KAPITEL I

HIGH THERMAL TOLERANCE OF PROTISTS APPEARING IN HEATED WASTEWATER

ABSTRACT

Here we describe the occurrence and temperature tolerance of different ciliate species that have adapted to extreme temperatures above 40°C. Two colpodids, one cyrtophorid, five hymenostomes, two hypotrichs, three stichotrichs, five peritrichs and one scuticociliate survived temperatures ranging up to 42°C. The ciliate species were isolated from effluents of heated, aerobic wastewater of paper mills, with an artificial softening at a pH of 7.5-8.5 and high water hardness (220-600 mg/l Ca). The organic pollution ranged from 178 to 2000 mg/l COD (chemical oxygen demand). Investigations of six different wastewater treatment plants revealed a certain overlap of common species as well as a relatively high variability of species composition which may indicate a random contamination. Most recorded species occurred at much higher temperatures than known from previous field studies. It seems possible that thermal adapted protists may be used to increase bacterial flocculation in thermal wastewater treatment.

Keywords: Ciliophora, Colpodida, Cyrtophorida, Hypotrichida, Oligotrichida, Peritrichida, thermophiles, waste water, activated sludge, protozoa

INTRODUCTION

Most ciliate species have been described to occur at temperatures up to 30 °C (see Foissner et al. 1991). Little is known about the adaptation of free-living ciliates to temperatures beyond 30°C (e.g. Baumgartner et al. 2003, Ketola et al. 2004). Recently, thermophile protist species, including some ciliate and flagellate species, have been described from hydrothermal vents (Atkins et al. 2002, Baumgartner et al. 2003, Brown & Wolfe 2006). Also from man-made environments such as heated wastewater, protists - especially pathogenic amoebae - have been observed (e.g. Penas-Ares et al. 1993, Rohr et al. 1998, Barbeau & Buhler 2001). According to our preliminary results, it seems that an astonishing variety of ciliate species may withstand high temperatures ($\geq$40°C) in wastewaters, which gave rise to the present investigations. Investigations of protist diversity in thermal habitats and its consequences in protist's tolerance to increasing temperatures could provide important information to predict potential future developments in natural habitats in the frame of global warming.

Protists play an essential part of complex microbial food webs (Azam et al. 1983, Fenchel 1987, Weisse et al. 1990, Arndt et al. 2003). The multiple functions of protists in natural food webs are used in conventional wastewater treatment (Curds 1973, Güde 1979). As bacterivores, they maximize bacterial production and thereby force mineralisation of organic compounds (e.g. Fenchel 1987, Fenchel 2002, Thelaus et al. 2008). Previous experiments showed, that without the presence of predators bacterial macro-structures through flocculation or sedimentation did not emerge (Curds 1973, Jürgens & Matz 2002). As a result high protist abundances caused a visible reduction of water dimness (Güde 1979). Today, protists and their accelerative flocculating effect are appreciated and hence supported in the conventional municipal sewage water treatment (e.g. Arregui et al. 2007). In contrast to this, heated wastewater (e.g. 40°C, e.g. from circular wastewater treatments), is generally treated with chemicals for a flocculation and sterilisation since the potential importance of microbial food webs at high temperatures is ignored.

Investigations of various extreme habitats in nature, including acetic hydrothermal environments of volcanic heated waters with temperatures up to 68°, revealed that ciliates (e.g. colpodids, oligohymenophores, scuticociliates), flagellates (bodonids) and amoebae (*Echinamoeba thermarum*) may occur under extreme thermal conditions (Baumgartner et al. 2003, Brown & Wolfe 2006). Studies of the impact of high temperatures on metabolic rate and body size showed, that various ciliates, flagellates and amoebae sustained up to 55°C, for instance in crust soils of deserts (Ernest et al. 2003, Darby et al. 2006).

With the expected increase in water temperatures in the course of global warming (IPCC 2007), natural environments in the temperate regions might be driven from macrofauna and microbe dominated food webs towards microbe dominated food webs in which ciliates play a vital role as consumers (Norf et al. 2007, in press). Thus it might become increasingly important to understand the adaptation of ciliates towards increasing temperatures. We investigated thermal wastewater contaminated from the environment regarding the occurrence of protists and their adaptation to high temperatures (above 40°C). Heated activated sludge basins represent extreme habitats with high temperatures and fluctuating values of pH and different other abiotic conditions (Althöfer and Feuersänger 2005, Feuersänger et al. 2008). Ciliate species isolated from natural habitats (River Rhine) and artificial thermal habitats (heated circular paper mill processing water) were compared regarding their tolerable temperature.

MATERIAL AND METHODS

The ciliate composition of several heated wastewater samples from paper mills was investigated and compared to study the occurrence of ciliate species at temperatures between 36-42°C. Several species could be isolated and cultured from these wastewaters. These cultures were used for detailed experiments on temperature tolerance. For comparative studies, additional ciliates were isolated and studied from the River Rhine. For

several thermal wastewater species, cultures could not be established. These were investigated regarding their maximum tolerable temperature in the original mixed populations from the activated sludge. These latter studies were carried out in bioreactors to keep conditions close to the original ones. Exemplarily, molecular studies of three ciliate clones, isolated from temperatures of 40°C, were carried out to study the relationship of isolated genotypes with those known from temperate habitats.

A) Investigations of species composition in heated wastewater

The heated wastewater samples were gathered from six paper mills possessing a thermal treatment facility, including an activated sludge basin: Fulda (Adolf Jass), Zülpich (Smurfit Kappa), Mayen (M. Weig), Düren (Schoellershammer), Viersen (Smurfit Kappa), Marsberg (Wepa). In addition, a municipal sewage treatment facility Cologne/Stammheim, which was running at 28°C without any thermal effluents, was chosen as a reference sample. Abiotic and biotic factors, such as temperature, COD (chemical oxygen demand), pH-value, water hardness and oxygen-content were recorded for this study (Table 1).

Table 1: Ecological characteristics of the seven different activated sludge basins (Fig.2). Average values of ecological conditions the isolates were taken from.

Wastewater facility[a]	COD [mg*l^{-1}]	Temperature [°C]	Ca content [mg*l^{-1}]	pH-value	[mg*l^{-1}]	Production type
FA: Viersen	698	39	262	7.4	3.2	cardboard (raw)
FB: Zülpich	1100	40	220	8	1.3	cardboard (fine)
FC: Düren	2000	36	290	7.8	2	inkjet paper
FD: Marsberg	1000	35	350	7	1.5	hygienic paper
FE: Mayen	188	28	362	7.5	unknown	paper and cardboard
FF: Fulda	338	27	600	7.6	2.4	paper and cardboard
MG: Stammheim	Unknown	25	unknown	8	3.2	municipal sewage

The water treatment program was similar in all facilities differing only by product type such as industrial cartonage or hygienic paper. Additionally, the quality of the recovered paper that was recycled and re-entered into the production process varied. These factors resulted in a variation in organic pollution and other abiotic factors in the wastewater (Althöfer & Feuersänger 2005). Aliquoted water samples were fixated with Bouin fixative and taxonomical identification on the morphospecies level was done using protargol staining (Skibbe 1994). In addition, aliquots were investigated by live observation. The species were morphologically determined using an inverse light microscope, including video- and photo-documentation.

A cluster analysis of the ciliate morphospecies compositions of the wastewater treatment facilities in relation to the quality of the wastewater was conducted using the Bray/Curtis similarity-test.

B) Investigations of temperature tolerance in mixed populations

Water samples (10 litres) from all wastewater treatment plants (see above) were kept at ambient temperature and were immediately filled into a bioreactor (five-litres-chemostat system) to keep the chemical and biological conditions as close as possible to the original conditions from the production facilities. In the course of longterm investigation environmental factors like temperature, transfer rate, ventilation (supply with oxygen), pH-value, water hardness and chemical oxygen demand (COD) were kept constant. Contamination during the injection of medium, overflow, ventilation or extraction of samples was avoided. In order to study the protists' tolerance towards heat, an accurate and stable temperature control unit was installed. Homogeneous temperature and medium distributions were realized by a ventilation system. The organic supply of the bioreactor was ensured by a proteose-pepton yeast extract soluted in WC medium (Guillard & Lorenzen 1972). The temperature was increased in steps of 1°C every 24 hours. At each temperature level the ciliate species composition was determined in subsamples (100µl) under an inverted microscope.

C) Investigations of temperature tolerance of selected ciliate species

Clonal cultures of *Colpoda steinii*, *Ophistonecta minima*, *Oxytricha sp.* and *Paramaecium caudatum* were established from the thermal activated sludge basin of the paper mill in Zülpich. The cultures were established using the liquid aliquot method (LAM). Isolated cells were cultivated at 36°C in 50ml-tissue–culture flasks in WC-medium (Guillard and Lorenzen 1972) supplied with a wheat grain as a carbon source for bacteria. The temperature effect on growth rates and mortality was investigated for a temperature range from 36 to 42°C. Cultures were started in tissue culture flasks at 36°C 10 days prior to the experiment. Tissue culture flasks (50ml, 10 replicates) were exposed in tempered water basins (20l-basins with adiabatic temperature gradient blocks, 2 level inverter GIR 2000 Pt (Conrad, Germany), accuracy $\pm$ 0.1°C). The temperature level was increased 1°C every 24 hours and subsamples (100µl) were counted at the experimental temperature under an inverted microscope. For sessile peritrichs, the counts were done on a square footage of 1 cm^2. For the live count, cilia, contractile vacuole and cytoplasm movement were registered as a vital sign. As a control to the stepwise temperature increase, 10 replicate flasks for each temperature level were maintained at the respective temperature for additional 5 days with inspections of every 24 hours. These latter flasks were used to determine the growth rates at the respective temperature. Constant growth rates within the first five days indicated that experiments were conducted at the exponential growth phase. The maximum tolerable temperature and the tolerable temperature at which growth rates were above zero for five days were determined. Growth rates were estimated assuming a linear growth development according to the following equation:

$\mu = (\ln N_t - \ln N_0) / (t_1-t_0)$;

N_0: Start-up-population at time 0, N_t: Population at the current time frame, t_0: Start-up-time, t_1: specific time frame.

For comparative studies of temperature tolerance of identical ciliate morphospecies, individuals from *Cyclidium glaucoma*, *Holosticha sp.* and the *Vorticella infusionum-complex*

were isolated from the activated sludge basin of the wastewater treatment in Zülpich as well as from the River Rhine at Cologne. The experiments were carried out as described above, except that the temperature spectrum laid between 26-32°C (Rhine) and 36-41°C (Zülpich). For the comparative analysis of temperature tolerance of different ciliate clones, the lethal temperature dose (LT50) was estimated as the temperature at which 50% of the population died. Estimates were based on regressions revealing the best fit to the data sets (10 replicates for each clone). LT50 values were compared for the different species using Student´s t-test.

D) Molecular investigation of selected ciliates

To check whether the heat adapted ciliate species belonged to the same 18S-rDNA genotypes known from temperate regions, the partial small sub unit (SSU) of rDNA was sequenced of three different heat adapted ciliate species. Ciliates were extracted using a micromanipulator. Cells were transferred to 27µl sterilized water and frozen at -20°C for three hours before PCR. The rDNA fragment was amplified using 18SFor and 18SRev primers at a concentration of 1.6 nM followed by a reamplification with the primer pair 42F and 18SRev-1. The PCR products were purified using the E.Z.N.A. Cycle-Pure-Kit (Peqlab, Erlangen, Germany). The sequencing of rDNA was done using Big Dye-Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) in accordance with the manufacturer's instructions. Primers used for sequencing were 42F and 590F for the forward strand and 600R and 1300R for the reverse strand (Nitsche and Arndt 2008). Alignments with SSU rDNA sequences of similar morphotypes available from genebank were carried out using ClustalX (Thompson *et al.* 1997), corrections were made manually with BioEdit. The distance model (JC69) for maximum likelihood (ML) analysis was determined by MrAIC (Nylander 2004) and the ML analysis computed by PhyML (Guindon and Gascuel 2003), using 100 replicates for the bootstrap analysis. Neighbour joining (NJ) was calculated using MEGA 3.1 (Kumar et al. 2004) using the JC model and 100 replicates for bootstrap analysis.

RESULTS

A) Investigations of species composition in heated wastewater

The thermal wastewater treatment facilities investigated showed special characteristics regarding abiotic conditions. Extreme values were reached especially regarding temperature, as well as chemical oxygen demand (COD), water hardness (calcium-content), pH-value and oxygen content (Table 1). 23 ciliate species were recorded in the activated sludge basins at temperatures ranging up to 40°C (Table 2). Ciliate abundances were only occasionally determined, but were generally above 50 ind./ml. A comparison of the ciliate community structure of the different wastewaters was done using a cluster analysis. The Bray/Curtis similarity revealed similar species compositions between Marsberg and Mayen, as well as between Zülpich and Fulda (Figure 1). The first cluster was characterized by relatively low temperatures (28°C-35°C) similar hardness (Ca-content: 350-365 mg/l) and similar pH-value (7-7.5). The second cluster did not show any analogies in temperature (27°C, 40°C) or calcium content (220 mg/l, 600 mg/l). Merely, the pH-value was at a similar level (7.6-8). The Bray/Curtis similarity indicated an overlap of the ciliate taxa within cluster 1 (Marsberg and Mayen) and cluster 2 (Zülpich and Fulda) of approximately 45 to 50%. Some ciliate morphospecies were common in many different thermal wastewaters (e.g. *Colpoda steinii*, *Chilodonella unicata*, *Cyclidium glaucoma*, *Paramaecium caudatum*, *Oxytricha sp.* and *Vorticella infusionum-complex*) while most species were recorded in only one or two wastewater treatment plants (Table 2). In addition to ciliates, several species of naked and testate amoebae and heterotrophic flagellates occurred in heated wastewater at temperatures up to 40°C (see Table 2).

Table 2: Recordings of protist morphospecies in wastewater treatment facilities

Morphospecies			Activated sludge basins, wastewater habitats						
			Viersen (39°C)	Zülpich (40°C)	Düren (36°C)	Marsberg (35°C)	Mayen (28°C)	Fulda (27°C)	Stammheim (20°C)
Ciliates	Colpodids	*?Colpoda simulans*	X						
		Colpoda steinii				X			X
	Cyrtophorids	*Chilodonella unicata*	X	X	X	X			X
	Hymenostomes	*Cyclidium glaucoma*		X			X	X	
		Dexiostoma campylum			X				
		Drepanonomas revoluta					X		
		?Ophryoglena sp.							X
		Paramaecium caudatum	X	X		X			
		Tetrahymena thermophila		X					
		Uronema sp.			X				
		Urostyla / Uroleptus sp.							X
	Hypotrichs	*Aspidisca cicada*			X		X		
		Euplotes aediculatus						X	
	Oligotrichs	*?Halteria grandinella*							X
	Stichotrichs	*Holosticha pullaster*		X					
		?Tachysoma pellionellum		X					
		Oxytricha longa		X		X	X		
	Peritrichs	*?Epistylis nympharum*							
		?Opercularia nutans						X	
		?Opercularia asymetrica		X	X				
		Ophistonecta minima		X					
		Vorticella infusionum-complex	X	X	X		X	X	
		Zoothamnium ramossissimum						X	
	Scuticociliates	*Philasterides armatus*	X						
Naked amoebae	Mayorellids	*Mayorella sp.*			X			X	
	Vannellids	*Vannella sp.*		X					
Testate amoebae	Arcellinids	*Centropyxis sp.*		X					X
	Arcellinids	*Cryptodifflugia sp.*		X					
	Arcellinids	*Arcella sp.*						X	
Hetero-trophic flagellates	Cercomonads	*Cercomonas sp.*							X
	Jakobids	*Histiona aroides*		X	X				
	Euglenids	*Peranema sp.*					X		
	Euglenids	*Entosiphon sulcatum*							X
	Protista incertae sedi	*Allantion sp.*	X						

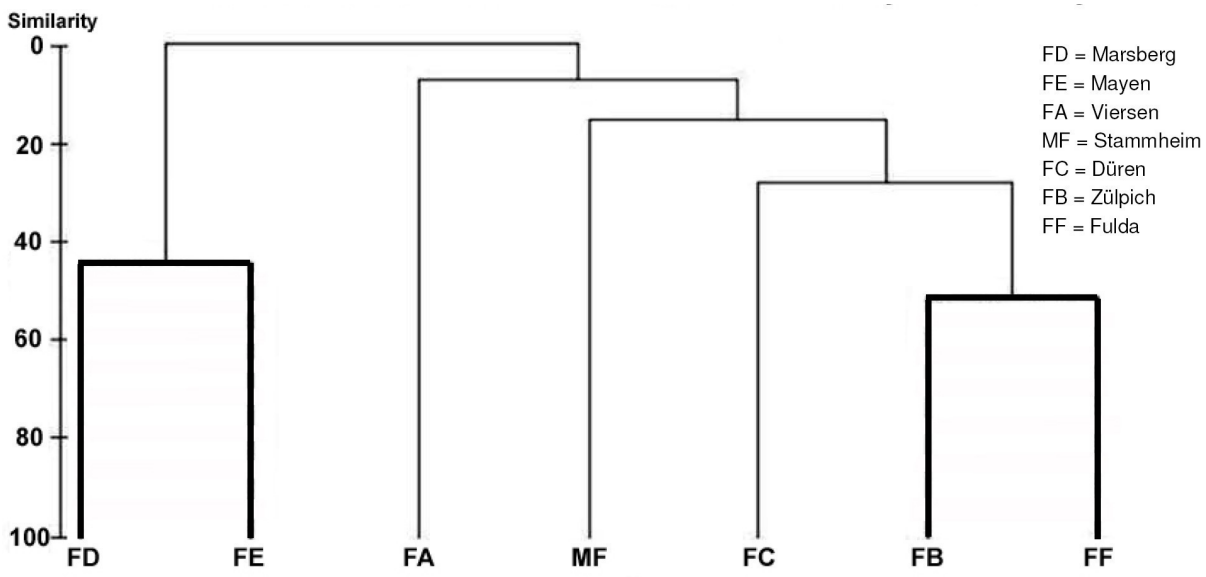

Figure 1: Cluster analysis of species composition from different wastewater habitats.

B) Investigations of temperature tolerance in mixed populations

The potential of several ciliate species to tolerate a further temperature increase compared to the wastewater temperature they were collected from was investigated in chemostats supplied with original wastewater. The temperature was increased in steps of 1°C every 24 hours. At each temperature level the ciliate species composition was determined to analyze which species could withstand higher temperatures. A large number ciliate species were registered at temperatures up to 42°C, *Ophistonecta minima* survived even 45°C for a short period of time (24 hours). Figure 2 summarizes the results and includes a comparison with former recordings of the respective ciliate species at high temperatures (Foissner et al. 1991). Except for *Ophistonecta minima* and *Aspidisca cicada*, all ciliates found in thermal wastewater were able to withstand much higher temperatures (up to 10°C) than previously reported.

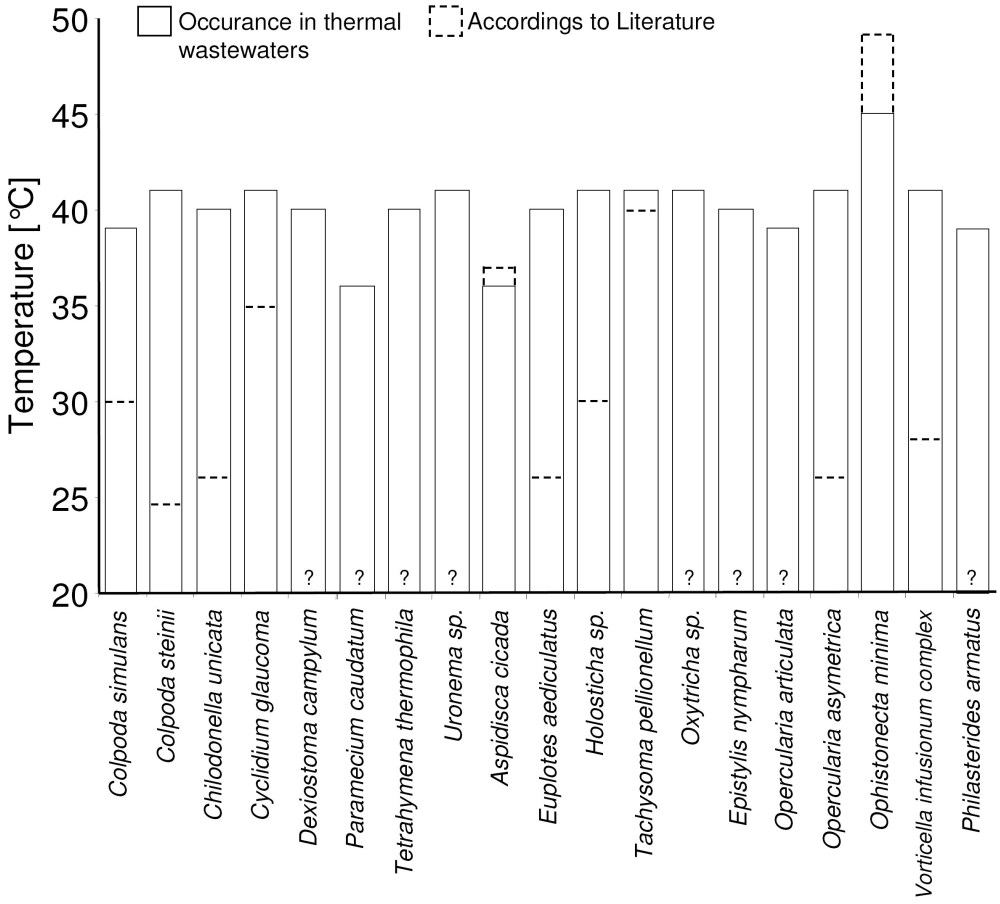

Figure 2: Comparison of heat tolerance between thermal wastewater and open freshwater habitats. The maximum tolerance published in literature is indicated by a horizontal dashed line. The absence of data on heat tolerance is indicated by "?".

C) Investigations of temperature tolerance of ciliate clonal cultures

For seven ciliate morphospecies, clonal cultures were established to determine temperature reaction norms and lethal temperatures. For three ciliate species, a comparison was possible with isolates from the River Rhine where temperatures do not exceed 28°C. The short-term survival (24 hours) at temperature ranges from 36 to 46°C varied for the different ciliate isolates (Figures 3-6). The *Paramecium* clone was the least resistant while *Ophistonecta* withstood a temperature increase up to 45°C. Most species isolated from thermal wastewater survived a temperature of up to 41°C. Long-term determinations of growth rates (Figures 3-6) indicated the ability of the ciliates to sustain their populations at high temperatures for a period of five days (Figure 7). Generally, long-term survival was estimated two to three degrees Celsius below the short-term survival. Isolates of the River Rhine showed on short- and long-term issues an average 8°C lower temperature tolerance

than isolates from thermal wastewater. The lethal temperature displayed in Table 3 indicated the same difference.

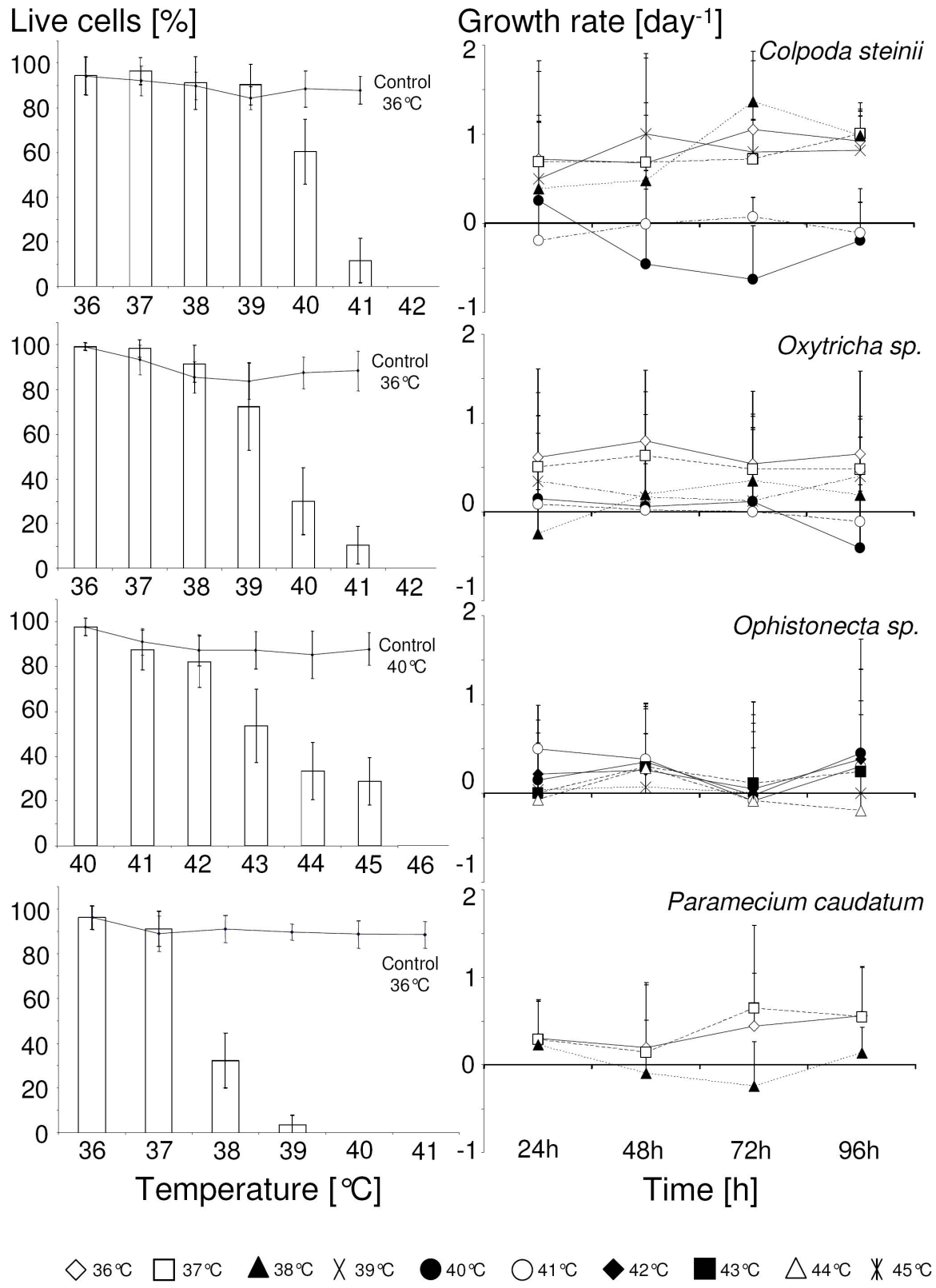

Figure 3: Survival and growth of ciliates isolated from thermal activated sludge, Zülpich. **Left:** Survival at different temperatures as percentage of the control. **Right:** Growth rates of isolates kept at the respective temperature for five days.

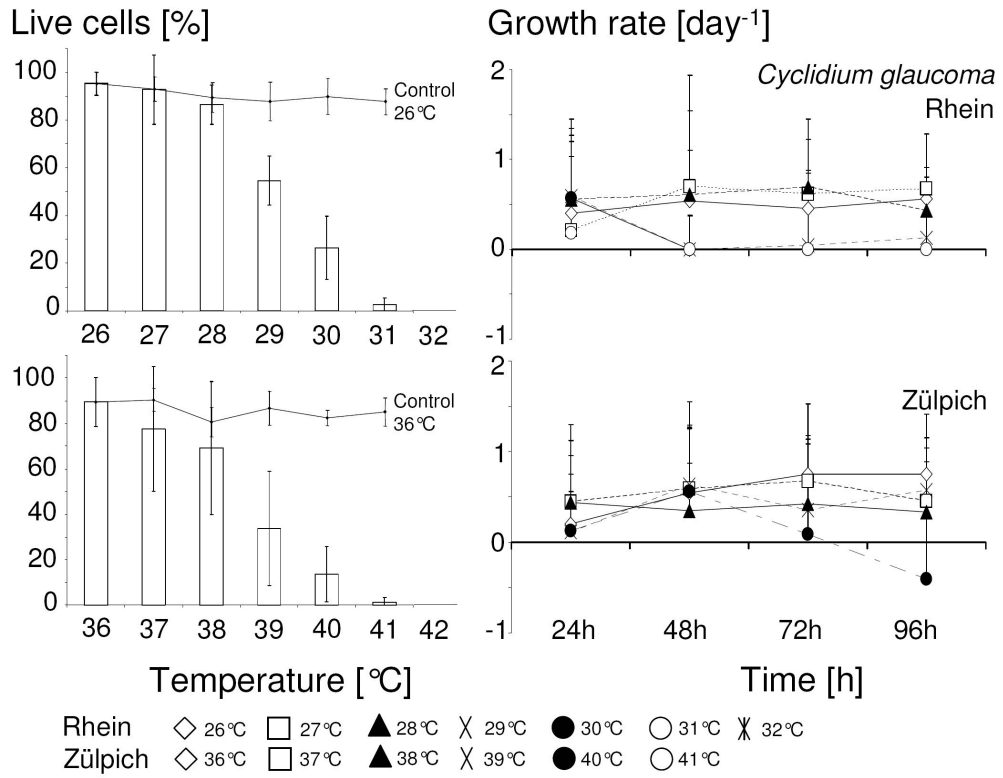

Figure 4: Survival and growth of *Cyclidium glaucoma* isolated from thermal activated sludge in Zülpich and from the R. Rhine. **Left:** Survival at different temperatures as percentage of the control. **Right:** Growth rates of isolates kept at the respective temperature for five days.

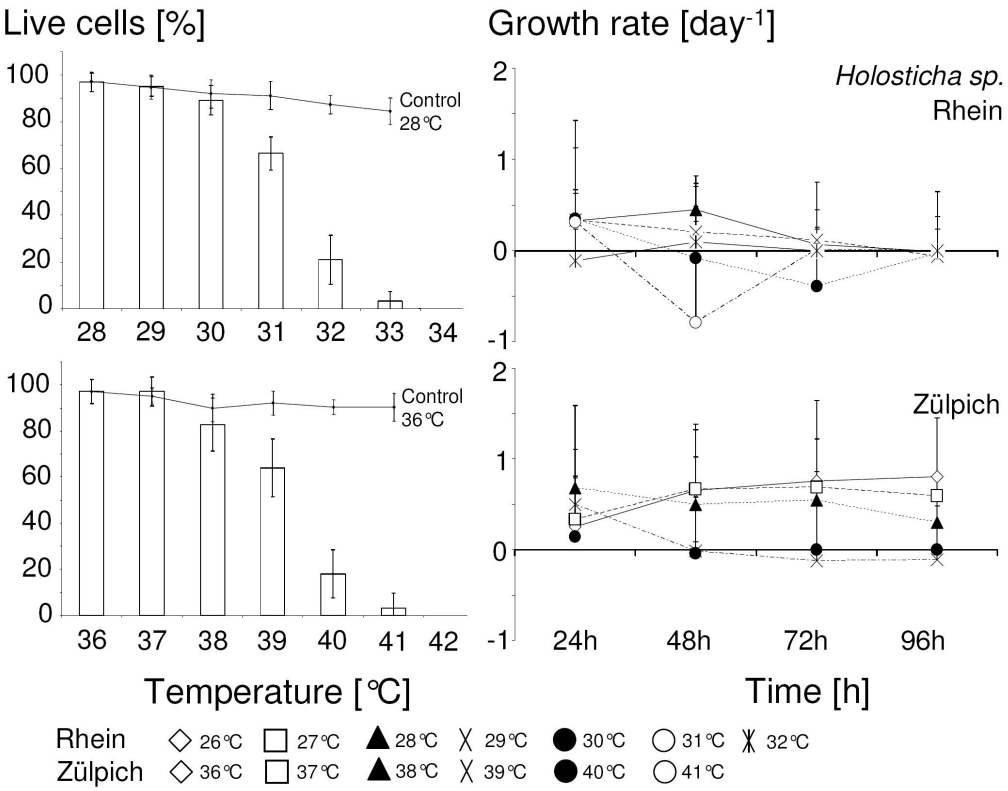

Figure 5: Survival and growth of *Holosticha sp.* isolated from thermal activated sludge in Zülpich and from the R. Rhine. **Left:** Survival at different temperatures as percentage of the control. **Right:** Growth rates of isolates kept at the respective temperature for five days.

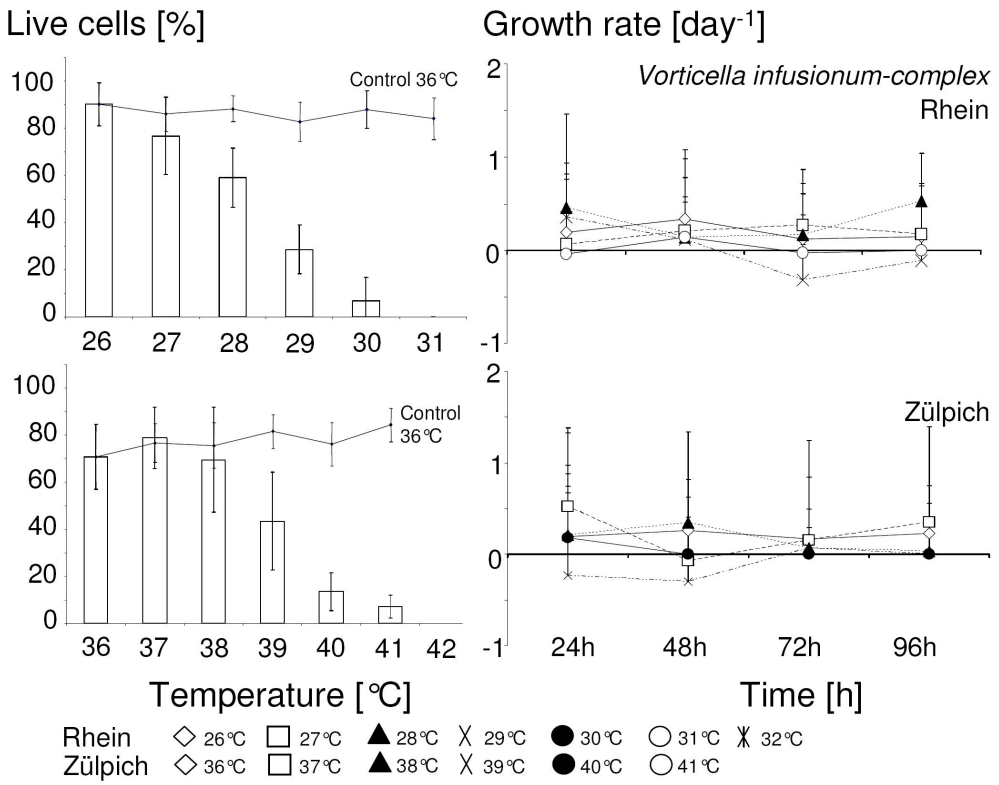

Figure 6: Survival and growth of *Vorticella infusionum* isolated from thermal activated sludge in Zülpich and from the R. Rhine. **Left:** Survival at different temperatures as percentage of the control. **Right:** Growth rates of isolates kept at the respective temperature for five days.

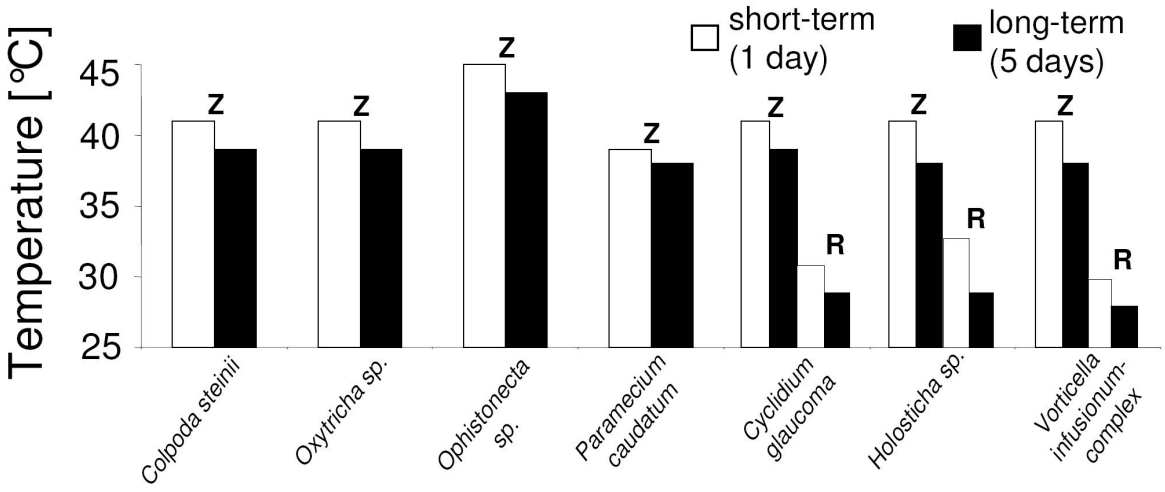

Figure 7: Comparison of short- and long-term temperature tolerance of ciliates isolated from thermal wastewater in Zülpich (Z) and from the River Rhine (R).

Table 3: Lethal temperature dose (LT50) for ciliate species isolated from thermal wastewater (Zülpich) and the River Rhine.

	Lethal temperature dose (LT50)			
	R. Rhein		Zülpich	
Morphospecies	LT50 [°C]	SD	LT50 [°C]	SD
Colpoda steinii	-	-	41.0	1.02
Ophistonecta sp.	-	-	41.0	0.70
Oxytricha sp.	-	-	37.6	1.95
Paramaecium caudatum	-	-	37.9	1.50
Cyclidium glaucoma	29.4	1.48	37.6	1.48
Holosticha sp.	30.2	2.06	37.0	1.03
Vorticella infusionum-complex	28.0	1.54	39.4	1.03

D) Molecular investigation for thermal ciliate isolates

A phylogenetic bootstrap analysis of the partial small sub unit (SSU) of rDNA was used to check whether heat adapted ciliate isolates from thermal wastewaters belonged to the same 18S-rDNA genotypes known from temperate regions. Three cultures were available for the study, *Cyclidium glaucoma*, *Ophistonecta minima* and *Oxytricha longa.* The analysis (Figure 8) revealed that all three strains were closely related (p-distances below 1%) to other genotypes available in genebank investigated from cultures of the temperate regions.

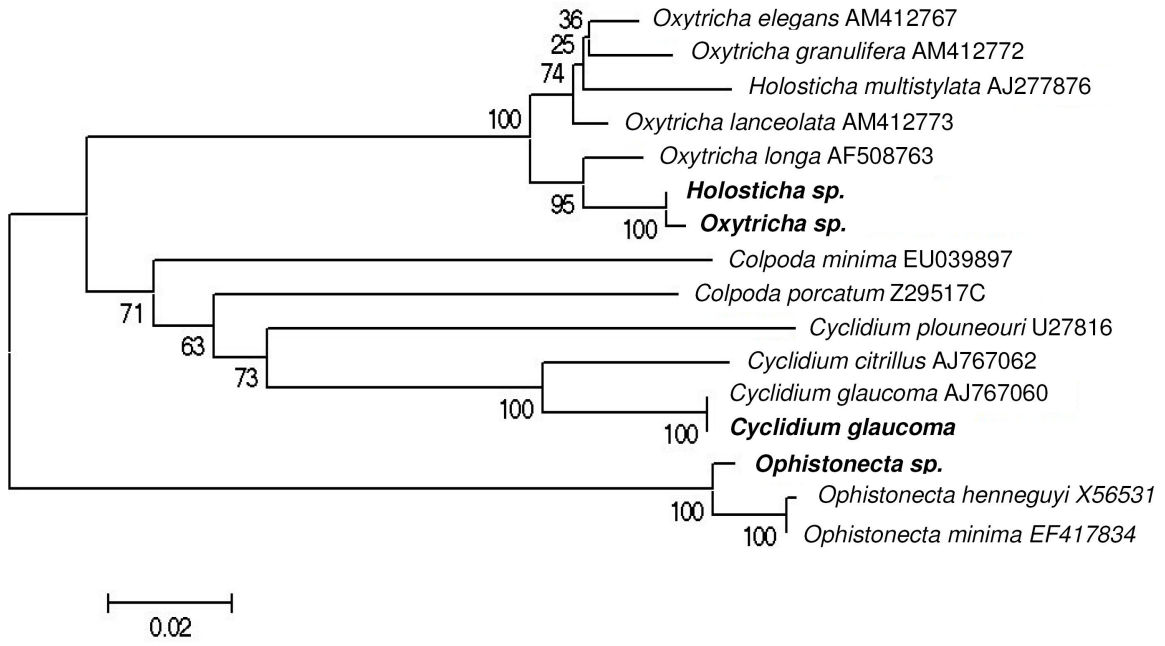

Figure 8: Phylogenetic bootstrap analysis (Neighbour Joining and Maximum Likelihood, JC69,100 replicates, numbers represent bootstrap values for NJ/ML) of partial SSU rRNA, Sequences from thermal wastewater (Zülpich) are shown bold.

DISCUSSION

1. Biodiversity

Only very few protists have been recorded from thermal habitats above 30°C (e.g. Atkins et al. 2002, Baumgartner et al. 2003, Brown and Wolfe 2006, Ketola et al. 2004). Our studies on the microfauna of heated activated sludge systems (40°C) revealed a much higher biodiversity among ciliate species than expected from literature reports. In all six paper mills, ciliates were found in high abundances (generally above 50 ind./ml). In addition, heterotrophic flagellates and various naked and testate amoebae were found in several activated sludge basins. Recorded ciliates belonged mainly to bacterivores and their presence should affect growth rates and morphology of bacterial communities (Feuersänger et al. 2008, Güde 1979, Jürgens and Matz 2002, Weitere and Arndt 2001). According to data acquired from literature (Foissner et al. 1991), some species were able to sustain much higher temperatures than previously reported. The similar morphology of ciliate isolates from natural and wastewater habitats suggested a close relationship between the populations which had to be tested by molecular studies.

2. Genotype and ecotype relationship

In order to support the morphological determination by video microscopy and protargol staining, partial SSU rDNA of *Cyclidium glaucoma*, *Ophistonecta minima* and *Oxytricha* sp. was sequenced. *Cyclidium glaucoma* was identical with strains isolated from temperate waters (Figure 8). The low genetic divergence (less then 2%) of *Ophistonecta* sp. and *Oxytricha longa* compared to isolates of temperate environments (Genbank) also indicates a close relationship to these genotypes. *Holosticha pullaster* was not yet sequenced for temperate waters. Further studies are necessary to investigate genes more related to heat tolerance ability (HSP synthesis, see below). Although the appearance of identical ciliate morphotypes recurring in natural water as well as in artificially heated wastewaters suggests an ubiquitous distribution (Finlay 2002), our data support the hypothesis that specific local adaptation may be common among cosmopolitan freshwater protists (Gächter and Weisse

2006, Weisse and Rammer 2006, Weisse et al. 2007). Some freshwater protists which reproduce predominantly asexually possess a (multi-) clonal population structure with globally divergent genotypes and phenotypes (Kusch et al. 2000). Thus, we suggest that the wastewater adapted ciliate morphospecies represent an intraspecific ecotype-variation. Our own previous studies of the heterotrophic flagellate *Histiona aroides* showed no morphological differences between isolates from Lake Schöhsee and those from heated wastewater at Zülpich. However, significant differences in the temperature tolerances of these two different clones were recorded (32°C Lake Schöhsee, 40°C Zülpich). Heated water isolates of *Histiona* did not show any reduction of temperature tolerance after long-term cultivation at room temperature. This may indicate that the temperature tolerance was genetically fixed. We assume that the ciliate isolates investigated in the present study also showed a genetically fixed heat adaptation. This has to be tested in future studies.

The fact that various ciliate, flagellate and amoebae taxa were able to survive in heated wastewater at temperatures up to 40°C lead to the assumption that temperature tolerance and long-term adaptation might be related to the synthesis of heat shock proteins (HSP). HSPs along with other chaperones are synthesised by the endoplasmatic reticulum in response to elevated temperature or other cellular stresses in protists (Germont et al. 1996, Hasegawa and Hashimoto 1999). For instance, HSP70 and HSP90 induce the functional folding and allow the implementation of functional proteins. By temporarily binding to hydrophobic residues exposed by stress, Hsp70 prevents these partially-denatured proteins from aggregating, and allows them to refold (Mayer and Bukau 2005).

3. Physiology: Short-term versus long-term temperature effects

Information about the lethal temperature dose (LT 50) for ciliate species is very rare. The temperature tolerance of thermal water isolates of *Cyclidium glaucoma*, *Holosticha pullaster* and *Vorticella infusionum*-complex significantly diverged from the freshwater isolates from River Rhine (Table 3). Decreasing growth rates at increasing temperatures might be related to the cost-intensive metabolic rate, which should for instance include the

synthesis of heat protecting proteins. In addition, there existed a temperature window of up to 3°C which comprised the difference between short-term (1 day) and long-term (5 days) temperature tolerance independent from the origin of ciliate isolates. We assume that this temperature frame lies within a general metabolic trade off. The correlation between temperature tolerance and membrane properties were studied for *Tetrahymena pyriformis* (Karsai et al. 1976) and *Paramecium aurelia* (Sasaki et al. 2006) describing the control of the membrane fluidity through the lipid metabolism and the desaturation of fatty acids. The membrane fluidity is regulated within the endoplasmatic reticulum. The temporary higher temperature tolerance of the ciliate species might be related to the increasing content of unsaturated fatty acids.

4. Conclusions

Our investigations of activated sludge in artificially heated wastewater of paper mills revealed a considerable number of protist species which are adapted to high temperatures. Thus, heat adapted protists could play the same important role for the flocculation of bacteria in heated activated sludge processes as it is known from moderately tempered basins (Güde 1979, Curds 1982). Bacteria, such as methanogens, which are commonly inoculated into thermal anaerobic wastewater treatments, have their optimal rate of metabolism at temperatures of about 50°C (Althöfer and Feuersänger 2005, Henze et al. 2002). The potentially high temperature tolerance of several protists would make them accessible to an additional thermal aerobic water treatment saving energy (Feuersänger et al. 2008). Further studies should be accompanied by more detailed molecular studies, especially regarding HSP70 and HSP90 chaperones, which have been proven to be a reliable marker for phylogenetic analyses (Budin and Philippe 1998, La Terza et al. 2001). Previous molecular investigations described the expression of HSP90 in response to varying temperatures for *Tetrahymena thermophila* (Ketola et al. 2004), where the synthesis is rapidly induced due to thermal stress.

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KAPITEL II

AUTECOLOGICAL COMPARISON OF JAKOBID MORPHOSPECIES (HETEROTROPHIC FLAGELLATES)-ISOLATES FROM NATURAL AND THERMAL WASTEWATER HABITATS AND THEIR PHYLOGENETIC RELATIONSHIP*

***** The molecular studies and the descriptive work of the investigated flagellate species were carried out by Dr. Nitsche.

ABSTRACT

Protists are commonly used in municipal sewage water treatment plants as bacterial consumers and flocculants. Very little is known about communities of heterotrophic flagellates (HNF) from wastewater treatment facilities. Morphologically indistinguishable HNFs were found in open water as well as in thermal wastewaters. Abundance and autecological characteristics of the morphospecies *Histiona aroides, Pseudohistiona africaniensis* and the assumingly new subspecies *Histiona aroides ?thermophilus* are subject to this study. Isolates from a lake, groundwater and thermal wastewater habitats were investigated regarding their tolerance to high temperatures. Jakobid isolates from thermal effluents of heated aerobe wastewater survived a pH – range of 6.8 - 7.5, high water hardness (- 220 mg/l Ca) and revealed a significantly higher temperature tolerance up to 40°C. Lake and groundwater isolates were only able to sustain a maximum temperature of 33°C for less than 24h.

According to recent results, jakobids are among the earliest protist groups diverging of eukaryotic lineages. They seem to be key organisms in the establishment of the mitochondrial symbiosis. The most bacterial-like mitrochondrial genomes were found in the group of Jakobida. Additionally we describe the morphology and molecular biology of *Histiona aroides ?thermophilus* using electron microscopy and an analysis of SSU rDNA. We also describe a new species and genus of Jakobidae isolated from South African groundwater, *Pseudohistiona africaniensis* gen. and sp. nov., which is a naked jakobid with a large ventral groove extending the length of the cell.

INTRODUCTION

Morphospecies are defined as organisms that look morphologically similar or identical. Presently it is discussed if the monophylum and the morphological characteristics reflect the present ecological niches occupied (Fenchel 1986, Finlay 2004). Selective factors that form the ecological niche, favour specific forms of mutations, which represent the adaptation and result in morphologically stable organisms (Fenchel 2001).

In detail, the temperature adaptation of clones from the same morphospecies can differ as previous studies on planktonic protist species showed (Weisse et al. 2001). Recent studies regarding the flagellate nanofauna (Arndt et al. 2003) and the reoccurrence of identical morphospecies (e.g. bodonids and euglenids) in various habitats, including deep sea samples from the Eastern Mediterranean Sea, supported the idea of either a vast spectrum of temperature and pressure tolerance, and/or a specific adaptation based for instance on the synthesis of essential proteins for temperature tolerance. Molecular investigations of *Ancyromonas sigmoides* and *Caecitellus parvulus* (Scheckenbach et al. 2005) pointed out, that the morphospecies concept might underestimate biodiversity and that the genetic variation within a morphospecies was surprisingly high.

For the autecological comparison we used jakobid morphospecies isolated from different natural and artificial habitats as model organisms to investigate temperature tolerance. *Histiona aroides* (Pascher 1942) was described as a colourless chrysophyte, occurring in mountain lakes of the Alps. The species was re-evaluated by Nicholls (1984) and later classified by O' Kelly (1993) as a member of the jakobids, characterized by a ventral feeding groove. *Histiona aroides* possesses an agile protoplast located in a colourless calyx formed theca. The protoplast is endued with a lip designed border and a singular flagellum. Protoplast and theca show multiple variations (Fig.1), while swarmers possess two flagellums.

The study of extreme habitats, such as the thermal effluents of heated activated sludge basins, offered the unique opportunity of finding cryptic species and to undertake

comparative autecological investigations to point out physiochemical differences between identical morphospecies. Although thermal wastewaters form an artificial habitat, previous studies revealed a high number of flagellate and ciliate taxa (Feuersänger et al. 2008, subm.), which also included *Histiona aroides ?thermophilus*. In this study we investigated the temperature response of 4 jakobid clones, isolated from natural freshwater habitats, including Lake Schöhsee (Plön, Germany), groundwater (Tokai, Capetown, RSA) and compared them with clones from artificially heated wastewaters (activated sludge basins, Zülpich and Düren, Germany). The established cultures contained *Histiona aroides* (Lake Schöhsee), *Pseudohistiona africaniensis* (Tokai) and *Histiona aroides ?thermophilus* (Düren and Zülpich). We determined the content of living cells and the growth rates during a stepwise temperature increase and identified short-term (24 hours) and long-term (5 days) temperature tolerances for each temperature level.

If specific local adaptation between the same morphospecies is found, it would support the hypothesis that genetic adaptation can occur over a short amount of time, since the activated sludge basins are running for only 10 to 15 years and are constantly supplied by freshwater.

Within the group of jakobids there are three families characterised: Histionidae (*Histiona*, Voight 1901; *Reclimonas*, Flavin and Nerad 1993), Jakobidae (*Jakoba*, Patterson 1990; *Andalucia*, Lara et al. 2006; *Seculamona*, Marx et al. 2003) and Malawimonadidae (*Malawiimonas*, O`Kelly and Nerad 1993). The group probably also includes the taxa *Stenocodon* and *Stomatochone* (Flavin and Nerad 1993, Patterson et al. 2002). Together with other groups including dipolomonads, retordamonads, heteroloposeids, and the taxa *Trimastix* and *Carpediemonas* they have been informally named "excavate taxa" (Simpson and Paterson 1990).

Recent results indicated that jakobids are among the earliest diverging of eukaryotic lineages (Edgcombe et al. 2001). Their utrastructural similarity to the retortamonads and, indirectly, diplomonads lead to the conclusion, that they were key organism in establishment

of the mitochondrial symbiosis. Preserved ancestral bacterial features that are not present in any other eukaryotes were found within the mitochondrial genomes of *Reclinomonas americana* and *Jakoba libera* (Lang et al. 1997). The most bacterial-like mitrochondrial genomes were found in the group of Jakobida. It retains more protein coding genes compared to those of other eukaryotes (Gray et al. 1999; 2004).

We isolated four strains of common jakobid genera *Histiona* from the surface waters of a German lake (Auer and Arndt 2001) and thermal wastewaters and we identified a new genus of the group from African groundwater. The characterisation of these isolates offered us the opportunity to study the morphological and phylogenetic relationships among jakobids in more detail. Up to our knowledge no member of jakobids has yet been described from thermal environments or groundwater.

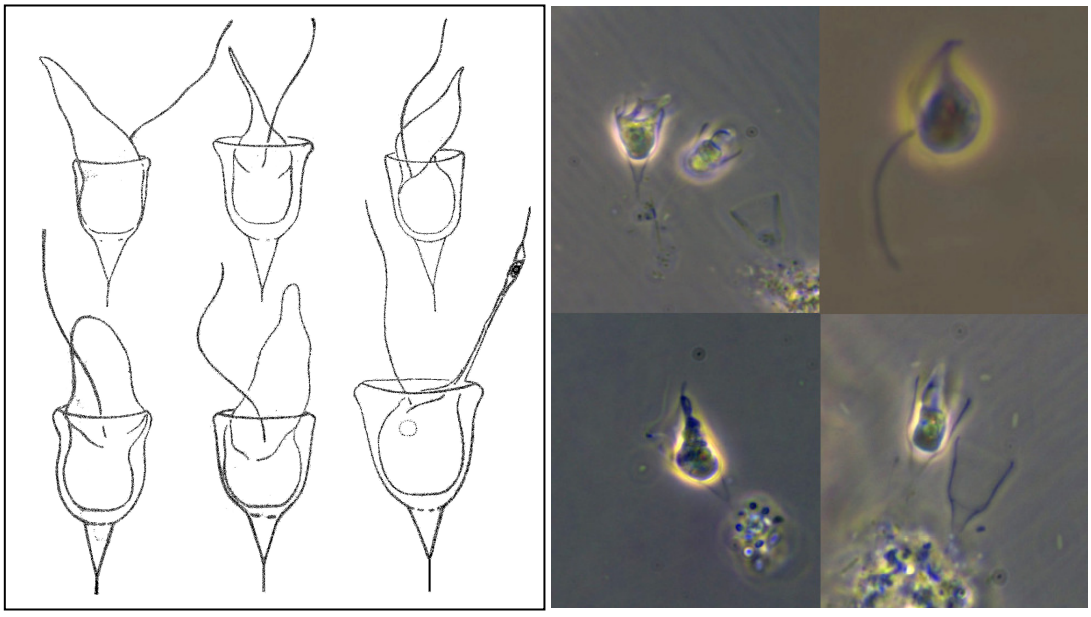

Figure 1: *Histiona aroides*. **Left:** Morphology of *Histiona aroides*, variation of lorica and ventral groove (Pascher, 1942). **Right:** Light-microscopical images of living cells. ***Upper left:*** *Histiona aroides* (Lake Schöhsee, Plön, Germany); ***upper right:*** *Pseudohistiona africanensis* (Groundwater, Tokai, Cape Town, Republic of South Africa), ***lower left:*** *Histiona aroides ?thermophilus* (Activated sludge, Düren, Germany); ***lower right:*** *Histiona aroides thermophila* (Activated sludge, Zülpich, Germany)

MATERIAL AND METHODS

Sampling sites. Samples of *Histiona aroides ?thermophilus* were isolated from activated sludge basins of paper mills in Zülpich and Düren, Germany. In addition to thermophile waste water isolates, cultures of *Histiona aroides* from Lake Schöhsee, Plön, Germany (HFCC 86) and *Pseudohistiona africaniensis* gen. and sp. nov. from groundwater (18°C, 12m depth) in Tokai (Cape Town), South Africa, were studied. The study showed that ecological parameters fluctuated within the activated sludge basins (Average values displayed in table 1). Before entering the activated sludge process, the wastewater passed the clearwater tank, where large debris was separated by various racks before proceeding to the acidulation. The acidulation prepared the water for further anaerobe bacterial treatment through nutrition supply, nitrogen and phosphorous. Additionally acidification bacteria eliminated calcium from recovered paper residues and transferred it into calcium hydrogen carbonate. Remaining carburetted compounds were converted by the anaerobe bacteria *Methanosarcina*, which leaves Methane as a sideline product (Althöfer 1999). The optimum temperature of operation was determined at 50°C (Henze and Harremoes 1983), which, after passing through the decalcification process, lead to the hydrothermal environment, the activated sludge basin (36-40°C).

Light microscopy. Samples were studied using a 100x water-immersion objective mounted on Zeiss Axioplan 200 Microscope.

Electron microscopy. Samples remained in the culture flask and were fixed using a 2% osmium tetroxide solution for 5 minutes followed by a dehydration series of ethanol with 30%, 50%, 60%, 70%, 80%, 90%, 96% and pure ethanol (each step was done three times and lasted 10 minutes) was carried out. After this procedure, a 50:50 hexamethyldisilazane (HMDS)-ethanol solution was applied for 30 minutes followed by pure HMDS for 30 minutes. Afterwards, the samples were allowed to dry. The bottom of the flasks was cut to appropriate size and stuck to a sample holder. SEM samples were sputtered with a 120Å layer of gold before examination by SEM (Hitachi S-520).

Autecological investigation. Clonal cultures of *Histiona aroides* were established from Lake Schöhsee (Plön, Germany), while clonal cultures of *Histiona aroides thermophilus* and *Pseudohistiona africanensis* were established from the thermal activated sludge basins of the paper mills (Zülpich, Düren) and the ground water of Tokai, RSA. The cultures were established using the liquid aliquot method (LAM). Isolated cells were cultivated 50ml-tissue–culture flasks in WC-medium (Guillard and Lorenzen 1972) supplied with a wheat grain as a carbon source for bacteria. The temperature effect on growth rates and mortality was investigated for a temperature range from 36 to 42°C for isolates from activated sludge and for a temperature range from 28 to 33°C for isolates from Lake Schöhsee and groundwater. Fresh cultures in tissue culture flasks were inoculated 10 days before the experiment. Tissue culture flasks (50ml, 10 replicates) were exposed in tempered water basins (20l-basins with adiabatic temperature gradient blocks, 2 level inverter GIR 2000 Pt (Conrad, Germany), accuracy $\pm$0.1°C). The temperature level was increased 1°C every 24 hours and cells were repeatedly counted on a previously marked square footage of 20mm² under an inverted microscope. For the live count the beat of the flagellum was determined as live sign. As a control to the stepwise temperature increase, 10 replicate flasks for each temperature level were maintained at the respective temperature for additional 5 days with inspections of every 24 hours. These latter flasks were used to determine the growth rates at the respective temperature. Constant growth rates within the first five days indicated that experiments were conducted at the exponential growth phase. The maximum tolerable temperature and the tolerable temperature at which growth rates were above zero for five days were determined. Growth rates were estimated assuming a linear growth development according to the following equation:

$\mu = (\ln Nt - \ln N0) / (t1-t0);$

N0: Start-up-population at time 0, Nt: Population at the current time frame, t0: Start-up-time, t1: specific time frame.

The lethal dose (LT50) defines the temperature, at which 50% of the tested cells died. The calculation is done according to the predefined regression of the values (surviving cells in percent).

Molecular biology. For each culture a DNA extraction was done. The cultured isolates were grown to high densities (10^4 cells · ml^1) and harvested by centrifugation in 50-ml tubes (Sarstedt) for 30 min at 4,000 *g* and 4°C. Each supernatant was discarded, and the cells were resuspended in 50 µl 1x Tris-EDTA buffer and transferred into PCR tubes. The collected cells were lysed and the genomic DNA was isolated using a cetyltrimethylammonium extraction method with phenol and chloroform (see Clark 1992). Amplification of the small-subunit (SSU) rRNA gene by PCR was performed in a 50µl reaction mixture containing each primer at a concentration of 0.1 µM, each desoxynucleoside triphosphate at a concentration of 200 µM, up to 100 ng genomic DNA, 2 mM $MgCl_2$, 1x reaction buffer, and 1 U AmpliTaq DNA polymerase (Applied Biosystems). General eukaryotic PCR primers (42F and 18SRev-1) were used for amplification of the SSU. The PCR products were purified using the E.Z.N.A. Cycle-Pure-Kit (Peqlab, Erlangen, Germany). The sequencing of rDNA was done using Big Dye-Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) in accordance with the manufacturer's instructions. Primers used for sequencing were 18SFor, 590F, 1280F, 600R, 1300R and 18SRev for the SSU.

Phylogenetic analyses. Alignments with other jakobid SSU rDNA sequences available from GenBank were carried out using ClustalX (Thompson et al. 1997) and corrections were made manually with BioEdit. The model (JC69) for maximum likelihood analysis was determined by MrAIC (Nylander 2004) and the ML analysis computed by PhyML (Guindon and Gascuel 2003), using 100 replicates for the bootstrap analysis. Neighbour joining (NJ) was calculated using MEGA 3.1 (Kumar et al. 2004) using the JC model and 100 replicates for bootstrap analysis.

RESULTS

1. Habitat characteristics.

The water purification process resulted in a pH-level between 7.8 and 8.0, an oxygen content between 1.3 and 2.0 mg/l, a calcium content of 220 – 310 mg/l, a conductivity of 1160 – 1208 µS and a COD ranging from 1100 to 2000 mg/l in the activated sludge basin. The large size of 2880 m^3, variable flow-rates estimated at 160m^3/h and a dwell period of 18h enabled different thermal layering. The average temperature was determined at 36-40°C, but due to being open to the atmosphere, climate variability is very likely. The ecological characteristics the isolates were taken from are displayed in Table 1.

Table 1: Ecological characteristics of the four different habitats. Average values of ecological conditions the isolates were taken from.

Origin of isolates	Temperature [°C]	Content O_2 [mg*l^{-1}]	pH-value	Conductivity [µS[	Content Ca [mg*l^{-1}]	Chemical Oxygen Demand [mg*l^{-1}]
Plön, GER	5	>12	8,6	239	unknown	unknown-
Tokai, RSA	15	>8	unknown	unknown	unknown	unknown
Düren, GER	40	1,3	8	1160	220	1100
Zülpich, GER	36	2	7,8	1208	310	2000

2. Physiology: Short-term versus long-term temperature effects

The temperatures comprised a wide spectrum from 5°C to 25°C for natural habitats and 20°C to 40°C for thermal wastewaters. The control cultures of *Histiona aroides* (HFCC86) and *Pseudohistiona africaniensis* were maintained at 28°C for 6 days and estimated a stable average survival rate of 82.9% (*Histiona*) and 85,3% (*Pseudohistiona*) (Fig. 2, left). For *Histiona aroides* the temperature increase resulted in increase of mortatility, the content of living cells decreased to 55% at 30°C, 15% at 32°C and led to extinction at 33°C (Fig.2, left). *Pseudohistiona africaniensis* the impact of the temperature increase proved to be almost linear and resulted in a decrease of the "live"-contingent to 0.6% living

cells at 32°C (Fig.2, left). Isolates from Zülpich and Düren sustained at solid 87.82% and 75.92% at 36°C for six days (Fig.2, left). Sudden impacts took place at 39°C (Düren: 39.3%; Zülpich: 38.4%) and 40°C (Düren: 13.2%; Zülpich: 8.5%). 41°C showed no surviving cells within the Zülpich isolates, while 0.4% of the Düren originated live cells persisted for 24 hours.

Estimated growth rates were determined by using an equation, founded on linear population expansion. The experiments comprised 96 hours, containing 4 equal temporal windows of 24 hours. HFCC 86 – Cultures indicated stable average growth - rates between 0.958 and 0.854 up to 30°C, while at 31°C the population kept expanding for merely 72 hours (Fig.2, right). The ground – water isolates (Fig.2, right) showed stable growth at an average of 0.566 at 28°C, but it only maintained stable at 29°C, between 0.496 and 0.699, for 72 hours. After 96 hours it approached close to zero. At 30°C it dropped from 0.399 to -0.464 within the first 48 hours. At a temperature level of 31°C the isolates did show a growth – rate of 0.125, but which was swiftly alleviated to zero after 72 hours. Temperatures beyond were not sustainable for the ground – water isolates. The wastewater isolates from Düren (Fig.2, right) and Zülpich (Fig.2, right) revealed stable growth – rates up to 38°C, located between 0.376 and 0.624 for Düren and between 0.21 and 0.882 for Zülpich. 39°C proved to be sustainable for the Düren – isolates for 24 hours (0.777) while the Zülpich isolates lasted 24 hours longer (0.566 at 24 hours and 0.726 at 48 hours). At 40°C both cultures displayed marginal, fluctuating growth – rates allocated around level zero. Higher temperatures indicated no further population growth. The short-term temperature tolerance (1 day) for each isolate was estimated by the maximum tolerable temperature, while the long-term temperature tolerance (5 days) was estimated by stable positive growth rates (Fig. 2).

The determination of the lethal temperature dose (LT50) revealed a significantly, approximately 10°C higher LT50 for wastewater isolates (Tab. 3). Independent from the

origin of the isolates, the growth rates showed a temporary temperature tolerance range, which comprised 3°C for the natural and 2°C for the artificial wastewater habitats.

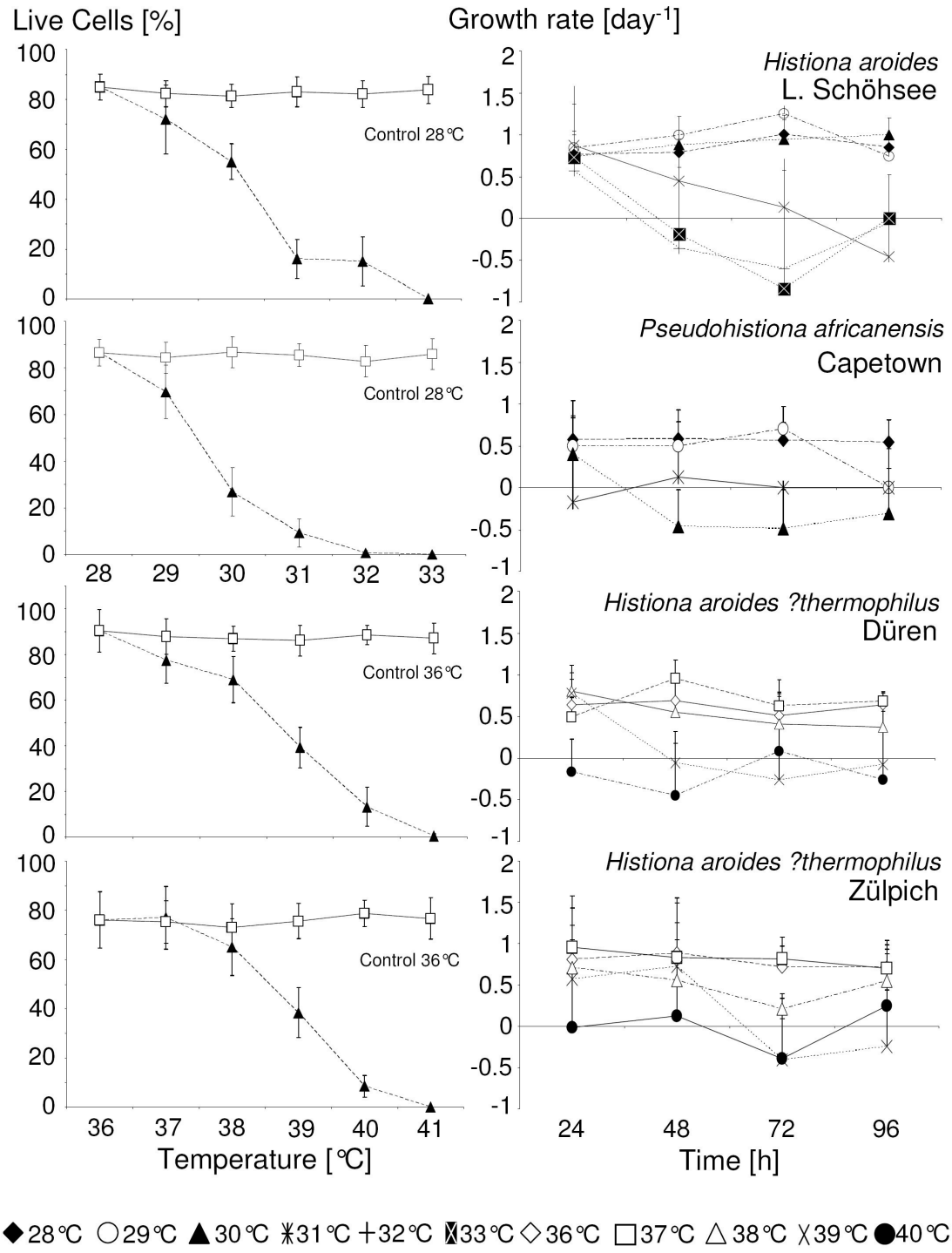

Figure 2: Left, count of living cells of *Histiona aroides, Pseudihistiona africanensis* and *Histiona aroides ?thermophilus*. from Lake Schöhsee, from groundwater, Tokai RSA, from activated sludge, Düren GER and from activated sludge, Zülpich GER. Temperature was increased 1°C/24h. **Right,** growth rates from *Histiona aroides*, *Pseudohistiona africaniensis*, *Histiona aroides ?thermophilus*.

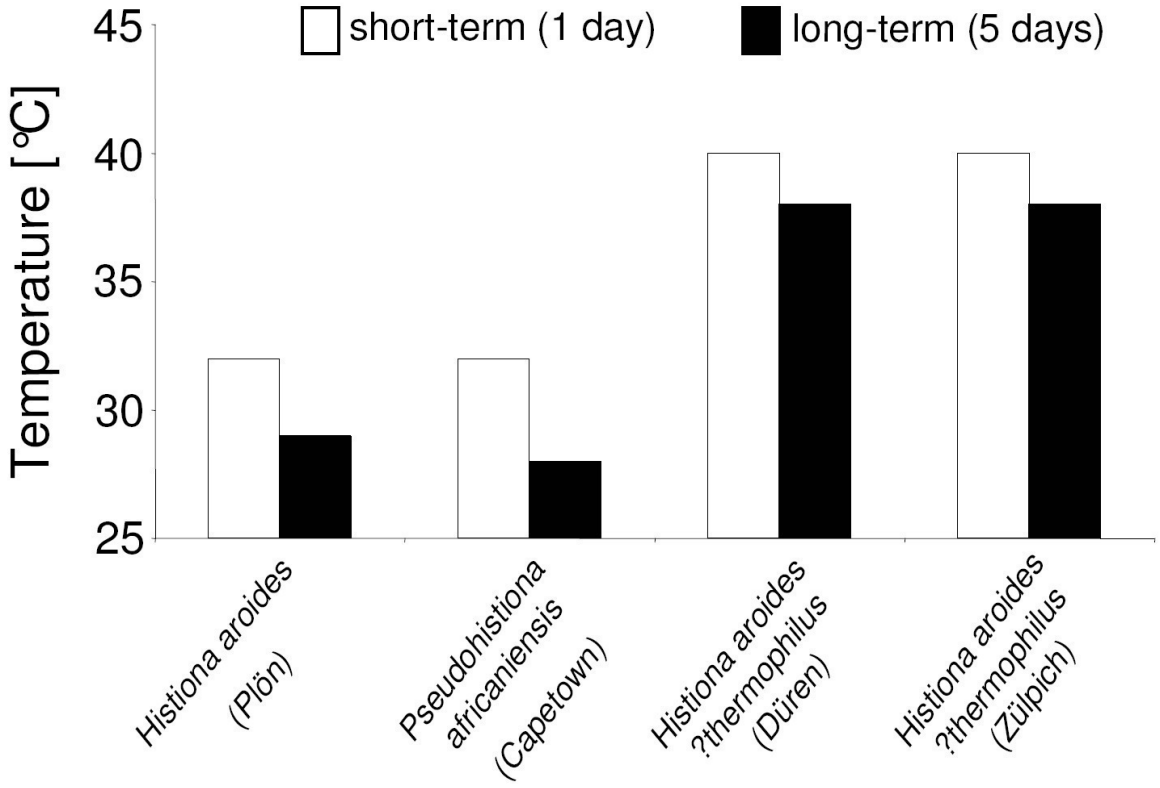

Figure 3: Comparison of short- and long-term temperature tolerance of jakobid isolates from natural habitats (Plön, Capetown) and thermal wastewater (Düren, Zülpich).

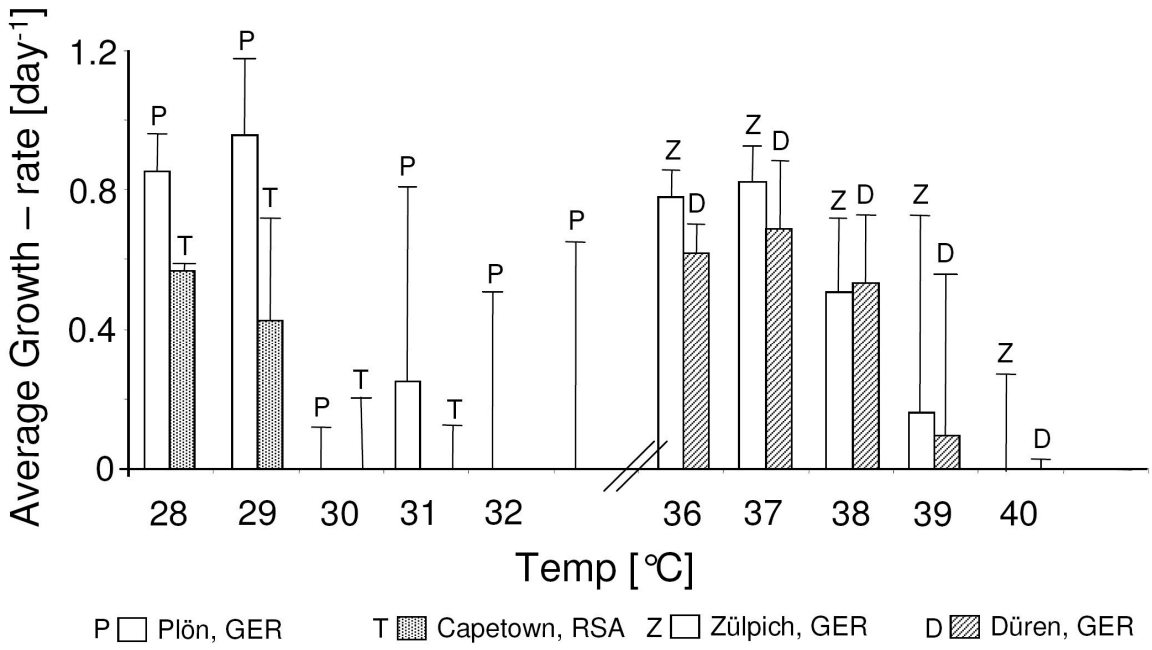

Figure 4: Comparison of Average Growth – rates. *Histiona aroides* (Plön, GER), *Pseudohistiona africaniensis* (Capetown, RSA), *Histiona aroides ?thermophilus* (Düren, Zülpich, GER). Negative growth rates neglected, except for positive SD.

Table 2: Average Growth – rates at each temperature level, rounded off to third decimal place. *Histiona aroides* (Plön, GER), *Pseudohistiona africaniensis* (Capetown, RSA), *Histiona aroides ?thermophilus* (Düren, Zülpich, GER).

	Temperature [°C]					
Origin of isolates	28	29	30	31	32	33
Plön, GER	0,854	0,958	0,890	0,25	- 0,115	- 0,076
Capetown, RSA	0,567	0,422	- 0,216	- 0,01	-	-
	36	37	38	39	40	41
Düren, GER	0,62	0,689	0,534	0,095	- 0,198	0
Zülpich, GER	0,779	0,824	0,506	0,161	- 0,009	0

Table 3: Lethal temperature dose (LT50) for jakobid morphospecies isolated from natural habitats (Plön/GER, Capetown/RSA) and from thermal wastewater (Düren/GER, Zülpich/GER).

	Lethal temperature dose (LT50)			
	Natural habitats		Artificially heated activated sludge basin	
Morphospecies	LT50 [°C]	SD	LT50 [°C]	SD
Histiona aroides (Plön)	30.3	1.64	-	-
Pseudohistiona africaniensis (Capetown))	30,4	1.12	-	-
Histiona aroides ?thermophilus (Düren)	-	-	39,7	1.40
Histiona aroides ?thermophilus (Zülpich)	-	-	39,7	1.34

3. Molecular biology and phylogenetic analysis

Morphological description of thermotolerant form of *Histiona aroides (?thermophilus)*

Diagnosis: Protist morphologically barely distinguishable from *Histiona aroides* except from a distinct variation in size (Tab. 4). The species specific stalked lorica measuring 5-19µm in diameter and 8-17µ in height, stalk 6-20µm long, was present as well as swarmers and cysts (Fig. 5). The ventral groove covered about two third of the protoplast which measured 3-6µm in length and 2-4µm in width. The flagella insert at the head of the groove. There are two flagella, one lying in the groove and one curving outwards (7-20µm long) from the point of insertion. The status as a subspecies was based on differences found in the SSU rDNA (Fig. 6) and autecological data (Feuersänger et al. subm.).

Etymology: *thermophilus* from Latin in reference to the origin from thermal wastewater.

Type location: Paper mills Smurfit Kappa Zülpich Paper (40°C) and Schoellershammer Düren (36°C) (February and March, 2007).

Holotype: The illustration of the specimen in Figure 1.

SSU rRNA: Partial SSU rRNA fragments were used for a FASTA search. The result assigned *Histiona aroides thermophilus* into the order of Jakobidae.

Description: The description of *Histiona aroides thermophilus* is based on the observation of cells using light and scanning electron microscopy. Cells are measuring 3-6µm in length and 2-4µm in width. The characteristic lorica sized 5-19µm in diameter and 8-17µ in hight, stalk 6-20µm. The shape of the lorica varies broadly as the form of the protoplast (Fig. 1, Fig. 5). The flagellum positioned in the groove is about half the length of the one curving outside (7-20µm). Swarmers are ovoid elongated with two prominent flagella measuring about the same size as the stalked forms. Cysts are ovoid (same size as stalked forms) with a significant protuberance on top of the cyst.

Tab. 4: Variation in size *of Histiona aroides* (Plön) and *Histiona aroides ?thermophilus* (Düren, Zülpich)

	Protoplast		**Shaft**	**Flagellum**	**Groove**		**Theka**	
Origin	*height*	*width*	*length*	*length*	*length*	*width*	*length*	*width*
Plön	4.9	3.08	8.45	14	5.38	2.83	11.6	9.85
	(±1.09)	(±0.60)	(±1.56)	(±4.32)	(±2.83)	(±0.80)	(±2.40)	(±1.68)
Düren	3.9	2.6	8.15	11.9	3.9	2.5	9.85	7.9
	(±0.60)	(±0.44)	(±1.65)	(±2.93)	(±1.09)	(±0.45)	(±2.00)	(±1.14)
Zülpich	4.25	3.05	9.5	13.25	4.2	2.35	11.65	9.9
	(±0.89)	(±0.22)	(±3.85)	(±3.3)	(±1.20)	(±0.55)	(±1.68)	(±2.59)

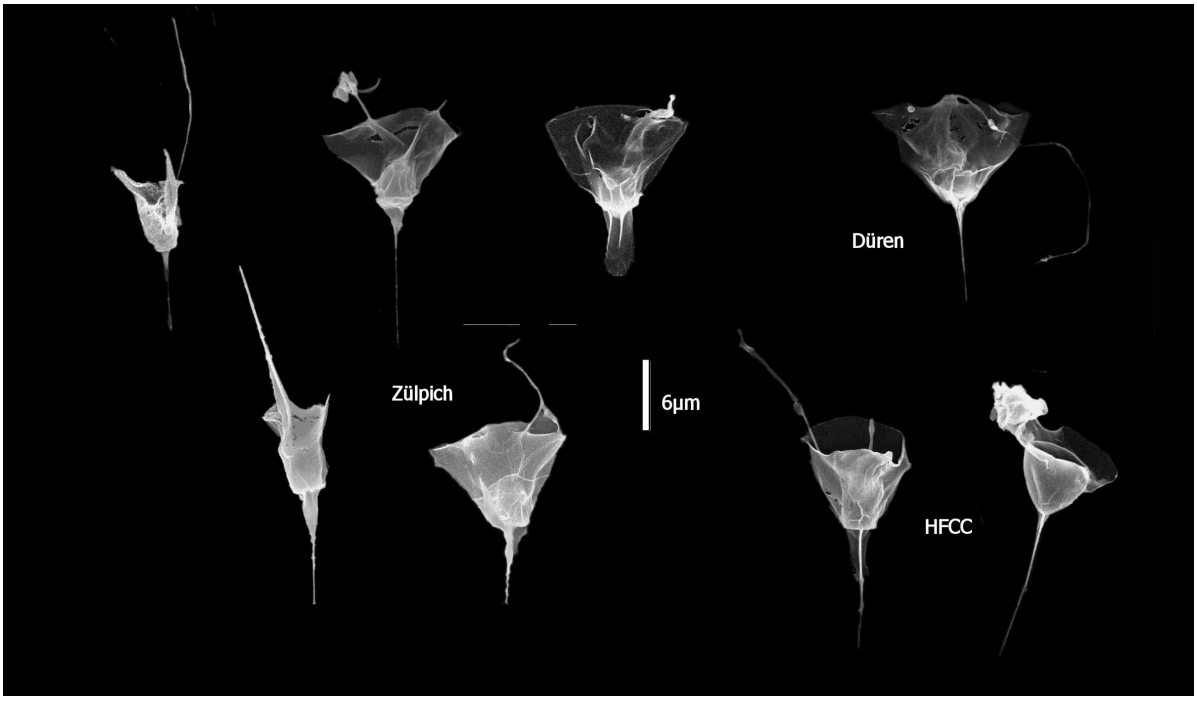

Figure 5: Electron-microscopical images of Histiona aroides (Lake Schöhsee, Plön) and *Histiona aroides ?thermophilus* (Activated Sludge, Düren and Zülpich)

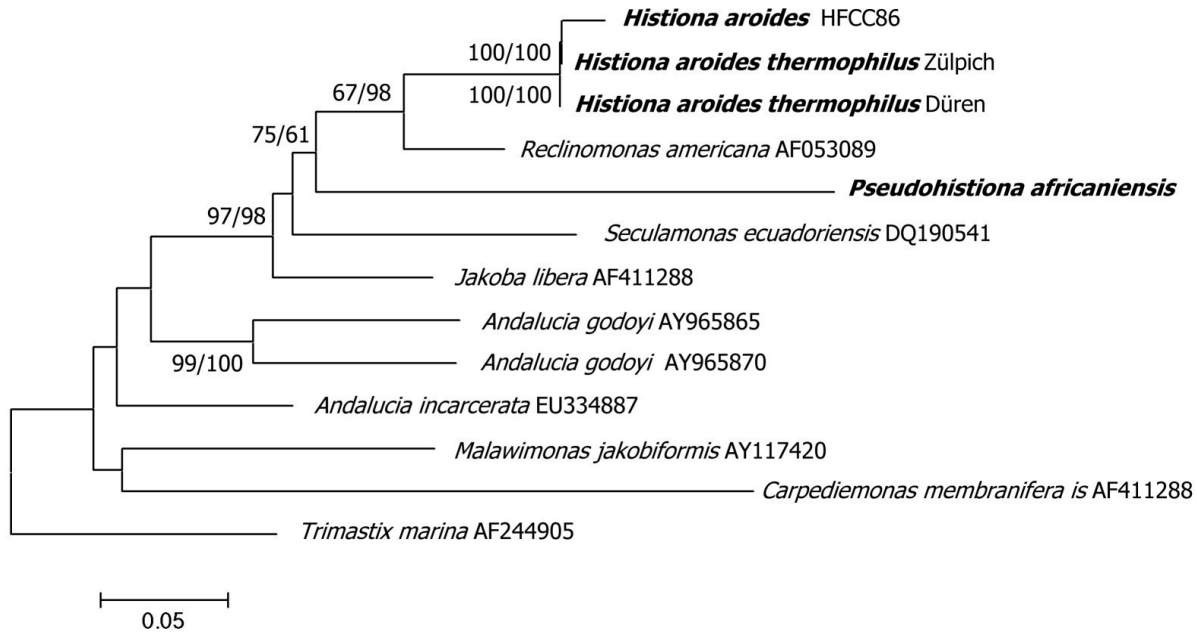

Figure 6: Genealogical tree according to the maximum likelihood analysis. Scale represents exchange of base pairs in percent.

DISCUSSION

Biodiversity

Based on a previous study of the microfauna of hydrothermal activated sludge systems, running at high temperatures (40°C) (Feuersänger et al. subm.) this work investigated temperature effects on survival rate and growth rate of jakobid morphospecies. Our studies revealed significant differences in short-term (1 day, Fig. 2 left) and long-term (5 days, Fig. 2 right) temperature tolerances between the isolates of natural habitats and thermal wastewaters. Whilst the cultures originating from the activated sludge basins showed a tolerance to up to 41°C, the lake and groundwater isolates were only able to sustain a maximum of 31°C.

Short-term versus long-term temperature effects

The decrease of living cells from the thermophile cultures (37-41°C), clearly diverged from the freshwater samples (Short-term: 32°C for *Histiona aroides*, respectively 30°C for *Pseudohistiona africaniensis*). To avoid temperature changes within one generation and to create statistically relevant data, each time period was defined 24 hours. According to the control cultures maintained at 28°C and 36°C and the appending average growth rates, we estimated the generation time distinct below 24 hours. While the freshwater samples showed stable growth rates up to 30°C (respectively 29°C for *Pseudohistiona africaniensis*) for a maximum of 96 hours, 31°C proved to be sustainable for 72 hours. Temperature above 31°C was lethal within the first 24 hours. Zülpich and Düren revealed stable positive growth rates till 38°C, 39°C was tolerable for 48 (Zülpich) and 24 (Düren) hours. The experiments, including growth rates at high temperatures, revealed for all isolates a difference of up to 3°C between short-term (1 day) and long-term (5 days) temperature tolerance. We assume this range reflects a general metabolic trade-off. According to our assumption, reduced reproduction might be related to the cost-intensive metabolic rate, which should include the synthesis of heat protecting proteins. Except for the pathogene *Trypanosoma brucei*, which is able to sustain temperatures beyond 40°C, data on heterotrophic flagellates regarding the

synthesis of HSP is very rare. In *Trypanosoma brucei* HSP 70 appears in a permanently activated state, and transcriptional induction of HSP 70 genes by heat shock does not occur (Lee 1998).

The temperature acclimation based on membrane fluidity control through the lipid metabolism and the desaturation of fatty acids was studied on the ciliate *Tetrahymena pyriformis* (Karsai et al, 1976). This study showed that membrane fluidity is a self-regulating mechanism, which is based on the content of desaturase molecules. Due to the slower metabolic rate at lower temperatures, for instance at 15°C, there is a much higher unsaturation (Karsai et al, 1976). The investigation of the total fatty acid content of *Thermophilus aquaticus* pointed out a significant increase according to the temperature raise (Nordström et al, 1992). Therefore the temporary higher temperature tolerance of 2°C above the LT50 of the jakobids (tab. 3) might be related to the total fatty acids as well and in conclusion relies on the lipid metabolism.

Molecular investigation and phylogenetic investigation

Our study adds another missing piece of information by presenting the four new 18S rDNA sequences. The basal systematic was confirmed by our additional sequences. Results from different studies often show conflicts between morphological and molecular phylogenetic analysis (Arisue et al. 2005; Simpson et al. 2006). These conflicts were not confirmed in our study as all members of the jakobids clustered together. Structural data suggest that *Malawimonas* is similar to jakobids (Simpson 2003), but it does not branch directly with jakobids in many phylogenetic analyses. The structural similarities with *Trimastix* and recent molecular data indicate a close relationship with *Trimastix* (Dacks et al. 2001). Therefore *Trimastix marina* was chosen as outgroup. The new species, *Pseudohistiona africaniensis,* branched clearly between *Reclinomonas* and *Seculamonas* away from the other Histionidae. Due to its similarity to *Histiona* it was called *Pseudohistiona*, though it lacks a lorica. Morphologically, it differed clearly from *Seculamonas* and *Reclinomonas* and therefore the new genus was established.

Histiona aroides ?thermophilus differed in its SSU only slightly from *Histiona aroides* but the high thermoresistance compared to the other *Histiona* strain gave reason to assign it as a new subspecies. Cultures showed the high thermotolerance (36-40°C) even when being cultivated for more than 100 generations at 10°C. So we concluded that the high thermotolerance was genetically fixed (Feuersänger et al. subm.). Further studies of other marker genes like HSP 90, alpha and beta Tubulin, EF1 and LSU would be necessary to resolve the phylogenetic tree even to a higher, more certain level.

Conclusions

The similar morphology of *Histiona* morphospecies between natural and heated artificial habitats supported the thesis that a distribution occurred either through air, or fresh water supply by nearby rivers. We hypothesise that the adaption of jakobid individuals to high temperatures was boosted by the high abundance within the freshwater source during the initiation of the basins and the inhomogenous temperature distribution. The size of 2880 m^3 and a dwell period of 18h also supported the formation of different thermal layers. Additionally the basin was exposed to climatic influences, so we expected a correlation between seasonal temperature fluctuations and the basin temperature. These factors allowed emersion and establishment of mutations among many generations and long-term adaptations. Although we could not find a genetic variation in the SSU of the rDNA, it was likely that a genetic variation on a different genome, for example HSP70 or HSP90, will be found in the future. Although the identical morphological appearance suggests an ubiquitous distribution (Finlay 2002), for instance through air, we can support the thesis that specific local adaptation is common among cosmopolitan freshwater protists (Gächter and Weisse 2006, Weisse and Rammer 2006). Therefore biodiversity, like already proven for ciliate species by Weisse and Rammer (2006), should not be assessed in numbers of morpho-species (Finlay and Fenchel 2004). If future molecular investigation identifies genetic differentiations between the clones, we suggest extending this concept to the jakobid flagellates.

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