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

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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 (T < 20°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 T-RFLP and clone-library analysis of partial 18S rRNA gene fragments and a *Hartmannella vermiformis*-specific q-PCR. In both supplies highly diverse eukaryotic communities were observed, including free-living protozoa, fungi, and metazoa. Sequences of protozoa clustered with Amoebozoa (10 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 was 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 q-PCR may be promising in elucidating the relationship between drinking water quality and the presence of specific protozoa.

INTRODUCTION

Free-living protozoa are ubiquitous in natural freshwater environments (7, 38, 51, 71), but also proliferate in engineered water systems including water treatment (3, 47, 70), distribution systems (6, 75) and tap water installations inside buildings (54, 69). Concentrations of protozoa, determined using cultivation methods and microscopy, ranging from <1 to 10^4 cells l^{-1} in treated water (3, 47, 70, 75) and from < 1 to 7×10^5 cells l^{-1} in distribution systems (6, 61, 64, 75), have been reported. Genera of free-living protozoa commonly observed in these systems and in tap water installations include *Acanthamoeba*, *Echinamoeba, Hartmannella, Platyamoeba, Vahlkampfia* and *Vannella* (47, 58, 69, 70). In warm water systems certain free-living protozoa serve as host for *Legionella pneumophila*, the etiologic agent of Legionnaires' disease, e.g. *Acanthamoeba* spp. (57), *Balamuthia mandrillaris* (62), *Echinamoeba exandans* (16), *Hartmannella* spp. (39, 56), *Naegleria* spp. (49, 57), *Tetrahymena* spp. (18, 33) and *Vahlkampfia jugosa* (56). High concentrations of *L. pneumophila* generally are associated with the proliferation of host protozoa in biofilms (38, 53). In addition, other amoeba-resistant potentially pathogenic bacteria have been observed in man-made aquatic environments (24), e.g. *Burkholderia* spp.(28) and *Mycobacterium* spp.(37). Free-living protozoa may enhance the multiplication of bacteria, serve as a transmission vector or serve as a shelter against unfavorable environmental conditions like the presence of disinfectants. Furthermore, certain free-living protozoa are human pathogens, e.g. *Naegleria fowleri* (81), *Balamuthia mandrillaris* (77) and *Acanthamoeba* spp. (12) can cause encephalitis. *Acanthamoeba* spp. have also been associated with keratitis in persons wearing contact lenses (31).

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 the microbial

71 communities. In turn, the community of free-living protozoa depends on the diversity and
72 abundance of bacteria in the biofilm and in the planktonic phase (26, 50, 51, 55, 63, 65).
73 Water quality is a critical factor for biofilm formation in distribution systems and in tap water
74 installations and therefore will affect the abundance and diversity of free-living protozoa in
75 these systems (72, 78). However, information about the presence and identity of free-living
76 protozoa in water supplies in relation to the quality of treated water is scarce, which may be
77 attributed to the limitations of microscopic techniques and cultivation methods for detection
78 and identification of these organisms, e.g. low detection limit and selective for specific
79 groups (19).
80 In this study, we applied a variety of cultivation-independent techniques, viz. quantitative
81 PCR, T-RFLP, cloning and sequencing of eukaryotic 18S rRNA gene fragments for the
82 detection and identification of free-living protozoa predominating in two unchlorinated
83 groundwater supplies. The concentration of dissolved natural organic matter (NOM) in
84 treated water at the plant in these supplies was < 0.5 mg C l^{-1} and 7.5 mg C l^{-1}, respectively,
85 covering the entire range of NOM concentrations in drinking water in The Netherlands. The
86 objectives of the study were: (i) elucidation of the identity and diversity of the free-living
87 protozoa predominating in these two different water supplies, and (ii) tracing the presence of
88 host protozoa for *L. pneumophila* and pathogenic free-living protozoa. The study revealed
89 that treated water and biofilms in the distribution system of both water supplies contained a
90 large variety of free-living protozoa, including protozoan hosts for *Legionella* bacteria.
91

92 **MATERIALS AND METHODS**

93 **Selected water supplies.** Two groundwater supplies in The Netherlands, distributing
94 drinking water with different NOM concentrations, were selected (Table 1). In supply A, with

an annual production of 5.6×10^6 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 (for details see supplemental material). The treated water (TW-A) contains a low concentration of NOM (<0.5 mg C l^{-1}), measured as non-purgable organic carbon (NPOC). In supply B, with an average annual production of 2.5×10^7 m^3 and a supply area of ca. 1000 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 rapid sand filtration (for details see supplemental material). The two stages of rapid sand filtration remove ammonia, iron, and manganese. The NOM concentration in the treated water (TW-B) is 7.9 mg C l^{-1}. Both water types are treated and distributed without chemical disinfection (73).

Sample collection. During all seasons of the year 2005, samples of treated water (TW) were collected at both plants. In September and October of the year 2005, samples of the biofilm (BF) in pipe segments of both distribution systems were taken. Distances between sample location and treatment plant ranged from 0.4 km to 6.0 km for supply A and from 17.1 km to 35 km for supply B. In July and in November of the year 2007, treated water and water from both distribution systems were collected. The samples of the distributed water (DW) were taken at the same locations where biofilms have been collected. The figure in the sample name indicates the location in the distribution system, e.g. the 1 in BF-A1 and DW-A1 indicate that the biofilm sample and the drinking water sample were collected at location 1 in supply A (for details see supplemental material). The water samples were stored at 4°C in sterile glass containers and processed within 24 hours. 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 PVC pipes (diameter 110 mm), after thoroughly cleaning of the outer surface, were removed and subsequently placed in plastic cylinders containing water from the distribution system. The samples were stored at 4°C and processed within 24 hours. The attached biomass at the inside surface was collected by swabbing ± 20 cm^2 with three sterile cotton swabs (Copan Innovation, Italy). These swabs were placed in 10 ml PBS and the biomass was removed from the swabs by four 2-min sonication steps in a water bath at a frequency of 40 kHz and an average power input of 0.015 W/ml (44). Total ATP concentrations, representing the active biomass, in biofilm and planktonic phase were determined by ATP analysis as described by Magic-Knezev et al. (44).

Water filtration and DNA extraction. Samples of 1.25 – 3 liter 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 TM Membrane (Millipore, Molsheim, France). Samples of biomass suspended in PBS were filtered through a 1.2-µm pore size and 25 mm diameter RTTP IsoporeTM Membrane (Millipore). DNA was isolated and purified using the Fast DNA® spin kit for Soil (BIO 101, Carlsbad, CA), following the instructions of the manufacturer with the exception that 2 ml tubes containing the lysing matrix E, sodium phosphate, MT buffer and filter with sample were processed in a FastPrep instrument (BIO 101) for two times for 30 s at the speed setting 5.5. The isolated DNA was eluted in 200 µl DNase/Pyrogen Free Water. Distilled water (DNAse and RNAse free) was taken 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 *H. 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 quantification of *H. vermiformis* populations.

Detection of *H. vermiformis* by quantitative PCR. Quantitative PCR assays were performed in 96-wells plates in an I-cycler iQ Multi-Color Real-time PCR detection system (Biorad, Veenendaal, The Netherlands) as described by Kuiper et al. (38). Experiments were performed in duplicate and using undiluted and tenfold diluted DNA extract as template. The quantification was based on a calibration curve of a suspension with a known number of *H. vermiformis* cells that was analyzed in different DNA dilutions in 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 System 9700 (Applied Biosystems, Nieuwerkerk aan de IJsel, The Netherlands) in a reaction mixture (50 µl) and 10 µl template DNA. The PCR was performed with 5% or 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 (3'FAM-labeled) Euk1a-f (68) and Euk516-r (2). 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 the DNA Clean & Concentrator-5 Kit (BaseClear, Leiden, The Netherlands) and redissolved in 20 µl of distilled water. The digestion reaction mixture (20 µl) contained 5U of HhaI (Promega), 2 µl Buffer C (Promega), 12.5 µl 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 redissolved in 15 µl DNA free-water. The restriction digest product (5 µl) was mixed with 15 µl loading buffer (15 µl Hi-Di formamide (Applied Biosystems) and 1 µl GS-500 ROX (Applied Biosystems) as internal standard). The injection time was 5 s for analysis of terminal restriction fragments (T-RFs) and the run time was 35 min. The fluorescently labeled T-RFs were analyzed by

166 electrophoresis on an ABI PRISM 310 Genetic analyzer (Applied Biosystems) in Genescan
167 mode. Electropherograms were imported into a genomic fingerprint analysis program,
168 Bionumerics v. 4.6 (Applied Maths, Sint-Martens-Latem, Belgium) and fragment sizes were
169 calculated. Banding patterns were compared using a densitometric curve-based method that
170 evaluates the position and intensity of the bands to generate pair wise similarity scores
171 (Pearson coefficient) that were subsequently used for cluster analysis.

172 **Cloning and sequencing of PCR product.** The identity of the predominant eukaryotes in the
173 treated water at the plant and in the biofilm of the distribution system was determined by
174 cloning and sequence analysis of approximately 550 bp 18S rRNA gene fragments amplified
175 with the primers Euk1a-f and Euk516-r. The PCR products were cloned using the pGEM-T
176 Easy Vector System II. The DNA insert of randomly selected positive clones were sequenced
177 using Euk1a-f and Euk516-r primers (BaseClear). 134 and 136 clones of the treated water
178 samples and 43 – 50 clones of the biofilm samples were analyzed.

179 **Phylogenetic analysis of partial sequences.** Operational taxonomic units (OTUs) were
180 defined as 18S rRNA gene sequences that shared ≥ 99% sequence similarity. The obtained
181 sequences of approximately 550 bp were compared to sequences in the National Center for
182 Biotechnology (NCBI)-database by BLAST search and were also imported and aligned into
183 the SSU Ref SILVA94 (52) database released in April 2008 using the ARB software package
184 (42). A distance matrix (no filter and no corrections) was calculated for all clones. This
185 distance matrix was used as an input file in the software program DOTUR (59). Operational
186 taxonomic units (OTUs) for the purpose of community analysis were defined by a 1%
187 difference in nucleic acid sequences, as determined using the furthest neighbor algorithm in
188 DOTUR. Similarity percentages were determined between complete and partial 18S rRNA
189 gene sequences in the SILVA database of closely related genera and species to the obtained

OTUs. The used partial sequences correspond to the amplified fragments with the primers Euk1a-f and Euk516-r. The OTU richness was estimated by the Chao1 estimator (11) and was calculated from randomized data as described by Hughes et al.(27).

The similarity of each OTU to 18S rRNA gene sequences in the SSU Ref SILVA 94 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 (excluded the highly variable positions 1-7) was used. The obtained sequences were divided in taxa based on the classification system of Cavalier-Smith (10) and the structure in the SSU Ref SILVA 94 database (52). Sequences with similarities lower than 75% to described species were excluded from further analysis.

Statistical analysis. The *F*-test, with log transformation of the concentrations, was used for determining 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.

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 l^{-1} and averaged 123.4 ± 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 ± 4.9 ng ATP l^{-1} and 4.7 ± 1.2 ATP ng l^{-1} in the distribution system, with biofilm concentrations of 334.7 ± 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 1). In

supply A the average temperature of the water samples collected from the distribution system in July was 14.8 ± 2.3 °C and 11.1 ± 0.6 °C in November. The average temperature of the distributed water in supply B in July was 13.6 ± 1.5 °C and 12.5 ± 0.8 °C in November. Hence, the temperature of the water in the distribution system increased during the summer. Summarized, the concentration of active biomass in supply B was higher than in supply A and both water types are 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 1). The fingerprints of the samples of each supply clustered together indicating that the water type affected the eukaryotic community (Fig. 1). 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 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.

Diversity of eukaryotes in treated water and in biofilms. Clone libraries were constructed for one treated water sample and for three biofilm samples from each supply. In total 545 partial 18S rRNA gene sequences of 550 bp were analyzed (Table 2). The results of the three biofilm samples were combined to compare the results with the treated water samples. All sequences showed the highest similarity to 18S rRNA gene sequences in the NCBI and SILVA database (release 94, April 2008), confirming the specificity for eukaryotic sequences of the primers used.

A total of 219 different OTUs (sequence similarity of ≥ 99%) were distinguished (Table 2). Eight OTUs were observed in more than one sample type, therefore, the addition sum of the OTUs in Tables 2, 3a and 3b will give excess values. The other 211 OTUs were unique for specific samples, demonstrating the high diversity of the eukaryotic communities in the two supplies. Table 3a shows that the coverage of the clone libraries for both supplies was almost similar and also the estimated total OTU richness was not significantly different between the two supplies. In treated water of both supplies, the free-living protozoa constituted that largest proportion (>48 %) of the obtained OTUs, with the fungi as the second largest number in supply A and the metazoa the second largest number in supply B (Table 2). In addition, protophyta and cellular 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% to described sequences in the SILVA database and remained unidentified (Table 2).

A total of 27 OTUs (12.3%) showed the highest similarity to a phylum 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 the highest similarity to a metazoan phylum 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 the highest similarity (99%) to the metazoan freshwater jellyfish *Craspedacusta sowerbyi*. Five OTUs (2.3%) clustered with protophyta or cellular

261 plants and one of these OTUs was obtained from BF-A and BF-B. The other four OTUs were
262 only obtained from supply A (Table 2). Four OTUs (1.8%) clustering with the protophyta
263 showed the highest similarity with species within the phylum Cryptophyta viz. *Plagioselmis*
264 *prolonga* (75.8% similarity), *Chlorella* sp. (76.5% similarity), *Staurastrum polymorphum*
265 (82.7% similarity) and *Goniomonas pacifica* (91.3% similarity). One OTU clustered within
266 the family of Poaceae (grasses).

267 **Identity and diversity of free-living protozoa in treated water and in biofilms.**

268 A total of 253 sequences (46.4%) and more than half of the obtained OTUs (127 OTUs)
269 showed the highest similarity to a free-living protozoan (Table 2). The coverage of the clone
270 libraries for supply A was lower than for supply B, but the estimated total OTU richness at
271 these supplies were not significantly different (Table 3b). The obtained OTUs had similarities
272 of 57% to 100% with eukaryotic sequences in the most recent release of the SILVA database
273 (release 94, April 2008). Similarity percentages for eukaryotic genera and species at the 18S
274 rRNA gene level have not yet been established. Therefore, similarity percentages of 18S
275 rRNA gene sequences most closely related to the same genus and species included in the
276 SILVA database were derived. Data of nine different genera of free-living protozoa revealed
277 that the minimum similarities ranged from 75% to 92%. For sequences in the SILVA
278 database most closely related to the cluster of *Hartmannella* (n = 19), *Acanthamoeba* (n =
279 211) and *Vorticella* (n = 7) minimum similarities of respectively 75.2%, 78.1% and 91.9%
280 were obtained. Minimum similarities, ranging from 86.6% (*Bodo saltans*, n = 23) to 99.7%
281 (*H. vermiformis*, n = 15), were calculated for sequences of seven protozoan species (not all
282 data shown) most closely related to those collected in this study. A total of 98 sequences (32
283 OTUs) had $\leq$ 75% similarity to sequences in the database and thus were considered
284 unidentifiable (Table 2). These unidentified OTUs showed a minimum similairy of 44.4%

and a maximum similarity of 99.3% to each other. Eleven of these OTUs, nine OTUs from supply A and two OTUs from supply B, clustered with each other with more than 95% similarity.

In the clone libraries of both supplies sequences clustering with seven protozoan phyla were observed (Fig. 2 and Table 4). 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 the highest similarity to a free-living protozoan were observed in both supplies demonstrating highly diverse protozoan communities in each supply (Table 4).

Occurrence of protozoan hosts and pathogenic free-living protozoa in treated water and biofilms. All samples of treated water, water from distribution systems and all samples of the biofilm in both supplies were analyzed for the presence of the *L. pneumophila* host *Hartmannella vermiformis* using quantitative PCR (38). In none of the samples, inhibition of the PCR amplification was observed. *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 l^{-1}), but was not detected in any of the DW-A samples, nor 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 l^{-1} and one (of 8) 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 l^{-1}) were significantly ($p < 0.05$) higher than the concentrations in November (median 4 cells l^{-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

309 to the family of *Hartmannellidae* (Table 4). A total of 2.2 % of the sequences representing
310 free-living protozoa obtained from supply A showed the highest similarity (85.9% – 89.3%)
311 to species within the genus of *Acanthamoeba*. Several *Acanthamoeba* spp. can serve as host
312 for *L. pneumophila* and other undesired bacteria (24). One OTU (0.8 %) obtained from TW-B
313 showed the highest similarity to *Echinamoeba thermarum* (85.7%), a potential host for *L.*
314 *pneumophila*. One OTU had the highest similarity to *Acanthamoeba polyphaga*, a potential
315 pathogen (Table 4).
316
317 **DISCUSSION**
318 **Analytical procedures.** To our knowledge, primers for the amplification of all free-living
319 protozoa included in public databases are not available. Therefore, we selected 18S rRNA
320 gene primers amplifying most but not all eukaryotic organisms represented in public
321 databases. Two genera serving as host for *L. pneumophila*, viz. *Naegleria* spp. and
322 *Valkampfia* spp., were not amplified with these primers. Recently a primer set for
323 vahlkampfiid amoeba has been developed for direct detection of *Acanthamoeba* spp.,
324 *Naegleria* spp. and *Vahlkampfia* spp. (13).
325 The variation in the T-RFLP fingerprints of treated water and biofilms exceeded the
326 reproducibility of the T-RFLP method, demonstrating differences in the involved eukaryotic
327 communities. However, a limitation of the T-RFLP method is that similar fragment lengths
328 may represent different sequences, implying that the diversity in the sample may be higher
329 than the number of observed fragments. The use of clone libraries for studying the diversity
330 of the eukaryotic communities and the estimation of the diversity estimation with the Chao1
331 index (11) also has a few limitations. In most eukaryotes, 18S rRNA genes are organized in
332 tandem repeat units (41) and the copy numbers differ significantly per genus, e.g. *H.*

333 *vermiformis* has about 1330 copies per cell (38) and *Acanthamoeba* spp. has about 600 copies
334 per cell (9) of the 18S rRNA gene. The clone libraries of the treated water and biofilm
335 samples were constructed using a fraction (5% to 6.7%) of the isolated DNA and therefore
336 only organisms with more than of 15 to 20 copies of the 18S rRNA gene per cell could be
337 represented in the clone libraries. Hence, the composition of the clone libraries does not
338 exactly reflect the composition of the involved eukaryotic communities. The effect of copy
339 numbers is most pronounced with multicellular eukaryotes containing more DNA (copies)
340 than unicellular eukaryotes, as is demonstrated in clone libraries of BF-B5 and BF-B6 with
341 an OTU with > 50% of the clone sequences representing metazoa (Table 2). Furthermore,
342 only the predominating sequences were analyzed (Table 3a and 3b).
343 **Identification of the obtained partial 18S rRNA gene sequences.** Similarity percentage at
344 the 18S rRNA gene level have not been published for members of eukaryotic genera and
345 species. For the genera of free-living protozoa most closely related to those observed in this
346 study similarities between 76% and 92% were derived from the 18S rRNA gene sequences in
347 the SILVA database. On species level similarities between 86.6% and 99.7% were calcultated
348 for sequences of a number of protozoan species most closely related to those collected in this
349 study. Morphologically well-defined ciliate species vary highly at the SSU rRNA sequences
350 level (30, 67). Hence, genera and species of many free-living protozoa may show relatively
351 high sequence diversities of the 18S rRNA gene. Therefore, with the division of sequences in
352 OTUs with 99% similarity almost all different species can be distinguished. The large
353 proportion of sequences with a relatively low similarity percentage to those included in the
354 databases further indicates that many eukaryotic organisms in fresh water and marine
355 environments are not yet described (5, 15, 46, 67, 70, 79). A total of 32 OTUs showed the
356 highest similarity to a specific eukaryote but clustered in the phylogenic tree with another

357 group of eukaryotic organisms. We used the information of these OTUs with the highest
358 similarity (blast search) for identification. These observations demonstrate that identification
359 of fresh-water protozoa is limited by the currently available database, but the large variety of
360 sequences retrieved in the present study will facilitate further investigations of free-living
361 protozoan communities in water supplies.

362 **Host protozoa for *Legionella spp.* and pathogenic free-living protozoa.** *H. vermiformis*, a
363 commonly observed protozoan host for *L. pneumophila* (17, 39, 80), was detected in both
364 supplies. This protozoan has also been observed in treated groundwater in Germany (34, 47),
365 using culture methods, in drinking water supplies (47, 70), in warm water supplies (54, 69)
366 and in surface water (38, 47, 70), demonstrating its ubiquitous presence in the fresh water
367 environment. However, *H. vermiformis* was not a predominant protozoan in the eukaryotic
368 communities in any of the samples in this study for which clone libraries were prepared. In
369 the distributed water *H. vermiformis* was only detected in supply B with higher
370 concentrations in the summer than in the autumn. The presence of *H. vermiformis* in supply B
371 is associated with an elevated level of active biomass and a high level of NOM.

372 A total of 2.2% of the protozoan sequences retrieved from supply A and 6.7% of the
373 protozoan sequences of supply B had the highest similarity to genera with one or more
374 protozoan species described as host for *L. pneumophila.* Water temperature in supplies A and
375 B were below 20ºC and thus were too low for growth of *L. pneumophila* (32), but uncultured
376 *Legionella* species, including *Legionella*-like amoeba pathogens (LLAP) (82) can multiply at
377 this temperature range in water supplies. Probably, a number of the detected free-living
378 protozoa serve as host for these uncultured *Legionella* bacteria. At elevated temperatures in
379 warm water installations *H. vermiformis* and *Acanthamoeba* spp, are available for promoting
380 growth of *L. pneumophila* and other undesired bacteria (24, 56, 80). *Acanthamoeba* spp. (12,

31, 48) and *H. vermiformis* (1) 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 cellular plants. Fungi, metazoa, cellular plants and protophyta were detected in the clone libraries of nearly all samples (Table 2). Fungi (25) and metazoa (75) can multiply in water treatment and distribution system (4). Some of the OTUs had the highest similarity to a fungus, e.g. *Aspergillus* spp., *Fusarium* spp. and *Cladosporium* spp., which have also been observed in drinking water in Slovakia (21), Norway (25) and Germany (23). A few OTUs had the highest similarity (> 99%) to pathogenic fungi, e.g. *Candida albicans* (14), but it is not possible to determine whether the obtained partial sequences represent pathogenic organisms. Metazoa such as Nematodes and Cnidarian's (e.g. freshwater jellyfish (74)) are common inhabitants of treated water in distribution systems and play a role in the food chain (4, 45, 46, 67, 75). 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 (51), 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 diversity in supplies A and B. The concentration of NOM in treated water of supply A (<0.5 ppm of C) is much lower than in supply B (7.9 ppm of C) (Table 1). This difference is reflected in the concentration of active biomass measured as ATP in these water types, viz. < 1 ng l^{-1} in TW-A and 10.6 g l^{-1} in 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 (60). Indeed, the fingerprints of TW-A showed more variation than the fingerprints of TW-B (Fig 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 3a).

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 3a and 4). This coverage index is low in comparison with the values derived for the community of small eukaryotes in an anaerobic aquifer (66%) (8) and in a mesotrophic lake (91%) (40), but higher than the value (22%) reported for eukaryotes in a suboxic and an oxid lake in France (67).

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 in 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 (Figure 1).

Diversity 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 (7, 50, 76). Due to their rapid response to environmental changes, free-living protozoa have been used as water quality indicators and the diversity of free-living protozoa generally increases with improved water quality (29, 34-36, 45). 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 diversity of the free-living protozoa was not significantly different between the two supplies (Table 3b).

429 Based on morphological studies, free-living protozoa have been divided in amoeba,
430 flagellates and ciliates (50, 76). Table 4 shows that representatives of these groups have been
431 identified with molecular techniques in the different samples. Microscopic studies have
432 shown that sand filters operating under similar conditions in water treatment systems
433 harbored different numbers and types of ciliates and amoeba (43, 70). Microscopic analysis
434 also showed that flagellates predominated (93%) in drinking water in an experimental
435 distribution system with pipes of concrete and PVC, supplied for four months with treated
436 water with a DOC-concentration of 2.3 mg C l^{-1} (64). However, in the biofilm no flagellates
437 but ciliates (52%) and amoeba (48%) were detected. In the present study many OTUs
438 observed in the biofilms had the highest similarity to flagellates, including *Cercomonas* spp.,
439 *Bodo saltans* and *Rhynchomans nasuta* (Table 4) (51).
440 Twelve OTUs of the clone libraries of supply A and 26 OTUs of the clone libraries of supply
441 B had the highest similarity to genera that have been used as indicator organisms in the
442 saprobic index for organic pollution (20, 66). A total of 87% of these organisms belong to
443 genera that indicate moderate pollution at a high dissolved oxygen content, e.g. *Hemiophrys*,
444 *Rhynchomonas*, and *Vorticella* (Table 4). However, elucidation of the relationship between
445 environmental conditions in water treatment and distribution, e.g. water composition, and the
446 occurrence of free-living protozoa is not possible because: (i) the communities are highly
447 diverse, (ii) species and genera boundaries of eukaryotes are yet unclear, (iii) little
448 information is available about the growth conditions of free-living protozoa, and (iv) the
449 diversity in the clone libraries is not proportional to the diversity of the protozoa in the
450 samples.
451 In conclusion, both groundwater supplies, with a large difference in the concentration of
452 NOM, highly diverse communities of free-living protozoa were observed. These communities

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

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691

692 **LEGENDS TO THE FIGURES**

693 FIG. 1. UPGMA dendogram of T-RFLP fingerprints of treated water (TW), biofilm (BF) from the
694 distribution system and distributed water (DW) of supply A and supply B. Samples of distributed
695 water were taken from different location in July and biofilm samples from different location were
696 taken in September and October.

697

698 FIG. 2.

699 a. Taxonomic distribution of free-living protozoa based on 18S rRNA gene clones retrieved from
700 treated water (TW) and biofilms (BF) of supply A and supply B. BF is total of the three analyzed
701 biofilm samples per supply.

702 b. Taxonomic distribution of the OTUs with the highest similarity to a free-living protozoan
703 retrieved from treated water (TW) and from biofilms (BF) of supply A and supply B. BF is total of
704 the three analyzed biofilm samples per supply.

705

706

707 TABLE 1. Quality characteristics of treated water at the treatment plants of supply A and supply B*

Parameter	**Treated water A**			**Treated water B**		
	Mean	*Min.*	*Max.*	*Mean*	*Min.*	*Max.*
Temperature (ºC)	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 (mg l^{-1})	6.4	5.6	7.8	5.9	3.9	8.3
HCO_3 (mg l^{-1})	98	92	124	282	273	308
Cl (mg l^{-1})	13	11	14	28	27	31
Ca (mg l^{-1})	35.4	32.9	39.6	32.7	25.7	52.8
Mg (mg l^{-1})	2.37	2.07	2.73	9.72	8.36	10.9
Total hardness (mmol l^{-1})	0.98	0.9	1.09	1.22	1.04	1.68
Fe (µg l^{-1})	< 20	< 20	< 20	25	< 20	73
Mn (µg l^{-1})	< 10	< 10	< 10	< 10	< 10	< 10
SO_4 (mg l^{-1})	16	13	19	< 10	< 10	< 10
NH_4 (mg l^{-1})	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
NPOC (mg C l^{-1})	0.33	< 0.3	0.49	7.9	7.6	8.3

708 * Mean, minimum and maximum values, based on routine monitoring over a period of one year.

709 TABLE 2. Classification of clones of eukaryotes retrieved from treated water and biofilms of supply A and supply B.

	Water supply A				Water supply B				Supply A and supply B	
	TW-A1[a]		BF-A[b]		TW-B1[a]		BF-B[b]		All analyzed samples	
Kingdom or subkingdom	**Number and % of the OTUs[c]**	**% of clones in library**	**Number and % of the OTUs[c]**	**% of clones in library**	**Number and % of the OTUs[c]**	**% of clones in library**	**Number and % of the OTUs[c]**	**% of clones in library**	**Number[d] and % of the OTUs**	**Number and % of clones in library**
Free-living protozoa	25 (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	1 (1.8)	0.7	5 (2.3)	9 (1.7)
< 75% similarity	13 (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)

710 [a] TW = treated water directly from plant,

711 [b] BF = Total of the three analyzed biofilm samples,

712 [c] Number of OTUs and percentage of the total number of OTUs per clone library,

713 [d] Total number of OTUs are compensated for OTUs which were obtained from more than one sample type.

714 TABLE 3a. Diversity of the eukaryotes in the clone libraries in supply A and supply B.

				Total OTU richness (Chao1[b] estimation)		
Supply	**No. of clones**	**No. of OTUs identified**	**Coverage index[a]**	**mean**	**lower limit**	**upper limit**
Supply A	272	108	39.7	159	136	204
Supply B	273	115	42.1	145	91	277
Total	545	219[c]	40.2	390	328	487

715 [a] Coverage index (number of OTUs /number of sequences × 100%) (22),

716 [b] The Chao1 index (11) was calculated with DOTUR (59),

717 [c] Total number of OTUs are compensated for OTUs which were obtained from more than one

718 sample type.

719 TABLE 3b. Diversity of the free-living protozoa in treated water (TW) at the plant and in biofilms
720 (BF) in the distribution systems of supply A and supply B.

				Total OTU richness (Chao1[b] estimation)		
Source	**No. of clones**	**No. of OTUs identified**	**Coverage index[a]**	**average**	**lower limit**	**upper limit**
Supply A	133	54[c]	40.6	113	81	187
TW-A	44	25	56.8	34	28	55
BF-A[d]	89	30	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[d]	253	127[c]	50.2	281	212	407

721 [a] Coverage index (number of OTUs /number of sequences × 100%) (22),
722 [b] The Chao1 index (11) was calculated with DOTUR (59),
723 [c] Total number of OTUs are compensated for OTUs which were obtained from more than one
724 sample type,
725 [d] Total of the three analyzed biofilm samples.
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730 TABLE 4. Classification of OTUs, clustering with free-living protozoa, obtained from treated water (TW) and biofilms (BF) in the
731 distribution system of water supply A and water supply B. OTUs were compared with sequences in the ARB database (10, 38).
732 [a] Most closely related sequence (accession no.) / closely related genus or species (accession no.). More than one accession number
733 indicates that more than one OTU had the highest similarity to the same genus or species, but to different sequence types.
734 [b] Total of the three analyzed biofilm samples.

Genus/species with highest similarity[a]	**Similarity (%)**	**Nr. of OTUs**	**No. of clones**			
			TW-A	**BF-A[b]**	**TW-B**	**BF-B[b]**
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.	89.0 / 88.4	1		1		

(AY749606)						
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) /	87.4 / 84.6	1		1		

Masisteria marina strain DFS1 (AF174371)					
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		
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	
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) / *Gonostomum namibiense* (AY498655)	97.5 / 96.9	1			1	
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 ciliate (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) / *Corallochytrium limacisporum* (L42528)	87.8 / 78.9	1			1	
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**

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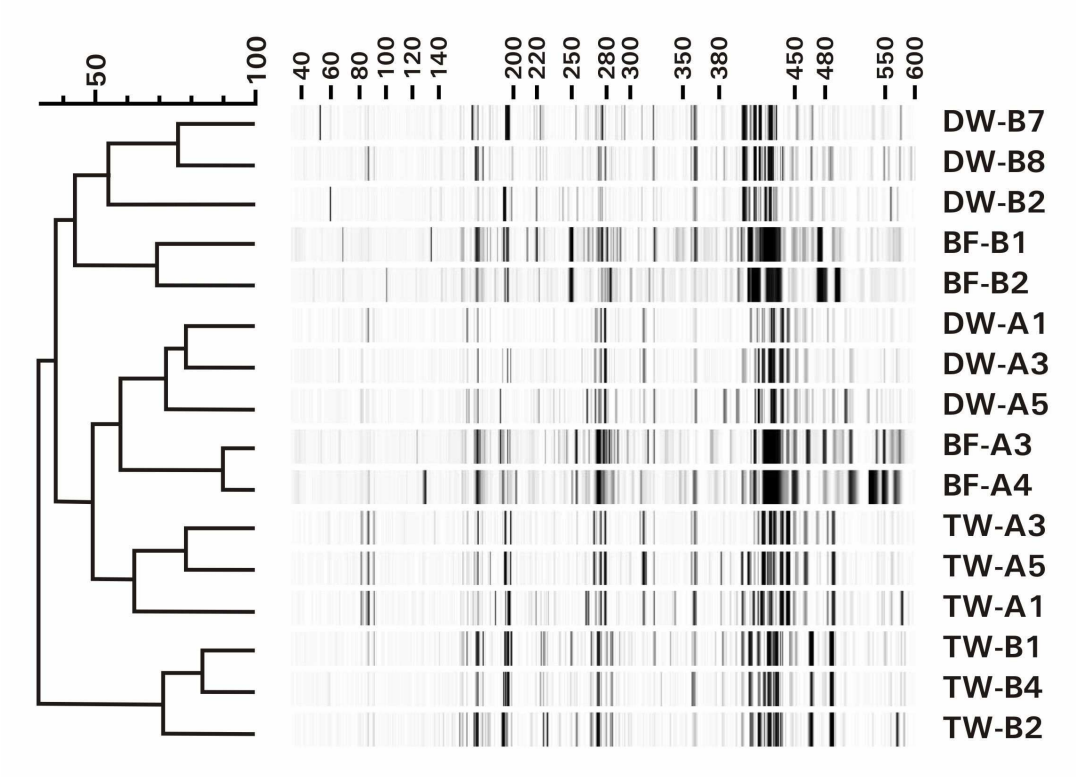

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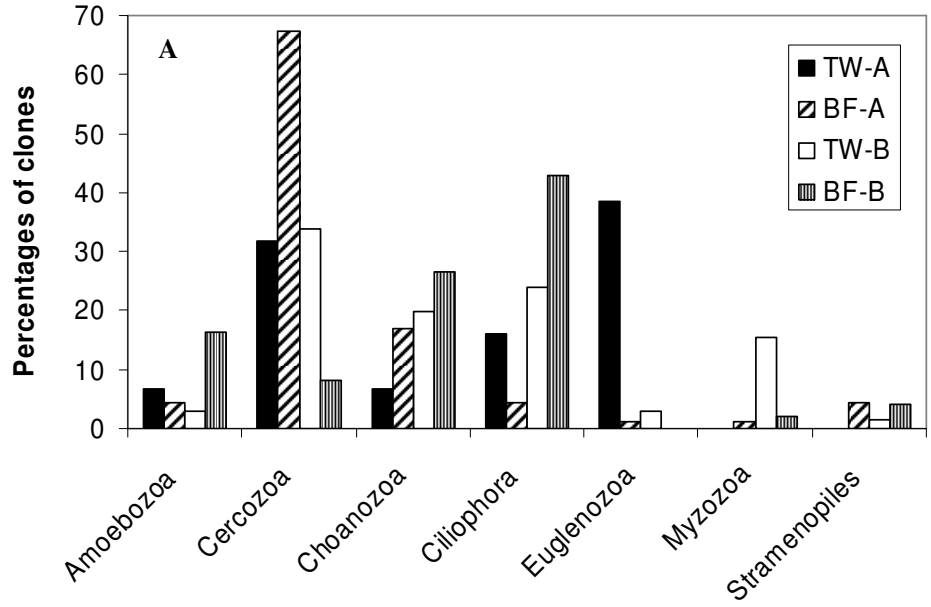

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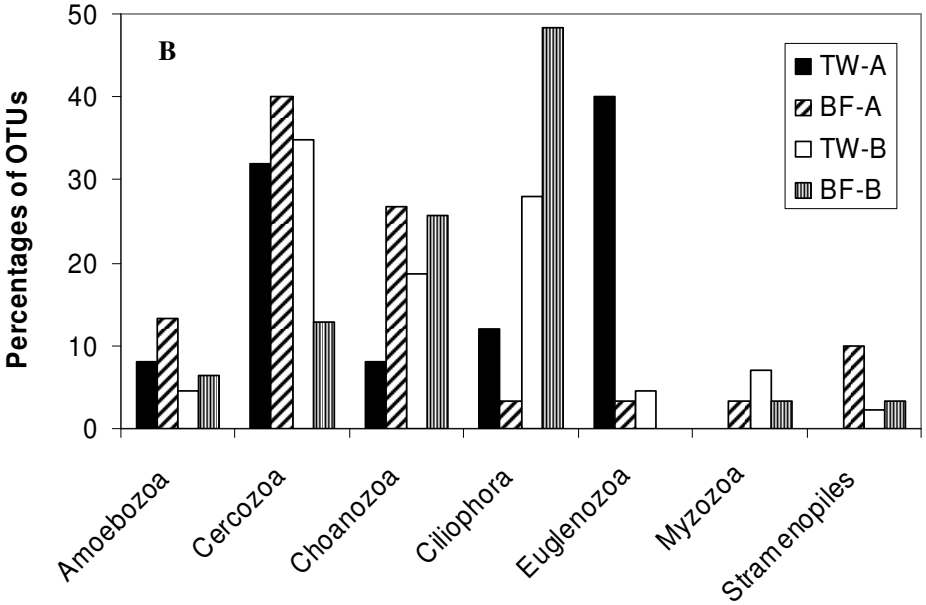

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