98.2	1	-	1	-	-
Total	**-**	**1**	**0**	**1**	**0**	**0**

Table D.10: Classification of OTUs clustering with free-living protozoa obtained from BBT flasks with cooling tower water at 37 and 42°C.

			No. of clones		
Organism(s) with highest similarity (GenBank accession no.)	**Similarity (%)**	**No. of OTUs**	**Flask I 37°C**	**Flask I 42°C**	**Flask II 42°C**
Amoebozoa	**-**	**10**	**11**	**4**	**1**
Uncultured eukaryote obtained from BBT with cooling water 5 after incubation at 37°C (GU970801)/ *Stenamoeba* sp. (EU377587)	99.8/84.8	1	5	-	1
Uncultured eukaryote obtained from BBT with biomass from limestone filter of groundwater supply after incubation at 37°C (GU970344)/ *Korotnevella stella* (AY686573)	94.0-95.5/ 93.8-95.4	3	3	-	-
Hartmannella vermiformis (AF426157)	100	1	2	-	-
Korotnevella stella (AY686573)	95.4, 96.5	2	1	1	-
Uncultured eukaryote obtained from BBT with cooling water 5 after incubation at 37°C (GU970344)/ *Korotnevella stella* (AY686573)	95.8, 95.5/ 95.4, 95.0	2	-	2	-
Uncultured eukaryote obtained from distributed water of supply CA-2 (HQ999192)/*Korotnevella stella* (AY686573)	85.8/85.3	1	-	1	-
Euglenozoa	**-**	**1**	**3**	**0**	**0**
Neobodo designis (AY998651)	98.3	1	3	-	-
Stramenopiles		**2**	**4**	**17**	**22**
Spumella-like flagellate JBC30 (AY651073)	99.8	1	4	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999655)/Spumella-like flagellate JBC30 (AY651073)	99.8/98.7	1	-	17	22
Total	**-**	**13**	**18**	**21**	**23**

Table D.11: Classification of OTUs clustering with free-living protozoa obtained from BBT flasks with river Rhine water incubated at 37 and 42°C.

			No. of clones		
Organism(s) with highest similarity (GenBank accession no.)	**Similarity (%)**	**No. of OTUs**	**Flask I 37°C**	**Flask I 42°C**	**Flask II 42°C**
Amoebozoa	-	**6**	**4**	**22**	**38**
Arachnula sp. (EU273440)	98.9	1	-	22	-
Uncultured eukaryote obtained from BBT with treated sewage incubated at 37°C (GU970535)/ *Diphylleia rotans* (AF420478)	99.8, 90.6/ 99.6, 90.6	2	3	-	-
Uncultured eukaryote obtained from BBT with river Rhine water incubated at 37°C (GU970502)/ *Neoparamoeba pemaquidensis* (AY183887)	99.3/91.6	1	1	-	-
Uncultured eukaryote obtained from BBT with cooling water 5 after incubation at 37°C (GU970896)/ *Lobosea* sp. (AB425946)	98.1/85.0	1	-	-	37
Uncultured eukaryote obtained from BBT with cooling water 5 after incubation at 37°C (GU970896)/*Rhinosporidium seeberi* (AF118851)	92.0/83.5	1	-	-	1
Cercozoa	**-**	**10**	**21**	**0**	**0**
Uncultured eukaryote obtained from distributed water of supply CA-1 (HQ620271)/uncultured cercozoan (AY620271)	97.9/97.7	1	1	-	-
Uncultured cercozoan (AY620271)/Uncultured eukaryote obtained from distributed water of supply CA-2 (HQ999500)	99.8/99.7	1	3	-	-
Uncultured eukaryote obtained from BBT with river Rhine water incubated at 37°C (GU970532)/ Uncultured cercomonad (EF024294)	99.6/87.6	1	7	-	-
Uncultured eukaryote obtained from BBT with river Rhine water incubated at 37°C (GU970532)/ *Telestula c.f. spiculicola* (FJ389265)	97.8/84.2	1	1	-	-
Uncultured eukaryote obtained from BBT with river Rhine water incubated at 37°C (GU970532)/ Uncultured cercozoan (AY620271)	97.8/87.3	1	1	-	-
Uncultured eukaryote obtained from distributed water of supply CA-2 (HQ999265)/*Paracercomonas saepenatans* (FJ790731)	95.6/94.4	1	1	-	-
Uncultured eukaryote obtained from distributed water of supply CA-1 (HQ999383/Uncultured cercozoan (AY620271)	92.3/92.0	1	1	-	-
Uncultured eukaryote obtained from BBT with biomass from limestone filter of groundwater supply after incubation at 15°C (GU970146)/ *Cercomonas metabolicus* (DQ211597)	96.3/93.9	1	1	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999652)/*Paracercomonas saepenatans* (FJ790731)	99.6/87.9	1	4	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999652)/*Cercomonas metabolicus* (DQ211597)	91.9/90.0	1	1	-	-
Choanozoa		**2**	**0**	**0**	**8**
Codonosigidae environmental sample (EF024012)/	96.0/94.9	1	-	-	3

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Table D.11 – continued from previous page

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones Flask I 37°C	Flask I 42°C	Flask II 42°C
Monosiga ovata (AF271999) *Reclinomonas Americana* (AY117417)	86.2	1	-	-	5
Stramenopiles	**-**	**2**	**10**	**0**	**0**
Uncultured freshwater eukaryote (AY919731)/ *Paramonas globosa* (AY520452)	98.4;95.2/ 96.6; 94.4	2	10	-	-
Total	**-**	**20**	**35**	**22**	**46**

Table D.12: Classification of OTUs clustering with free-living protozoa obtained from BBT flasks with tap water I incubated at 20, 30, 37 and 42°C.

			No. of clones			
Organism(s) with highest similarity (GenBank accession no.)	**Similarity (%)**	**No. of OTUs**	**Flask I 20°C**	**Flask II 30°C**	**Flask II 37°C**	**Flask I 42°C**
Amoebozoa	**-**	**3**	**3**	**5**	**3**	**1**
Hartmannella vermiformis (AY680840)	100	1	2	4	2	-
Uncultured eukaryote obtained from BBT with water from warm tap water installation B2 in distribution area of groundwater supply B after incubation at 37°C (GU970755)/ *Hartmannella vermiformis* (AY680840)	99.7/96.1	1	1	1	1	-
Uncultured Sarcosomataceae (EF023872)/ *Sphaerastrum fockii* (AY749614)	96.2/95.7	1	-	-	-	1
Eugelenzoa		**2**	**0**	**8**	**0**	**1**
Bodonidae sp.(AY753625)	99.8	1	-	8	-	-
Petalomonas cantuscygni (U84731)	87.4	1	-	-	-	1
Stramenopiles	**-**	**4**	**14**	**10**	**21**	**4**
Uncultured eukaryote obtained from distributed water of supply CA-3(HQ999674)/Spumella-like flagellate JBC30 (AY651073)	99.4/98.0	1	10	10	21	3
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999674)/ *Poterioochromonas stipitata*(AF123295)	98.6/95.3	1	1	-	-	-
Epipyxis pulchra (AF123298)	96.5	1	3	-	-	-
Aphanomyces invadans (AF396684)	99.6	1	-	-	-	1
Total	**-**	**9**	**17**	**23**	**24**	**6**

Table D.13: Classification of OTUs clustering with free-living protozoa obtained from BBT flasks with tap water II incubated at 20, 30, 37 and 42°C.

			No. of clones			
Organism(s) with highest similarity (GenBank accession no.)	**Similarity (%)**	**No. of OTUs**	**Flask I 20°C**	**Flask II 30°C**	**Flask II 37°C**	**Flask I 42°C**
Amoebozoa	**-**	**10**	**19**	**15**	**24**	**12**
Acanthamoeba sp. (AB425952); (AY549562)	99.8; 96.4	2	-	7	-	8
Acanthamoeba polyphaga (AF019051)	83.2	1	-	-	-	1
Hartmannella vermiformis (AY680840)	100	1	12	-	21	1
Echinamoeba exundans (AF293895)	99.2	1	1	-	-	-
Saccamoeba limax (AF293902)	96.2	1	-	-	-	1
Stenamoeba sp. (EU377587)	92.0	1	1	-	-	-
Uncultured eukaryote obtained from BBT with water from warm tap water installation A1 in distribution area of groundwater supply (GU970784)/*Lobosea* sp. (AB425946)	99.1; 96.4/ 90.4; 86.7	2	5	8	2	1
Uncultured marine eukaryote (EF526997)/ *Hartmannella vermiformis* (AY680840)	97.9/96.5	1	-	-	1	-
Cercozoa	**-**	**2**	**0**	**0**	**0**	**2**
Soil flagellate (AY965864)/ *Paracercomonas crassicauda*(FJ790725)	99.1/96.4	1	-	-	-	1
Uncultured alveolate (EU910603)/ *Platyreta germanica*(AY941200)	90.0/85.1	1	-	-	-	1
Ciliophora	**-**	**1**	**0**	**0**	**0**	**1**
Uncultured oligohymenophorid ciliate (AY821923)/ *Miamiensis avidus* (AY642280)	96.1/91.8	1	-	-	-	1
Myzozoa	**-**	**1**	**0**	**0**	**0**	**2**
Uncultured marine eukaryote (EF526851)/ *Takayama cf. pulchellum* (AY800130)	89.9/89.6	1	-	-	-	2
Stramenopiles	**-**	**6**	**3**	**2**	**0**	**1**
Uncultured eukaryote obtained from treated water of groundwater supply B (EU860793)/ *Paraphysomonas imperforate*(Ef432518)	99.4/99.1	1	1	-	-	-
Uncultured chrysophyta (AY821972)/ *Chrysamoeba mikrokonta* (AF123287)	98.8/98.1	1	1	-	-	-
Uncultured eukaryote obtained from BBT with biomass from limestone filter of groundwater supply after incubation at 15°C (GU970138)/ Spumella-like flagellate (DQ388541)	99.0/98.9	1	1	-	-	-
Uncultured freshwater eukaryote (AY919756)/ *Oikomonas* sp. (AY520450)	94.8/94.2	1	-	1	-	-
Uncultured eukaryote (AB275091)/ *Oikomonas* sp. (AY520450)	98.4/97.5	1	-	1	-	-
Uncultured eukaryote obtained from BBT with biomass from limestone filter of groundwater supply after incubation at 37°C (GU970352)/ *Rhizidiomyces apophysatus* (AF163295)	99.6/88.6	1	-	-	-	1
Total	**-**	**20**	**22**	**17**	**24**	**18**

Table D.14: Classification of OTUs clustering with fungi obtained from BBT flasks with cooling tower water (CTW; 37°C), river Rhine water (RRW; 37 and 42°C), tap water I (TW-I; 42°C) and tap water II (TW-II; 30 and 42°C).

			No. of clones					
Organism(s) with highest similarity (GenBank accession no.)	**Similarity (%)**	**No. of OTUs**	**CTW 37°C**	**RRW 37°C**	**RRW 42°C**	**TW-I 42°C**	**TW-II 30°C**	**TW-II 42°C**
Basidiomycota	**-**	**1**	**0**	**0**	**0**	**0**	**0**	**2**
Uncultured Boletaceae (EF024590)	96.9	1	-	-	-	-	-	2
Ascomycota	**-**	**21**	**28**	**0**	**0**	**2**	**5**	**0**
Uncultured Banisveld eukaryote (EU091865)	95.4	1	4	-	-	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999654)/Uncultured Banisveld eukaryote (EU091865)	97.2, 98.3/ 84.3, 85.5	2	7	-	-	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999649)/Uncultured Banisveld eukaryote (EU091865)	96.2-98.3/ 83.0-84.3	3	3	-	-	-	-	-
Uncultured eukaryote obtained from BBT with river Rhine water incubated at 37°C (GU970493)/ Uncultured Banisveld eukaryote (EU091865)	97.3/86.0	1	2	-	-	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999649)/Uncultured fungus (EU162635)	96.2/86.3	1	1	-	-	-	-	-
Uncultured eukaryote obtained from BBT with cooling water 1 after incubation at 37°C (GU970904)/ uncultured fungus (EU162635)	93.3/87.6	1	1	-	-	-	-	-
Uncultured eukaryote obtained from BBT with cooling water 1 after incubation at 37°C (GU970885; GU970888)/ Uncultured Banisveld eukaryote (EU091865)	93.8-98.6/ 87.0-90.4	8	8	-	-	-	-	-
Uncultured eukaryote obtained from distributed water of supply CA-2 (HQ999280)/Uncultured eukaryote obtained from BBT with treated sewage after inoculation at 37°C (GU970556)/ *Ochromonas* sp. (EF165109)	91.2 92.3/80.8	1 1	1 1	- -	- -	- -	- -	- -
Uncultured eukaryote obtained from distributed water of supply CA-3 (HQ999779)/*Pichia jadinii*(EF550447)	99.8/99.8	1	-	-	-	2	-	-
Uncultured eukaryote obtained from BBT with river Rhine water incubated at 37°C (GU970634)/ *Saccinobaculus ambloaxostylus*(DQ525706)	99.6/67.0	1	-	-	-	-	5	-
Unidentified	**-**	**6**	**0**	**13**	**1**	**0**	**0**	**1**
Uncultured Eimeriidae (EF023703)	93.2	1	-	-	1	-	-	-
Uncultured eukaryote obtained from BBT with treated sewage incubated at 37°C (GU970554)/	93.0/92.5	1	-	4	-	-	-	-

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Table D.14 – continued from previous page

Organism(s) with highest similarity (GenBank accession no.)	Similarity (%)	No. of OTUs	No. of clones: CTW 37°C	RRW 37°C	RRW 42°C	TW-I 42°C	TW-II 30°C	TW-II 42°C
Uncultured Banisveld eukaryote (EU091865)								
Uncultured eukaryote obtained from BBT with river Rhine water incubated at 37°C (GU970601)/ Uncultured eukaryote (AJ130857)	97.1/91.0	1	-	1	-	-	-	-
Uncultured fungus (DQ244017)	97.1, 92.2	2	-	8	-	-	-	-
Uncultured eukaryote obtained from BBT with biomass from sand filter-bed of surface water supply D after incubation at 37°C (GU970695)/ Uncultured fungus (AB468674)	99.8/96.6	1	-	-	-	-	-	1
Total	**-**	**28**	**28**	**13**	**1**	**2**	**5**	**3**

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Summary

Free-living protozoa in drinking water supplies: community composition and role as hosts for *Legionella pneumophila*

A large variety of free-living protozoa is present in aquatic environments and these organisms are also ubiquitous in drinking water supplies in (warm) tap water installations inside buildings. Free-living protozoa feed on bacteria and other microorganisms and have a large impact on the microbial communities in drinking water supplies and tap water installations. A number of bacteria, including opportunistic human pathogens such as *Legionella pneumophila* and *Burkholderia pseudomallei* are able to survive and/or replicate within certain protozoa, which serve as hosts. In addition, certain free-living protozoa can be human pathogens themselves. However, information about the occurrence and the conditions for growth of these organisms including water quality is limited, which in part may be attributed to the limitations of microscopic techniques and cultivation methods that have been used in the past for their detection and identification. In the studies described in this thesis, molecular methods were applied to identify communities of free-living protozoa in drinking water supplies and to determine which conditions enhance growth of free-living protozoa. Furthermore, these molecular methods were used to identify predominating protozoan hosts, both known and not yet described, for *L. pneumophila*. The eukaryotic communities were studied using terminal restriction fragment length polymorphism (T-RLFP) and clone library analyses of partial 18S rRNA gene fragments and qPCR assays for *Acanthamoeba* spp. and *Hartmannella vermiformis*.

Predominant free-living protozoa were identified in the treated water and in distribution system biofilms of two drinking water supplies in the Netherlands with water temperatures below 18°C **(Chapter 2)**. Both supplies use groundwater as their source, but the treated water of supply A contains low concentrations of active biomass ($<$ 1 ng ATP liter^{-1}) and natural organic matter (NOM, $<$ 0.5 mg C liter^{-1}), whereas the treated water of supply B contains elevated concentrations of ATP (10 ng ATP liter^{-1}) and NOM (7.9 mg C liter^{-1}). In both supplies, highly diverse eukaryotic communities were observed, including free-living protozoa, fungi, and metazoa. In total 127 operation taxonomic units (OTUs, each OTU containing sequences with $\geq$ 99% similarity) related to free-living protozoa were identified, clustering with Amoebozoa, Cercozoa, Choanozoa, Ciliophora, Euglenozoa, Myzozoa and Stramenopiles. In the two supplies, the protozoan communities were predominated by different OTUs, but the estimated values for protozoan richness did not differ significantly, despite the differences in

concentrations of NOM and ATP in the treated water. *H. vermiformis*, a described host for *L. pneumophila* was observed in both supplies, but was not a predominant protozoan in the treated water and the distribution system biofilms. The high level of NOM in supply B corresponded to an elevated level of active biomass and to elevated concentrations of *H. vermiformis*, as measured using qPCR, in distributed water. *H. vermiformis* was absent in most samples of supply A with low concentrations of ATP and NOM.

A biofilm batch test (BBT) was used to elucidate the identity of protozoan hosts for *L. pneumophila* under conditions resembling those in engineered water systems **(Chapter 3)**. Samples of 600 ml collected from 21 engineered freshwater systems, with added polyethylene cylinders to promote biofilm formation, were inoculated with *L. pneumophila* and subsequently incubated at 37°C for 20 days. During incubation, growth of *L. pneumophila* was determined, with a specific qPCR method, in the BBT systems and subsequently predominating free-living protozoa were identified. Growth of *L. pneumophila* was observed in 16 of 18 water types when the host protozoan *H. vermiformis* was inoculated. Twelve of the tested water types supported growth of *L. pneumophila* or indigenous *L. anisa* without inoculated *H. vermiformis*. In 12 of the 19 BBT flasks *H. vermiformis* was indicated as host, based on the ratio between maximum concentrations of *L. pneumophila* and *H. vermiformis*, determined with qPCR, and the composition of the eukaryotic clone libraries. None of the other protozoa serving as hosts in *in vitro* studies were detected in the BBTs. In several tests with growth of *L. pneumophila*, the protozoa *Diphylleia rotans*, *Echinamoeba thermarum* and *Neoparamoeba* sp. were identified as candidate hosts. *In vitro* studies are needed to confirm their role as hosts for *L. pneumophila*.

The richness and identity of free-living protozoa and other small eukaryotes were determined in three supplies, with treated water at temperatures of about 30°C, in the Caribbean region **(Chapter 4)**. Seawater is treated with distillation and/or reverse osmosis for desalination and the treated water contains low concentrations of NOM ($<$ 0.5 mg C liter^{-1}). Cultivable *L. pneumophila* and *H. vermiformis* were observed in all three supplies. OTUs with the highest similarity to the potential or candidate hosts *Acanthamoeba* spp., *E. exundans*, *E. thermarum* and *Neoparamoeba* sp. were detected as well. In total, 59 OTUs related to free-living protozoa were identified and the estimated protozoan richness did not differ significantly between the three supplies. In supply CA-1, the concentration of *H. vermiformis* correlated with the concentration of *Legionella* spp. and clones related to Amoebozoa predominated (82%) the protozoan community. These observations, the low turbidity ($<$ 0.2 NTU) and the varying ATP concentrations (1 to 12 ng liter^{-1}) suggest that the growth of microorganisms in the distribution system was promoted by biofilms. Ciliophora represented 25% of the protozoan OTUs in supply CA-2 with elevated ATP concentrations (max. 55 ng liter^{-1}) correlating with turbidity (max. 62 NTU) related to corroding iron pipes. Cercozoan types predominated (70% of protozoan clones) in supply CA-3 with ATP concentrations $<$ 1 ng liter^{-1} and turbidity $<$ 0.5 NTU in most samples of distributed water. The absence of *H. vermiformis* in most samples from supply CA-3 suggests that growth of this amoeba is limited at ATP concentrations $<$ 1 ng liter^{-1}.

To assess the effects of water temperature on indigenous free-living protozoan communities, various water types were incubated in the BBT system at 20, 30, 37 and 42°C **(Chapter 5)**. Duplicate water samples of 1.8 liter of the river Rhine, a cooling tower and two tap water installations were inoculated with *L. pneumophila*, as described in **Chapter 3**, to identify host protozoa for *L. pneumophila*. The incubation of the four water types at the four temperatures in the BBT system caused shifts in the composition of protozoan communities. Only two of the 53 OTUs related to free-living protozoa obtained from the four freshwater types at day 0 were also detected after incubation. Multiplication of indigenous *Acanthamoeba* spp. was observed with specific qPCR methods, at 20 and 30°C, with only limited growth in one flask at 37°C. *H. vermiformis* multiplied at 20, 30, and 37°C, but at 42°C proliferation was only observed in one water type. Growth of organisms related to Amoebozoa and Stramenopiles was observed at all four temperatures, whereas Cercozoan and Euglenozoan types predominated in the BBT system incubated at 37 and 30°C, respectively. Yet-undescribed sequence types of *L. pneumophila* multiplied in river water and cooling tower water incubated at 42°C. The Amoebozoan *Arachnula* sp. was identified as candidate host for *L. pneumophila* at 42°C, but *in vitro* studies with this organism are needed for confirmation.

The results of the investigations revealed that highly diverse communities of free-living protozoa are present in the drinking water supplies in the Netherlands and in the Caribbean **(Chapter 6)**. However, information about the conditions favoring the growth of specific free-living protozoa in drinking supplies and (warm) tap water installation is still in its infancy. *H. vermiformis*, which was the most commonly observed protozoan host for *L. pneumophila*, was detected in all five investigated supplies, in one of which it was the predominating free-living protozoan. This organism also was the most frequently observed host for *L. pneumophila* in samples, derived from a variety of engineered water systems and incubated in the BBT system. *Acanthamoeba* spp. were observed only incidentally in the investigated supplies and were unable to compete with *H. vermiformis* in most BBT flasks.

Measures for limiting growth of *H. vermiformis* in engineered freshwater systems will very likely also limit the growth of other amoebae, which can serve as hosts for pathogenic bacteria end/or which have pathogenic traits. Growth of *H. vermiformis* was found to be limited in two supplies at active biomass concentrations < 1 ng ATP liter^{-1} in combination with NOM concentrations < 0.5 mg C liter^{-1}. However, production of drinking water with such low concentrations of NOM and ATP is not achievable in most cases. *L. pneumophila* can grow at temperatures ≥ 25°C. Therefore, reducing the potential for growth of free-living amoebae in water supplies and in water installations requires combinations of measures in water treatment (NOM removal), during distribution (prevention of sediment accumulation or disinfection), and in installations for warm tapwater (temperature control, prevention of stagnation, copper-silver ionization). Quantification of specific species of free-living protozoa, such as *H. vermiformis*, using qPCR can be used to determine the efficacy of these measures.

Samenvatting

Vrijlevende protozoa in drinkwater: samenstelling van gemeenschappen en de rol als gastheer voor *Legionella pneumophila*

Vrijlevende protozoa komen algemeen voor in het water en dus ook in installaties voor de bereiding van drinkwater, in distributiesystemen en in installaties voor (warm)kraanwater in gebouwen. Deze organismen voeden zich voornamelijk met bacteriën en spelen een belangrijke rol in gemeenschappen van micro-organismen en invertebraten in zoetwatermilieus. Verschillende bacteriën, waaronder ook ziekteverwekkers zoals *Legionella pneumophila* en *Burkholderia pseudomallei* overleven en/of vermeerderen zich in vrijlevende protozoa die als gastheer fungeren. Daarnaast bezitten enkele vrijlevende protozoa ziekteverwekkende eigenschappen voor de mens. Informatie over de invloed van de waterkwaliteit op het voorkomen en de groei van vrijlevende protozoa is zeer beperkt. Dit komt onder andere door de beperkingen van de microscopische technieken en de kweekmethoden die gebruikt zijn om protozoa te detecteren en te identificeren. Voor de studies beschreven in dit proefschrift, zijn kweek-onafhankelijke moleculaire methoden toegepast om de gemeenschappen van vrijlevende protozoa te identificeren in verschillende typen drinkwater. Daarnaast zijn deze technieken toegepast om te bepalen onder welke condities groei van bepaalde vrijlevende protozoa plaats vindt. Verder zijn reeds bekende, maar ook niet eerder beschreven gastheerprotozoa voor *L. pneumophila* geïdentificeerd met moleculaire technieken. De gemeenschappen van eukaryoten zijn bestudeerd met behulp van de volgende moleculaire technieken: (i) terminal restriction fragment length polymorphism (T-RFLP), (ii) kloonbanken met 18S rRNA gen fragmenten en (iii) kwantitatieve PCR (qPCR) voor *Acanthamoeba*-soorten en *Hartmannella vermiformis*.

Dominante vrijlevende protozoa zijn geïdentificeerd in het reine water en in de biofilm uit het distributiesysteem van twee productiebedrijven in Nederland (**Hoofdstuk 2**). Beide bedrijven produceren drinkwater van grondwater, en de temperatuur van de geanalyseerde monsters was lager dan 18°C. Het afgeleverde drinkwater van productiebedrijf A heeft zeer lage gehaltes aan actieve biomassa ($<$ 1 ng ATP liter^{-1}) en natuurlijk organisch materiaal (NOM, $<$ 0,5 mg C liter^{-1}), terwijl productiebedrijf B water levert met hoge concentraties van actieve biomassa (10 ng ATP liter^{-1}) en NOM (7,9 mg C liter^{-1}). Een grote verscheidenheid aan eukaryoten, inclusief vrijlevende protozoa, schimmels en metazoa is aangetroffen in het reine water en in de biofilms van beide bedrijven. In totaal zijn 127 operational taxonomic units (OTUs, iedere OTU bevat DNA-sequenties die onderling minimaal 99%

overeenkomen) gerelateerd aan vrijlevende protozoa geïdentificeerd. Deze OTUs vallen binnen de fyla Amoebozoa, Cercozoa, Choanozoa, Ciliophora, Euglenozoa, Myzozoa en Stramenopiles. Bij beide bedrijven domineren verschillende typen vrijlevende protozoa, maar de geschatte rijkdom aan vrijlevende protozoa verschilt niet tussen deze bedrijven, ondanks de grote verschillen in NOM en ATP in het drinkwater. *H. vermiformis*, een reeds bekende gastheer voor *L. pneumophila*, is waargenomen bij beide productiebedrijven, maar was geen dominante protozo in het reine water of in de biofilms uit het distributiesysteem. Verhoogde concentraties van *H. vermiformis*, bepaald met qPCR, werden aangetroffen in het gedistribueerde water van productiebedrijf B. *H. vermiformis* is niet aangetroffen in de meeste monsters van het drinkwater van productiebedrijf A met lage concentraties aan ATP en NOM.

Een biofilm batch test (BBT) is gebruikt om de identiteit van gastheerprotozoa voor *L. pneumophila* te bepalen onder omstandigheden die overeenkomen met condities in door de mens gemaakte watersystemen (**Hoofdstuk 3**). Volumes van 600 ml, afkomstig van 21 zoetwatersystemen, waaraan *L. pneumophila* is toegevoegd, zijn gedurende 20 dagen geïncubeerd bij 37°C. Cilinders van polyethyleen zijn toegevoegd om groei van biofilm te bevorderen. Tijdens de incubatie in de BBT-flessen is met een specifieke qPCR methode bepaald of groei van *L. pneumophila* plaatsvond, en vervolgens zijn de dominante vrijlevende protozoa geïdentificeerd. Groei van *L. pneumophila* is waargenomen in 16 van de 18 watertypen waaraan ook de gastheerprotozo *H. vermiformis* was toegevoegd. In 12 van de onderzochte watertypen is groei van *L. pneumophila* of van *Legionella anisa* waargenomen zonder dat *H. vermiformis* was toegevoegd. In 12 van de 18 BBT-flessen is *H. vermiformis* geïdentificeerd als gastheer, gebaseerd op de verhouding tussen maximale concentraties van *L. pneumophila* en *H. vermiformis*, bepaald met qPCR, en de samenstelling van de eukaryotische kloonbanken. Geen van de andere vrijlevende protozoa, die beschreven zijn als gastheer aan de hand van *in vitro* experimenten, zijn waargenomen in de BBT-flessen met groei van *L. pneumophila*. In enkele testen waarin groei van *L. pneumophila* is waargenomen, zijn de vrijlevende protozoa *Diphylleia rotans*, *Echinamoeba thermarum* en *Neoparamoeba*-soort geïdentificeerd als meest waarschijnlijke ("kandidaten") gastheer. *In vitro* experimenten moeten bevestigen of deze protozoa als gastheer kunnen dienen voor *L. pneumophila*.

De rijkdom en identiteit van vrijlevende protozoa en andere eukaryoten zijn bepaald in het reine en gedistribueerde water van drie verschillende drinkwaterbedrijven in het Caribische gebied (**Hoofdstuk 4**). De temperatuur van het reine water was hier rond 30°C. Bij deze bedrijven wordt zeewater ontzout door middel van destillatie en/of omgekeerde osmose waardoor het reine water lage concentraties aan NOM bevat ($<$ 0.5 mg C liter^{-1}). *H. vermiformis* en kweekbare *L. pneumophila* zijn waargenomen in alle drie de voorzieningsgebieden. Ook zijn OTUs met de meeste overeenkomst met de gastheerkandidaten *Acanthamoeba*-soorten, *Echinamoeba exundans*, *Echinamoeba thermarum* en *Neoparamoeba*-soort aangetroffen. In totaal zijn 59 OTUs van vrijlevende protozoa geïdentificeerd en de geschatte rijkdom aan vrijlevende protozoa verschilt niet tussen de drie voorzieningsgebieden. In het voorzieningsgebied CA-1 correleert de concentratie van *H. vermiformis* met concentraties van gekweekte *Legionella*-soorten. De gemeenschappen van vrijlevende protozoa in dit voorzieningsgebied werden ook gedomineerd (82%) door klonen gerelateerd aan Amoebozoa. Deze waarnemingen, in combinatie met de lage troebelheid ($<$0.2 NTU) en de variërende concentraties van ATP (1 tot 12 ng liter^{-1}), duiden op

de aanwezigheid van biofilms die groei van micro-organismen bevorderen. In het voorzieningsgebied van drinkwaterbedrijf CA-2 behoort 25% van de OTUs van vrijlevende protozoa tot de Ciliophora. Verhoogde concentraties van ATP (max. 55 ng liter^{-1}) correleren met troebelheid (max. 62 NTU) die waarschijnlijjk samenhangt met corrosie van gietijzeren leidingen in het distributiesysteem van dit bedrijf. Vrijlevende protozoa behorende tot het Cercozoan-fylum domineren (70% van de klonen gerelateerd aan de vrijlevende protozoa) in het voorzieningsgebied van drinkwaterbedrijf CA-3 met lage concentraties van ATP ($<$ 1 ng liter^{-1}) en troebelheid ($<$ 0.5 NTU) in de meeste onderzochte monsters van het gedistribueerde water. De afwezigheid van *H. vermiformis* in de meeste monsters van voorzieningsgebied CA-3 kan een aanduiding zijn dat groei van deze amoebe beperkt is bij concentraties van ATP lager dan 1 ng liter^{-1}.

Om te bepalen welke invloed de temperatuur van het water heeft op de gemeenschappen van vrijlevende protozoa, zijn vier watertypen geïncubeerd in een BBT-systeem bij 20, 30, 37 en 42°C (**Hoofdstuk 5**). Evenals in de experimenten die zijn beschreven in **Hoofdstuk 3** was ook hier *L. pneumophila* toegevoegd aan de watermonsters, die afkomstig waren uit de rivier de Rijn, een koeltoren en twee binneninstallaties. Van ieder watertype is 1.8 liter in tweevoud geïncubeerd bij vier temperaturen in de BBT-flessen. Deze incubatie veroorzaakte bij alle vier de temperaturen een verschuiving in de samenstelling van de gemeenschappen van vrijlevende protozoa in de onderzochte watertypen in de BBT-flessen. Slechts twee van de 53 OTUs, gerelateerd aan vrijlevende protozoa, en geïsoleerd uit de vier watertypen op dag 0, zijn ook waargenomen na incubatie. Groei van in het water aanwezige *Acanthamoeba*-soorten is waargenomen met de selectieve qPCR-methoden tijdens de incubatie bij 20 en 30°C, terwijl bij 37°C slechts beperkte groei in één fles is waargenomen. *H. vermiformis* is gegroeid bij 20, 30 en 37°C, maar bij 42°C vond alleen vermeerdering van dit organisme plaats in het water uit de river de Rijn. Groei van organismen gerelateerd aan Amoebozoa en Stramenopiles is waargenomen tijdens de incubatie bij alle vier de temperaturen, terwijl vrijlevende protozoa behorende tot de fyla Cercozoa en Euglenozoa domineerden in de BBT-systemen na incubatie bij respectievelijk 37 en 30°C. Tijdens de incubatie bij 42°C van het Rijnwater en koeltorenwater trad groei op van stammen van *L. pneumophila* waarvan de DNA-sequenties nog niet eerder van beschreven zijn. Een *Arachnula*-soort, behorende tot het fylum Amoebozoa, is geïdentificeerd als waarschijnlijke gastheer voor *L. pneumophila* bij 42°C, maar *in vitro* experimenten met dit organisme zijn nodig om dit te bevestigen.

Uit de studies beschreven in dit proefschrift komt naar voren dat zeer diverse gemeenschappen van vrijlevende protozoa zijn waargenomen in het reine water en in het distributiesysteem zowel in Nederland als ook in het Caribische gebied (**Hoofdstuk 6**). Echter, informatie over de factoren die de groei van specifieke vrijlevende protozoa bevorderen in distributiesystemen en (warm)waterinstallaties, is nog zeer beperkt. Protozoa die bekend staan als gastheer voor *L. pneumophila* zijn niet de meest voorkomende vrijlevende protozoa in vier van de vijf onderzochte voorzieningsgebieden. *H. vermiformis* is in beide regio's de meest frequent aangetroffen gastheerprotozo (amoebe) in het drinkwater. Dit organisme vermeerdert zich in biofims bij temperaturen tussen de 20 and 42°C. Maatregelen die tot een beperking van de groei van *H. vermiformis* in door de mens gemaakte watersystemen leiden, zullen waarschijnlijk ook de groei van andere amoeben terugdringen. Ook een aantal

andere amoebensoorten kunnen als gastheer dienen voor pathogene bacteriën en enkele van deze amoeben bezitten ziekteverwekkende eigenschappen voor de mens. Groei van *H. vermiformis* is zeer beperkt bij lage concentraties van actieve biomassa ($<$ 1 ng ATP liter^{-1}) in combinatie met lage NOM concentraties($<$ 0.5 mg C liter^{-1}). Het produceren van drinkwater met zulke lage concentraties aan NOM en ATP is niet haalbaar voor de meeste bedrijven. Bij watertemperaturen $\geq$ 25°C kan groei van *L. pneumophila* optreden. Daarom zijn maatregelen nodig bij de drinkwaterbereiding (verwijdering van NOM) in combinatie met maatregelen in het distributiesysteem (verhinderen van sedimentvorming) en in (warm)waterinstallaties (thermisch beheer; beperken van stagnatie; toepassing van fysische en chemische beheersmaatregelen) om de groei van vrijlevende amoeben te beperken. De ook in dit proefschrift gebruikte moleculaire methoden voor de kwantitatieve detectie van vrijlevende protozoa kunnen worden toegepast om de effectiviteit van dergelijke maatregelen te beoordelen.

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Curriculum Vitae

Rinske Valster werd geboren op 3 november 1979 in de Wieringermeer. In 1997 behaalde zij het HAVO-diploma aan de R.S.G. Wiringherlant in Wieringerwerf. In datzelfde jaar begon zij met de studie Lerarenopleiding voor Biologie aan de Noordelijke Hogeschool Leeuwarden. Hierbij gaf ze in het vierde jaar les als leraar in opleiding aan de onderbouw van de HAVO en VMBO op de C.S.G. Anna Maria van Schurmann in Franeker. In 2001 rondde zij deze studie af en begon aan de studie Biologie aan Wageningen Universiteit met als specialisatie plantenbiologie. Gedurende deze studie is haar interesse voor microbiologie ontstaan, waardoor twee afstudeervakken in deze richting werden uitgevoerd. Het eerste afstudeervak naar de interactie tussen 2,4-DAPG producerende *Pseudomonas*-soorten en inheemse micro-organismen in de rhizosfeer van tarwe, werd uitgevoerd bij de vakgroep Fytophatologie aan de Wageningen Universiteit. Vervolgens heeft ze een afstudeeronderzoek uitgevoerd bij KWR Watercycle Research Institute, met als onderwerp de detectie en identificatie van vrijlevende protozoa, die als gastheer kunnen dienen voor *Legionella*-soorten in drink- en oppervlaktewater. Naar aanleiding hiervan begon ze in 2005 aan haar promotieonderzoek, ook bij KWR Watercycle Research Institute, getiteld: "Vrijlevende protozoa in drinkwater: samenstelling van gemeenschappen en de rol als gastheer voor *Legionella pneumophila*", wat heeft geresulteerd in dit proefschrift.

Rinske Valster was born on November 3rd, 1979 in the Wieringermeer. In 1997 she completed secondary school at the HAVO-level at the R.S.G. Wiringherlant in Wieringerwerf. In the same year, she started studying to become a biology teacher at the Noordelijke Hogeschool Leeuwarden. In the fourth year of her study, she teached as a trainee teacher in the lower three classes of HAVO and VMBO secondary school at the C.S.G. Anna Maria van Schurmann in Franeker. In 2001 she finished this study and started studying Biology at Wageningen University with a specialization in plant biology. She became interested in microbiology and carried out two theses in this direction. The first one took place at the Phytopathology group of Wageningen University, were she studied the interaction between 2,4-DAPG producing *Pseudomonas* spp. and indigenous microorganisms in the rhizosphere of wheat. Her second thesis was performed at KWR Watercycle Reseach Institute, about the detection and identification of free-living protozoa, which can serve as host for *Legionella* spp., in drinking and surface water. Consequently, she started her PhD-research in 2005, again at KWR Watercycle Research Institute, entitled: "Free-living protozoa in drinking water supplies: community composition and role as hosts for *Legionella pneumophila*", which resulted in this dissertation.

VLAG PhD Education form

Overview of completed training activities:

Discipline-specific courses:	
The Art of Modelling	2006
Drinkwatervoorziening voor managers	2006
General ARB course (Ribocon)	2006
Advanced Microscopy and Vital Imaging	2007
General courses:	
Project and Time Management	2006
VLAG PhD Week	2006
Techniques for writing and presenting a scientific paper	2006
Presentation skills	2007
Meetings:	
6^{th} International Conference *Legionella* 2005, Chicago, USA (poster presentation)	2005
KWR-Workshop: "Moleculaire technieken in drinkwater" (oral presentation)	2007
Vitens-Workshop: "Microbiologisch onderzoek bij grondwaterproductiebedrijven" (oral presentation)	2007
12^{th} International Symposium on Microbial Ecology, Cairns, Australia (poster presentation)	2008
rRNA technology workshop, Bremen, Germany	2008
Conference *Legionella* 2009, Paris, France (oral presentation)	2009
DPW-Workshop: "*Legionella*", (oral presentation)	2009
Legionella congres, Stichting Veteranenziekte Nederland	2009
WNAA-Workshop: "*Legionella*", Aruba, Netherlands Antilles (oral presentation)	2009
Optional activities:	
Preparing research proposal	2005
Teaching obligations:	
Microbial ecology (MIB31306), Practicum	2006
Microbial ecology (MIB31306), Excursion KWR	2006–2009
Bachelor thesis, student supervision	2007

List of publications

Peer-reviewed articles:

- Valster, R. M., B. A. Wullings, G. Bakker, H. Smidt, and D. van der Kooij (2009), "Free-living protozoa in two unchlorinated drinking water supplies, identified by phylogenic analysis of 18S rRNA gene sequences", *Appl. Environ. Microbiol.*, 75, 4736-4746.
- Valster, R. M., B. A. Wullings, and D. van der Kooij (2010), "Detection of protozoan hosts for *Legionella pneumophila* in engineered water systems by using a biofilm batch test", *Appl. Environ. Microbiol.*, 76, 7144-7153.
- Valster, R. M., B. A. Wullings, R. van den Berg, and D. van der Kooij (2011), "Relationships between free-living protozoa, cultivable *Legionella* spp. and water quality parameters in three drinking water supplies in the Caribbean", submitted to *Appl. Environ. Microbiol.*
- Valster, R. M. B. A. Wullings, and D. der Kooij, "Effect of temperature on the communities of free-living protozoa in four freshwater types in a biofilm batch system", submitted to *Appl. Environ. Microbiol.*
- Kuiper, M. W., R. M. Valster, B. A. Wullings, H. Boonstra, H. Smidt, and D. van der Kooij (2006), "Quantitative detection of the free-living amoeba *Hartmannella vermiformis* in surface water by using real-time PCR", *Appl. Environ. Microbiol.*, 72, 5750-5756.

Other publications:

- Valster, R., B. Wullings, G. Bakker, and D. van der Kooij (2005), "Detectie en identificatie van vrijlevende protozoa in drinkwater", *H2O*, 25/26, 47–49 *(in Dutch)*.
- Valster, R., B. Wullings, S. Voost, G. Bakker, H. Smidt, and D. van der Kooij (2006), "Detection and identification of free-living protozoa present in drinking water". In: *Legionella: state of the art 30 years after its recognition. Proceedings of the 6th International Conference on Legionella.*, 427-430.
- Valster, R. (2008), "Elucidation of relationships between free-living protozoa, *Legionella pneumophila* and biofilm concentrations in drinking water", *Delft Cluster Magazine, no. 5: "Drinking water: Transfer of knowledge"*, 6–9.
- Valster, R. (2009), "Elucidation of relationships between protozoa, *L. pneumophila* and biofilm concentrations in tap-water installations", *Afsluitend magazine van Delft Cluster "Duurzame gebiedsinrichting"*, p. 62 *(in Dutch)*.

Addendum

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The cover is a photo of a *Vorticella* species, belonging to the phylum of Ciliophora, and made available by iStockphoto.