DIVERSITY OF PROTISTS

WITH SPECIAL EMPHASIS ON CHRYSOMONADS:

MORPHOLOGICAL AND MOLECULAR DIVERSITY, DISTRIBUTION PATTERNS AND FUNCTIONAL DIFFERENTIATION

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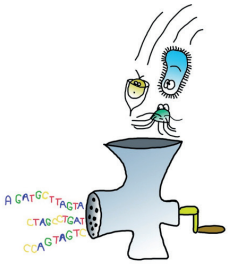

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Summary in English:

Protistan diversity is tremendous. And it is largely understudied. Although the microbial sphere is the basis of all life and the driver of ecosystem functions, most attention is given to the organisms that one can observe with the bare eye. Therefore, the biology, the diversity and the functional differentiation of microbial eukaryotes is dramatically understudied. Many ecological hypotheses have not yet been tested for protists. The extent of protistan diversity and its relevance to ecosystems are largely unknown. Addressing protistan diversity ideally requires a comprehensive approach including alpha-taxonomy, phylogenetics, phylogeography and molecular diversity as well as functional and physiological diversity. This thesis aims at this broad interdisciplinary approach to protist biodiversity. Small colourless flagellates are among the most problematic protists as the scarceness of characters makes species identification questionable. Such small flagellates, specifically chrysomonads, are, therefore, a consequent starting point for investigating protistan diversity. As a detailed study of the cryptic diversity within small heterotrophic colourless chrysophytes, I reveal polyphyly within this group of organisms in this work and, thereby, set the ground for assessing their true diversity in the field based on molecular signatures. Addressing protistan distribution patterns again is usually restricted by a limited and scattered set of sampling sites. Based on an unequalled sample set focusing on European protist communities, I demonstrate differential occurrence patterns in different protistan metagroups among habitat types. Last but not least, the vast diversity of protists raises the question for their coexistence and potential functional differentiation. I address this last aspect with a metatranscriptomic approach, revealing the degree of functional redundancy within protistan communities. Thus, in this thesis I reveal protistan diversity starting from species delimitation to large scale patterns of protistan diversity and distribution and further to the functional role and functional differentiation of protists.

Zusammenfassung in Deutsch:

Protisten sind extrem divers, ihre Diversität ist bislang jedoch nur wenig untersucht. Obwohl mikrobielle Organismen die Basis allen Lebens darstellen und das Funktionieren von Ökosystemen sichern, gilt die meiste Aufmerksamkeit doch denjenigen Organismen, die man mit bloßem Auge sehen kann. Somit sind die Lebensweise und Diversität, sowie die funktionelle Differenzierung einzelliger Eukaryoten kaum bekannt. Ebenso sind viele ökologische Hypothesen für Protisten bislang nicht getestet. Das Ausmaß protistischer Diverstät und dessen Bedeutung für Ökosysteme ist weitgehend unverstanden. Die Aufklärung protistischer Diversität verlangt idealerweise eine Herangehensweise, die Alpha-Taxonomie, Phylogenetik, Phylogeographie und molekulare Diversität ebenso wie funktionelle und physiologische Diversität umfasst. Die vorliegende Arbeit wendet diese breite interdisziplinäre Herangehensweise an, um die Biodiversität von Protisten zu untersuchen. Kleine farblose Flagellaten gehören zu den problematischsten Protisten, da sie kaum markante morphologische Merkmale aufweisen und somit eine sichere Identifikation von Arten fragwürdig wird. Solche kleinen Flagellaten, insbesondere innerhalb der Chrysophyceen, sind daher ein folgerichtiger Anfangspunkt für die Untersuchung der Diversität von Protisten. In der detaillierten Untersuchung kryptischer Diversität innerhalb der kleinen, heterotrophen, farblosen Chrysophyceen zeige ich in dieser Arbeit die vorhandene Polyphylie in dieser Gruppe von Organismen auf und lege damit die Grundlage, ihre wahre Diversität in Feldstudien anhand molekularer Signaturen zu erforschen. Im Weiteren wird die Untersuchung der Verbreitungsmuster von Protisten in Diversitätsstudien meist durch zu wenige oder nur unzusammenhängende Probenahmestellen verhindert. In dieser Arbeit kann ich jedoch basierend auf einem der größten Probensets europäischer Protistengemeinschaften zeigen, dass verschiedene Großgruppen innerhalb der Protisten in verschiedenen Habitattypen unterschiedliche Verteilungsmuster aufweisen. Und nicht zuletzt wirft die enorme Diversität innerhalb der Protisten auch die Frage von Koexistenz und potentieller funktioneller Differenzierung auf. Ich wende mich diesem letzten Aspekt mit Hilfe einer metatranskriptomischen Herangehensweise zu, die den Grad funktioneller Redundanz innerhalb von Protistengemeinschaften aufdeckt. Damit kann ich in dieser Arbeit Protistendiversität in unterschiedlichen Aspekten auflösen: angefangen von der Artabgrenzung zwischen Protisten bis hin zu großskaligen Diversitäts- und Verbreitungsmustern und weiter zur funktionalen Rolle und Differenzierung von Protisten in Ökosystemen.

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And I much thank Hannah for her patience with me and this work and for her love.

CONTENTS

LIST OF ABBREVIATIONS

units	nm	nanometre
	µm	micrometre
	mm	millimeter
	µl	microliter
	ml	milliliter
	L	liter
	µM	micromole
	M	mole
	µg	microgram
	mg	milligram
	g	gram
	µS	microsiemens
	µE	microeinstein
	°C	degree Celcius
	s and sec	second
	min	minute
	h	hour
	bp	base pairs
	Mbp	megabase pairs
	asl	above sea level
	m. ü. A.	metres above the Adria
others	Na	sodium
	NaCl	sodium chloride
	Cl	chloride
	Ca	calcium
	K	potassium
	Cu^{+}	copper ion
	Ag^{+}	silver ion

AgNO3	silver nitrate
AgNP	silver nanoparticles
P_{tot} and TP	total phosphate
P_{dis}	dissolved phosphate
DN	dissolved nitrogen
DOC	dissolved organic carbon
SO4	sulfate
DRSi	dissolved reactive silicon
NO3-N	nitrate
NH4-N	ammonium
HCO3	hydrogen carbonate
DAPI	4,6-diamidino-2-phenylindole
UV-light	ultra violet light
avg	average
pw	pathway
ID	identification
KO	control
Q	quality
HQ	high quality
no	number
pl.	plate (with images)
sp.	species
n. sp.	new species
n. g./gen.	new genus
n. fam.	new family
n. ord.	new order
nov. comb.	new combination
DNA	deoxyribonucleic acid
cDNA	complementary DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA

mRNA	messanger RNA
PCR	polymerase chain reaction
HNF	heterotrophic nanoflagellates
SEM	scanning electron microscope
TEM	transmission electron microscope
OTU	operational taxonomic unit
SSU	small subunit
LSU	large subunit
ITS	internal transcribed spacer
COX	cyclooxygenase-1
SD	standard deviation
FDR	false discovery rate
GLM	generalized linear model
PCA	principal component analysis
CA	correspondence analysis
CCA	canonical correspondence analysis
RDA	redundancy analysis
TCA cycle	tricarboxylic acid cycle (=citric acid cycle)
KEGG	Kyoto Encyclopedia of Genes and Genomes
Uniprot	Universal Protein Ressource
NCBI	National Center for Biotechnology Information
CCAP	Culture Collection of Algae and Protozoa
SAG	Sammlung von Algenkulturen Göttingen
BGBM	Botanischer Garten und Botanisches Museum Berlin-Dahlem

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1) INTRODUCTION

The earth is amazingly diverse in living organisms as well as in habitats – and both are linked. Abiotic and biotic factors form and characterize habitats and make, thus, life of specific organisms possible. Organisms themselves interact with their environment and often change conditions in the habitat, forming new niches for others to come. Ecology, therefore, is fluid driving evolutionary processes on a larger time scale. The measurement and investigation of biodiversity as its unit allows both insights into ecological and evolutionary changes. Biodiversity research is, however, biased in more than one way:

- What is known about biodiversity (i.e. ecological theories) is mainly derived from multicellular organisms, mostly land plants and metazoa, leaving the microbial sphere unmentioned and possibly wrongly represented.
- Biodiversity is mostly counted in 'species', a term of unclear definition as various species concepts with quite different implications exist in rivalry.
- Changes in biodiversity are often described as long-term events (except when being catastrophic) drawing on long life cycles and generation times.

In times of a man-made decline of biodiversity (Boenigk et al. 2015; Cardinale et al. 2012; Barnosky et al. 2011), the understanding of biodiversity patterns and of the processes driving diversity is of tremendous importance. However, not multicellular organisms (being more or less our size and visible by the bare eye!) are the basis of ecosystems, but microbes of enormous phylogenetic and functional diversity (van der Heijden et al. 2008). They drive and fuel the system and their diversity and ecology has to be thought of with care in order not to be preoccupied by existing knowledge. Taking into account the importance of microbial life, their diversity is surely understudied. This work, thus, means to be a contribution to the understanding of microbial diversity, in establishing new methods of investigation and in presenting unknown patterns of diversity.

Diversity of protists

Protists (i.e. single cell eukaryotic organisms – and aggregates of them) are present and diverse in most aquatic and terrestrial habitats – literally in every drop of water irrespective of its place (Triadó-Margarit & Casamayor 2012; Bates et al. 2013; del Campo & Massana 2011). As photosynthetic algae they are responsible for half of the global oxygen production (Field et al. 1998) and as heterotrophic protozoa they prey on bacteria and other protists (Boenigk & Arndt 2002; Sherr & Sherr 2002). Protistan diversity has been lumped together and largely underestimated in the past (Stackebrandt & Ebers 2006). Protists (especially protozoa) have been thought to form their

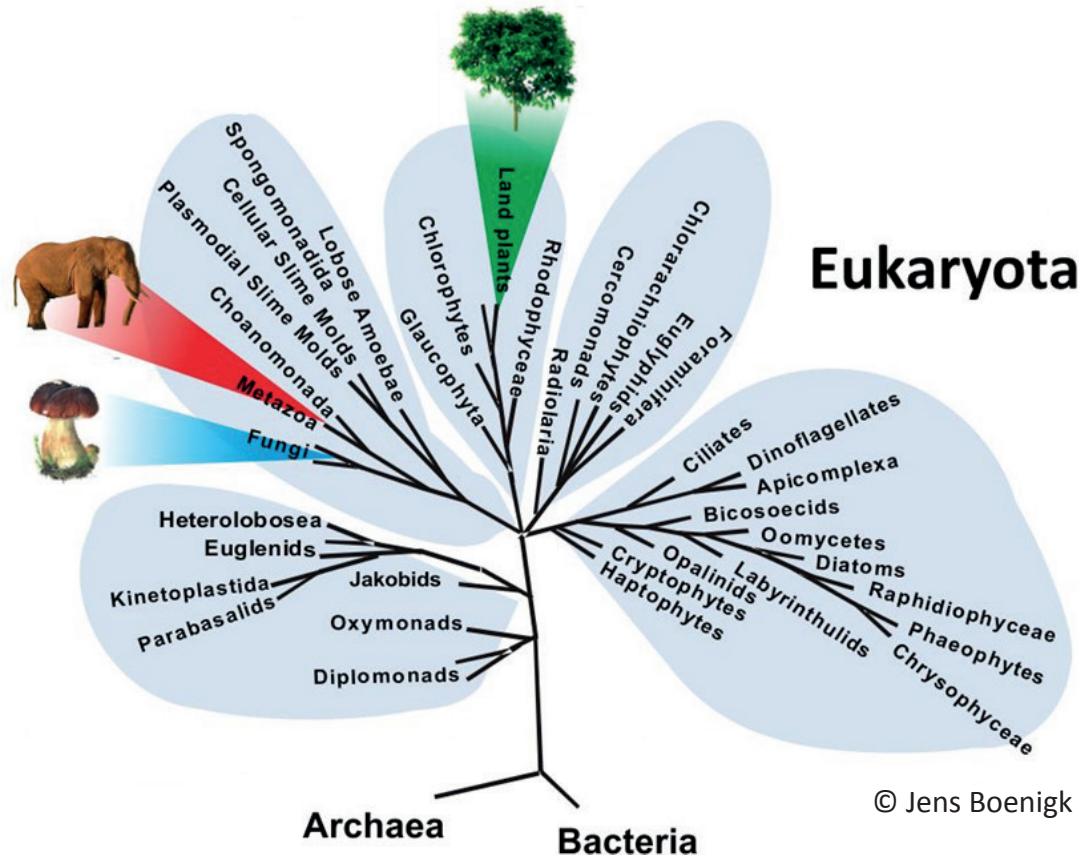


Figure 1.1. The immense diversity of protists. The graph shows the multitude of different protistan lineages within Eukaryota. Land plants, metazoa and fungi are not opposed to a realm of protists, but are, as three out of many lineages, part of the diverse tree of eukaryotes.

own amorphous sphere opposed to plants and animals within the eukaryotic tree. Only with the arrival of molecular methods did this view of a separated protistan sphere change for good. Now, the eukaryotic tree shows a multitude of strongly diversified protistan lineages (Adl et al. 2012) that only in a few cases also developed multicellularity – i.e. in plants and animals, but also e.g. in Phaeophyceae. Plants and animals are, thus, declassified as only two of many eukaryotic lineages in terms of evolutionary diversification (Fig. 1.1). Moreover, microbial life is not known for long. With the introduction of the first microscopes at the end of the 17th century, life in the microbial scale was first encountered and the first descriptions of organisms now being visible and recognizable followed (van Leeuwenhoek in Dobbel 1932; Müller 1773). Here, prokaryotic and eukaryotic cells – as well as cells at all – were neither known nor distinguished and the description of organisms was mainly based on what visual characteristics could be grasped. This was the strategy applied with multicellular organisms and it was kept using microscopes of ever higher resolution up to the use of electron microscopy. As visibility was the main character of investigation, mainly larger and more abundant taxa stood in the centre of protistan research. There is, however, a much larger diversity in protists – taxa of low abundances, cryptic taxa of scarce and similar morphology and taxa of

limited distribution (Stoeck et al. 2009; Caron & Countway 2009; Huss et al. 1999; Moreira et al. 2004; Sommaruga 2001). This enlargement of the protistan sphere could mainly be discovered with the help of molecular methods, especially high-throughput sequencing enabling for the in-depth study of protists and 'making visible' many more taxa of protistan communities (Lecroq et al. 2011; Medinger et al. 2010).

What is a species? – morphological and molecular attempts to protists

The basic unit of biodiversity, 'a species', unfortunately is not easy to grasp which is also the reason why species estimates are problematic (Mora et al. 2011). Different surveys and investigation in biological research classify species differently as contradicting species concepts are used (Claridge et al. 1997; Mallet 2006), some of which are the biological species concept, the morphological species concept and phylogenetic species concept. The concept 'species' is classically thought of as bearing clear-cut border lines to other organisms on the same level. In the biological species concept, fertile reproduction characterizes a species (mainly used for animals) (Corbet 1997), in the morphological species concept, a combination of (mostly) visible traits draws the line (mainly used for higher plants) (Gornall 1997). However, the clear-cut border line itself has been identified as a major problem when dealing with the term and concept of 'species' (Ereshefsky 1992; Boenigk et al. 2011). It reflects the wish to order and clear up the denomination of an organism once and for all, but the attempt fails when species do not follow the rules or different species concepts contradict each other as e.g. when geographically separated 'species' interbreed when brought together or morphologically clearly separated 'species' produce hybrid forms.

In protists, traditionally morphology has been used to differentiate species (and consequently the morphospecies concept was widely used), drawing on some (by no means all) aspects of a phenotype (John & Maggs 1997). This is most probably due to the visible approach of observing protistan life, however, missing e.g. the organisms' physiology, ecological tolerance or reproduction (also being part of the phenotype) (Koch & Ekelund 2005; Lowe et al. 2005; Boenigk et al. 2007). Since the advent of molecular methods in the 1990s, this strategy of species denomination in protists has been challenged by a genotypic approach, using the genetic information of specific loci on the genome or the genome as a whole to differentiate between organisms. Molecular methods have elucidated 'cryptic species' of indistinguishable morphology within protists having been lumped together before (Huss et al. 1999; Moreira et al. 2004; Boenigk 2008a).

Environmental studies including the recent high-throughput sequencing approaches use operational taxonomic units (OTUs) to draw on sequence clusters generated from a multitude of sequences from molecular screening (Green et al. 2004; Schloss & Handelsmann 2005). Thereby, OTUs are not meant to precisely reflect species by the one or the other species concept, but to propose a comparable and usable unit in e.g. ecological surveys. OTUs are usually defined by fixed sequence similarity cut-off levels. They are therefore sensitive to the phylogenetic resolution of the chosen sequence. The resolution of the highly conserved SSU gene (coding for the small subunit

of ribosomes), which is most often used in molecular diversity studies, is, however, too low to distinguish between many well-established species and OTUs based on the SSU presumably lump several species into one OTU (Bass & Boenigk 2011).

Thus, different concepts of protistan species are in use and different approaches to delimit species are current practice. This complex situation of species delimitation is further blurred by the environmental approaches relying on approximate substitutes for species such as OTUs. Sensible knowledge, however, can be gained by all of them in their proper context and combined approaches promise to grasp protistan diversity in even higher precision (Scoble & Cavalier-Smith 2014). To be aware of the chosen level of resolution, thereby, is one major aspect in protistan diversity research.

Where are all the species? – patterns of protistan distribution

Protistan diversity is much larger than thought. Molecular studies, using various primers and thereby augmenting the sequence yield in samples, also imply that numbers of protists are supposedly much higher than those of plants and animals (Moreira & López-García 2002; Cavalier-Smith 2004; Countway et al. 2005). Estimates of the actual total diversity are, however, difficult to make. This is mostly due to protistan occurrence patterns in the environment in time and space. Taxon diversity in a single molecular sample is already enormous, but taken at only one point in time it misses taxon succession in diurnal as well as seasonal intervals (Rodriguez Zaragoza et al. 2005; Gilbert et al. 2009; Nolte et al. 2010). The same can be assumed for the geographical distribution of protists. On a global scale, the spatial distribution of protists has been controversially discussed. Positions reach from a potential global distribution (‘Everything is everywhere’) going hand in hand with the assumed worldwide dispersal of protists, where only the ecology of the habitat selects for specific taxa (Baas-Becking 1934; Fenchel et al. 1997), to biogeographical restriction and endemicity (Sommaruga 2001; Sonntag et al 2011). Molecular methods and the collection of found taxa within databases support the whole range of distribution patterns supporting a ‘moderate endemicity’ of protists (Foissner 2006; Foissner 2008; Hughes Martiny et al. 2006). Some taxa are found worldwide and in diverse habitats (Finlay et al. 2006; Darling & Wade 2008), others appear highly restricted and will not reappear in other samplings (Bass et al. 2007; Darling & Wade 2008). The reasons for such spatial differentiation can be manifold just as in multicellular organisms, e.g. endemism (Sommaruga 2001), limitations in dispersal (Telford et al. 2006) or biogeographic history (Vyverman et al. 2007). Moreover, protists that appear in many different samples and sampling campaigns are likely to be of ubiquitous ecology. Strongly habitat-specific taxa are, however, easily missed in samplings. Molecular studies always detect such rare taxa – also called the ‘rare biosphere’ – in the sampled habitats, even augmenting with the depth of sequencing (Pedrós-Alió 2006; Caron 2009; Stoeck et al. 2010). By the given reasons, the evaluation of protistan diversity faces the problem of undersampling (Bass & Boenigk 2011). Therefore, to get closer to the actual picture of protistan distribution as well as to estimates of diversity and diversity patterns, large scale samplings and surveys are needed. As patterns of diversity within protists can be expected to be quite differing,

the investigation of specific lineages (i.e., for example, metagroups within Eukarya) may shed light on general ecological trends. Moreover, with the help of large scale molecular datasets, theories derived from multicellular organisms can be tested (Tringe et al. 2005; Bik et al. 2012) and protistan diversity can be evaluated in a larger biological context.

What are the different taxa doing? – functional diversity and differentiation in protists

Even more complex than 'mere' occurrence is the functional diversity of protists in their habitats, i.e. being present does not mean to necessarily perform the same physiological processes and play the same role in an ecosystem. Not assuming such fine-tuned differences in functionality, the relatively high species richness and co-occurrence of similar taxa in seemingly uniform environments such as freshwater lakes was formulated as the 'plankton paradox' (Hutchinson 1961). Thereby, many protistan taxa obviously share one and the same niche in their habitat. However, when looking not merely at the similar morphology of the taxa, but rather on their functional behaviour and physiology, differences appear and diversify the high species richness. The probably best investigated functional differentiations are linked to feeding and food web ecology (Boenigk & Arndt 2002; Šimek et al. 2013) – as e.g. in feeding mode, size preferences of feeding particles or specific food specialisation – or to the microbial food web in general. Protists show diverse forms of energy uptake ranging from phototrophy to heterotrophy and multiple intermediate forms of mixotrophy. As well, biotic interactions within microbial food webs are manifold explaining for the co-occurrence of many morphologically similar taxa at a time. Therein, functional groups are not ordered taxonomically as in heterotrophic animals and phototrophic plants. Not seldom are diverse feeding types realized in one and the same protistan lineage as in Chrysophyceae (Graham et al. 2009; Hausmann et al. 2003). Taxonomic diversity is, therefore, not identical with functional diversity in protists and different taxa or metagroups can potentially take over the same function in an ecosystem (Allison & Martiny 2008). Under which conditions and to what extent such functional redundancy in protists occurs, is an open research question. Molecular sequencing in terms of metatranscriptomics is potentially able to grasp such changes within protistan communities focusing on the active part of the community and thereby showing changes in the functioning of an ecosystem or even the actual contribution of specific taxa to ecosystem functioning (Daniel 2005; Muller et al. 2014; Aylward et al. 2015).

Methodical and conceptual advances in protistology

Methods in protistology have come a long way. Protistology (as we call it today) effectively started when van Leeuwenhoek first encountered living organisms on a smaller scale in 1674 (Dobbel 1932). The key to this encounter was a microscope magnifying objects, as in this case organisms, to a scale recognizable for the human eye. With improvements in microscopy (higher resolution, advances in microscopic techniques), cells were recognized as the units which tissues and organisms

consist of (Schwann 1839; Schleiden 1838), also preparing the way for the acknowledgement of single cell organisms. Electron microscopy with its even higher resolution allowed for the study of ultrastructural characters and had its main influence in the second half of the 20th century. With the introduction of PCR (polymerase chain reaction) in 1983, however, the focus of investigation in protistology turned away from visible characters to genetic information (Mullis 1990; Saiki et al. 1988), the latest advances being high-throughput sequencing technologies appearing at the turn of the millennium. These sequencing strategies are able to work both parallel and in depth and, therefore, have the potential to screen large amounts of samples and reveal even rare sequences within them (Hall 2007). Competing high-throughput sequencing strategies are currently on the market, however, all offering deep and massive sequencing of samples (Kircher & Kelso 2010; Loman et al. 2012). In using these strategies and molecular methods in general, however, the unit of diversity changes. As sequences are used to draw back on specific organisms, a reference databank (such as GeneBank) is needed to identify organisms within a sample. This is not too much a difference to identifying cells under the microscope with the help of a morphological key. And as with small heterotrophic colourless chrysophytes, microscopy might even come to its limits not being able to distinguish different taxa due to the lack of morphological features. High-throughput sequencing on the other hand shows the whole diversity of sequence variation (that also exists within species) and on the contrary faces the problem of combining sequences to a meaningful taxon (Huse et al. 2010), at best on species level. For the difficulties involved here, molecular methods work with the term 'OTU' (operational taxonomic unit) and will identify the similarity of an OTU with a reference sequence in the databank (Green et al. 2004). Sequences are, thereby, identified as affiliated with a distinct specific species or, as being more or less closely related to the reference sequence, as e.g. belonging to the same family or order. In doing so, molecular methods are able to screen protistan communities in depth and in large numbers and reveal unknown taxa and lineages whenever used (Capraso et al. 2012; Degnan & Ochman 2012). They have shown and continue to do so that protistan diversity is much larger than formerly thought.

In this work, I investigated protistan diversity by light microscopy, electron microscopy, Sanger sequencing, amplicon high-throughput sequencing and metatranscriptomic sequencing. The idea is to use sequencing and high-throughput sequencing not to replace microscopic methods, but to apply the newer methods for questions that microscopy cannot answer. In this work, this was the investigation of supposedly cryptic taxa ('cryptic species') of small heterotrophic colourless chrysophytes, the investigation of protistan diversity patterns in a large scale sampling (232 samples from diverse sites and habitats) and the establishment of metatranscriptomics for protists and the subsequent use of this method for a comparative analysis of protistan diversity and activity patterns in a Europe wide dataset. In this sense, I used sequencing where the eyes could no more see.

Chrysophytes as a model taxon for in-depth investigation

For an in-depth investigation of protistan diversity, chrysophytes qualify by multiple reasons as

a model taxon. The group has been split apart for most of its taxonomic history, as chrysophytes comprise both photosynthetic and non-photosynthetic organisms, and has only been united on the basis of phylogenetic information. Traditionally, photosynthetic chrysophytes ('golden algae') have been dealt with by phycologists and were described by the botanical code (Graham et al. 2009). Heterotrophic chrysophytes, on the contrary, have been dealt with as chrysomonads by protozoologists and were described by the zoological code (Hausmann et al. 2003). It is, however, evident that the terms 'botanical' and 'zoological' do not meet the requirements of the group as chrysophytes show a much broader range of nutritional strategies. They also comprise many mixotrophic taxa which use different kinds of feeding to different extents. Also, feeding strategies of heterotrophic chrysophytes have been described as manifold (Boenigk & Arndt 2002). In this functional differentiation, chrysophytes are among the dominant flagellates in many habitats (del Campo & Massana 2011; Lara et al. 2011; Weitere & Arndt 2003) and show high ecological relevance. Heterotrophic chrysophytes are one of the most important grazers of bacteria-sized microorganisms (Finlay & Esteban 1998) and photosynthetic chrysophytes do an important part of primary production in oligotrophic waters (Wolfe & Siver 2013). Chrysophytes, moreover, are relatively sensitive to environmental changes (Lotter et al. 1997) and as such, are used as model species in ecophysiological research (Boenigk 2008; Montagnes et al. 2008) and as a tool in palaeolimnological studies (Facher & Schmidt 1996). In this work, I conduct an in-depth study of strains of small heterotrophic chrysophytes which by their similar morphology are lumped together as '*Spumella*-like flagellates' in ecological surveys, much in contrast to their diverse ecophysiology. Moreover, I also pay special attention to chrysophytes within the different molecular investigations of total protistan diversity in this work and, thereby, elucidate chrysophyte distribution patterns and functional diversity.

The objectives of this work

The investigations undertaken in this work using the different methods of protistan research will be presented in five chapters (chapters 2-6 of this work). In these, I address the species diversity of protists (chapter 2), protistan distribution patterns (chapters 3 & 4) as well as the functional diversity of protists (chapters 5 & 6). Introduction, methods, results and discussion are given respectively in the different chapters.

Chapter 2 investigates assumed cryptic diversity and polyphyly within small heterotrophic colourless chrysophytes. The investigation uses microscopy (light microscopy, transmission electron microscopy and scanning electron microscopy) and sequencing (Sanger sequencing of SSU, LSU, ITS and Cox1) to evaluate the diversity of the organisms and possibly conclude a new taxonomic naming and positioning. Thus, it sets the basis to end the assumed lumping of small heterotrophic colourless chrysophytes in ecological studies.

Chapter 3 investigates protistan diversity and distribution patterns in a large scale amplicon dataset of various ecologies. 232 samples from soil, fresh, brackish and sea water as well as from

experimental set-ups are compared statistically in their community composition. Hereby, also a new method of data transformation had to be implemented in order to deal with the multitude of zero counts in the data matrices. The huge number of samples within this dataset makes it possible to use the investigation as a proof of principle for molecular amplicon surveys and investigate protistan distribution patterns only possible in such numbers.

Chapter 4 investigates protistan diversity in an alpine elevation gradient. The 32 lakes of this study are a subset of the above mentioned 232 samplings and have likewise been studied by means of amplicon sequencing. Here, ecological hypotheses derived from multicellular organisms are tested for their applicability in protists, namely the reduction of species richness and the change in community composition with elevation.

Chapter 5 investigates the effect of silver pollution on freshwater protists by means of metatranscriptomic analyses. This ecophysiological experiment likewise is a proof of principle of metatranscriptomics in protists. Silver as a pollutant is increasingly measured in the environment with its effect on protistan communities being unclear. In investigating the non-lethal response of the communities to silver pollution, possible effects in natural waters can be predicted and prevented.

Chapter 6 investigates protistan diversity and activity patterns comparatively. Within a set of 21 Europe-wide distributed freshwater lakes, metatranscriptomics are used to screen for the co-occurrence of protistan lineages, their activity patterns and the ecological parameters of the investigated lakes. In using metatranscriptomics in an environmental context, the method is also tested in its potential as a general means of water quality and ecosystem health assessment.

As outlined for the individual chapters above, the aims of this work are:

- to investigate cryptic diversity in small heterotrophic colourless chrysophytes,
- to illuminate distribution and diversity patterns within the protistan sphere,
- and to establish and use metatranscriptomics in protists as a tool of ecosystem assessment and diversity research.

Many of the investigations of this dissertation have been carried out in cooperation with other working groups. At the end of each chapter (chapters 2-6), I will, therefore, make clear their part in the respective projects.

2) SMALL BUT MANIFOLD – HIDDEN DIVERSITY IN '*SPUMELLA*-LIKE FLAGELLATES'

ABSTRACT

Colourless, non-scaled chrysophytes comprise morphologically similar or even indistinguishable flagellates which are important bacterivors in water and soil crucial for ecosystem functioning. However, phylogenetic analyses indicate a multiple origin of such colourless, non-scaled flagellate lineages. These flagellates are often referred to as '*Spumella*-like flagellates' in ecological and biogeographic studies. Even though this denomination reflects an assumed polyphyly, it obscures the phylogenetic and taxonomic diversity of this important flagellate group and, thus, hinders progress in lineage- and taxon-specific ecological surveys. The smallest representatives of colourless chrysophytes have been addressed in very few taxonomic studies although they are among the dominant flagellates in field communities. In order to overcome the blurred picture and set the field for further investigation in biogeography and ecology of the organisms in question, we studied a set of strains of specifically small, colourless, non-scaled chrysomonad flagellates by means of electron microscopy and molecular analyses. They were isolated by a filtration-acclimatisation approach focusing on flagellates of around 5 µm. We present the phylogenetic position of eight different lineages on both the ordinal and the generic level. Accordingly, we describe the new genera *Apoikiospumella, Chromulinospumella, Segregatospumella, Cornospumella* and *Acrispumella* Boenigk et Grossmann n. g. and different species within them.

2.1 INTRODUCTION

Non-scaled, colourless chrysomonad flagellates (=non-scaled, colourless chrysphytes) are major phagotrophs in freshwater and soil food webs (Boenigk and Arndt 2002; del Campo and Massana 2011; Ekelund et al. 2001; Finlay and Esteban 1998; Richards and Bass 2005) and are among the dominant feeders on bacteria (Berninger et al. 1991; Šimek et al. 2013).

Scaled, colourless chrysomonad flagellates have recently been revised, specifically the genus *Paraphysomonas* and the newly erected genus *Clathromonas* (Scoble and Cavalier-Smith 2014). Colourless chrysomonad taxa lacking surface scales seem to be at least as diverse (Boenigk 2008b). Due to the lack of surface scales, these latter taxa are even harder to distinguish based on morphology. As one consequence, these flagellates have often been merged as *Spumella* spp. (Boenigk 2008a; Boenigk et al. 2005) irrespective of the molecular diversity and polyphyly of this group (Boenigk 2008a) calling for major revisions. As rDNA phylogenies suggest, colourless, non-scaled chrysomonad flagellates are not monophyletic, but have lost photosynthesis at least five times independently in different lineages and, thus, became *Spumella*-like in morphology (Boenigk 2008a; Boenigk et al. 2005; Cavalier-Smith and Chao 2006; Stoeck et al. 2008). The terms '*Spumella*-like flagellates', '*Spumella*-like cells' or '*Spumella* sensu Cienkowsky' often used in the ecological literature hint at the current uncertainties and the potential polyphyly of the genus *Spumella*

(Berglund et al. 2005; Boenigk et al. 2005; Charvet et al. 2012; Lepère et al. 2006). Eventually, this genus must be divided into several genera (Scoble and Cavalier-Smith 2014; Boenigk 2005; Findenig et al. 2010).

2.1.1 Generic affiliation of colourless, non-scaled chrysomonad taxa

Several colourless, non-scaled chrysophyte genera have been described including *Spumella* Cienkowsky 1870, *Monas* Müller 1773, *Oikomonas* Kent 1880 (also *Eucomonas* or *Oicomonas*), *Synoikomonas* Skuja 1964 and *Paramonas* Kent 1880. Several further generic names have been proposed but are considered to be synonyms of one of the above names, specifically *Heterochromulina* Pascher 1912 and *Heterochromonas* Pascher 1912.

The genus *Monas* Müller 1773 is one of the first flagellate genera ever described (Müller 1773; 'Vermis inconspicuus, simplicissimus, pellucidus, punctiformis' [inconspicuous, most simple, transparent, and punctiform worm]). Organisms affiliated with most major branches of eukaryotes and even bacteria have subsequently been lumped into this genus. Later, many of these organisms have been removed, however, still leaving the genus *Monas* as a polyphyletic collection of species and considerable doubts about the identity of its lectotype, *Monas mica* Müller 1773, selected by Diesing (1850).

Spumella Cienkowski 1870 has been synonymised with *Monas* Müller 1773, but Silva (1960) and later Preisig et al. (1991) recommended that the genus *Monas* should be abandoned because of the uncertainties pertaining to the identity of its lectotype. The strains of *Spumella* investigated so far were all characterised by a naked cell surface; heterokont flagella emerging from an apical depression with mastigonemes on the long flagellum, while the short flagellum is naked; and mitochondria with tubular cristae. A number of other structures differed between strains; for instance, they may or may not possess a leucoplast, a flagellar swelling, an eyespot or mucocysts (Bruchmüller 1998; Preisig and Hibberd 1983). Only a few studies have investigated the stomatocysts in colourless, non-scaled chrysomonad flagellates with a *Spumella*–like morphology (Cienkowsky 1870; Belcher and Swale 1976; Yubuki et al. 2008; Findenig et al. 2010). Cyst morphology, as well as scale morphology in scaled chrysophycean taxa, is believed to be species-specific and thus a suitable criterion for differentiating species (Sandgren 1991; Findenig et al. 2010). Based on the morphology of stomatocysts, Findenig et al. (2010) designated an epitype for *Spumella vulgaris* Cienkowsky 1870, i.e. for the type species of the genus.

Heterochromonas Pascher 1912 has been proposed as a generic name for biflagellated, colourless, and non-scaled chrysomonad flagellates based on the assumption that these flagellates are the colourless counterpart of *Ochromonas*, i.e. a phylogenetic sister group differing by the reduction of the plastids. Pascher (1912) and Bourrelly (1957) suggested to use the genus name *Heterochromonas* for those flagellates of the *Monas* / *Spumella* morphodeme with known stomatocysts. In contrast, Skuja (1939, 1948, 1956) rejected cyst formation as a criterion for the separation of genera. From a taxonomic perspective, the generic name *Heterochromonas* is a synonym of *Spumella,* and as *Spumella* has priority, this generic name can be rejected. From a phylogenetic point of view,

this generic name should be used for a colourless sister taxon of the clade comprising the type species of *Ochromonas* Wysotzki 1887 (i.e. *O. triangulata* Wysotzki 1887). No sequence data exist for *O. triangulata* and the type strain is unavailable and has never been found again. However, *Ochromonas moestrupii* Andersen 2011 is very similar to *O. triangulata* and has even been proposed as a promising candidate for an epitype (Andersen 2011). Based on the presumable monophyly of *O. triangulata* and *O. moestrupii,* the molecular affiliation of the type species of *Ochromonas* can, therefore, be deduced even though molecular data for the type species itself are missing. *Heterochromonas* may be considered sister to the clade comprising *O. moestrupii,* which clusters close to *Dinobryon* in phylogenetic analyses, but not with one of the strains described herein.

Related to the above case of the genus *Heterochromonas* is the potential inclusion of some colourless, non-scaled chrysomonad flagellates within the genus *Ochromonas*. Wysotzki (1887) described two species from which *Ochromonas triangulata* Wysotzki was designated as lectotype for the genus by Bourrelly (1957). The polyphyly of *Ochromonas*, as shown in molecular studies, indicates that there are several genera that have a similar morphology (i.e. they are naked single cells with two heterodynamic flagella, but otherwise are distinctive genetically) (Andersen 2011). Nomenclaturally, the name *Ochromonas* will be applied to the clade containing the type species (Andersen 2011). Despite intensive investigations of the type locality (Lake Veisovo, a salt lake in the Ukraine) by Andersen and co-workers, the type species could not be found again. As there are neither sequence data nor type material in order to make such sequence data available, the molecular identity of the type species cannot be verified. It is, however, very likely that *Ochromonas moestrupii* is a very close relative as it resembles *Ochromonas triangulata* in several ways (Andersen 2011); furthermore, both species originate from salt water. Based on the close resemblance of both species, *O. moestrupii* has been proposed as a potential epitype and is, therefore, the currently best choice for rooting the genus *Ochromonas* in molecular trees (Andersen 2011). Species which are affiliated with *Ochromonas,* but are phylogenetically affiliated with other clades, therefore, probably do not belong into the genus *Ochromonas* and await taxonomic revision.

For heterotrophic, non-scaled chrysomonad flagellates with a largely reduced second flagellum the generic names *Oikomonas* Kent 1880 (also *Eucomonas* or *Oicomonas*) and *Heterochromulina* Pascher 1912 have been proposed: *Oikomonas* is regarded as the colourless counterpart of *Chromulina* Cienkowsky 1870 by some authors, however, the identity of the genus is not clear (Silva 1960; Preisig et al. 1991). For both genera, type material of the type species is not available for molecular analyses, however, strains affiliated with the type species of *Oikomonas* have been isolated later, and these latter strains have been sequenced. Based on these sequence data, *Oikomonas* spp. (with *Oikomonas mutabilis* Kent 1880 as the type species) cluster as a sister group to *Chromulina* spp. (with *Chromulina nebulosa* Cienkowsky 1870 as the type species) (Cavalier-Smith and Chao 2006). Pascher (1912) proposed the generic name *Heterochromulina* for colourless non-scaled chrysomonad flagellates with a very short second flagellum which are sister to *Chromulina*. However, as *Oikomonas* Kent has priority over *Heterochromulina* Pascher the latter name is only a synonym and has been included in *Oikomonas* (Preisig et al. 1991). From a phylogenetic point of view,

Heterochromulina is sister to the clade comprising *Chromulina nebulosa*. However, as *Oikomonas* spp. cluster as a sister clade to *Chromulina* and *Oikomonas* has priority over *Heterochromulina*, the latter name must be regarded as a synonym to *Oikomonas* and should not be further used to avoid confusion.

The genus *Paramonas* Kent 1880 has been erected for uniflagellate taxa formerly affiliated with the genus *Monas* Müller, which have a distinct oral aperture. A type species has not been designated by Kent, but Cavalier-Smith and Chao (2006) designated *Paramonas globosa* as type for the genus providing further morphological and molecular data based on an ATCC isolate of this species. Based on the phylogenetic data, the genus *Paramonas* belongs to the Pseudodendromonadales (Bicoecia) (Cavalier-Smith and Chao 2006).

The genus *Physomonas* Kent 1880 and its type species *Physomonas socialis* (Ehrenberg) Kent 1880 differ considerably from the flagellates described herein (Kent 1880; as *Bodo socialis* in Ehrenberg 1832). *Physomonas* is usually attached to the substratum by a thread-like pedicle, and food uptake takes place at all parts of the periphery. In contrast, food uptake in small, colourless chrysomonad flagellates occurs only at the anterior part of the organisms near the flagellar roots, and thread-like pedicles do not occur in these flagellates. More importantly, the mode of spore formation is very different. A subdivision of the body into many spores, as described for *Physomonas,* has never been observed for the flagellates described herein and the genus *Physomonas* can be excluded.

The genus *Pedospumella* Boenigk et Findenig has recently been erected for small, colourless, non-scaled chrysomonad flagellates based on molecular data and cyst morphology. The vegetative cell of the type species *Pedospumella encystans* Findenig et Boenigk 2010 is similar to that of *Spumella vulgaris* (Cienkowsky) Findenig et Boenigk 2010, however, molecular data clearly show that *Pedospumella* forms a separate clade not related to the clade containing *Spumella*.

Similarly, the genus *Poteriospumella* Boenigk et Findenig 2010 has been erected for phylogenetically divergent lineages based on sequence information. Phylogenetically, this genus is the sister taxon to *Poterioochromonas* Scherffel 1901, but differs in lacking the characteristic stalk of *Poterioochromonas* as well as in lacking photosynthesis. Furthermore, considerable differences in gene sequence data support *Poteriospumella* as a separate genus.

The genus *Apoikia* Kim et al. 2010 has been erected by Kim et al. (2010) with *A. lindahlii* Skuja 1956 as the type species using *Monas lindahlii* Skuja 1956 as basionym. Molecular data show that this taxon and related strains form a separate clade (i.e. the Apoikiida) within Chrysophyceae (Scoble and Cavalier-Smith 2014). The genus is described as colonial with the cells growing in colourless mucilage. As none of our isolates grow in a colony or are surrounded by mucilage, this generic name can also be rejected for any of our isolates.

2.1.2 Species identity of colourless, non-scaled chrysomonad flagellates

As outlined above, most of the strains described in this study are not affiliated with one of the described genera. However, we need to consider whether one of our strains may belong to a

previously described species, which (based on the above considerations) would then need to be transferred to another genus. More than 100 of such colourless, non-scaled chrysomonad taxa have been formally described. The described species affiliated with the above genera deviate from the strains described in this study (see supplement for *Monas* spp. and *Spumella* spp.). Due to the high degree of morphological similarities of colourless, non-scaled chrysomonad taxa, molecular data seem inevitable for differentiating species and genera. However, for most described species no sequence data or cultures are available. Exceptions are the type species of the genera *Pedospumella*, *Poteriospumella*, *Spumella* sensu Boenigk et Findenig, and *Apoikia* Kim et al. 2010 as well as few other strains all of which differ in SSU rRNA gene sequence from the taxa described herein.

Most of the taxa described to date have a cell size of 12 µm or more. This is in contrast to the dominance of colourless chrysomonad flagellates of around 5 µm or less in ecological studies over recent decades. Ecological as well as phylogenetic research has increasingly focused on these small representatives. The disproportionate number of descriptions of large species is likely due to isolation protocols. A recent example is the isolation of predominantly scaled strains by Scoble (pers. comm.) in contrast to the isolation of predominantly non-scaled strains by Boenigk (own unpublished data). Similar biases are to be expected between different past and recent isolation approaches, specifically considering enrichment cultures which made up for the majority of previous studies versus direct isolation approach and dilution techniques such as filtration-acclimatisation approaches. The latter approaches exclude large taxa by a filtration step and allow for successful isolation and cultivation of otherwise sensitive strains by gradual acclimatisation (Hahn et al. 2004; Boenigk et al. 2005).

Boenigk and co-workers isolated several strains of colourless, non-scaled chrysomonad flagellates with cell sizes around 5 µm (between 1.2 and 8.6 µm) based on such filtration-acclimatisation protocols (Boenigk et al. 2005). In order to address the diversity of these small colourless non-scaled chrysomonad flagellates, we studied 16 clonal cultures which are, based on molecular data, placed within 8 different genera. We describe 6 new species and introduce 3 new taxonomic combinations.

Even though our isolates differ from all taxa described to date and, therefore, must be considered as new species, some of the formerly described species may be related to the taxa described herein and may eventually need to be transferred to one of the new genera. This holds true specifically for taxa for which electron microscopic data are available but molecular data are missing. Future molecular studies on these strains – as far as available – may shed light on their phylogenetic position.

Taking into account the present level of research, we hypothesize that colourless, non-scaled chrysophytes are far more diverse than currently documented. We further hypothesize that microscopic methods (LM, SEM, TEM) are unable to resolve this diversity and, thus, molecular methods must be integrated in taxonomic and systematic studies of this group. We hypothesize that the phylogeny of '*Spumella*-like flagellates' is supported by analyses of different genetic loci

(SSU, LSU, ITS, COX1) and suggest to routinely integrate molecular analyses in ecological and biogeographical studies of this group and of nanoflagellates in general.

2.2 METHODS

2.2.1 Cultivation of strains

The investigated strains were obtained from the culture collection at the University of Duisburg-Essen and originate from geographically and ecologically distinct sampling sites worldwide (for details see Table 2.1). Clonal cultures have been established by serial dilution and enrichment culturing (Boenigk et al. 2005; Boenigk et al. 2006). The strains are permanently cultivated in IB-medium (Hahn et al. 2003) supplemented with a sterilised wheat grain at 15 °C and in a light-dark cycle of 14:10 hours with 65 µE. When grown solely in inorganic medium and better light conditions, all strains die. To achieve high abundances (up to 200,000 individuals per ml) for EM fixation and DNA isolation, strains were fed with bacteria (gammaproteobacterium *Limnohabitans pelagia*, strain CB5, GenBank synonym *Vibrio pelagius*, from Lake Constance, see Hahn and Höfle, 1998). Three axenic strains (JBC07, JBM10, JBNZ41) were cultivated in NSY-medium (Hahn et al. 2003) achieving equally high abundances without bacterial prey.

2.2.2 Light microscopy and electron microscopy

Light microscopic analyses were carried out with a Nikon Eclipse Ti inverse microscope (Nikon Corporation, Tokyo, Japan). Cultures were observed in specially prepared thin bottom petri dishes (Boenigk and Arndt 2000) and documented using NIS-Elements Basic Software.

In order to detect ultrastructural features on whole cells not visible by light microscopy, both SEM (scanning electron microscopy) and TEM (transmission electron microscopy, applied on positive-stained specimens) were applied. Furthermore, ultrathin sections were produced for six of the chosen strains [i.e. for strains 199hm (*Spumella vulgaris*), JBM10 (*Poteriospumella lacustris*), JBNZ41, JBC07, AR4D6 and JBMS11 (*Pedospumella encystans*)]. Electron microscopy was carried out with a Zeiss EM10 at 60KV for ultrathin sections and positive-stained specimens and with a Zeiss 940A for SEM. Strains were cooled down before fixation and fixed with 2.5% glutaraldehyde (final concentration) in 0.1M Hepes buffer (pH = 7.2) for 1 hour on ice. Positive contrast was carried out on an EM copper grid with 1% aqueous uranyl acetate for 30–60 s. For ultrathin sections, fixed cells were pelleted by centrifugation at 2,000 *g*, stained with 1% osmium tetroxide in 0.1 M Hepes buffer (pH = 7.2) on ice, dehydrated in an acetone concentration series (30%, 50%, 75%, 90%, 100%, 100%) for 15 min at room temperature respectively and embedded in Spurr Epoxy Resin (Spurr 1969). Ultrathin sections were obtained with a Leica UCT ultramicrotome and poststained on grids with 1% aqueous uranyl acetate for 6 min and lead citrate (after Venable and Coggeshall 1965) for 4 min at room temperature. For SEM, strains were filtered on polycarbonate filters (0.2 µm), critical point dried with CO_2 in a Balzers CPC 030 and sputtered with gold in an Emitech K 550 sputter coater.

Table 2.1. Strains of *Spumella* morphology used in this study + *Spumella* ‚species' and strains for which sequences were available (bolt print for herein investigated strains and newly designated species)

strain designation	origin	accession no. SSU	accession no. ITS	accession no. LSU	accession no. COX1	BGBM no.	SAG no.	species designation (former designation)
JBM08	Austria, Lake Mondsee [1) 2)]	AY651098	KF697325	KF697336	KF697353	B 40 004 1261	SAG 2428	***Apoikiospumella mondseeiensis* n.sp. (*Spumella* spec.)**
JBM18	Austria, Lake Krottensee [1)]	AY651092	EF577169	x	KF697355	x	x	x
JBC27	People's Republic of China, Small pond in Huqiu [2)]	AY651093	KF697326	KF697335	KF697351	B 40 004 1262	SAG 2429	***Chromulinospumella sphaerica nov.comb.* (*Spumella* spec.)**
AR3A3	Austria, River Fuschler Ache [3)]	GU073467	KF697322	KF697337	KF697345	B 40 004 1263	SAG 2432	***Segregatospumella dracosaxi* n.sp. (*Spumella* spec.)**
N1846	Japan, Tsukuba, freshwater pond	x	x	x	x	x	x	x
JBNA45	USA, Hawaii, freshwater [2)]	DQ388541	EF577173	KF697332	KF697356	x	x	*Spumella* spec. (*Spumella* spec.)
JBNZ39	New Zealand, Shallow tarn near Karangarua [1) 2)]	AY651088	KF697324	KF697331	KF697357	B 40 004 1264	SAG 2434	***Spumella lacusvadosi* n.sp. (*Spumella* spec.)**
AR4A6	Austria, River Fuschler Ache [3)]	GU073468	KF697328	KF697344	x	B 40 004 0670	SAG 2321	*Spumella rivalis*
194f	Antarctica, Alexander Island, freshwater [2)]	DQ388551	EF577179	KF607330	x	x	x	*Spumella* spec.
JBL14	Austria, puddle in Lunz [1) 2)]	AY651086	EF577172	KF697329	x	B 40 004 1265	SAG 2433	***Spumella bureschii nov.comb* (*Spumella* spec.)**
199hm	Antarctica, Davis Valley, freshwater [2) 3)]	DQ388552	EF577180	KF697342	x	B 40 004 0672	SAG 2322	*Spumella vulgaris*
JBM10	Austria, Small artificial pond in Mondsee, Karlsgarten [1) 2) 3)]	AY651074	EF577166	KF697339	KF697354	B 40 004 0673	SAG 2323	*Poteriospumella lacustris*
JBNZ41	New Zealand, Lake Aviemore [1) 2)]	AY651075	EF577167	KF697340	KF697358	x	x	***Poteriospumella lacustris* (*Spumella* spec.)**
JBC07	People's Republic of China, Lake Tai Hu [1) 2)]	AY651097	EF577165	KF697341	KF697350	x	x	***Poteriospumella lacustris* (*Spumella* spec.)**
AR4D6	Austria, River Fuschler Ache [3)]	GU073469	KF697327	KF697343	KF697361	B 40 004 1266	SAG 2430	***Cornospumella fuschlensis* n.sp. (*Spumella* spec.)**
JBAF33	Tanzania, Msimbazi River [1) 2)]	AY651077	x	KF697338	KF697359	B 40 004 1267	SAG 2427	***Acrispumella msimbaziensis* n.sp. (*Spumella* spec.)**
1006	Antarctica, Signy Island, soil [2)]	DQ388558	EF577176	KF697333	x	x	x	***Pedospumella encystans* (*Spumella* spec.)**
JBMS11	Austria, soil, Mondsee near ‚Rauchhaus' [1) 2) 3)]	AY651083	KF697323	KF697334	KF697352	B 40 004 0671	SAG 2324	*Pedospumella encystans*
JBAS36	Nepal, Nag Pokhari, Kathmandu [1) 2)]	AY6510	x	x	x	x	x	*Pedospumella* spec. (*Spumella* spec.)
JBCS23	People's Republic of China, soil, near Badaling [2)]	AY651081	EF577170	x	KF697349	B 40 004 1268	SAG 2431	***Pedospumella sinomuralis* n.sp. (*Spumella* spec.)**
JBAF35	Kenya, River Sagana [2)]	AY651071	x	x	x	x	x	*Oicomonas* spec. (*Spumella* spec.)
"*Spumella obliqua*"	Germany, Lake Constance	AJ236860	x	x	x	x	x	x
„*Spumella danica*"	Denmark, Jutland, soil	AJ236861	x	x	x	x	x	x
CCAP 955/1	UK, Girton, soil	AJ236859	x	EF681931	x	x	x	***Pedospumella elongata* nov.comb. (*Spumella elongata*)**

[1]for further details see Boenigk et al. 2005; [2]for further details see Boenigk et al. 2006; [3]for further details see Findenig et al. 2010

2.2.3 DNA isolation, PCR and sequencing

Genetic analyses were carried out with both conservative (SSU, 5.8S, and LSU) and more variable (ITS) nuclear gene sequences, as well as COX1. We isolated DNA from the cultures using BioBudget DNA mini-kit (BioBudget Technologies GmbH, Krefeld, Germany) and carried out PCR in a 33-cycle-programme (5-1-2-3 min; see Table 2.2) (Bock et al. 2011) with a BioRad MyCycler (Bio-Rad Laboratories, USA). Sequencing was provided by Beckman Counter Genomics (Takeley, UK). For both PCR and sequencing, different forward and reverse primer combinations per locus were tested until sufficient and comparable results were achieved (see Table 2.2).

2.2.4 Phylogenetic analysis

Sequences were edited with DNADragon 1.5.2 (Hepperle 2012) and aligned in BioEdit Sequence Alignment Editor 7.1.3.0 (Hall 2011) using the ClustalW algorithm (default settings) and manual editing by eye. The SSU alignment (1,491 bp) follows a compilation of sequences (provided by J.M.Scoble) covering all known lineages of Chrysophyceae. Sequences of *Spumella* strains were added using *Sellaphora blackfordensis* and *Nannochloropsis granulata* as outgroups. For ITS1+5.8S+ITS2 (1,009 bp), LSU (1,034 bp) and COX1 (399 bp), mainly sequences of '*Spumella*-like flagellates' were aligned (see Table 2.1 for accession numbers) and an unrooted phylogeny was inferred. As a proof of principle for polyphyly, two photosynthetic chrysophycean species were added. The ITS sequences (ITS1+5.8S+ITS2) of the different *Spumella* strains showed such great diversity that proper alignment and phylogenetic inference was only possible for the 5.8S (156 bp). Phylogenetic trees and corresponding robustness measures (bootstrap analyses with 1,000 replicates, and posterior probabilities) were inferred for all loci with MEGA 5.0.1.102 (Tamura et al. 2011) for neighbour joining and maximum parsimony, with Treefinder (Jobb 2004) for maximum likelihood (ML) and with MrBayes 3.2.1 (Ronquist et al. 2012) for Bayesian analyses. Models for Bayesian analyses were calculated by MrModeltest (SSU and LSU: GTR+I+gamma, 5.8S: SYM+gamma, COX1: F81+I+gamma) in PAUP* 4.0b10 (Swofford 2002). The most similar models available in Treefinder were used for the ML analysis. Bayesian analyses were performed until passing a threshold of 0.01 for the average standard deviations of split frequencies between two runs (sample frequency = 0.01).

Table 2.2. Primers used in this study

Primer	Sequence 5'-3' (melting temperature)	Target	Reference
18SF1 (forward)	AATCTGGTTGATCCTGCCAG (58.7 °C)	SSU	Katana et al. 2001
18SR1 (reverse)	TGATCCTTCTGCAGGTTCACCTA (61.6 °C)	SSU	mod. a. Katana et al. 2001
1420F (forward)	CAGGTCTGTGATGCCC (57.3 °C)	ITS	Rogers et al. 2006
ITSF (forward)	CGTAACAAGGTTTCCGTAGG (57 °C)	ITS	Barth et al. 2006
ITSR (reverse)	TCCTCCGCTTACTGATATGC (56.9 °C)	ITS	Barth et al. 2006
ITS055R (reverse)	CTCCTTGGTCCGTGTTTCAAGACGGG (68.6 °C)	ITS	Marin et al. 1998
ITS2R (reverse)	CCTCACGGTACTTGTTC (53.7 °C)	ITS	An et al. 1999
25F (forward)	ACCCGCTGAATTTAAGCATATA (53.5 °C)	LSU	Jo et al. 2011
1440R (reverse)	TGCTGTTCACATGGAACCTTTC (59.1 °C)	LSU	Jo et al. 2011
2160R (reverse)	CCGCGCTTGGTTGAATTC (58.2 °C)	LSU	Jo et al. 2011
CoChryF (forward)	TCTACTAAyCATAAAGATATCGG (~50 °C)	COX1	Jost unpublished
Cox1BR (reverse)	ACGGTAAACATATGATGAGCCCAAAC (59.9 °C)	COX1	Jost et al. 2010

2.3 RESULTS

2.3.1 General findings

All investigated strains are small chrysomonad flagellates with a mean diameter of 1.2 to 8.6 µm that lack pigmentation and scales (Figs. 2.1 and 2.2). The cells have a smooth surface. They have one long apical anteriorly oriented flagellum that is, approximately, two to three times the length of the cell body and a second shorter flagellum. The long apically anterior-oriented flagellum is always covered by tripartite mastigonemes as revealed by positive contrast (TEM). For some strains, smaller hairs on these mastigonemes are visible (strains 1006, JBM/S11, AR4D6, JBM10). Strain JBC27 shows smaller hairs between the mastigonemes on the long flagellum. However, the short flagellum is always naked (Fig. 2.3). Absolute as well as relative measurements of cell bodies (>30 individuals from actively growing cultures), flagella length and mastigoneme length in SEM, positive contrast, and light microscopy reveal differences in cell size and shape. However, differences in size and shape within strains are comparable in magnitude to those among the different strains and are, thus, not conclusive for delimitation of the investigated strains (compare Table 2.3). All investigated strains are considerably smaller than most of the described species (compare Supplementary Table S2.1). A presumptive plastid was observed close to the nucleus in all investigated strains (Fig. 2.4 circles). The presence of thylakoids was observed only in strain AR4D6 (Fig. 2.4), although all strains lacked visible pigmentation and failed to grow at low food concentrations, independent of light

regime. All investigated strains are very similar to each other based on morphology (Figs. 2.1 to 2.4) whereas molecular data clearly show that they cluster in different branches within Chrysophyceae and are unrelated to each other (Fig. 2.5). Despite the high morphological similarity between these small non-scaled chrysomonad taxa, they differ considerably from the species described to date (Supplementary Table S2.1) except for the cases outlined below. Whereas the morphological diversity of large colourless, non-scaled chrysomonad flagellates seems considerable, it is much less conclusive regarding the smallest fraction of these flagellates.

2.3.2 Distinct strains

Based on SSU rRNA sequence data, all investigated strains cluster within the Chrysophyceae (Fig. 2.5). The strain JBC27 clusters within the order Chromulinales, the strains JBM08 and JBM18 cluster within a clade comprising the genus *Apoikia*. The strain AR3A3 is not closely related to any of the known orders within Chrysophyceae, but clusters separately. Together with an *Oikomonas* species, it forms a neighbouring lineage to the Synurales. All other investigated strains cluster within the order Ochromonadales. Phylogenetic trees calculated based on 5.8S rRNA gene sequences, LSU rRNA gene sequences and COX1 gene sequences are much less conclusive due to the sparseness of sequence data available in public databases. Nevertheless, the tree topologies largely correspond to those of the SSU rRNA tree. The three clusters within Ochromonadales can be identified accordingly in the 5.8S, the LSU and the COX1 phylogeny though their relative position changes (Figs. 2.6 to 2.8).

Within Ochromonadales, the strains JBNA45, JBNZ39, 194f and JBL14 cluster within clade C2, which is currently regarded as genus *Spumella* (Cienkowsky) Boenigk et Findenig. Based on the molecular distance to previously described species (*Spumella vulgaris*, *Spumella rivalis*, and *Spumella obliqua*), all but strain JBNA45 must be considered as new species. Even though strain JBNA45 also differs in the SSU rRNA sequence from its closest relative (i.e. *Spumella obliqua*), these differences are small (0.1% difference). It is therefore uncertain whether this strain must be considered a different species or as a variant of *Spumella obliqua*.

The strains JBNZ41, JBC07, JBAF33 and AR4D6 also cluster within Ochromonadales, but within clade C3. Hereof, the strains JBNZ41 and JBC07 are nearly identical in all investigated gene sequences to *Poteriospumella lacustris* (0.000% in SSU, LSU, 5.8S and Cox). We, therefore, assign these strains to this species. The next known relatives are species affiliated with the genus *Poterioochromonas* with an SSU rRNA sequence difference of 4.4–4.7%. Strain JBAF33 strongly deviates from its next relative (*Uroglena* sp.) with 3.1% sequence difference in SSU rRNA. Based on this strong sequence difference, and the clear morphological and physiological (autotrophic versus heterotrophic) differences, we designate this strain as type for the genus *Acrispumella* n. gen. Strain AR4D6 strongly deviates from its closest relatives (i.e. *Ochromonas sphaerocystis* and *Ochromonas danica*). As the latter two species do not cluster with *Ochromonas moestrupii*, i.e. with the clade presumably comprising the type species of *Ochromonas*, strain AR4D6 cannot be affiliated with the genus *Ochromonas* and we, therefore, designate this strain as type for the genus *Cornospumella* n. gen.

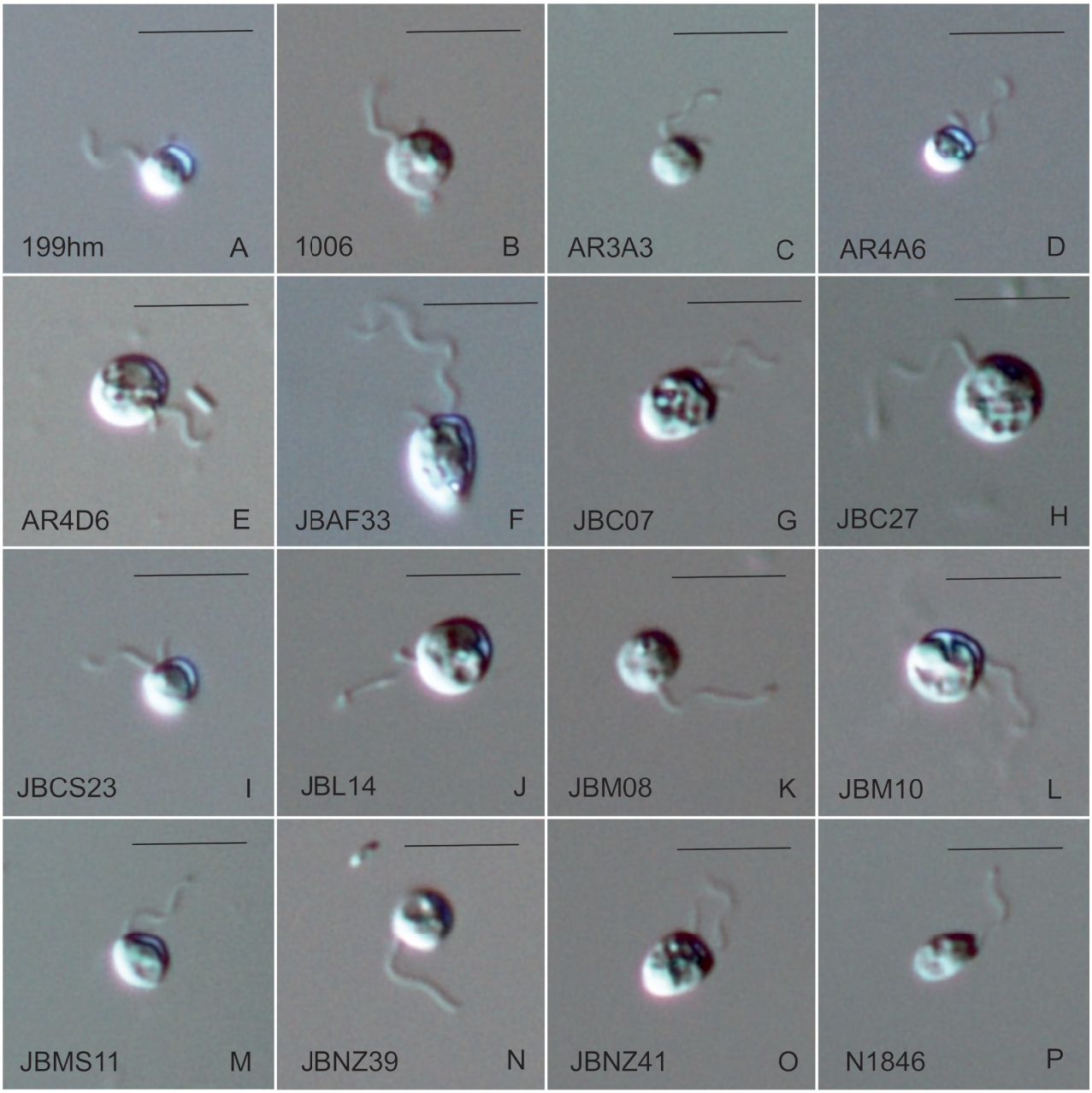


Figure 2.1. Light microscopic images (A-P) showing vegetative cells of strains of colourless non-scaled chrysophytes. (A). 199hm = *Spumella vulgaris.* **(B).** 1006 = *Pedospumella encystans.* **(C)** AR3A3 = *Segregatospumella dracosaxi* n. gen. n. sp. **(D).** AR4A6 = *Spumella rivalis.* **(E).** AR4D6 = *Cornospumella fuschlensis* n. gen. n. sp. **(F).** JBAF33 = *Acrispumella msimbaziensis* n. gen. n. sp. **(G).** JBC07 = *Poteriospumella lacustris.* **(H).** JBC27 = *Chromulinospumella sphaerica* n. gen. nov. comb. **(I).** JBCS23 = *Pedospumella sinomuralis* n.sp. **(J).** JBL14 = *Spumella bureschii* nov. comb. **(K).** JBM08 = *Apoikiospumella mondseeiensis* n. gen. n. sp. **(L).** JBM10 = *Poteriospumella lacustris.* **(M).** JBMS11 = *Pedospumella encystans.* **(N).** JBNZ39 = *Spumella lacusvadosi* n. sp. **(O).** JBNZ41 = *Poteriospumella lacustris.* **(P).** N1846. Scale bars = 10 µm for A-P.

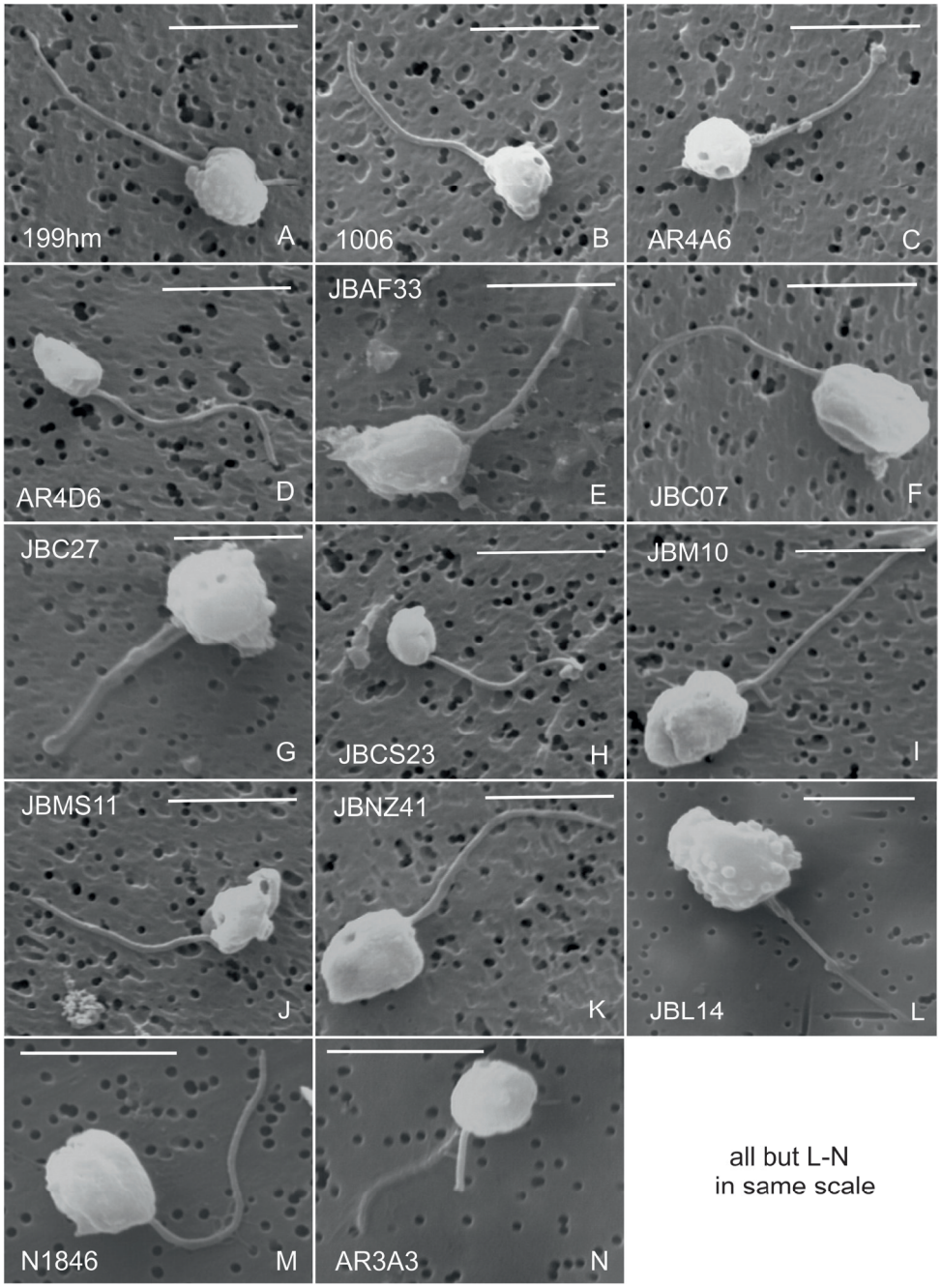


Figure 2.2. SEM images (A-N) showing vegetative cells of strains of *Spumella* morphology. (A). 199hm = *Spumella vulgaris*. **(B).** 1006 = *Pedospumella encystans*. **(C).** AR4A6 = *Spumella rivalis*. **(D).** AR4D6 = *Cornospumella fuschlensis* n. gen. n. sp. **(E).** JBAF33 = *Acrispumella msimbaziensis* n. gen. n. sp. **(F).** JBC07 = *Poteriospumella lacustris*. **(G).** JBC27 = *Chromulinospumella sphaerica* n. gen. nov. comb. **(H).** JBCS23 = *Pedospumella sinomuralis* n. sp. **(I).** JBM10 = *Poteriospumella lacustris*. **(J).** JBMS11 = *Pedospumella encystans*. **(K).** JBNZ41 = *Poteriospumella lacustris*. **(L).** JBL14 = *Spumella bureschii* nov. comb. **(M).** N1846. **(N).** AR3A3 = *Segregatospumella dracosaxi* n. gen. n. sp. Scale bars = 5 µm for A-N.

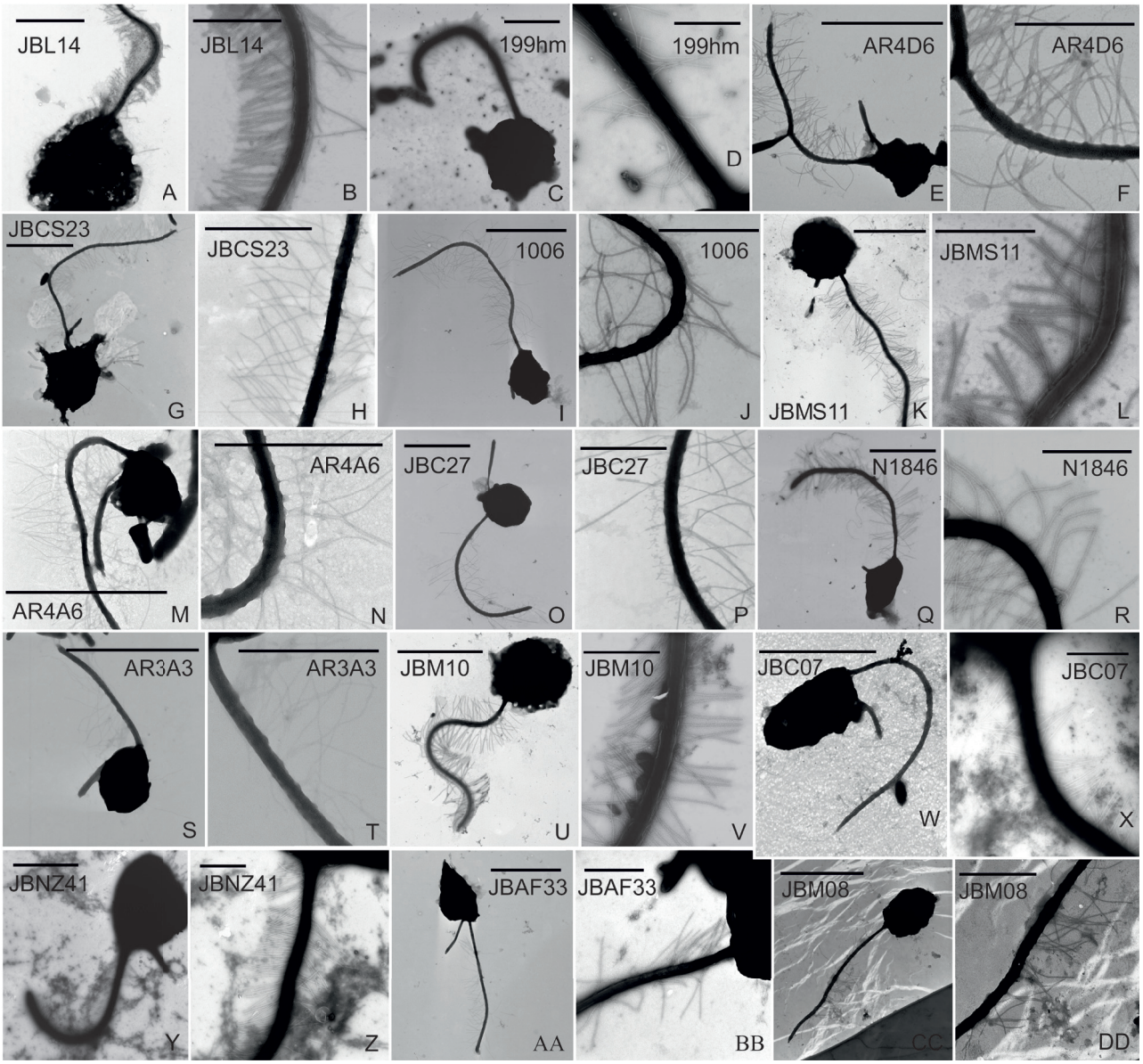


Figure 2.3. TEM images (positive contrast) (A-DD) showing vegetative cells of strains of colourless non-scaled chrysophytes. Two images per strain are given respectively: 1. whole cell, 2. zoom on mastigonemes of large flagellum. **(A+B).** JBL14 = *Spumella bureschii* nov. comb. **(C+D).** 199hm = *Spumella vulgaris.* (**E+F).** AR4D6 = *Cornospumella fuschlensis* n. gen. n. sp. **(G+H).** JBCS23 = *Pedospumella sinomuralis* n. sp. **(I+J)** 1006 = *Pedospumella encystans*. **(K+L).** JBMS11 = *Pedospumella encystans*. **(M+N).** AR4A6 = *Spumella rivalis*. **(O+P).** JBC27 = *Chromulinospumella sphaerica* n. gen. nov. comb. **(Q+R).** N1846. **(S+T).** AR3A3 = *Segregatospumella dracosaxi* n. gen. n. sp. **(U+V).** JBM10 = *Poteriospumella lacustris*. **(W+X).** JBC07 = *Poteriospumella lacustris*. **(Y+Z).** JBNZ41 = *Poteriospumella lacustris*. **(AA+BB).** JBAF33 = *Acrispumella msimbaziensis* n. gen. n. sp. **(CC+DD).** JBM08 = *Apoikiospumella mondseeiensis* n. gen. n. sp. Scale bars = 3 µm for A, C, E, G, I, K, M, O, Q, S, U, W, Y, AA, CC; Scale bars = 1 µm for B, D, F, H, J, L, N, P, R, T, V, X, Z, BB, DD.

Table 2.3. Measurements of strains

species epithet	strain	positive contrast (TEM): cell body (µm)	SEM: cell body (µm)	Light microscopy: cell body (µm) (also see Boenigk et al. 2005 and Findenig et al. 2010)	positive contrast (TEM): long flagellum (µm)	SEM: long flagellum (µm)	Light microscopy: long flagellum (µm) (also see Boenigk et al. 2005 and Findenig et al. 2010)
Apoikiospumella mondseeiensis	**JBM08**	2.0	x	2.9 - 4.1	6.0	x	5.9 - 12.0
Chromulinospumella sphaerica	**JBC27**	2.0	2.55 - 3.65	4.7 - 7.0	6.9	6.4 - 6.9	3.7 - 15.0
Segregatospumella dracosaxi	**AR3A3**	1.2 - 1.9	2.5 - 2.6	2.1 - 4.0	2.9 - 3.4	5.0 - 7.6	4.2 - 8.0
Spumella lacusvadosi	**JBNZ39**	x	x	1.5 - 5.2	x	x	0.7 - 10.4
Spumella rivalis	**AR4A6**	1.4 - 3.45	2.2 - 2.4	2.7 - 3.9	4.4 - 6.3	7.7 - 8.7	5.4 - 7.8
Acrispumella msimbaziensis	**JBAF33**	2.05 - 2.8	3.45 - 4.8	3.0 - 4.5	5.7 - 8.4	8.8 - 17.4	5.8
Spumella bureschii	**JBL14**	2.85 - 5.15	3.2 - 4.45	3.8 - 7.1	6.9 - 11.0	5.5 - 9.3	7.6 - 14.2
Pedospumella sinomuralis	**JBCS23**	2.45	1.85 - 2.35	1.8 - 2.2	9.5	4.5 - 7.1	4.2
Poteriospumella lacustris	**JBNZ41**	1.85 - 3.9	3.1 - 3.9	2.3 - 2.8	4.8 - 9.0	6.2 - 10.0	3.7 - 3.8
Poteriospumella lacustris	**JBC07**	1.65 - 5.4	3.35 - 4.8	3.3 - 4.4	5.2 - 9.4	5.9 - 9.5	5.0
Pedospumella encystans	**1006**	1.2 - 1.5	2.2 - 3.3	x	3.5 - 6.5	5.0 - 10.8	x
Spumella vulgaris	**199hm**	3.35 - 3.85	2.4 - 3.55	3.2 - 5.9	8.5 - 10.3	8.5 - 10.4	6.4 - 11.8
Cornospumella fuschlensis	**AR4D6**	1.25	2.1 - 3.15	2.8 - 4.7	4.6	4.3 - 7.0	5.6 - 9.4
Pedospumella encystans	**JBMS11**	1.95 - 2.7	1.75 - 2.9	3.0 - 6.2	5.0 - 10.0	4.3 - 9.6	6.0 - 12.4
Poteriospumella lacustris	**JBM10**	2.25 - 3.9	2.95 - 3.6	3.0 - 5.0	5.0 - 10.0	6.0 - 8.4	4.5 - 10.0
‚*Spumella*-like flagellate‘	**N1846**	1.65 - 4.35	2.55 - 3.7	x	4.6 - 11.4	8.5 - 10.8	x

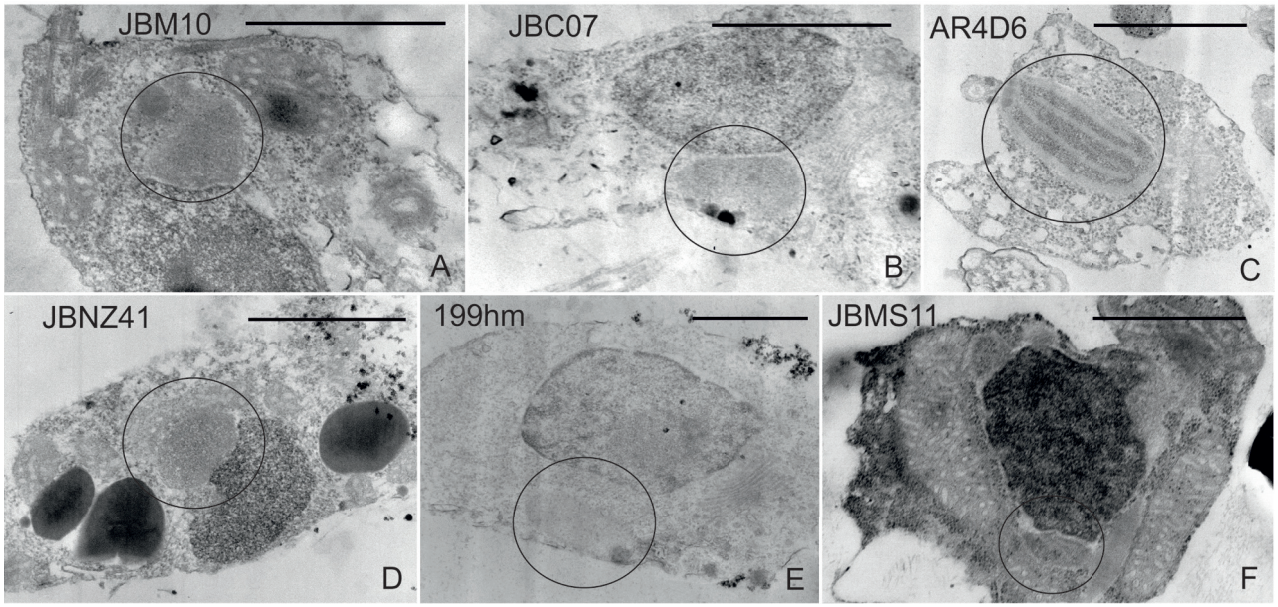


Figure 2.4. TEM images (ultrathin sections) (A-F) showing cell interior of strains of colourless non-scaled chrysophytes. Core-associated plastidal organells are highlighted. **(A).** JBM10 = *Poteriospumella lacustris*. **(B).** JBC07 = *Poteriospumella lacustris*. **(C).** AR4D6 = *Cornospumella fuschlensis* n. gen. n. sp. **(D).** JBNZ41 = *Poteriospumella lacustris*. **(E).** 199hm = *Spumella vulgaris*. **(F).** JBMS11 = *Pedospumella encystans.* Scale bars = 1 µm for A-F.

A third clade within Ochromonadales (C1-clade) comprises taxa affiliated with the genus *Pedospumella*. Strain 1006 is nearly identical in sequence data to the type strain and is, therefore, considered as affiliated with the type species *Pedospumella encystans*. *Spumella elongata* differs considerably in cell size and in the ratio of cell body and long flagellum from *Pedospumella encystans* and is, therefore, regarded a different species, which must, however, be recombined to *Pedospumella elongata* nov. comb. The strains JBC23 and JBAS36 cluster in a sister clade to these former two species with a sequence difference of 0.7–2.2%. They cluster together with a sequence labelled *Spumella danica* which is, however, a nomen nudum, i.e. the organism has not formally been described (cf. Bruchmüller 1998; see supplement).

The strain AR3A3 clusters within Chrysophyceae together with a strain designated as *Oikomonas* sp. (as this strain does not cluster with *Chromulina* this designation must be regarded as incorrect), but without statistical support to reveal the next related lineages. We designate this strain as type for the genus *Segregatospumella* n. gen. within family Segrataceae n. fam. and order Segregatales n. ord.

Within the order Chromulinales, strain JBC27 clusters separately from the only colourless genus within Chromulinales [i.e. *Oikomonas* (=*Heterochromulina*)]. In contrast to the genus *Oikomonas*, in which only one flagellum is visible in the light microscope, strain JBC27 has a second flagellum clearly visible by light microscopy. *Cyclonexis annularis* Stokes 1886, a colonial photosynthetic chrysophyte, is the next relative to strain JBC27 based on available sequence data. Based on the high sequence difference of 7% from its next relative and the morphological differences, we designate this strain as type for the genus *Chromulinospumella* nov. gen.

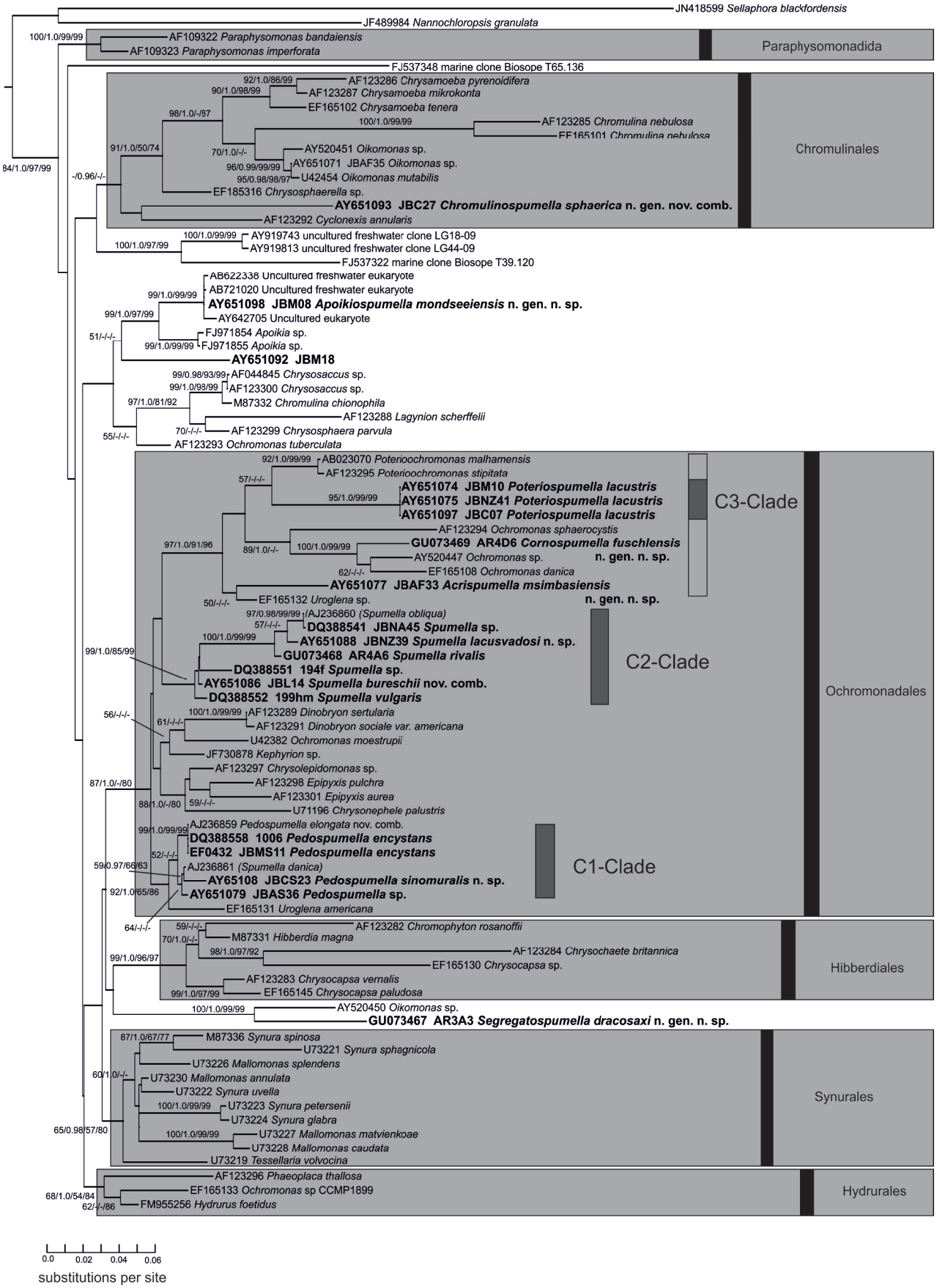


Figure 2.5. Maximum-likelihood phylogeny based on SSU sequences showing the investigated strains of colourless non-scaled chrysophytes (bolt print) within Chrysophyceae. Numbers at nodes give bootstrap values and posterior probabilities in following order: maximum-likelihood / bayesian / maximum-parsimony / neighbour-joining (values >50 are shown; posterior probabilities >0.95).

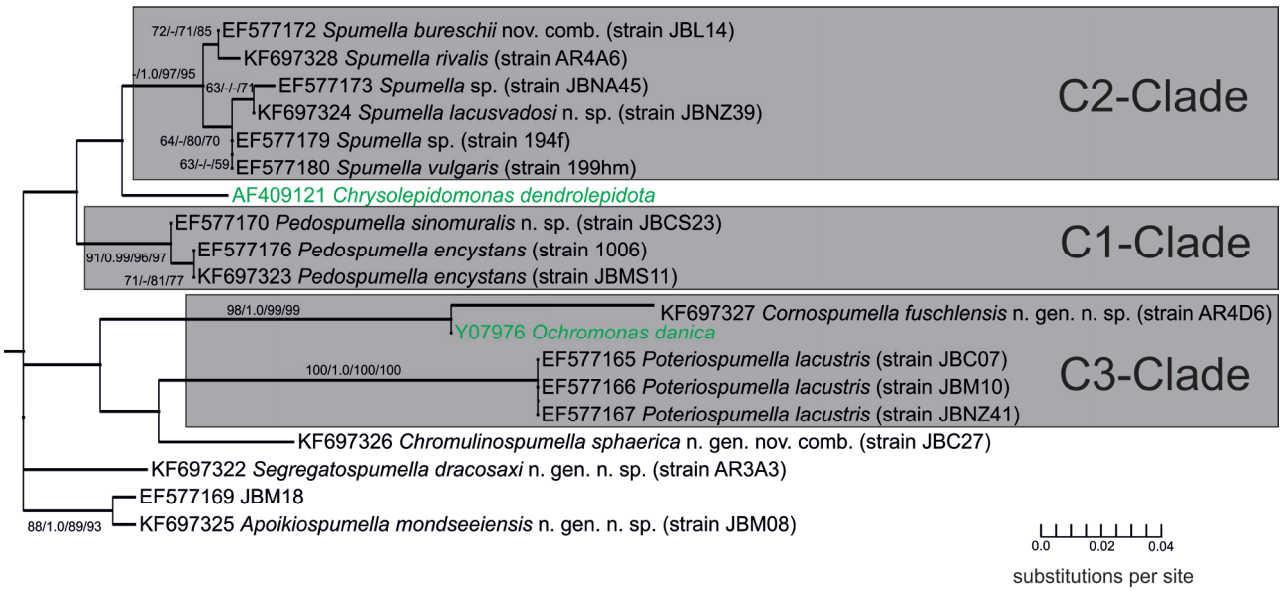


Figure 2.6. Maximum-likelihood phylogeny based on 5.8S sequences of strains of colourless non-scaled chrysophytes. Numbers at nodes give bootstrap values and posterior probabilities in following order: maximum-likelihood / bayesian / maximum-parsimony / neighbour-joining (values >50 are shown; posterior probabilities >0.95). Two additional photosynthetic chrysophycean species (in green) show polyphyly of colourless non-scaled chrysophytes.

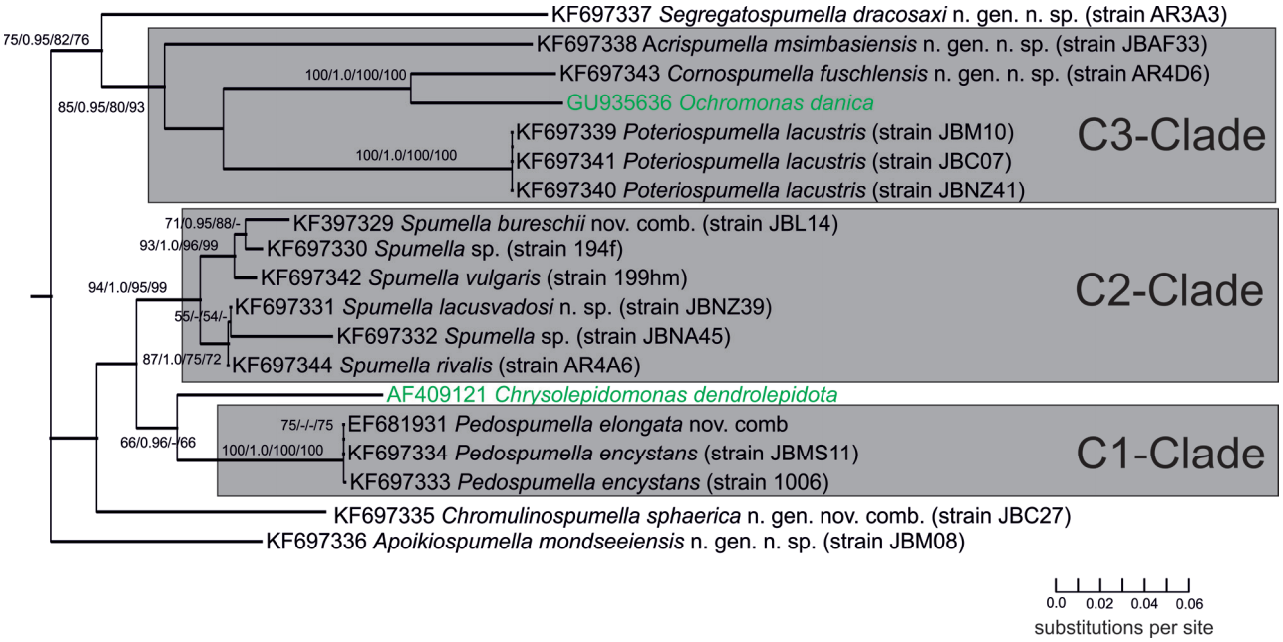


Figure 2.7. Maximum-likelihood phylogeny based on LSU sequences of strains of colourless non-scaled chrysophytes. Numbers at nodes give bootstrap values and posterior probabilities in following order: maximum-likelihood / bayesian / maximum-parsimony / neighbour-joining (values >50 are shown; posterior probabilities >0.95). Two additional photosynthetic chrysophycean species (in green) show polyphyly of colourless non-scaled chrysophytes.

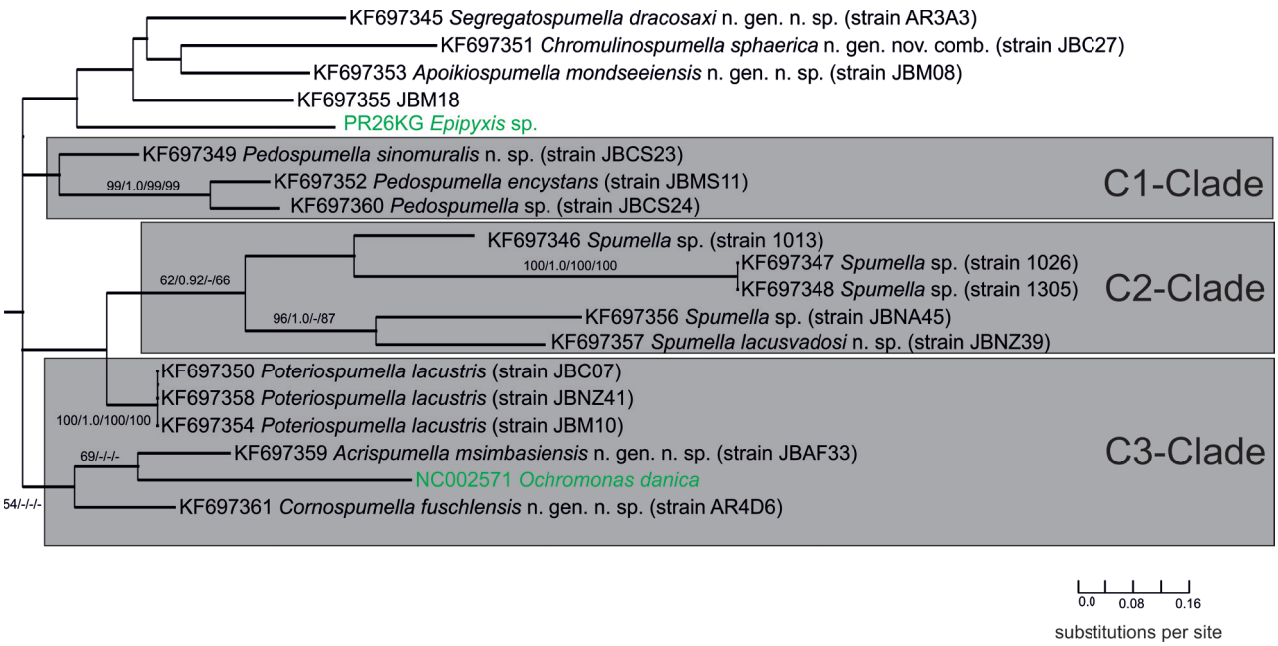


Figure 2.8. Maximum-likelihood phylogeny based on COX1 sequences of strains of colourless non-scaled chrysophytes. Numbers at nodes give bootstrap values and posterior probabilities in following order: maximum-likelihood / bayesian / maximum-parsimoy / neighbour-joining (values >50 are shown; posterior probabilities >0.95). Two additional photosynthetic chrysophycean species (in green) show polyphyly of colourless non-scaled chrysophytes.

The strains JBM08 and JBM18 cluster within the clade comprising *Apoikia* with strain JBM08 being the next relative to *Apoikia* with a molecular difference of 2.5–2.7% in the SSU rRNA. However, *Apoikia* is a genus of colonial flagellates embedded in mucilage, and the second flagellum is clearly visible. In contrast, strain JBM08 is unicellular and the second flagellum is hardly visible in the light microscope. As its morphology strongly deviates from that of the genus *Apoikia* and its sequence also shows considerable differences, we designate strain JBM08 as type of the genus *Apoikiospumella* nov. gen.

2.4 DISCUSSION

2.4.1 Taxonomy and phylogeny

Colourless, non-scaled chrysomonad flagellates have been described and named since Müller's description of *Monas* species in 1773 (see Boenigk 2008b and references therein). However, most of the described flagellates which can be considered as colourless, non-scaled chrysomonad flagellates differ from the strains described in this study (see Supplementary Table S2.1). Carefully considering the taxonomic literature, we thus come to the conclusion that only two of the observed strains in this study (i.e. strains JBC27 and JBL14) can be identified with one of the existing description (for details see Table S2.1). Many taxa, originally described as *Monas* spp., have already been reclassified to other protistan groups or cannot be assigned with Chrysophyceae (Boenigk 2008b; Table S2.1). The described species which are affiliated with Chrysophyceae (including those

for which such an affiliation is unclear, but cannot be rejected) differ from the strains investigated in this study. Quite a number of described species do not match the strains investigated herein for the details provided, such as being pigmented as for *Monas bicolor* (Ehrenberg 1832) or having a gelatinous sheath as for *Monas coronifera* (Skuja 1948).

The characters of *Spumella vulgaris* as described by Cienkowsky raised doubts about the purity of the type culture. Some characters such as non-contractile spines may even hint to species possibly affiliated with other chrysomonad genera such as *Paraphysomonas* whereas other characters such as the flexible cell surface observed in some individuals presumably contradict such an affiliation. The stomatocyst as described by Cienkowsky (1870) corresponds well with cysts produced by flagellates which are today predominantly considered to be *Spumella* sp. It needs to be noted that flagellates producing stomatocysts of the size as described by Cienkowsky can be much smaller than the vegetative cells described by Cienkowsky (Findenig et al. 2010). Again, this underpins doubts about the purity of the type culture of Cienkowsky. Based on the similarity of the lectotype with stomatocysts of an extant flagellate, Findenig et al. (2010) designated an epitype for *Spumella vulgaris*. Thus, the epitype of *Spumella vulgaris* corresponds to the original description of the stomatocyst by Cienkowsky (1870), and the vegetative cells correspond to what is today considered to be *Spumella*. It needs to be stated, however, that the vegetative cell of the epitype (Findenig et al. 2010) deviates in some characters to those provided by Cienkowsky (1870). A future discovery of a taxon with all the properties described by Cienkowsky may prove this basic assumption by Findenig et al. (2010) wrong and, thus, would require a transfer of the taxa affiliated with the genus *Spumella* Cienkowsky sensu Findenig and Boenigk into a new genus. Considering the doubts about the purity of the original culture of Cienkowsky, the choice of Findenig et al. (2010) seems justified, conserves the generic name for those flagellates which are today regarded as *Spumella*, and lays a sound basis for revising the colourless, non-scaled chrysomonad flagellates.

The flagellates investigated herein were morphologically very similar. Specifically, size is not a criterion allowing for the differentiation of the distinct investigated strains: all strains were small and intraspecific variation of size was high. The investigated strains were, however, considerably smaller than most described taxa. This difference in size between the investigated strains and most described taxa is presumably due to differences in the isolation protocol. While most previous studies applied enrichment techniques, we used a filtration-acclimatisation approach (i.e. selecting for flagellates of around 5 µm and smaller). We furthermore demonstrate plastidal remains in all strains that we investigated as TEM ultrathin sections (compare images in Fig. 2.4). We show plastidal remains in four different genera (independent heterotrophic lineages within Chrysophyceae) and expect the other described genera to also bear reduced plastids (TEM ultrathin sections were not carried out here).These remains support the assumption that heterotrophic chrysomonad flagellates derive from phototrophic ancestors.

The flagellates formerly lumped as being '*Spumella*-like' in morphology – despite being morphologically hardly or not at all distinguishable – show high polyphyly in phylogenetic analyses. Our analyses support the polyphyly of these flagellates (Boenigk et al. 2005; Scoble and Cavalier-Smith

2014) and the erection of the genera *Pedospumella* and *Poteriospumella* by Findenig et al. (2010). As well, we show that a further subdivision within the identified three clades of Ochromonadales is required to adequately differentiate between lineages of small colourless, non-scaled chrysomonad flagellates that evolved independently. We furthermore identify lineages that are not part of the three described clades within Ochromonadales having evolved in the chrysophycean order Chromulinales, in a cluster together with the genus *Apoikia* and in one yet undescribed clade. We, therefore, describe flagellates with a *Spumella*-like morphology also outside Ochromonadales. This finding is in accordance with the observed huge diversity in the ITS1 and ITS2 sequences of the strains indicating only a distant relationship. On the basis of the above analyses, we agree with Findenig et al. (2010) in abandoning the generic name *Monas* and suggest the exclusive use of the genus names *Spumella* sensu Findenig, *Pedospumella, Poteriospumella* (see Findenig et al. 2010) and the generic names introduced herein, namely *Apoikiospumella, Chromulinospumella, Segregatospumella, Acrispumella* and *Cornospumella* (see 'Taxonomic Summary'). As none of our isolates cluster within the clade comprising *O. moestrupii* or as a sister to this clade, both from a taxonomic and a phylogenetic point of view, the generic names *Ochromonas* and *Heterochromonas* can be rejected for any strain described in this study. However, we consider it likely that the *Ochoromonas* clade comprising *O. moestrupii* also has a colourless sister clade – specifically because *O. moestrupii* has been reported to sometimes divide into two cells one of which lacks a plastid (Andersen 2011). The generic name *Heterochromonas* should, thus, be reserved for such a sister clade according to the original proposition by Pascher (1912). Several former *Spumella* spp. are, thus, reclassified within this paper. Other *Spumella* spp. have been described in detail by electron microscopic methods, but can be separated from the herein described taxa based on either molecular data (*Spumella obliqua* sensu Mylnikov et al. 2007), deviant morphology (*Spumella sociabilis* sensu Mylnikov and Mylnikova 2005; colony-forming), (*Spumella gregaria* Tanichev 1993; length of flagella), (*Spumella termo* sensu Tanichev 1993; forms exovacuoles) or size (*Spumella sphaerophora*: sensu Mylnikov and Mylnikova 2005). The description of *Spumella subterana* (Tanichev and Karpov 1995) is not valid and must, therefore, be considered as nomen nudum. Even though these latter taxa differ from the strains investigated herein, they may be related to one of them. A re-examination of these latter strains with molecular methods may shed light on their phylogenetic position possibly requiring a transfer to another genus.

Such polyphyly in morphologically similar or indistinguishable groups has also been reported for several other protistan lineages, prominent examples of which are the Trebouxiophyceae of *Chlorella*-like morphology (Huss et al. 1999) and of *Dictyosphaerium*-like morphology (Krienitz et al. 2010), the choanoflagellates affiliated with Codonosigidae (Nitsche et al. 2011), the kinetoplastids of *Bodo*-like morphology (Moreira et al. 2004), and the pigmented chrysophytes of an *Ochromonas*-like morphology (Andersen et al. 1999). Thus, polyphyly of protistan morphodemes, rather than being unexpected and rare, is currently disclosed for more and more organisms.

2.4.2 Ecological and evolutionary scenarios behind the multiple evolution of small colourless, non-scaled chrysomonad flagellates

The high molecular diversity between the flagellate taxa investigated herein is not reflected by a respective morphological differentiation indicating multiple convergent evolution of this morphodeme. Despite morpholol gical similarity, the investigated strains may differ considerably in ecology, ecophysiology and geographical distribution (Boenigk 2008a; Nolte et al. 2010).

The occurrence of multiple convergent evolution raises the question of reasons for the success of this morphology. The optimisation of nutrition and the trade-off between photosynthesis on the one hand and consumption of small bacteria, specifically ultramicrobacteria, may be the key behind this evolution: According to the predator-prey theory, a reduction of the plastid and with that a reduction of overall cell size increases the capture efficiency of small prey (DeCastro et al. 2009). The loss of pigmentation and the evolution of small bacterivorous forms may, therefore, be driven by the availability of differential food sources (i.e. large versus small bacteria) and the availability of nutrients and light for photosynthesis (DeCastro et al. 2009).

Although being very similar in aspects such as general feeding behaviour (Boenigk et al. 2005) or tolerance to pH (generally 3.15 to ~11) (Pfandl et al. 2009), comparative research on different strains of small, colourless, non-scaled chrysomonad flagellates (named therein '*Spumella* spec.' or '*Spumella*-like flagellates' and specified by strain names) reveals inequality in many respects including in temperature as well as salinity tolerance (Pfandl et al. 2009), in specific feeding behaviour and food selection (Boenigk et al. 2004; Šimek et al. 2013), in toxicity level (Boenigk and Stadler 2004), seasonal appearance (Nolte et al. 2010), required habitat character (Boenigk 2005) and in global distribution (Boenigk et al. 2006). These inequalities, however, have been observed on different taxonomic levels. Tolerance to salinity varies within '*Spumella*-like flagellates' from 1–6 g $NaCl*L^{-1}$, but shows a comparable range of tolerances within cluster C3 of Ochromonadales itself from strain 1-8-A1 tolerating up to 2 $g*L^{-1}$ to strain JBC30 tolerating up to 6 $g*L^{-1}$ (Pfandl et al. 2009 – also compare species descriptions herein for specific data). Such high variance in phylogenetically closely related strains rather points to a quick evolutionary change concerning salinity. High intraclade variation has also been observed in comparable research of strains JBM10, JBC07 and JBNZ41, which show almost a 100% similarity in SSU sequences. JBC07 has proved to be more toxic to zooplankton than strains JBM10 and JBNZ41 (Boenigk and Stadler 2004). In feeding experiments, strain JBC07 in comparison grows the fastest on large bacteria, whereas strain JBNZ41 grows faster than JBC07 and JBM10 on two ultramicrobacterial strains. Unlike strains JBC07 and JBNZ41, strain JBM10 grows equally well on all offered food bacteria (Boenigk et al. 2004). In contrast to these findings, temperature tolerance as well as habitat preference seem to be more stable within clades indicating slower evolutionary change. This is most striking in clades C1, C2 and C3 of Ochromonadales. Clade C1 comprises mainly soil-associated strains, whereas clades C2 and C3 are mostly aquatic – C3 mostly from eutrophic and shallow waters, C2 from aquatic environments ranging from large lakes to puddles (Boenigk 2005). Differences in temperature tolerance of the three clades are most striking showing an overlapping, but unambiguous differentiation from

cold- to warm-adapted. Clade C2, being the most cold-adapted of the three, grows well between 5–10 °C whereas C1 and C3 hardly grow or even die (Boenigk et al. 2006). Correspondingly and based on environmental data from samplings on a global scale as well as from seasonal succession studies (spring to autumn), clade C2 can be characterised as cold-temperate, C1 as temperate-moderately-warm and C3 as temperate-warm (Nolte et al. 2010), including endemic adaptation as for strains of clade C2 originating from the Antarctica (Boenigk et al. 2006). On the level of genera of '*Spumella*-like flagellates' (described by Findenig et Boenigk 2010 and Grossmann et Boenigk herein), a preliminary ecological characterisation would thus be as follows:

Genus *Spumella*: predominantly cold-temperate, early-year (in temperate zone), freshwater

Genus *Poteriospumella*: predominantly temperate-warm, late-year, freshwater, rather eutrophic

Genus *Cornospumella*: predominantly temperate-warm, late-year, freshwater

Genus *Acrispumella*: predominantly temperate-warm, late-year, freshwater

Genus *Pedospumella*: predominantly temperate-moderately-warm, mid-year, mostly soil

Genus *Apoikiospumella*: freshwater, slow-growing

Genus *Chromulinospumella*: freshwater

Genus *Segregatospumella*: freshwater

These genus-level characterisations are rough generalisations, and variation between species is to be expected. Published data so far, however, provide evidence that ecological and physiological differences between different genera and between different species of small, colourless, non-scaled chrysomonad flagellates do exist. The practice of lumping such organisms in ecological studies, such as occurs with '*Spumella*-like flagellates', is therefore questionable. It may be justified with respect to rough predator-prey interactions such as gross feeding rates on bacterioplankton (Boenigk 2005). However, different species feed preferentially on different bacteria (Šimek et al. 2013) and are adapted to different habitat characteristics. Future ecological investigations should, therefore, consider the different genera and preferentially also the different species separately.

Distribution patterns of protists have been controversially discussed (Fenchel et al. 1997: 'Everything-is-everywhere'; Foissner 2006 and Foissner 2008: 'moderate endemicity') taking into account the organisms' small size and potential dispersal as well as local and regional dynamics of habitat enclosure. For the investigated groups of organisms, taxa of presumable worldwide distribution (e.g. *Poteriospumella lacustris*) as well as presumable endemics (such as the cold-adapted Antarctic strains) are known. Furthermore, the finding of low-abundance taxa (Nolte et al. 2010) revisits the question of dispersal and distribution patterns. Due to the lack of mere numbers of investigations of this group of organisms, such issues can currently not be answered satisfactorily – despite the group being one of the most abundant flagellates in aquatic as well as soil environments, and with that being among the most abundant eukaryotes on earth. Given the high diversity specifically

in freshwaters, large-scale sampling campaigns specifically focusing on freshwaters and soils would, therefore, be most desirable to further address this issue. Such studies should, however, be accompanied by analyses addressing the functional differentiation between these genera and species in the field as well as by further taxonomic analyses of such flagellates. As with respect to diversity, we are currently certainly just scraping the tip of an iceberg. The taxonomic amendment presented herein should provide a sound basis for addressing the yet enigmatic diversity of small, colourless, non-scaled chrysomonad flagellates.

Due to the high degree of morphological convergence between the different groups, we propose and call for using molecular phylogenetic information rather than morphology for the classification of such flagellates. Currently, the SSU as a conserved sequence for a gross classification and ITS sequences as variable sequences allowing for species discrimination seem to be the best choice with a good database reference. As shown, different molecular loci reveal a concordant phylogeny. Small, colourless, non-scaled chrysomonad flagellates are not limited to the order Ochromonadales. We describe the new genera *Apoikiospumella,* as a neighbour to the genus *Apoikia*, *Chromulinospumella* within the order Chromulinales and the genus *Segregatospumella* of uncertain connection within Chrysophyceae with their type species, respectively. For the genus *Segregatospumella*, we consequently describe the new family Segregataceae and the new order Segregatales. Within Ochromonadales, we describe two new genera with their respective type species. We describe and recombine additional species within the already known genera *Spumella* sensu Findenig and *Pedospumella* of Ochromonadales. Furthermore, three of the investigated strains cannot be regarded as independent species lineages and are, therefore, included in previously described species, namely strains JBNZ41 and JBC07 to *Poteriospumella lacustris* (Boenigk et Findenig 2010) and strain 1006 to *Pedospumella encystans* (Boenigk et Findenig 2010). According to their position in the SSU phylogeny, we include two further strains respectively in the genera *Spumella* and *Pedospumella*, namely strain JBNA45 (‘*Spumella*-like flagellate’) as *Spumella* spec. and strain JBAS36 (‘*Spumella*-like flagellate’) as *Pedospumella* spec. However, the species delimitation of these strains in not clear and we, therefore, do not formally describe them. Accordingly, strain JBAF35 changes its taxonomic affiliation from ‘*Spumella*-like flagellate’ to *Oikomonas* spec.

The two species names *Spumella obliqua* and *Spumella danica* are only represented by sequence information in GeneBank (submitted in 1999). The publication containing their descriptions and naming remains unpublished. Both names are, therefore, invalid. In the phylogenetic analysis undertaken herein, the sequence of ‘*Spumella obliqua’* clusters within the genus *Spumella* and the sequence of ‘*Spumella danica’* within the genus *Pedospumella*.

As Chrysophyceae are a mixed class of phototrophic, mixotrophic and heterotrophic organisms, we describe all species and genera under the ICN and ICZN both whenever possible. In cases where the two codes contradict each other, we follow the ICN. For the description of higher taxa, we likewise apply the ICN.

2.5 TAXONOMIC SUMMARY

Sar Adl et al. 2012

Stramenopiles Patterson 1989, emend. Adl et al. 2005

Class: Chrysophyceae Pascher 1914

Order: Apoikiida Boenigk et Grossmann n. ord.

Diagnosis: Non-photosynthetic, non-scaled, bacterivorous chrysophycean bi-flagellates. Colonial as well as solitary living species. Order containing only the family Apoikiaceae. Distinct from other orders within Chrysophyceae by the gene sequences (SSU, ITS, LSU, COX1) of the described species within the order: *Apoikia lindahlii* Kim et al. 2010 (Basionym *Monas lindahlii* Skuja 1956) and *Apoikiospumella mondseeiensis* n. g. n. sp. (described below).

Type of the order: Apoikiaceae Boenigk et Grossmann n. fam.

Etymology: 'Apoikiida' draws on the only known family of the new order: 'Apoikiaceae'.

Family: Apoikiaceae Boenigk et Grossmann n. fam.

Diagnosis: Non-photosynthetic, non-scaled, bacterivorous chrysophycean bi-flagellates. Colonial as well as solitary living species. Family containing the genera *Apoikia* and *Apoikiospumella*. Distinct by the gene sequences (SSU, ITS, LSU, COX1) of the two known species within the family: *Apoikia lindahlii* Kim et al. 2010 (Basionym *Monas lindahlii* Skuja 1956) and *Apoikiaspumella mondseeiensis* n. g. n. sp. (described below).

Type of the family: *Apoikia lindahlii* Kim et al. 2010 (Basionym *Monas lindahlii* Skuja 1956)

Etymology: 'Apoikiaceae' draws on the genus '*Apoikia*'.

Apoikiospumella Boenigk et Grossmann n. g.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Distinct phylogenetic lineage related to the genera *Apoikia* (SSU sequence difference: 2.5-2.7%), *Chrysosphaera* (4.3%) and *Chrysosaccus* (5%).

Typus generis: ***Apoikiospumella mondseeiensis*** Boenigk et Grossmann n. sp.

Etymology: Feminine. '*Apoikio-*' indicates the phylogenetic affiliation to the genus *Apoikia* and '*-spumella'* the taxonomic point of origin as so-called '*Spumella*-like flagellate'.

Apoikiospumella mondseeiensis Boenigk et Grossmann n. sp.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Vegetative cells mostly spherical, sometimes elongated or posteriorly pointed, 2-7.1 µm in diameter. Long flagellum up to 12 µm in length bearing tripartite mastigonemes (compare images Fig. 2.1(K), Fig. 2.3(CC+DD) in this paper). Second flagellum short and attached to the cell body so that scarcely visible in light microscopy. Distinct from its closest relative *Apoikia* sp. (i.e. *Apoikia lindahlii*) as non-colonial and not covered in mucilage, as well as in the SSU sequence by 2.5-2.7% sequence difference.

Distinct – on at least species level – from other '*Spumella*-like flagellates' of comparable morphology (as those described herein) by its gene sequences of SSU, ITS, LSU and COX1 (compare trees Figs. 2.5, 2.6, 2.7, 2.8).

Holotype: Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1261 (formaldehyde fixation of strain JBM08).

Type habitat: Mesotrophic freshwater lake.

Type locality: Austria, Lake Mondsee, 47°52'0 N, 13°20'0 E, 500 m asl.

Etymology: The species epithet '*mondseeiensis*' hints at the species' place of origin from Lake Mondsee, Austria.

Gene sequences: NCBI accession no. AY651098 (SSU), no. KF697325 (ITS), no. KF697336 (LSU), no. KF697353 (COX1); all from strain JBM08.

Further reference: SAG number 2428 (from strain JBM08); strain JBM08 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information: In experiments: temperature maximum of 30.7 °C, salinity maximum of 1 g $NaCl*L^{-1}$, pH tolerance between 3.15 and 10.9 (Pfandl et al. 2009); all data from strain JBM08.

Order: Chromulinales Pascher 1910

Family: Chromulinaceae Engler 1897

Chromulinospumella Boenigk et Grossmann n. g.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Distinct phylogenetic lineage within the order Chromulinales of Chrysophyceae related to the genus *Cyclonexis* (SSU sequence difference: 7%).

Typus generis: ***Chromulinospumella sphaerica*** (Valkanov) Boenigk et Grossmann nov. comb.

Etymology: Feminine. '*Chromulino-*' indicates the phylogenetic affiliation to the order Chromulinales and '*-spumella*' the taxonomic point of origin as so-called '*Spumella*-like flagellate'.

Chromulinospumella sphaerica (Valkanov) Boenigk et Grossmann nov. comb.

Basionym: *Monas sphaericus* Valkanov (1925).

Emended diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated, 2-8.6 µm in diameter. Long flagellum up to 15 µm in length bearing tripartite mastigonemes. Additionally, smaller hairs in-between the mastigonemes, inserting at the plasma membrane of the long flagellum (compare images Fig. 2.1(H), Fig. 2.2(G), Fig. 2.3(O+P) in this paper). Distinct from the genus *Cyclonexis* (closest relative) as neither photosynthetic nor building ring shaped colonies, as well as in the SSU sequence by 7% sequence difference.

Distinct – on at least species level – from other '*Spumella*-like flagellates' of comparable morphology (as those described herein) by its gene sequences of SSU, ITS, LSU and COX1 (compare trees Figs. 2.5, 2.6, 2.7, 2.8).

Holotype: Figure 7 in Valkanov (1925), designated by Valkanov.

Epitype (designated herein): Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1262 (formaldehyde fixation of strain JBC27).

Type habitat: Small pond.

Type locality: People's Republic of China, Huqiu, 31°20'05 N, 120°34'27E, 4 m asl.

Gene sequences: NCBI accession no. AY651093 (SSU), no. KF697326 (ITS), no. KF697335 (LSU), no. KF697351 (COX1); all from strain JBC27.

Further reference: SAG number 2429 (from strain JBC27); strain JBC27 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information: In experiments: temperature maximum of 33.6 °C, salinity maximum of 3 g $NaCl*L^{-1}$, pH tolerance between 3.15 and 10.9 (Pfandl et al. 2009); all data from strain JBC27.

Order: Segregatales Boenigk et Grossmann n. ord.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellates. Order only containing the family Segregataceae. Distinct from other orders within Chrysophyceae by the gene sequences (SSU, ITS, LSU, COX1) of the known species *Segregatospumella dracosaxi* n.sp. (described below) and the SSU sequence AY520450 (NCBI accession number).

Type of the order: Segregataceae Boenigk et Grossmann n. fam.

Etymology: 'Segregatales' draws on the only known family of the new order: 'Segregataceae'.

Family: Segregataceae Boenigk et Grossmann n. fam.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellates. Family containing the genus *Segregatospumella*. Distinct by the gene sequences (SSU, ITS, LSU, COX1) of the known species within the family: *Segregatospumella dracosaxi* n. sp. (described below) and the SSU sequence AY520450 (NCBI accession number).

Type of the family: *Segregatospumella* Boenigk et Grossmann n. g.

Etymology: 'Segregataceae' draws on the genus: '*Segregatospumella*'.

Segregatospumella Boenigk et Grossmann n. g.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Distinct and independent phylogenetic lineage within Chrysophyceae.The only close relative is the NCBI sequence AY520450 with an SSU sequence difference of 4.7%.

Typus generis: ***Segregatospumella dracosaxi*** Boenigk et Grossmann n. sp.

Etymology: Feminine. '*Segregato-*' indicates the phylogenetic unrelatedness to all other known lineages of Chrysophyceae and '*-spumella*' the taxonomic point of origin as so-called '*Spumella*-like flagellate'.

Segregatospumella dracosaxi Boenigk et Grossmann n. sp.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated or posteriorly pointed, 1.2-5.6 µm in diameter. Long flagellum up to 8 µm in length bearing tripartite mastigonemes (compare images Fig. 2.1(C), Fig. 2.2(N), Fig. 2.3(S+T) in this paper). Stomatocysts 3.6-5.5 µm in diameter and with a smooth surface (compare Findenig et al. 2010). Distinct from its closest relative AY520450 (NCBI accession number) by 4.7% sequence difference in the SSU sequence.

Distinct – on at least species level – from other '*Spumella*-like flagellates' of comparable morphology (as those described herein) by its gene sequences of SSU, ITS, LSU and COX1 (compare trees Figs. 2.5, 2.6, 2.7, 2.8).

Holotype: Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1263 (formaldehyde fixation of strain AR3A3).

Type habitat: Small freshwater stream.

Type locality: Austria, River Fuschler Ache near Mondsee, 47°50' N, 13°16' E, 500 m asl.

Etymology: The species epithet '*dracosaxi*' hints at the species' place of origin in Austria near the mountain 'Drachenwand'.

Gene sequences: NCBI accession no. GU073467 (SSU), no. KF697322 (ITS), no. KF697337 (LSU), no. KF697345 (COX1); all from strain AR3A3.

Further reference: SAG number 2432 (from strain AR3A3); strain AR3A3 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Order: Ochromonadales Pascher 1910

Family: Ochromonadaceae Lemmermann 1899

Cornospumella Boenigk et Grossmann n. g.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Clusters within clade C3 in Ochromonadales and forms a distinct phylogenetic lineage with the closest relatives being *Ochromonas sphaerocystis* (AF123294) and *Ochromonas danica* (EF165108) with an SSU sequence difference of 6.1% (*O. sphaerocystis*) and 3.4% (*O. danica*).

Typus generis: ***Cornospumella fuschlensis*** Boenigk et Grossmann n. sp.

Etymology: Feminine. '*Corno-*' hints at the typical hook-like projections of the stomatocysts (as described in Findenig et al. 2010) and '*-spumella*' indicates the taxonomic point of origin as so-called '*Spumella*-like flagellate'.

Cornospumella fuschlensis Boenigk et Grossmann n. sp.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated or posteriorly pointed, 1.3-6.9 µm in diameter. Long flagellum up to 9.4 µm in length bearing tripartite mastigonemes. Additionally, smaller hairs on mastigonemes. Plastidal, non-pigmented compartment showing thylakoidal inner folding (compare images Fig. 2.1(E), Fig. 2.2(D), Fig. 2.3(E+F), Fig. 2.4(C) in this paper). Stomatocysts

with distinctly deformed shape and hook-like projections, 4.35-5.7 µm in length and 3.96-5.45 µm in width (compare Findenig et al. 2010). Distinct from its closest relatives *Ochromonas sphaerocystis* and *Ochromonas danica* as not being photosynthetic. Sequence difference in the SSU sequence: 6.1% (*O. sphaerocystis*) and 3.4% (*O. danica*).

Distinct – on at least species level – from other '*Spumella*-like flagellates' of comparable morphology (as those described herein) by its gene sequences of SSU, ITS, LSU and COX1 (compare trees Figs. 2.5, 2.6, 2.7, 2.8).

Holotype: Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1266 (formaldehyde fixation of strain AR4D6).

Type habitat: Small freshwater stream.

Type locality: Austria, River Fuschler Ache near Mondsee, 47°50' N, 13°16' E , 500 m asl.

Etymology: The species epithet '*fuschlensis*' hints at the species' place of origin from the river Fuschler Ache near Mondsee, Austria.

Gene sequences: NCBI accession no. GU073469 (SSU), no. KF697327 (ITS), no. KF697343 (LSU), no. KF697361 (COX1); all from strain AR4D6.

Further reference: SAG number 2430 (from strain AR4D6); strain AR4D6 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Acrispumella Boenigk et Grossmann n. g.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate with a typical pointy elongation of the cell body at its posterior end. Clusters within clade C3 in Ochromonadales and forms a distinct phylogenetic lineage with the closest relative being a *Uroglena* species (EF165132) with an SSU sequence difference of 3.1%.

Typus generis: ***Acrispumella msimbaziensis*** Boenigk et Grossmann n. sp.

Etymology: Feminine. '*Acri-*' hints at the typical pointy shape of the cell body and '*-spumella*' indicates the taxonomic point of origin as so-called '*Spumella*-like flagellate'.

Acrispumella msimbaziensis Boenigk et Grossmann n. sp.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Cells typically elongated with pointed posterior end, 2-7.7 µm in diameter (mean of length and width). Long flagellum up to 17.4 µm in length bearing tripartite mastigonemes (compare: images Fig. 2.1(F), Fig. 2.2(E), Fig. 2.3(AA+BB) in this paper). Distinct from its closest relative *Uroglena* sp. as not photosynthetic, with a sequence difference in the SSU sequence of 3.1%.

Distinct – on at least species level – from other '*Spumella*-like flagellates' of comparable morphology (as those described herein) by its gene sequences of SSU, LSU and COX1 (compare trees Figs. 2.5, 2.6, 2.8).

Holotype: Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1267 (formaldehyde fixation of strain JBAF33).

Type habitat: Freshwater river.

Type locality: Tanzania, Msimbazi River, 5°15'0 S, 38°49'60 E, 151 m asl.

Etymology: The species epithet '*msimbaziensis*' refers to the species' place of origin from the river Msimbazi in Tanzania.

Gene sequences: NCBI accession no. AY651077 (SSU), no. KF697338 (LSU), no. KF697359 (COX1); all from strain JBAF33.

Further reference: SAG number 2427 (from strain JBAF33); strain JBAF33 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information: In experiments: temperature maximum of 34.6 °C, salinity maximum of 4 g $NaCl*L^{-1}$, pH tolerance between 3.15 and 10.9 (Pfandl et al. 2009); all data from strain JBAF33.

Spumella (Cienkowsky) Findenig et Boenigk

Spumella bureschii (Valkanov) Boenigk et Grossmann nov.comb.

Basionym: *Monas bureschii* Valkanov (1925)

Emended diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated or posteriorly pointed, 2.9-7.4 µm in diameter. Long flagellum up to 14.2 µm in length bearing tripartite mastigonemes (compare images Fig. 2.1(J), Fig. 2.2(L), Fig. 2.3(A+B) in this paper). Stomatocysts 5.17-8.09 µm in diameter, spherical to slightly oval with deep conical pore (compare Findenig et al. 2010). Distinct phylogenetic lineage within the genus *Spumella* with an SSU sequence difference of 0.7% to Spumella vulgaris and 1.2% to Spumella sp. (DQ388558).

Holotype: Figure 18 in Valkanov (1925), designated by Valkanov.

Epitype (designated here): Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1265 (formaldehyde fixation of strain JBL14).

Type habitat: Puddle water.

Type locality: Austria, Lunz, 47°51'0 N, 15°03'0 E, 884 m asl.

Gene sequences: NCBI accession no. AY651086 (SSU), no. EF577172 (ITS), no. KF697329 (LSU); all from strain JBL14.

Further reference: SAG number 2433 (from strain JBL14); strain JBL14 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information: In experiments: temperature maximum of 29.1 °C (Boenigk 2006), salinity maximum of 6 g $NaCl*L^{-1}$, pH tolerance between 3.15 and 11.2 (Pfandl et al. 2009); all data from strain JBL14.

Spumella lacusvadosi Boenigk et Grossmann n. sp.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated or posteriorly pointed, 1.5-7.1 µm in diameter. Long flagellum up to 10.4 µm in length bearing tripartite mastigonemes (compare image Fig. 2.1(N) in this paper). Distinct phylogenetic lineage within the genus *Spumella* with an SSU sequence difference of 1% to *Spumella* sp. (DQ388541) and 1% to *Spumella rivalis*.

Holotype: Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1264 (formaldehyde fixation of strain JBNZ39).

Type habitat: Shallow tarn.

Type locality: New Zealand, near Karangarua, 43°37'0 S, 169°46'0 E, 1118 m asl.

Etymology: The species epithet '*lacusvadosi*' hints at the species' origin from a shallow mountain lake.

Gene sequences: NCBI accession no. AY651088 (SSU), no. KF697324 (ITS), no. KF697331 (LSU), no. KF697357 (COX1); all from strain JBNZ39.

Further reference: SAG number 2434 (from strain JBNZ39); strain JBNZ39 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information: In experiments: temperature maximum of 36.7 °C (Boenigk 2006), salinity maximum of 4 g $NaCl*L^{-1}$, pH tolerance between 3.15 and 10.9 (Pfandl et al. 2009); all data from strain JBNZ39.

Pedospumella Findenig et Boenigk

Pedospumella sinomuralis Boenigk et Grossmann n. sp.

Diagnosis: Non-photosynthetic, non-colonial, non-scaled, bacterivorous chrysophycean bi-flagellate. Cells mostly spherical, sometimes elongated or posteriorly pointed, 1.9-5.6 µm in

diameter. Long flagellum up to 9.5 µm in length bearing tripartite mastigonemes (compare images Fig. 2.1(I), Fig. 2.2(H), Fig. 2.3(G+H) in this paper). Distinct phylogenetic lineage within the genus *Pedospumella* with an SSU sequence difference of 1% to *Pedospumella* sp. (AY651079) and 0.8% to *Spumella danica*.

Holotype: Botanical Garden and Botanical Museum Berlin Dahlem, no. B 40 004 1268 (formaldehyde fixation of strain JBCS23).

Type habitat: Soil.

Type locality: People's Republic of China, near Badaling, 40°20'15 N, 115°58'10 E, 795 m asl.

Etymology: The species epithet '*sinomuralis*' refers to the species' place of origin in China close to the Great Wall.

Gene sequences: NCBI accession no. AY651081 (SSU), no. EF577170 (ITS), no. KF697349 (COX1); all from strain JBCS23.

Further reference: SAG number 2431 (from strain JBCS23); strain JBCS23 in the culture collection of Jens Boenigk at University Duisburg-Essen.

Additional information: In experiments: temperature maximum of 29.1 °C (Boenigk 2006), salinity maximum of 5 g $NaCl*L^{-1}$, pH tolerance between 3.15 and 11.2 (Pfandl et al. 2009); all data from strain JBCS23.

Pedospumella elongata (Stokes) Boenigk et Grossmann nov.comb.

Basionym: *Physomonas elongata* Stokes (1886).

Synonym: *Spumella elongata* (Stokes) Belcher et Swale (1976), *Monas elongata* (Stokes) Lemmermann (1910).

Emended Diagnosis: (See descriptions as *Physomonas elongata* in Stokes 1886 and as *Spumella elongata* in Belcher and Swale 1976 and sequence information as *Spumella elongata* in Bruchmüller 1998 and Wylezich et al. 2010 – the latter two available in GenBank). Distinct from its closest relative *Pedospumella encyctans* in the larger cell body (8-11.2µm) and the ratio of cell body and long flagellum (long flagellum as long as or shorter than the cell body).

Lectotype: Figure 1 in Stokes (1886), designated here by Grossmann and Boenigk.

Epitype: Strain CCAP 955/1 (formally designated here following Belcher and Swale).

Type habitat: Soil.

Type locality: UK, Girton (Cambridgeshire), 1974 (by Belcher and Swale).

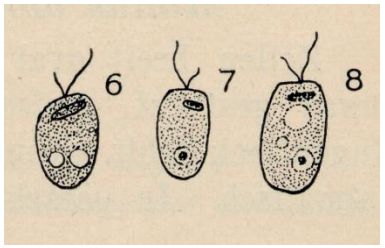
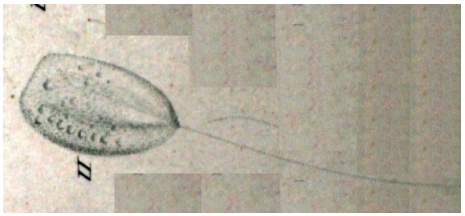
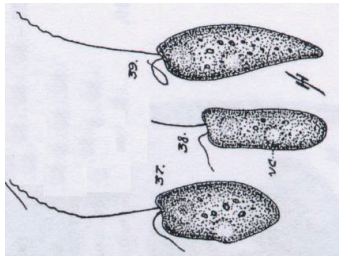
Gene sequences: NCBI accession no. AJ236859 (SSU) and NCBI accession no. EF681931 (LSU); all from strain CCAP 955/1.

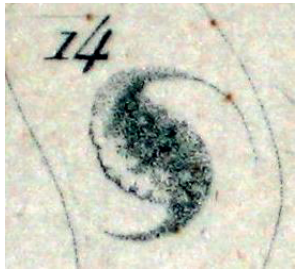
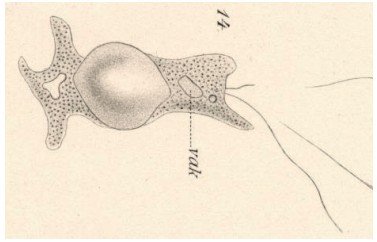
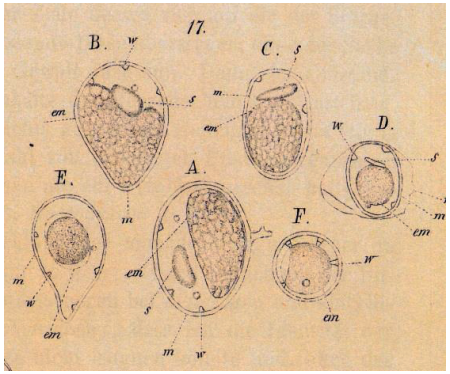
REMARKS

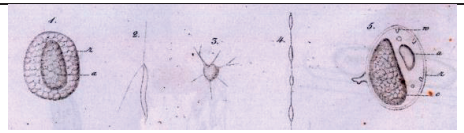
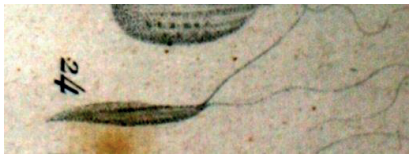
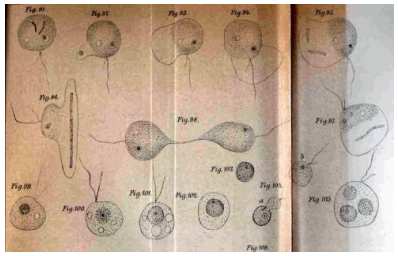
I submitted chapter 2 as first author to the Journal of Eukaryotic Microbiology; the manuscript is accepted for publication.

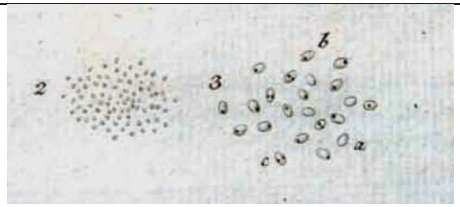
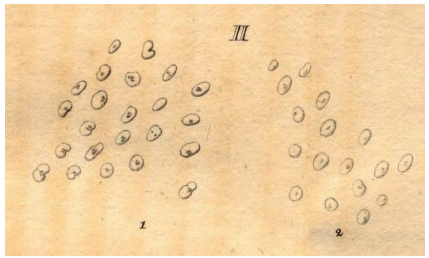
The electron microscopy shown in this chapter was carried out at the University of Stuttgart under the guidance of PD Dr. Michael Schweikert.

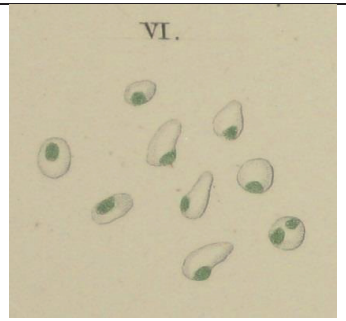
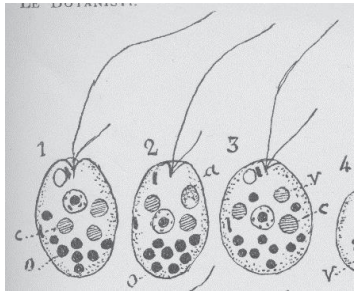
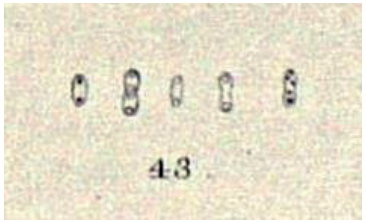
Supplementary table S2.1. List of *Monas* and *Spumella* species

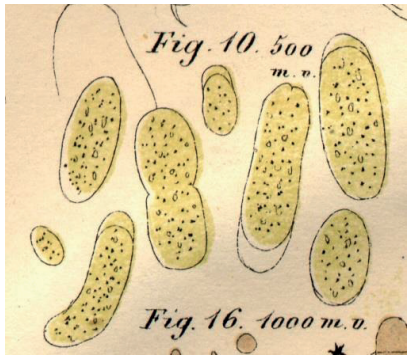
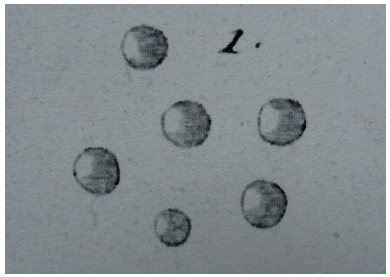
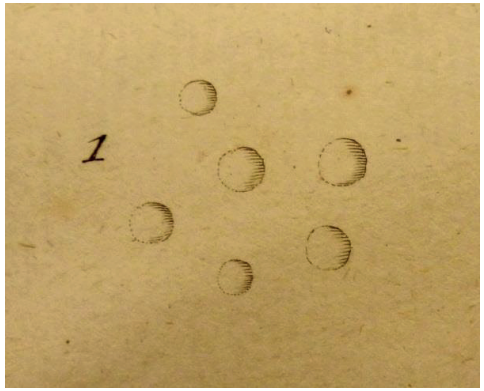
Species designation	First description by	Image proof and notes	Literature references	Assessment of species (in regard to the strains described herein)
Monas abrupta	Skvortzow 1932	 (Skvortzow)	**Skvortzow** B.W. (1932) Flagellaten aus der Nordmandschurei, *Archiv für Protistenkunde*, 77: 522-527 (incl. Tab.).	Not one of the strains described herein as long flagellum too short
Monas abscissa / abscissum	Diesing 1850 without image → in Dujardin 1841 as *Cyclidium abscissum*	 (Dujardin, pl.IV, fig.11)	**Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35. **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les à l'aide du microscope*, Paris (planche IV).	Reclassified as: *Petalomonas abscissa* (Dujardin) Stein 1878 (see Boenigk, 2008)
Monas affinis	Skuja 1948	 (Skuja)	**Skuja** H. (1948) Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden. *Symbolae Botanicae Upsalienses,* IX: 308-309 (Tafel XXXIV).	Not one of the strains described herein as cell body and flagellum too large (cell: 17-20µ)

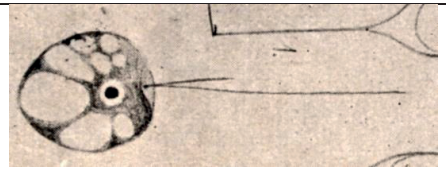
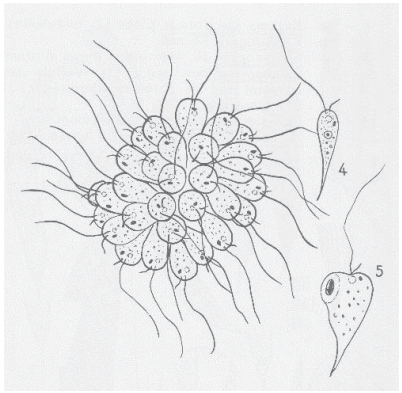
Monas agilis	Diesing 1850 without image → in Dujardin 1841 as *Trepomonas agilis*	 (Dujardin, pl. III, fig. 14)	**Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35. **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris (planche III).	Reclassified as: *Trepomonas agilis*, Dujardin 1841 (see Boenigk, 2008)
Monas amoebina	Meyer 1897	 (Meyer)	**Meyer** Hans (1897-1898) Untersuchungen über einige Flagellaten. *Revue Suisse de Zoologie et Annales du Musée d'Histoire Naturelle de Genève*. Tome V: 43-89 (+ plate 2).	(see Boenigk, 2008) Not one of the strains described herein as pigmented
Monas amyli	Cienkowsky 1858, 1865	 (Cienkowsky 1858, pl.XVII)	**Cienkowsky** L. (1858) Ueber meinen Beweis für die Generatio primaria, *Bulletin de la Classe Physico-Mathématique de l'Académie Impériale des Sciences de St.-Pétersbourg*, Tome XVII: 81-95 (+Tab. XVII). **Cienkowsky** L. (1865) Beiträge zur Kenntnis der Monaden. *Archiv für Mikroskopische Anatomie*. 1: 203-232 (+ Tafel XII).	Not one of the strains described herein (→ cells also show amoeboid appearance)

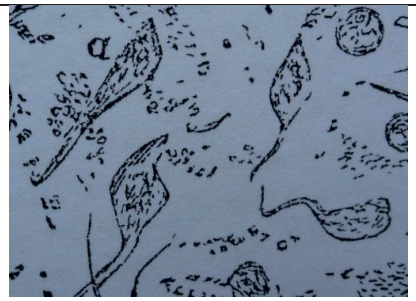
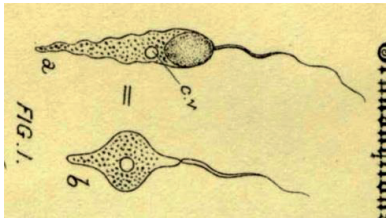
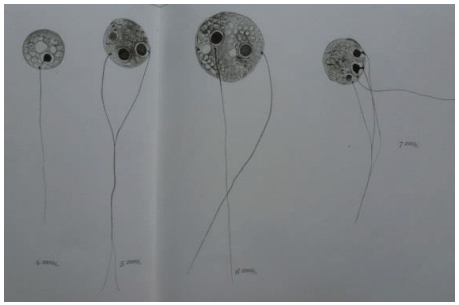
		 (Cienkowsky 1865, pl. XII)		
Monas anatis	Davaine C. 1875 (in Dechambre) (found in duck and chicken)	No image	In: **Dechambre** A. (ed.) (1875) *Dictionnaire Encyclodédique des Sciences Médicales*, Deuxième Série, Tome Neuvième: 122.	Not one of the strains described herein as found in duck intestines
Monas angusta	Diesing 1850 without image → in Dujardin 1841 as *Heteromita angusta*	 (Dujardin, pl.IV, fig. 24)	**Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35. **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris (planche IV).	Reclassified as: *Colpodella angusta* (Dujardin) Simpson & Patterson 1986 (see Boenigk, 2008)
Monas arhabdomonas	Meyer 1897 (also mentioned in Lemmermann 1910) without image → in Fisch 1885 as *Arhabdomonas vulgaris*	 (Fisch, pl. IV, fig. 91-105)	**Meyer** Hans (1897-1898) Untersuchungen über einige Flagellaten. *Revue Suisse de Zoologie et Annales du Musée d'Histoire Naturelle de Genève*. Tome V: 43-89. **Lemmermann** E. (1910) *Kryptogamenflora der Mark Brandenburg und angrenzender Gebiete*, Gebrüder Borntraeger, Leipzig. **Fisch** F. (1885) Untersuchungen über einige Flagellaten und verwandte Organismen. *Zeitschrift für wissenschaftliche Zoologie*. 42: 47-125 (+Tafel IV).	Not one of the strains described herein (→ ingesting algae, cell: ~15µm)
Monas atomus	Müller 1786 / Ehrenberg 1830		**Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p. 2 (+Tab I). **Ehrenberg** Ch.G. (1830) Beiträge zur	Not one of the strains described herein as living in salt water

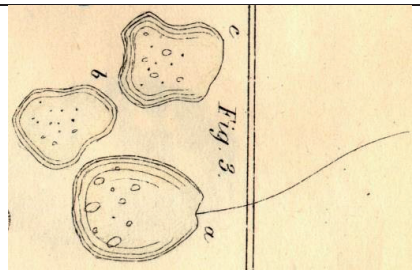
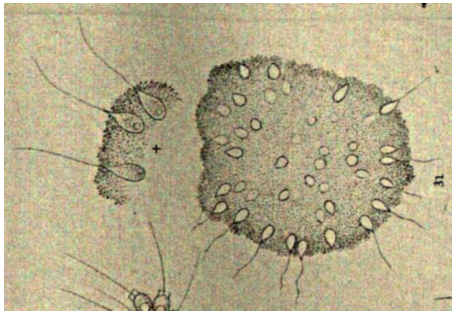
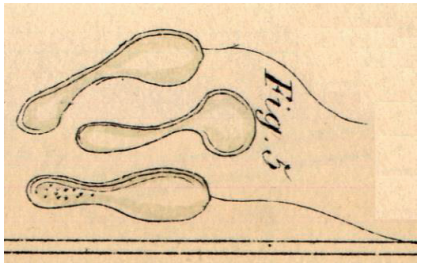
		 (Müller, pl.I, 2-3)  (Ehrenberg)	Kenntniss der Organisation der Infusorien und ihrer geographischen Verbreitung, besonders in Sibirien, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 37 (+Tab. I).	
Monas attenuata	Dujardin 1841	 (Dujardin, pl.III, fig. 12)	**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286 (planche III).	Not one of the strains described herein as morphology differing (→ 'pocks' on cell surface, cell: 16µm)
Monas attenuata var. *oblonga*	Diesing 1850 without image → in Dujardin 1841 as *Monas oblonga* without image	No image	**Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35. **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286.	Not one of the strains described herein as flagella and vacuoles different
Monas bicolor	Ehrenberg 1832 (image 1838)		**Ehrenberg** Ch.G. (1832) Über die Entwickelung und Lebensdauer der Infusionsthiere; nebst ferneren Beiträgen zu einer Vergleichung ihrer organischen	Not one of the strains described herein as pigmented

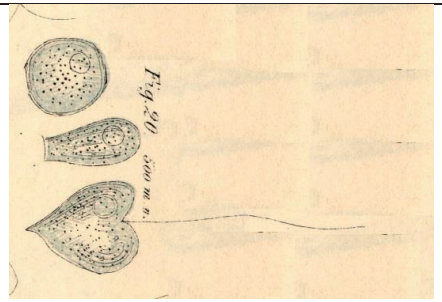
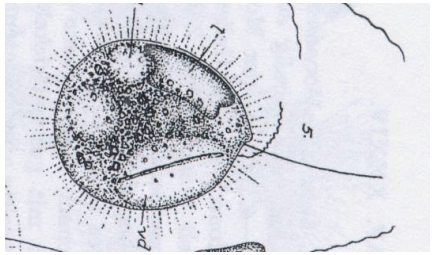
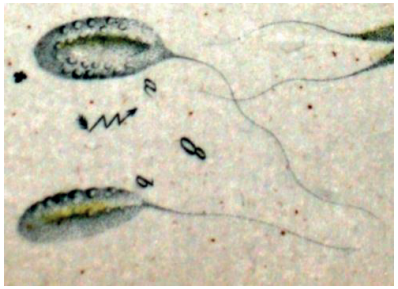
		 (Ehrenberg)	Systeme, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 56-59. **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	
Monas biocellata	Dangeard 1934	 (Dangeard)	**Dangeard** P.-A. (1934) Mémoire sur l'*Apistonema submarinum* sp. nov. et considerations generals sur la structure des Protozoaires et des Protophytes, *Le Botanist*: 310-318 (+planche XXVIII).	Not one of the strains described herein as feeding on algae (cell: 20µm)
Monas bipunctaca	Fresenius 1858	 (Fresenius)	**Fresenius** G. (1858) Beiträge zur Kenntniss mikroskopischer Organismen, *Abh. Senckenb. Naturf. Ges.*, 2 : 211-242 (227) (+ Tafel X).	Reclassified as belonging to fungi (see Boenigk, 2008)
Monas botulus	Perty 1852		**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein as pigmented

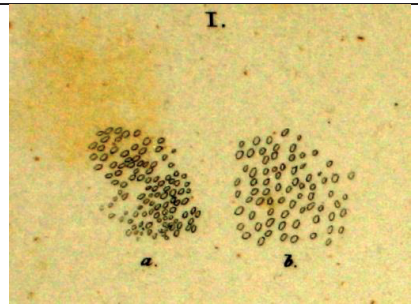
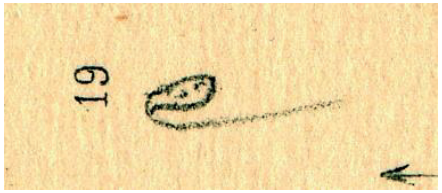
		 (Perty)		
Monas bulla	Bory de St.Vincent 1824 → in Müller 1786 as *Cyclidium bulla*	 (Bory, pl. V, fig. 1)  (Müller, pl. XI, 1)	**Bory de Saint-Vincent** et al. (1824) *Encyclopédie Méthodique, Histoire Naturelle des Zoophytes, ou Animaux Rayonnés*, Tome Second: 548-550 (+planches 1, 2, 5 from Tome Premier!). **Müller** Othone Friderico (1773) *Vermium terrestrium et fluviatilium.* Hauniae et Lipsiae, p.49-50. **Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p. 78 (+Tab XI).	Not one of the strains described herein as morphology different (shown and decribed are vegetative cells, not resting stages) (→ no flagella, cells too regularly circular)
Monas	Valkanov 1925		**Valkanov** A. (1925) Beitrag zur Kenntnis	

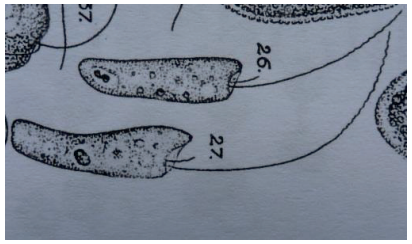
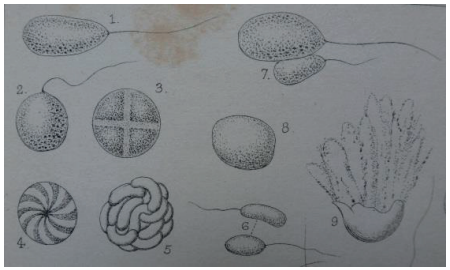
bureschii		 (Valkanov)	der Flagellaten von Bulgarien, Izg. Bulg. Bot. Druzh., 1:109 (+Tab. 2).	Identified with strain JBL14 from this study; emended and recombined to: *Spumella bureschii* (Valkanov) Boenigk et Großmann nov. comb.
Monas captiva	Bourrelly 1987	 (Bourrelly)	**Bourrelly** P. (1987) Algues d'eau douce des mares d'alpage de la region de Lunz am See, Autriche, *Bibliotheca Phycologica*, Gebrüder Borntraeger, Berlin: 25 (+ planche I).	Not one of the strains described herein as colonial
Monas caviae	Davaine C. 1875	No image	In: **Dechambre** A. (ed.) (1875) *Dictionnaire Encyclodédique des Sciences Médicales*, Deuxième Série, Tome Neuvième: 122.	Reclassified as: *Trichomonas caviae* (Davaine) Doflein 1901 (see Boenigk, 2008)
Monas cholerica	Davaine C. 1875 (in Dechambre) → as *Cercomonas urinarius* (Bodo and Hassall) in the same		In: **Dechambre** A. (ed.) (1875) *Dictionnaire Encyclodédique des Sciences Médicales*, Deuxième Série, Tome Neuvième: 122. **Hassall** Arthur Hill (1859) On the Development and Signification of Vibrio lineola, Bodo urinarius, and on Certain	Invalid binomen

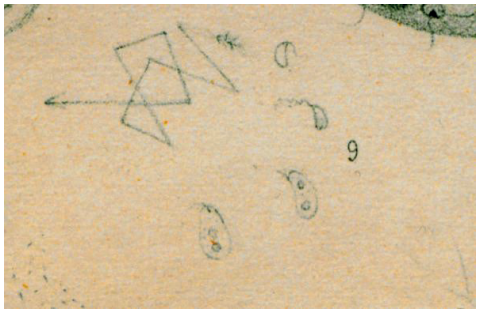
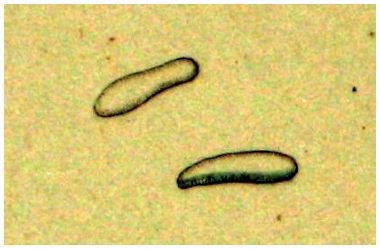
	encyclopedia→ in Hassall 1859 as *Bodo urinarius*	 (Hassall, S. 504)	Fungoid and other Organic Productions Generated in Alkaline and Albuminous Urine. *The Lancet*, November 19: 503-506 (incl. figure 1, p. 504).	
Monas claviculus	Maskell 1887	 (Maskell)	**Maskell** W.M. (1887) On the Freshwater Infusoria of the Wellington District, *Transactions of the New Zealand Institute*, Vol. XX: 4-5 (+plate I).	Not one of the strains described herein (cell body irregularly and largely elongated)
Monas communis	Braune 1913	 (Braune)	**Braune** R. (1913) Untersuchungen über die im Wiederkäuermagen vorkommenden Protozoen. *Archiv für Protistenkunde*, 32: 111-170 (+Tafel 3).	Reclassified as: *Caecomyces communis*, Oroin 1976 (see Boenigk, 2008)
Monas concava	Dujardin 1841 without image → Perty 1852		**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286.	Not one of the strains described herein (cells irregularly shaped and of cell boundaries of solid appearance, cell:

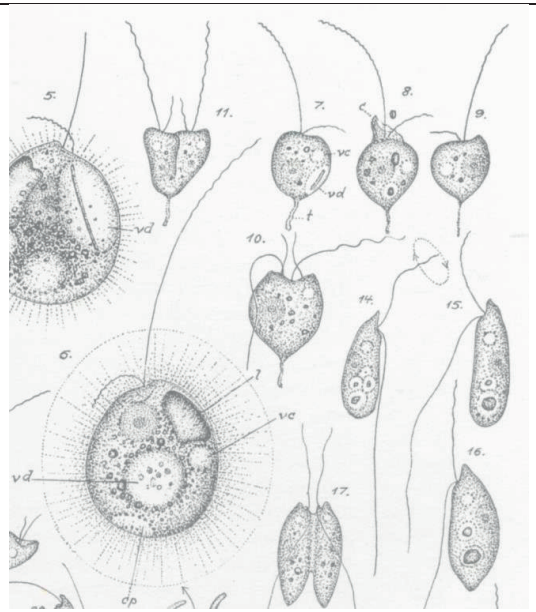
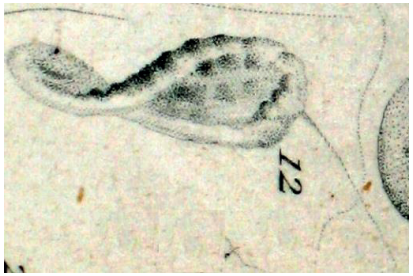
		 (Perty)	**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	12.5µm)
Monas consociata	Fresenius 1858	 (Fresenius)	**Fresenius** G. (1858) Beiträge zur Kenntniss mikroskopischer Organismen, *Abh. Senckenb. Naturf. Ges.*, 2 : 211-242 (227) (+ Tafel X).	Reclassified as: *Phalansterium consociatum* (Fresenius) Cienkowsky 1870 (see Boenigk, 2008)
Monas constricta	Dujardin 1841 without image → Perty 1852	 (Perty)	**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286. **Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein as morphology different (cells flattened)
Monas cordata	Perty 1852		**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein (cells heart-shaped, cell: 25µm)

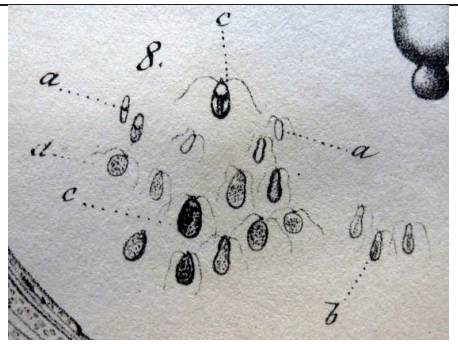
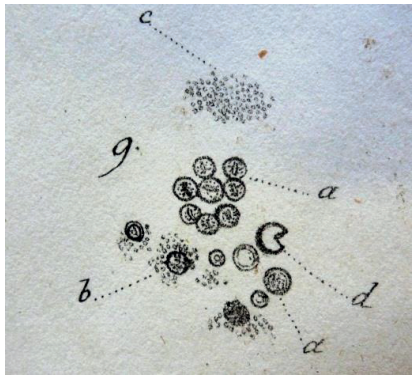
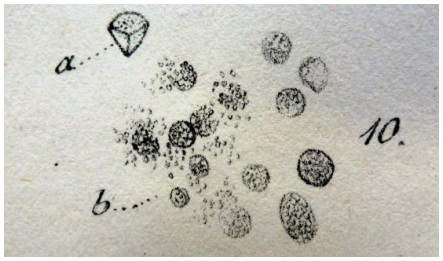
		 (Perty)		
Monas coronifera	Skuja 1948	 (Skuja)	**Skuja** H. (1948) Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden. *Symbolae Botanicae Upsalienses,* IX: 309-310 (+Tafel XXXV).	Not one of the strains described herein (cells in gelatinous sheath, ingesting algae)
Monas crassa	Diesing 1850 without image → in Dujardin 1841 as *Cyclidium crassum*	 (Dujardin, pl.III, fig.8)	**Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35. **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris (planche III).	Not one of the strains described herein ('pocks' on cell surface, cells flattened, cell: 16.7µ)
Monas crepusculum	Ehrenberg 1832 (image 1838)		**Ehrenberg** Ch.G. (1832) Über die Entwickelung und Lebensdauer der Infusionsthiere; nebst ferneren Beiträgen zu einer Vergleichung ihrer organischen Systeme, *Abhandlungen der königlichen*	Not one of the strains described herein as description of bacteria

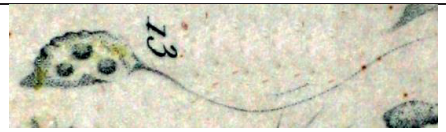
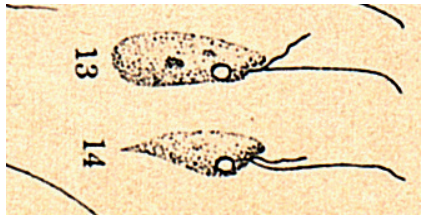
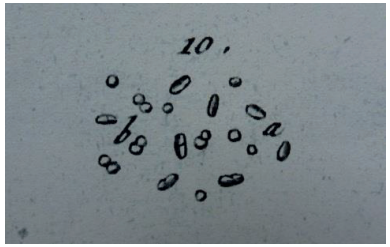
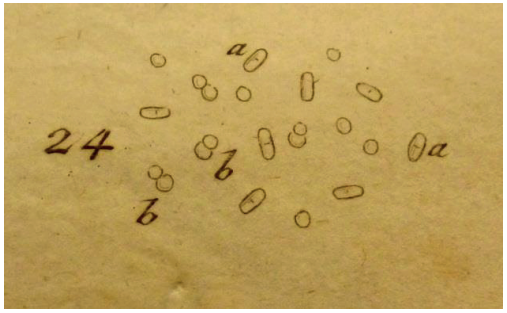
		 (Ehrenberg)	*Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 56-59. **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	
Monas cunillus	de Fromentel 1874	 (de Fromentel, pl.XXIII)	**de Fromentel** E. (1874) *Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues*, Paris: 325 (planches XXIII - XXVII).	Not one of the strains described herein (contractile vacuole large and clearly visible)
Monas cylindrica	Ehrenberg 1838 ≠ Schulz 1856 ≠ Skuja 1956	 (Ehrenberg)	**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I). **Schulz** A. (1856) Beiträge zur Kenntniß der Infusorien des Herzogthums Nassau. *Jahrbücher des Vereins für Naturkunde im Herzogthum Nassau*, 11: 1-12 (+Tafel I). **Skuja** H. (1956) Taxonomische und biologische Studien über das Phytoplankton schwedischer Binnengewässer, *Nova Acta Reg. Soc. Sc. Ups.*, Ser.IV, Vol.16, No.3: 317-21 (+Taf. LVI).	Not one of the strains described herein (strongly elongated, cell:23.5µ)

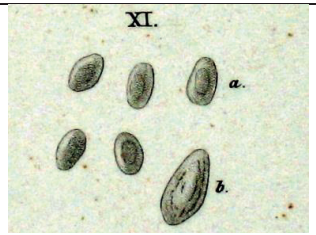
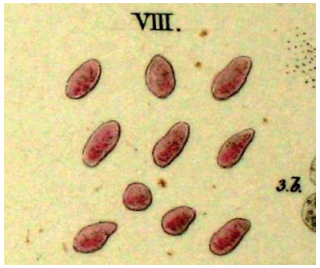
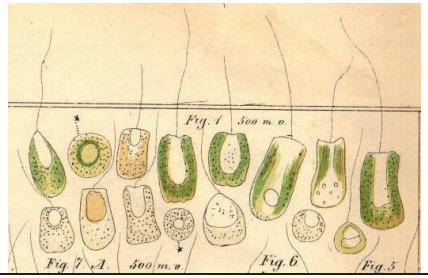
		 (Schulz, pl. I)  (Skuja)		
Monas dallingeri	Kent 1880-81	 (Kent, pl. XIII)	**Kent** W. S. (1880/81) *A Manual of the Infusoria: including a description of all known flagellate, ciliate, and tentaculiferous protozoa, British and foreign, and an account of the organization and affinities of the sponges*, London, David Bogue: 233 (plate XIII, XIV).	Not one of the strains described herein as only one flagellum
Monas dangeardii	Lemmermann 1913 (synonym: *Monas vulgaris*, Dangeard 1910)	No image	**Lemmermann** E. (1913) Notizen über Flagellaten, Arch. Hydrobiol., 8: 565-66. **Dangeard** P.-A. (1910) Etudes sur le développement et la structure des organismes inférieurs, avec 33 planches. *Le*	Recombination from *Spumella vulgaris* (Cienkowsky)

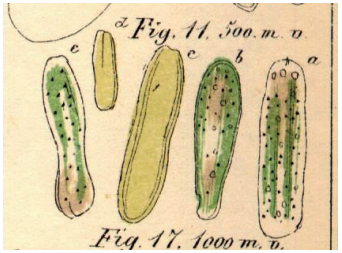
			Botaniste, Onzième Série, Mai 1910: S.141ff (+ planches XX-XXII).	
Monas depressa	de Fromentel 1874	 (de Fromentel, pl.XXV)	**de Fromentel** E. (1874) *Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues*, Paris: 328 (planches XXIII - XXVII).	Not one of the strains described herein (cells bended like bodonids)
Monas deses	Ehrenberg 1838	 (Ehrenberg)	**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Not one of the strains described herein (too elongated, cell: 22.5µ)
Monas dinobryonis	Skuja 1948		**Skuja** H. (1948) Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden. *Symbolae Botanicae Upsalienses,* IX: 310-311 (Tafel XXXV).	Not one of the strains described herein (→ protrusion for food uptake and plasma stalk for adhesion)

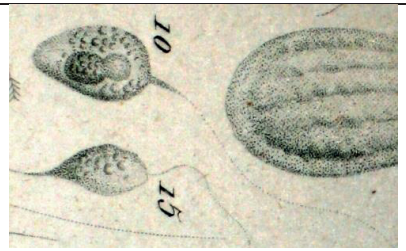
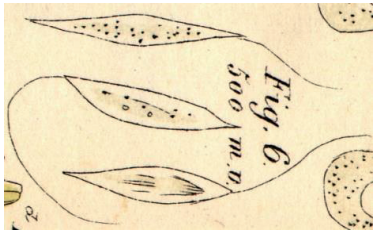
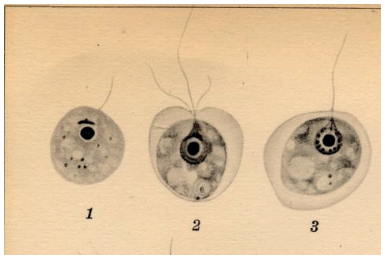
		 (Skuja, pl. XXXV, 7-11)		
Monas distorta	Diesing 1850 without image → in Dujardin 1841 as *Cyclidium distorta*	 (Dujardin, pl.IV, fig.12)	**Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35. **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris (planche IV).	Not one of the strains described herein as cells flattened
Monas dunalii	Joly 1840 / Diesing 1850, see also: Dunal 1838, *Protococcus/ Haematococcus salinus*		**Joly** N. (1840) *Histoire d'un petit crustacé*, Boehm et Comp., Montpellier (+ planche III). **Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35.	Reclassified as: *Dunaliella salina* (Dunal) Teodoresco 1904 (see Boenigk, 2008)

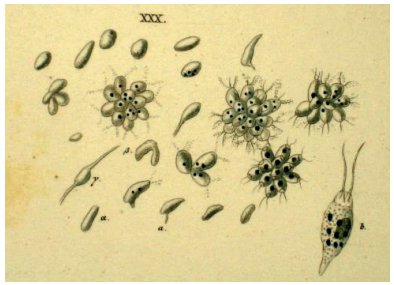
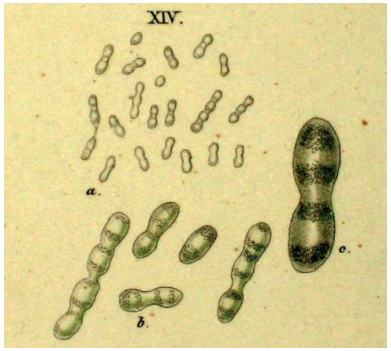
		 (Joly)  (Joly)  (Joly)		
Monas elongata	Dujardin 1841 ≠ Lemmermann 1910 (image in		**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces*	Recombined technically incorrect as:

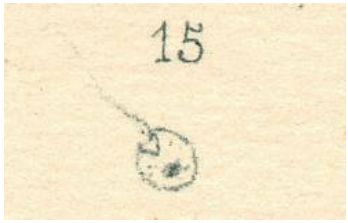
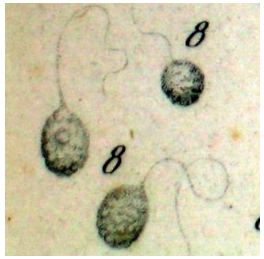
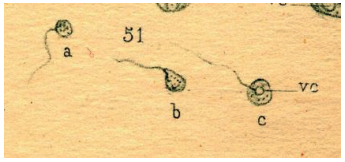
	Stokes 1888 as *Physomonas elongata*)	 (Dujardin, pl.III, fig.13)  (Stokes)	*animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286 (planche III). **Lemmermann** E. (1910) *Kryptogamenflora der Mark Brandenburg und angrenzender Gebiete*, Gebrüder Borntraeger, Leipzig. **Stokes** Alfred C. (1886) Notices of New Fresh-Water Infusoria.-V. *The American Monthly Microscopical Journal*, Vol. VII No.5: 81-86 (incl. fig.). **Stokes** Alfred C. (1888) *Journal of the Trenton Natural History Society 1888*: 81ff (+plate I).	*Spumella elongata* (Stokes) Belcher et Swale 1976 (see Boenigk, 2008) thus invalid; recombined in this study as: *Pedospumella elongata* (Stokes) nov.comb.
Monas enchelioides	Bory de St.Vincent 1824 (encyclopedic) → in Müller 1786 as *Enchelis intermedia*	 (Bory, pl. II, fig. 10)  (Müller, pl. IV, 24)	**Bory de Saint-Vincent** et al. (1824) *Encyclopédie Méthodique, Histoire Naturelle des Zoophytes, ou Animaux Rayonnés*, Tome Second: 548-550 (+planches 1, 2, 5 from Tome Premier!). **Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p. 28 (+Tab IV).	Not one of the strains described herein as morphology completely different (→ morphology and cell division similar to that of bacteria)
Monas enchelys	Ehrenberg 1838		**Ehrenberg** Ch.G. (1838) *Die*	

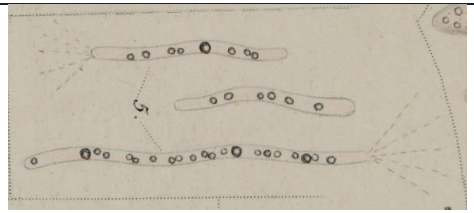
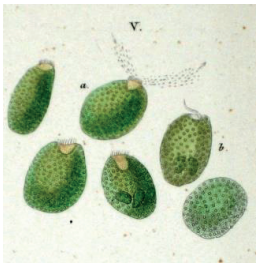
		 (Ehrenberg)	*Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Not one of the strains described herein (appearance of solid surface, cell: 22.5-28.5µ)
Monas enchyloides/ encheloides	Morren 1835	No image	**Morren** Ch. (1835) Essais pour déterminer l'influence qu'exerce la lumière, sur la manifestation, et les développemens des êtres végétaux et animaux, dont l'origine avait été attribuée à la génération directe, spontanée ou équivoque. *Annales des Sciences Naturelles,* Série 2, Tome 3: 5ff.	Invalid
Monas erubescens	Ehrenberg 1838	 (Ehrenberg)	**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Reclassified as belonging to fungi (see Boenigk, 2008) Not one of the strains described herein as pigmented
Monas excavata	Perty 1852		**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein as pigmented

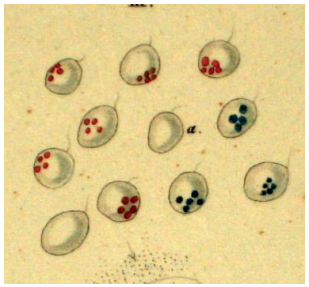
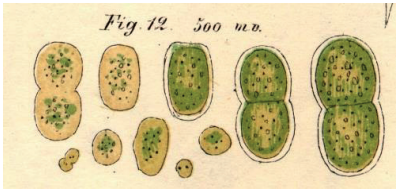
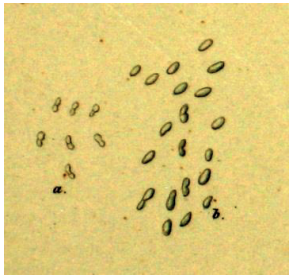
		(Perty)		
Monas fallax	Warming 1875/76	 (Warming)	**Warming** Eug. (1875) Om nogle ved Danmarks Kyster levende Bakterier, *Videnskabelige Meddelelser fra den naturhistoriske Forening i Kjöbenhavn*, Utgivne af Selskabets Bestyrelse, Kjöbenhavn, 1875-76: 363-70 (+Tab. X).	Not one of the strains described herein (cells with dark interior containing white shining compartments)
Monas farcimen	Perty 1852	 (Perty)	**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein as pigmented
Monas flavicans	Ehrenberg 1838	 (Ehrenberg)	**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Reclassified as: *Chromulina flavicans*, Provazek 1900 (see Boenigk, 2008)
Monas fluida	Dujardin 1841		**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286	Not one of the strains described herein ('pocks' on cell surface)

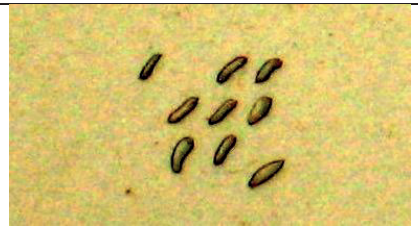
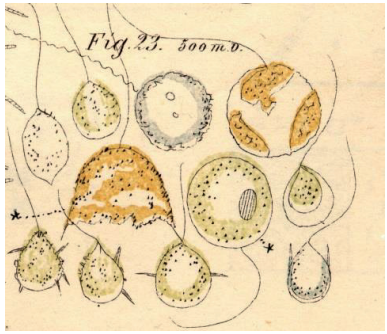
		 (Dujardin, pl.IV, fig.10)	(planche IV).	
Monas foliolum	Perty 1852	 (Perty)	**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein (cells of pointy, almost cristal-like shape, cell: 22.5µ)
Monas gallionella	Stiebel 1839	No image	**Stiebel** S.F. (1839) Ueber den Bau und das Leben der grünen Oscillatorie (Lysogonium taeniodes). *Museum Senckenbergianum: Abhandlungen aus dem Gebiete der beschreibenden Naturgeschichte, von Mitgliedern der Senckenbergischen Naturforschenden Gesellschaft in Frankfurt*, M. 3: 81-90.	Not one of the strains described herein (cells colonial, forming lines of cells)
Monas gelatinosa	Nägler 1912	 (Nägler)	**Nägler** K. (1912) Über Kernteilung und Fortpflanzung von *Monas gelatinosa* n. sp., *Archiv für Protistenkunde*, Band XXVII: 315-26 (+Tafel 15).	Not one of the strains described herein (cells in gelatineous bag)

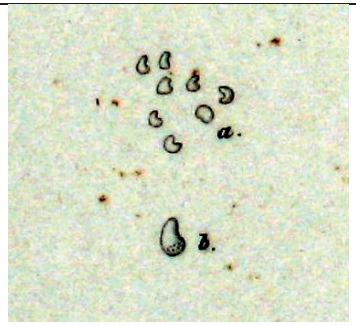
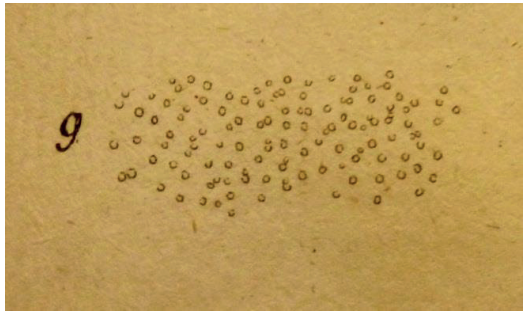
Monas gibbosa	Dujardin 1841	No image	**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286.	Not one of the strains described herein (cells irregularely shaped showing hunches)
Monas glaucoma	Ehrenberg 1828 (synonym: *Uvella glaucoma* and *Volvox glaucoma* in Symbolae physicae, p.23)	 (Ehrenberg)	**Ehrenberg** Ch.G. (1828) Die geographische Verbreitung der Infusionsthierchen in Nord-Afrika und West-Asien, beobachtet auf Hamprich und Ehrenbergs Reisen, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 10 (incl. Tab.). **Ehrenberg** Ch.G., Hemprich F.G. (1828) *Symbolae Physicae*, Berolini: 23.	Not one of the strains described herein (cells colonial, showing appendices)
Monas gliscens	Ehrenberg 1838 ≠ Shuttleworth 1840	 (Ehrenberg)  (Shuttleworth)	**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I). **Shuttleworth** R.J. (1840) Nouvelles observations sur la matière colorante de la neige rouge. *Bibliothèque Universelle de Genève*, Tome 25: 383-406 (+planche).	Not one of the strains described herein (shape different)

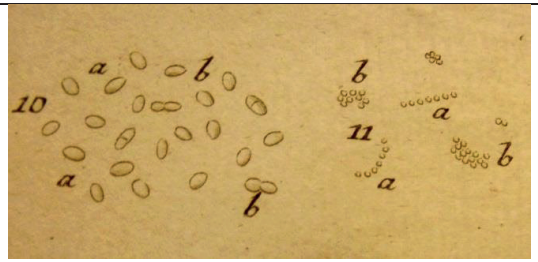
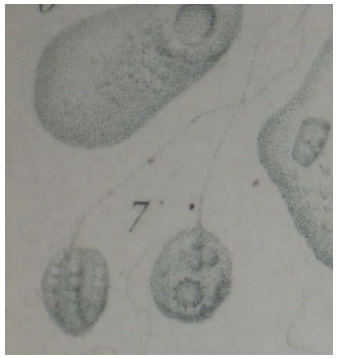
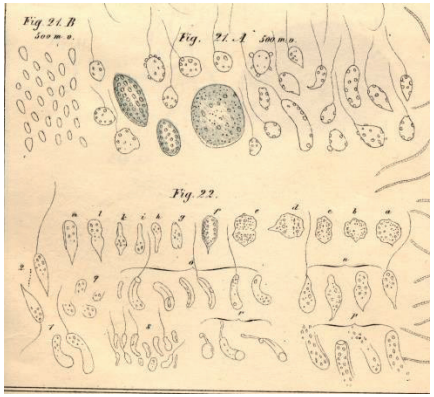
Monas globosa	de Fromentel 1874	 (de Fromentel, pl.XXIII)	**de Fromentel** E. (1874) *Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues*, Paris: 325 (planches XXIII - XXVII).	Not one of the strains described herein as pigmented
Monas globosus	Dujardin 1841 (as *Monas globulus* in the text part)	 (Dujardin, pl.IV, fig.8)	**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286 (planche IV).	Invalid
Monas globulus	de Fromentel 1874 → in Dujardin 1841 as *Monas globosus/ globulus*	 (deFromentel, pl.XXIII)	**de Fromentel** E. (1874) *Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues*, Paris: 326 (planches XXIII - XXVII). **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286 (planche IV).	Not one of the strains described herein as living in salt water
Monas gracilis	Warming 1875/76		**Warming** Eug. (1875) Om nogle ved Danmarks Kyster levende Bakterier, *Videnskabelige Meddelelser fra den*	Not one of the strains described herein

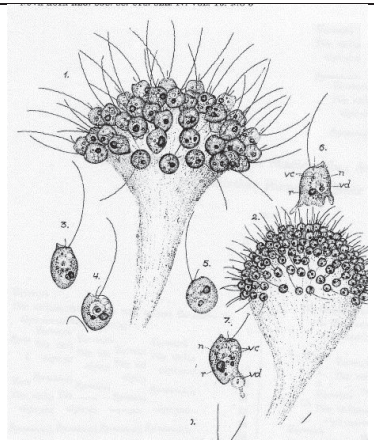
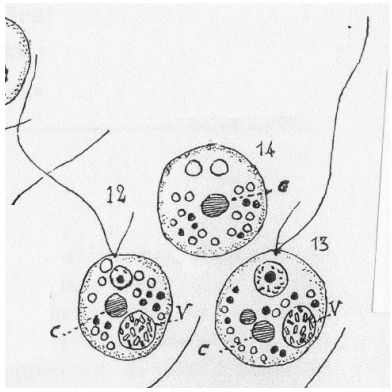
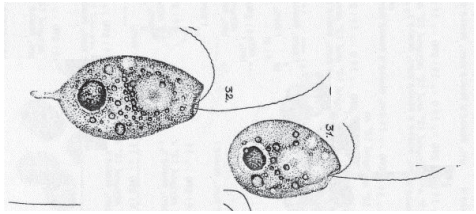
		 (Warming)	*naturhistoriske Forening i Kjöbenhavn*, Utgivne af Selskabets Bestyrelse, Kjöbenhavn, 1875-76: 331-32 (+Tab. VII).	(tube-like)
Monas grandis	Ehrenberg 1834 (image 1838)	 (Ehrenberg)	**Ehrenberg** Ch.G. (1834) Beitrag zur Erkenntniss grosser Organismen in der Richtung des kleinsten Raumes, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 253-54. **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Reclassified as: *Coelomonas (Vacuolaria) grandis* (Ehrenberg) Stein 1878 (see Boenigk, 2008)
Monas granulum	Diesing 1850 without image → in Dujardin 1841 as *Heteromita granulosa*	 (Dujardin, pl.IV, fig.23)	**Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35. **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris (planche IV).	Not one of the strains described herein (cells with 'pocks' on the surface)
Monas guttula	Ehrenberg 1830/38		**Ehrenberg** Ch.G. (1830) Beiträge zur Kenntniss der Organisation der Infusorien und ihrer geographischen Verbreitung, besonders in Sibirien, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 37 (+Tab. I). **Ehrenberg** Ch.G. (1838) *Die*	Reclassified as: *Spumella guttula* (Ehrenberg) Kent 1881 (see Boenigk, 2008)

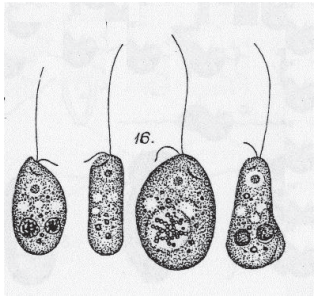
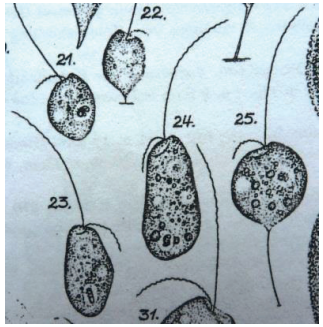
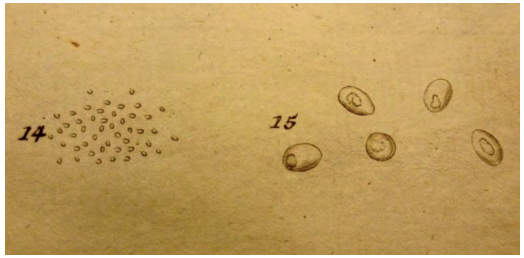
		(Ehrenberg)  (Ehrenberg)	*Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	
Monas hilla	Perty 1852	 (Perty)	**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein as pigmented
Monas hyalina	Ehrenberg 1838	 (Ehrenberg)	**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Not one of the strains described herein as living in salt water
Monas inanis	Ehrenberg 1828 (image 1838)		**Ehrenberg** Ch.G., Hemprich F.G. (1828) *Symbolae Physicae*, Berolini: 15-16. **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18	Not one of the strains described herein (cells too elongated)

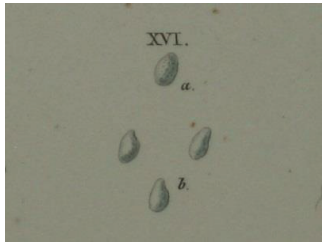
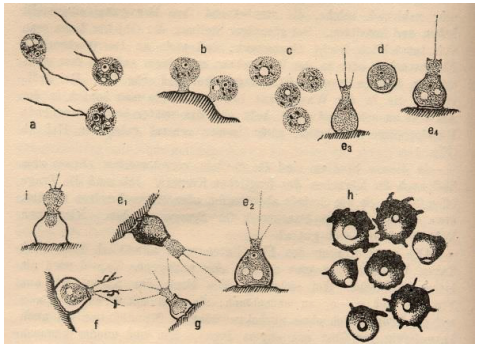
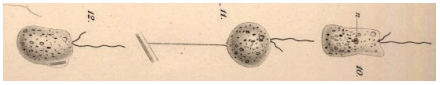
		 (Ehrenberg)	(+Tafel I).	
Monas intermedia	Ehrenberg 1838 (without image) → in Müller 1786 as *Enchelis intermedia*	 (Müller)	**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I). **Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p.28 (Tab IV).	Not one of the strains described herein as morphology completely different (→ morphology and cell division similar to that of bacteria)
Monas intestinalis	Dujardin 1841	No image	**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286.	Not one of the strains described herein (cells too elongated, cell: 17µ)
Monas irregularis	Perty 1852	 (Perty)	**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein as pigmented
Monas kolpoda	Ehrenberg 1832		**Ehrenberg** Ch.G. (1832) Über die	

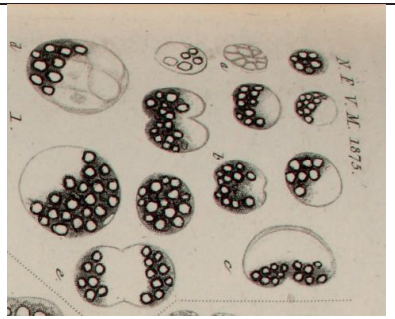
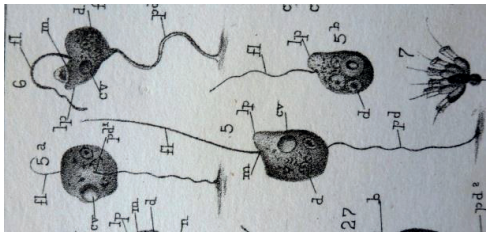
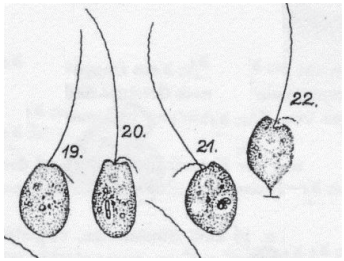
	(image 1838)	 (Ehrenberg)	Entwickelung und Lebensdauer der Infusionsthiere; nebst ferneren Beiträgen zu einer Vergleichung ihrer organischen Systeme, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 56-59. **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Not one of the strains described herein (cells of bended shape like bodonids)
Monas lamellula	Müller 1786	 (Müller, pl. I, 16/17)	**Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p.7 (+Tab I).	Not one of the strains described herein (cells too elongated)
Monas lens	Müller 1773/1786 (also see: Dujardin 1841 and Perty 1852, here: var. *astasioides* and var. *curvata*)		**Müller** Othone Friderico (1773) *Vermium terrestrium et fluviatilium.* Hauniae et Lipsiae, p.26-27. **Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p.4 (+Tab I). **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286 (planche IV). **Perty** M. (1852) *Zur Kenntnis kleinster*	Not one of the strains described herein as living in salt water

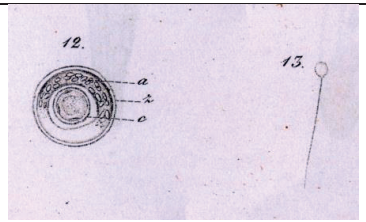
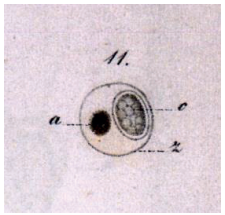
		 (Müller 1786, pl. I, 9-11)  (Dujardin, pl.IV, fig.7)  (Perty)	*Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	
Monas lindahlii	Skuja 1956		**Skuja** H. (1956) Taxonomische und	

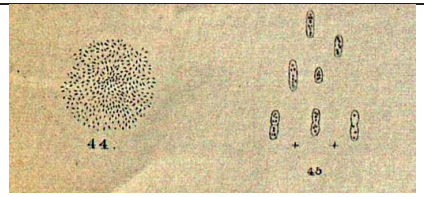
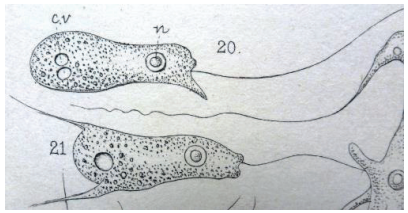
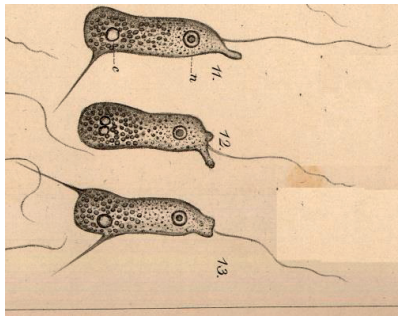
		 (Skuja)	biologische Studien über das Phytoplankton schwedischer Binnengewässer, *Nova Acta Reg. Soc. Sc. Ups.*, Ser.IV, Vol.16, No.3: 317-21 (+Taf. LVII).	Reclassified as: *Apoikia lindahlii* (Skuja) nov.comb. (Kim et al. 2010)
Monas longicilia	Dangeard 1934	 (Dangeard)	**Dangeard** P.-A. (1934) Mémoire sur l'*Apistonema submarinum* sp. nov. et considerations generals sur la structure des Protozoaires et des Protophytes, *Le Botanist*: 310-318 (+planche XXVIII).	Not one of the strains described herein as morphology differing (flagella too long, i.e. 3 to 4 times the length of the cell body)
Monas maior	Skuja 1956	 (Skuja)	**Skuja** H. (1956) Taxonomische und biologische Studien über das Phytoplankton schwedischer Binnengewässer, *Nova Acta Reg. Soc. Sc. Ups.*, Ser.IV, Vol.16, No.3: 317-21 (+Taf. LVI).	Not one of the strains described herein as cells too large (cell: 20-40µ)

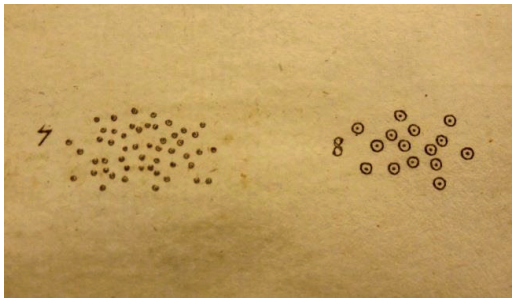
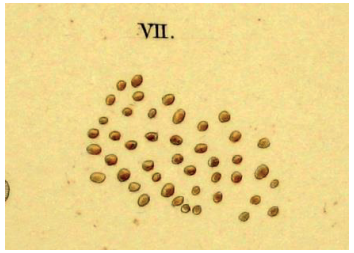
Monas marina	Uhlela 1911	No image	**Uhlela** V. (1911) Ultramikroskopische Studien über Geisselbewegung, *Biologisches Centralblatt*, Band XXX + XXXI (Fortsetzung): 654.	Not one of the strains described herein as living in salt water
Monas mediovacuolata (and var. *facilis*)	Skuja 1939 (1956)	 (Skuja)  (Skuja)	**Skuja** H. (1939) Beitrag zur Algenflora Lettlands II. *Acta Horti Botanici Universitatis Latviensis*, Latvijas Universitates Botaniska Darza Raksti, XI/XII: 41-169 (+Tab III). **Skuja** H. (1956) Taxonomische und biologische Studien über das Phytoplankton schwedischer Binnengewässer, *Nova Acta Reg. Soc. Sc. Ups.*, Ser.IV, Vol.16, No.3: 317-21 (+Taf. LVI).	Not one of the strains described herein (cell shape too elongated, cell: 16-20µ)
Monas mica	Müller 1773/1786 (also see Ehrenberg 1838)		**Müller** Othone Friderico (1773) *Vermium terrestrium et fluviatilium.* Hauniae et Lipsiae, p.27. **Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p.6 (+Tab I). **Ehrenberg** Ch.G. (1838) *Die*	Not one of the strains described herein as cells too large (cell: 18.8-22.5µ)

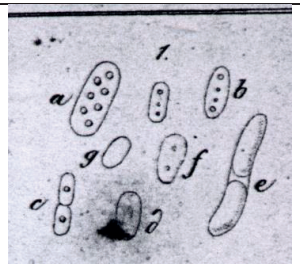
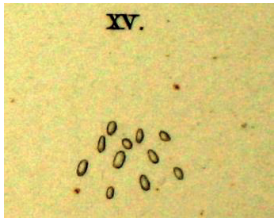
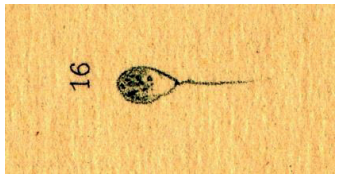
		(Müller, pl. I, 14/15)  (Ehrenberg)	*Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	
Monas micropora	Gicklhorn 1920	 (Gicklhorn)	**Gicklhorn** J. (1920) Notizen über einen Eisenflagellaten (*Monas micropora* nov. spec.), *Archiv für Protistenkunde*, Band XLI: 242-48 (incl. Abb.).	Not one of the strains described herein (amoeboid stages)
Monas minima	Meyer 1897	 (Meyer)	**Meyer** Hans (1897-1898) Untersuchungen über einige Flagellaten. *Revue Suisse de Zoologie et Annales du Musée d'Histoire Naturelle de Genève*. Tome V: 43-89 (+ plate 2).	(see Boenigk, 2008) Not one of the strains described herein as contractile vacuole clearly visible
Monas mülleri	Warming 1875/76		**Warming** Eug. (1875) Om nogle ved Danmarks Kyster levende Bakterier, *Videnskabelige Meddelelser fra den naturhistoriske Forening i Kjöbenhavn*, Utgivne af Selskabets Bestyrelse,	Reclassified as: *Thiovolum majus*, Hinze 1913

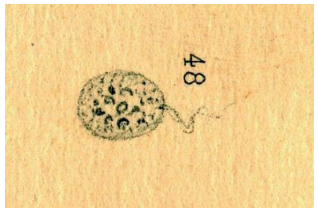
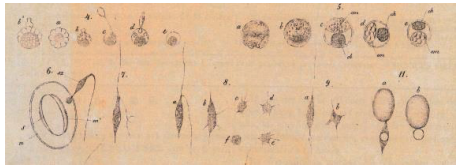
		 (Warming)	Kjöbenhavn, 1875-76: 363-70 (+Tab. X).	(see Boenigk, 2008)
Monas neglecta	James-Clark 1866 (also see: Skuja 1956)	 (James-Clark, pl. IX)  (Skuja)	**James-Clark** H. (1866) On the Spongiae Ciliatae, as Infusoria Flagellata; or, Observations on the Structure, Animality and Relationship of Leucosolenia Botryoides Bowerbank. *Memoirs read before the Boston Society of Natural History, Boston Journal of Natural History*, NewYork/London. Vol. I, Part I: 305-340 (+ plate IX/X). **Skuja** H. (1956) Taxonomische und biologische Studien über das Phytoplankton schwedischer Binnengewässer, *Nova Acta Reg. Soc. Sc. Ups.*, Ser.IV, Vol.16, No.3: 317-21 (+Taf. LVI).	Reclassified as: *Pseudobodo tremulans*, Griessmann 1913 (see Boenigk, 2008)
Monas nephrodes	Maggi 1893	No image	**Maggi** L. (1893) Alcuni nuovi protisti. *Rendiconti/Istituto Lombardo di Scienze e Lettere*, Milano. Band 26 (10), Serie 2: 354.	Not one of the strains described herein (cells are kidney-shaped)
Monas	Kent 1880-81		**Kent** W. S. (1880/81) *A Manual of the*	

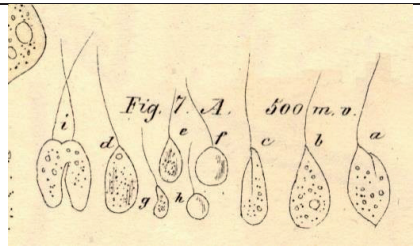
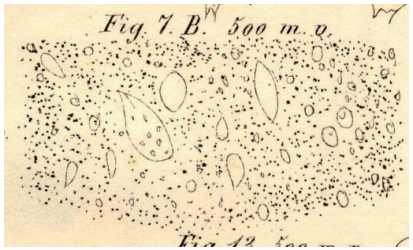
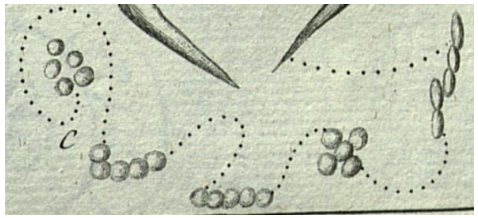
nittelarum	without image → in Cienkowsky 1865 as *Pseudospora nittelarum*	 (Cienkowsky)  (Cienkowsky)	*Infusoria: including a description of all known flagellate, ciliate, and tentaculiferous protozoa, British and foreign, and an account of the organization and affinities of the sponges*, London, David Bogue: 237. **Cienkowsky** L. (1865) Beiträge zur Kenntnis der Monaden. *Archiv für Mikroskopische Anatomie*. 1: 203-232 (+ Tafel XII).	Not one of the strains described herein (flagellum too long)
Monas nodosa	Dujardin 1841	 (Dujardin, pl. IV, fig. 9)	**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286 (planche IV).	Not one of the strains described herein (cells with irregularly 'bubbly' surface)
Monas nodulosa	Diesing 1850 → in Dujardin 1841 as *Cyclidium nodulosum* without image	No image	**Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35. **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris.	Not one of the strains described herein as cells too large (cell: 50.1µ)
Monas oberhauseri	Fresenius 1858		**Fresenius** G. (1858) Beiträge zur Kenntniss mikroskopischer Organismen, *Abh.*	Not one of the strains

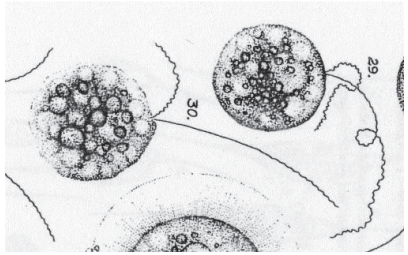
		 (Fresenius)	*Senckenb. Naturf. Ges.*, 2 : 211-242 (227) (+ Tafel X).	described herein (cells too elongated, cell: 12-21.7µ)
Monas obesa	Kent 1880-81 → in Stein 1878 as *Cercomonas obesa*	 (Kent, pl. XIII)  (Stein, pl.I)	**Kent** W. S. (1880/81) *A Manual of the Infusoria: including a description of all known flagellate, ciliate, and tentaculiferous protozoa, British and foreign, and an account of the organization and affinities of the sponges*, London, David Bogue: 236 (plate XIII, XIV). **Stein** Friedrich Ritter v. (1878) *Der Organismus der Infusionsthiere*, III. Abtheilung, I. Hälfte, Leipzig (Tafel I).	Not one of the strains described herein (cells too elongated and with spine-like protrusions)
Monas obliqua	Schewiakoff 1892	No image	**Schewiakoff** W. (1892) Ueber die geographische Verbreitung der Süsswasser-Protozoën, Verh. Naturh. Med. Ver. Heidelberg, Ser.2/4: 544-567 (550).	Not one of the strains described herein as contractile vacuole clearly visible sequence in NCBI (*Spumella obliqua*) not from type material (*Monas obliqua*),

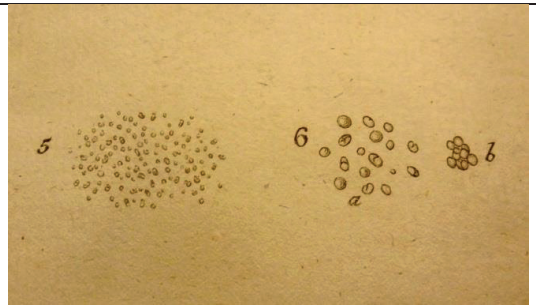
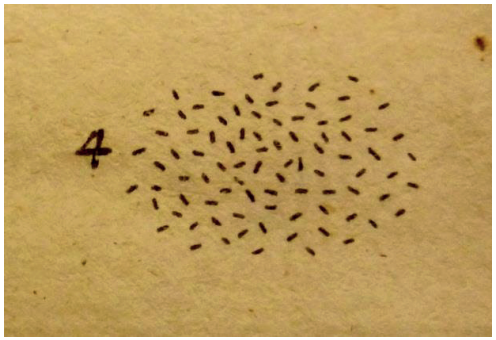
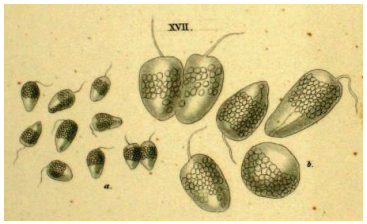
				affiliation with type material unclear
Monas oblonga	Dujardin 1841	No image	**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris.	Not one of the strains described herein as flagella and vacuoles different
Monas ocellus	Müller 1786	 (Müller, pl. I, 7/8)	**Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p.3-4 (+Tab I).	Not one of the strains described herein as pigmented
Monas ochracea	Ehrenberg 1832 (image 1838)	 (Ehrenberg)	**Ehrenberg** Ch.G. (1832) Über die Entwickelung und Lebensdauer der Infusionsthiere; nebst ferneren Beiträgen zu einer Vergleichung ihrer organischen Systeme, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 56-59. **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Reclassified as: *Sphaleromantis ochracea* (Ehrenberg) Pascher 1910 (see Boenigk, 2008)
Monas okeni(i)	Ehrenberg 1838 (image in Eichwald 1847)		**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I). **Eichwald** E. (1847) Erster Nachtrag zur	Reclassified as: *Chromatium okenii* (Ehrenberg) Perty 1852

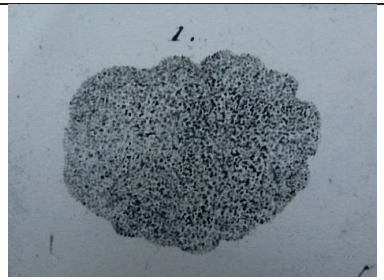
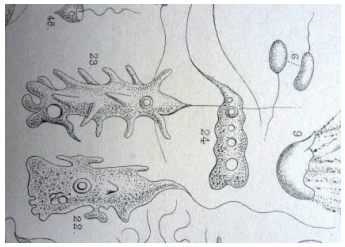
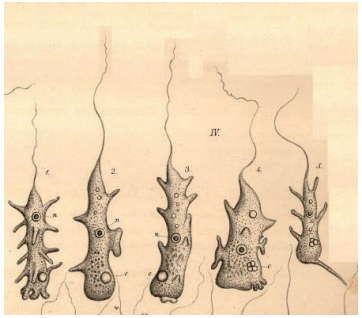
		 (Eichwald)	Infusorienkunde Russlands, *Bulletin de la Société impériale des Naturalistes de Moscou*, Tome XX, Première Partie, Moscou: 293 (+Tab. VIII).	(see Boenigk, 2008)
Monas ovalis	Ehrenberg 1832 (image 1838)	 (Ehrenberg)	**Ehrenberg** Ch.G. (1832) Über die Entwickelung und Lebensdauer der Infusionsthiere; nebst ferneren Beiträgen zu einer Vergleichung ihrer organischen Systeme, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 56-59. **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Not one of the strains described herein as living associated with genus *Anodonta* (Bivalvia)
Monas ovata	Diesing 1850 without image → in Dujardin 1841 as *Heteromita ovata* ≠ de Fromentel 1874 (here: *Monas ovata=ovalis*)	 (Dujardin, pl.IV, fig.22)  (deFromentel)	**Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35. **Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris (planche IV). **de Fromentel** E. (1874) *Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues*, Paris: 326 (planches XXIII - XXVII).	Reclassified as: *Cercomonas ovata* (Dujardin 1841) Tong, Vers, Patterson, 1997 (see Boenigk, 2008)

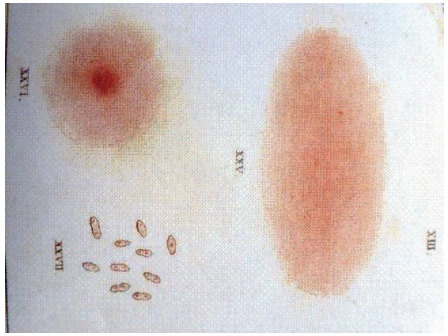
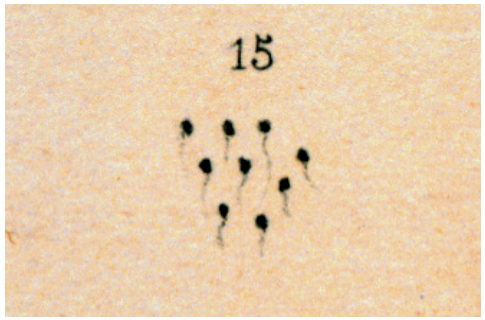
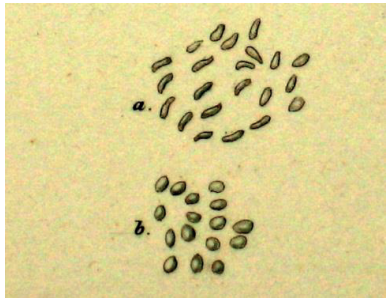
Monas ovulum	Göze (or Götze or Goeze) 1783	No image	**Götze** Johann August Ephraim (1783) Fortsetzung der Beobachtungen und Versuche des Hrn. Abt Spallanzani, die Infusionsthierchen betreffend - Anmerkungen zu diesen Beobachtungen von dem Hrn. Pastor Götze. *Wittenbergisches Magazin auf das Jahr 1783*, Zweytes Stück: 290-297.	Not one of the strains described herein as pigmented
Monas ovum	de Fromentel 1874	 (de Fromentel, pl.XXIII)	**de Fromentel** E. (1874) *Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues*, Paris: 326 (planches XXIII - XXVII).	Not one of the strains described herein as heavily granulated
Monas parasitica	Cienkowsky 1858	 (Cienkowsky)	**Cienkowsky** L. (1858) Ueber meinen Beweis für die Generatio primaria, *Bulletin de la Classe Physico-Mathématique de l'Académie Impériale des Sciences de St.-Pétersbourg*, Tome XVII: 81-95 (+Tab. XVII).	Not one of the strains described herein (cells too elongated)
Monas partita	Perty 1849	No image	**Perty** M. (1849) Mikroskopische Organismen der Alpen und der italienischen Schweiz, *Mittheilungen der Naturforschenden Gesellschaft in Bern*: 153-176 (168).	Reclassified as: *Katodinium monadicum*, Javornicky 1970 (see Boenigk, 2008)
Monas pileatorum	Perty 1852		**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein (flagellum beats only at the tip)

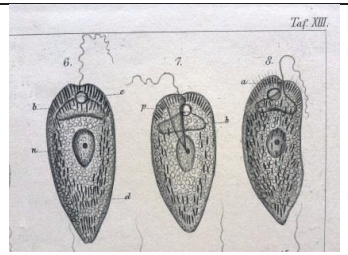
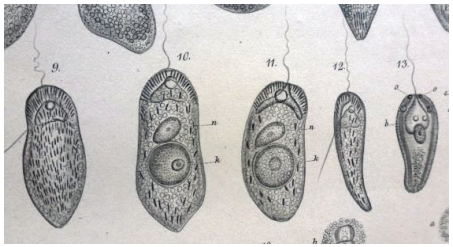
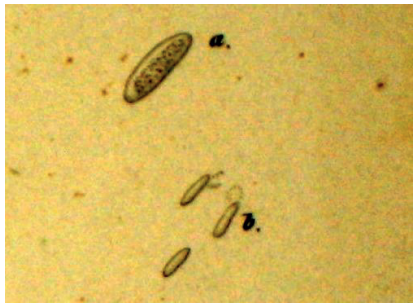
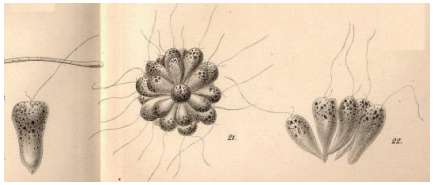
		 (Perty)  (Perty)		
Monas polytoma	Ehrenberg 1830	No image	**Ehrenberg** Ch.G. (1830) Beiträge zur Kenntniss der Organisation der Infusorien und ihrer geographischen Verbreitung, besonders in Sibirien, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 64.	Reclassified as: *Polytoma uvella* (Ehrenberg) Ehrenberg 1832 (see Boenigk, 2008)
Monas precatoria	Bory de St.Vincent 1824 (image in Gleichen 1778)	 (Gleichen, pl.XVII, D IIIc)	**Bory de Saint-Vincent** et al. (1824) *Encyclopédie Méthodique, Histoire Naturelle des Zoophytes, ou Animaux Rayonnés*, Tome Second: 548-550. **Gleichen** Wilhelm Friedrich Freiherr von (1778) *Abhandlung über die Saamen= und Infusionsthierchen, und über die Erzeugung; nebst mikroskopischen Beobachtungen des Saamens der Thiere, und verschiedener Infusionen*, Nürnberg:	Not one of the strains described herein (cells colonial as perls on a chain)

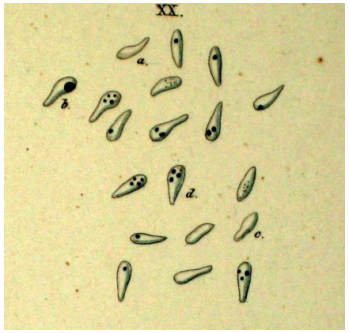
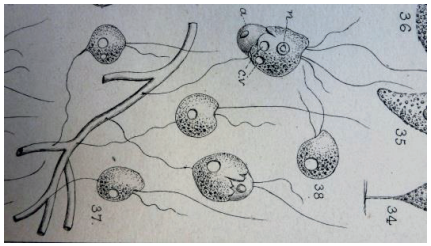
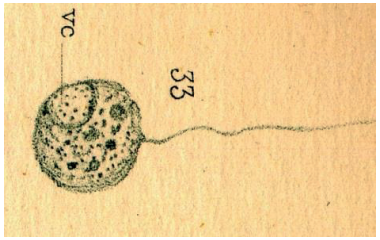
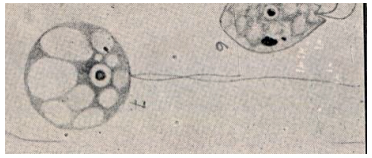
		 (Gleichen, pl.XVII, G I)	134 (pl. XVII, D III c und G I).	
Monas prodigiosa	Ehrenberg 1848	No image	**Ehrenberg** Ch.G. (1848) *Bericht über die zur Bekanntmachung geeigneten Verhandlungen der Königlich Preußischen Akademie der Wissenschaften zu Berlin. 26. Oktober. Gesamtsitzung der Akademie*. 349-362.	Reclassified as: *Serratia marcescens*, Bizio 1823 (see Boenigk, 2008)
Monas profunda	Skuja 1956	 (Skuja)	**Skuja** H. (1956) Taxonomische und biologische Studien über das Phytoplankton schwedischer Binnengewässer, *Nova Acta Reg. Soc. Sc. Ups.*, Ser.IV, Vol.16, No.3: 317-21 (+Taf. LVI).	Not one of the strains described herein (flagella vibrating over the whole length)
Monas pudica	Pringsheim 1951 (see *Spumella pudica* 1946?)	nom. prov. No image No description, only record of organism in culutre collection → no more available	**Pringsheim** E.G. (1951) Die Sammlung von Algen-, Flagellaten- und Mooskulturen am Botanischen Institut der Universität Cambridge, *Archiv für Mikrobiologie*, Band 16: 1-17.	invalid
Monas pulvisculus/um	Müller 1786 (also see		**Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae,	Reclassified as:

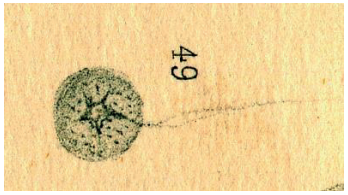
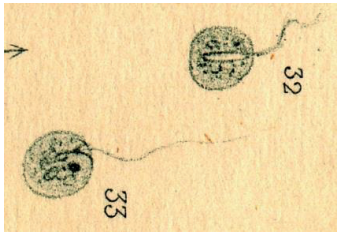
	Ehrenberg 1832)	 (Müller, pl. I, 5/6)	p.7-8 (+Tab I). **Ehrenberg** Ch.G. (1832) Über die Entwickelung und Lebensdauer der Infusionsthiere; nebst ferneren Beiträgen zu einer Vergleichung ihrer organischen Systeme, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 56-59.	*Chlamydomonas reinhardtii*, Dangeard 1888 (see Boenigk, 2008)
Monas punctum	Müller 1786 ≠ Ehrenberg 1838 ≠ Bory de St.Vincent 1824/25	 (Müller, pl. I, 4)  (Ehrenberg)	**Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p.3 (+Tab I). **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I). **Bory de Saint-Vincent** et al. (1824) *Encyclopédie Méthodique, Histoire Naturelle des Zoophytes, ou Animaux Rayonnés*, Tome Second: 548-550 (+planches 1,2,5 from Tome Premier!).	Reclassified as: *Distigma proteus*, Ehrenberg 1831, sensu Stein 1878 pro parte (see Boenigk, 2008)

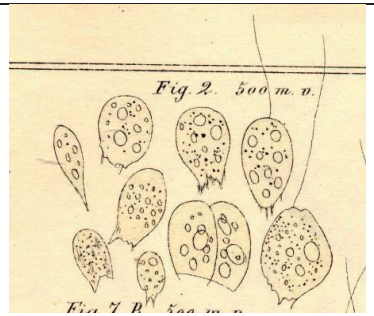
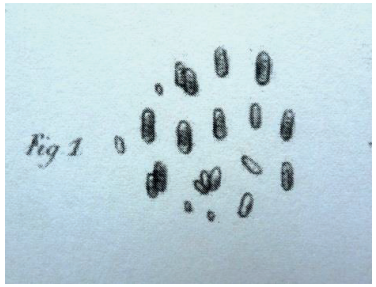
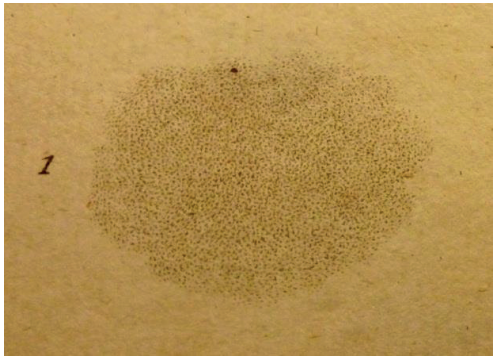
		 (Bory, pl. I, fig. 1)		
Monas ramulosa	Kent 1880-81 → in Stein 1878 as *Cercomonas ramulosa*	 (Kent, pl. XIII, fig. 22-24)  (Stein, pl.I)	**Kent** W. S. (1880/81) *A Manual of the Infusoria: including a description of all known flagellate, ciliate, and tentaculiferous protozoa, British and foreign, and an account of the organization and affinities of the sponges*, London, David Bogue: 235 (plate XIII, XIV). **Stein** Friedrich Ritter v. (1878) *Der Organismus der Infusionsthiere*, III. Abtheilung, I. Hälfte, Leipzig (Tafel I).	Not one of the strains described herein (cells amoeboid)
Monas redivina	Davaine C. 1875 (in Dechambre)	No image	In: **Dechambre** A. (ed.) (1875) *Dictionnaire Encyclodédique des Sciences Médicales*, Deuxième Série, Tome Neuvième: 125.	Not one of the strains described herein (flagellum six times as long as cell body)

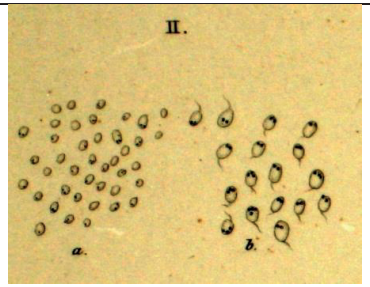
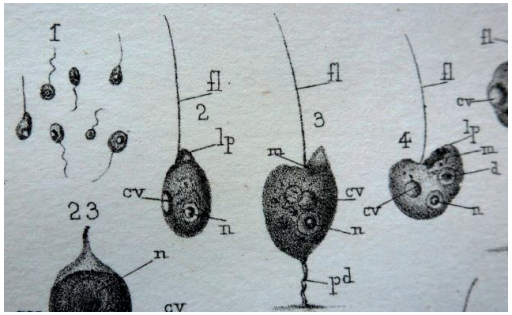
Monas rosea	Morren 1841 (4eme)	 (Morren, pl.V)	**Morren** Ch. (1841) *Recherches physiologiques sur les hydrophytes de Belgique. Quatrième mémoire: Recherches sur la rubéfaction des eaux*. S.5-47 (+planche V).	Reclassified as: *Chromatium okenii* (Ehrenberg) Perty 1852 (see Boenigk, 2008)
Monas rubra	de Fromentel 1874	 (de Fromentel, pl.XXVII)	**de Fromentel** E. (1874) *Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues*, Paris: 328 (planches XXIII - XXVII).	Not one of the strains described herein as pigmented
Monas scintillans	Ehrenberg 1828 (image 18 38)	 (Ehrenberg)	**Ehrenberg** Ch.G., Hemprich F.G. (1828) *Symbolae Physicae*, Berolini: 15-16. **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Not one of the strains described herein as morphology differing (cells too elongated)
Monas semen	Ehrenberg 1853		**Ehrenberg** Ch.G. (1853) *Bericht über die zur*	

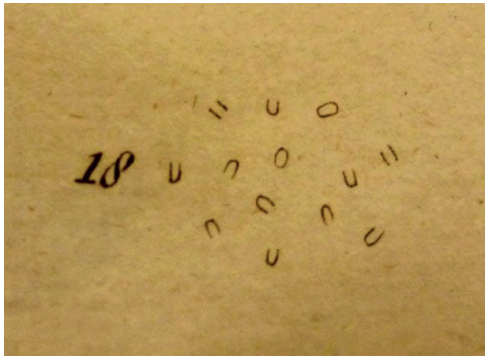
	without image → in Stein 1878 as *Raphidomonas semen* (Taf. XIII)	 (Stein, pl.XIII)  (Stein, pl.XIII)	*Bekanntmachung geeigneten Verhandlungen der Königlich Preußischen Akademie der Wissenschaften zu Berlin. 14. März. Sitzung der physikalisch-mathematischen Klasse*: 178-194. **Stein** Friedrich Ritter v. (1878) *Der Organismus der Infusionsthiere*, III. Abtheilung, I. Hälfte, Leipzig (Tafel XIII).	Not one of the strains described herein (flagella vibrating over the whole length, cell: 50.1µm)
Monas simplex	Ehrenberg 1828 (image 1838)	 (Ehrenberg)	**Ehrenberg** Ch.G., Hemprich F.G. (1828) *Symbolae Physicae*, Berolini: 15-16. **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Not one of the strains described herein (cells too elongated, cell: 12.5 µm)
Monas sociabilis	Meyer 1897	 (Meyer)	**Meyer** Hans (1897-1898) Untersuchungen über einige Flagellaten. *Revue Suisse de Zoologie et Annales du Musée d'Histoire Naturelle de Genève*. Tome V: 43-89 (+ plate 2).	Reclassified as: *Spumella sociabilis* (Meyer) Stein 1976 (see Boenigk, 2008)

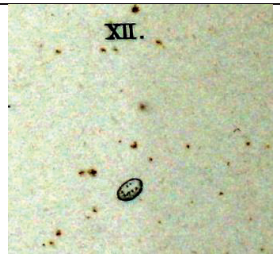
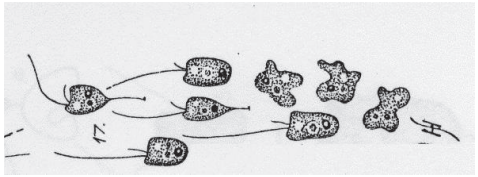
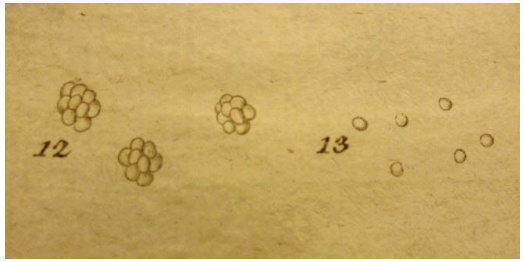
Monas socialis	Ehrenberg 1838 ≠ Lemmermann 1910 without image → in Kent 1880-81 as *Physomonas socialis* (Taf. XIV)	 (Ehrenberg)  (Kent, pl.XIV)	**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I). **Lemmermann** E. (1910) *Kryptogamenflora der Mark Brandenburg und angrenzender Gebiete*, Gebrüder Borntraeger, Leipzig. **Kent** W. S. (1880/81) *A Manual of the Infusoria: including a description of all known flagellate, ciliate, and tentaculiferous protozoa, British and foreign, and an account of the organization and affinities of the sponges*, London, David Bogue (plate XIV) .	Reclassified as: *Spumella socialis* **(invalidly)** (see Boenigk, 2008) Not one of the strains described herein as morphology differing (cells attaching to substrate with a stalk, cell: 15.9µm)
Monas sphaerica	de Fromentel 1874	 (de Fromentel, pl.XXIV)	**de Fromentel** E. (1874) *Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues*, Paris: 329 (planches XXIII - XXVII).	Not one of the strains described herein as contractile vacuole clearly visible
Monas sphaericus	Valkanov 1925	 (Valkanov)	**Valkanov** A. (1925) Beitrag zur Kenntnis der Flagellaten von Bulgarien, Izg. Bulg. Bot. Druzh., 1:109 (+Tab. 2).	Identified with strain JBC27 from this study; emended and recombined to:

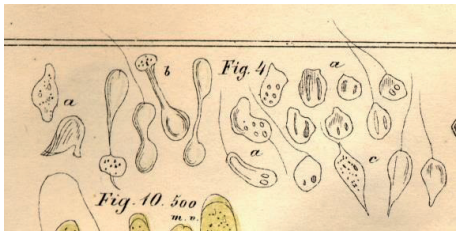
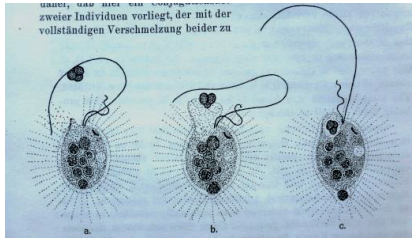
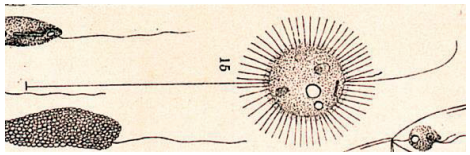
				Chromulinospumella sphaerica (Valkanov) Boenigk et Großmann nov. comb.
Monas stellata	de Fromentel 1874	 (de Fromentel, pl.XXIV)  (de Fromentel, pl.XXIV)	**de Fromentel** E. (1874) *Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues*, Paris: 326 (planches XXIII - XXVII).	Not one of the strains described herein as showing star-shaped structure
Monas stigmatica	Pringsheim (in Lowndes 1944)	No image No description Without date or name of Pringsheim's publication! Strain from Culture Collection, Cambridge → no more available	**Lowndes** A.G. (1944) The Swimming of *Monas stigmatica* Pringsheim and *Peranema trichophorum* (Ehrbg.) Stein and *Volvox* sp. – Additional Experiments on the Working of a Flagellum. *Proceedings of the Zoological Society of London*, vol. 114, part 3: 325-338.	Invalid
Monas succisa	Perty 1852		**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein (cells show theca-like

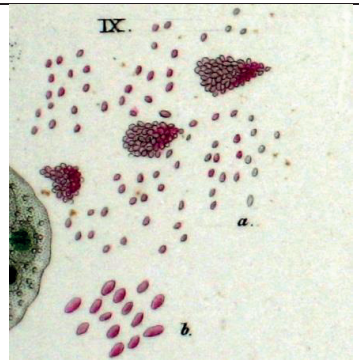
		 (Perty)		appearance, cell: 12.5-15µ)
Monas sulphuraria	Fontan & Joly 1845	 (Fontan and Joly)	**Fontan** A. & **Joly** N. (1844/45) Note sur une nouvelle espèce d'annimalcule infusoire (*Monas sulphuraria*), qui colore en rouge les sources sulfureuses accidentelles de Salies (Haute-Garonne) et d'Enghien (Seine-et-Oise). *Mémoires de l'Académie Royale des Sciences, Inscriptions et Belles-Lettres de Toulouse*. Troisième Série, Tome Premier: 116-124 (+planche).	Not one of the strains described herein (cells are red coloured)
Monas termo	Müller 1773/1786 (also see Ehrenberg 1830/38 and James-Clark 1866)	 (Müller, pl. I, 1)	**Müller** Othone Friderico (1773) *Vermium terrestrium et fluviatilium.* Hauniae et Lipsiae, p.25-26. **Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p.1 (+Tab I). **Ehrenberg** Ch.G. (1830) Beiträge zur Kenntniss der Organisation der Infusorien und ihrer geographischen Verbreitung, besonders in Sibirien, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 37 (+Tab. I).	Reclassified as: *Oicomonas termo* (James-Clark) Kent 1880-81 (see Boenigk, 2008)

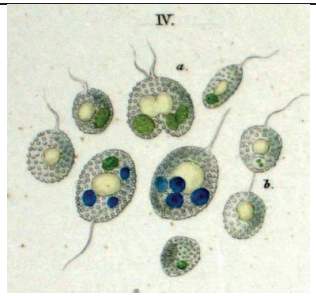
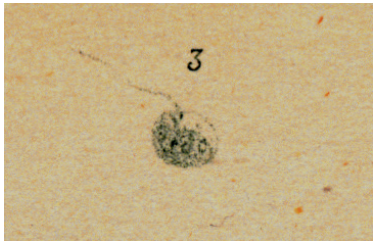
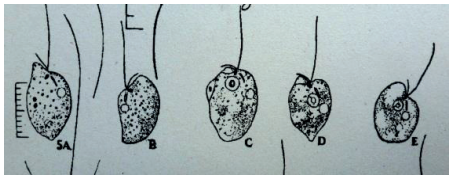
		 (Ehrenberg)  (James-Clark, pl. IX)	**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I). **James-Clark** H. (1866) On the Spongiae Ciliatae, as Infusoria Flagellata; or, Observations on the Structure, Animality and Relationship of Leucosolenia Botryoides Bowerbank. *Memoirs read before the Boston Society of Natural History, Boston Journal of Natural History*, New York/London. Vol. I, Part I: 305-340 (+ plate IX/X).	
Monas tetrarhynchus	Diesing 1850 without image	No image	**Diesing** C.M. (1850) *Systema Helminthum*, vol.I, Vindobonae: 22-35.	Reclassified as: *Polytoma tetrarhynchus* Schmarda, 1850 (see Boenigk, 2008)
Monas tingens	Ehrenberg 1838		**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Not one of the strains described herein as pigmented

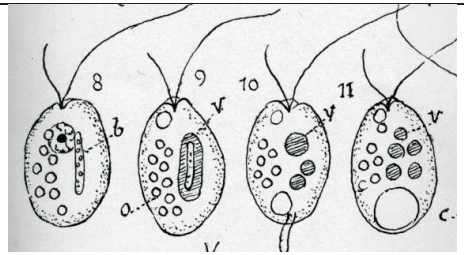
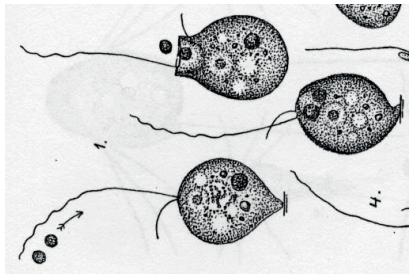
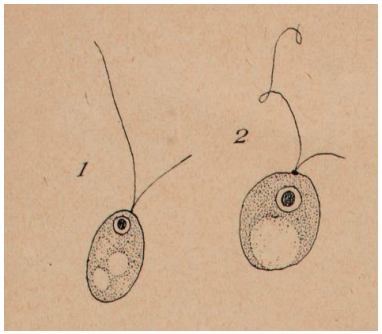
		 (Ehrenberg)		
Monas tranquilla	Müller 1786	 (Müller, pl.I, 18)	**Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p.6-7 (+Tab I).	Not one of the strains described herein (square shape)
Monas truncata	Fresenius 1858	 (Fresenius)	**Fresenius** G. (1858) Beiträge zur Kenntniss mikroskopischer Organismen, *Abh. Senckenb. Naturf. Ges.*, 2 : 211-242 (227) (+ Tafel X).	Reclassified as: *Goniomonas truncata* (Fresenius) Stein 1878 (see Boenigk, 2008)
Monas umbra	Ehrenberg 1832 (image 1838)		**Ehrenberg** Ch.G. (1832) Über die Entwickelung und Lebensdauer der Infusionsthiere; nebst ferneren Beiträgen zu einer Vergleichung ihrer organischen Systeme, *Abhandlungen der königlichen*	Not one of the strains described herein as pigmented

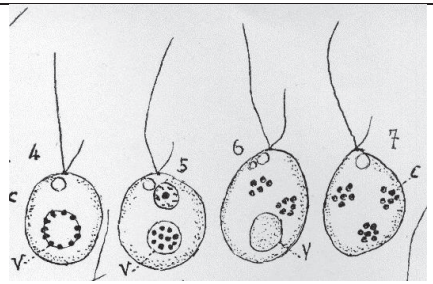
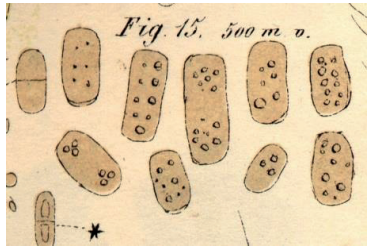
		 (Ehrenberg)	*Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 56-59. **Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	
Monas uniguttata	Skuja 1939	 (Skuja)	**Skuja** H. (1939) Beitrag zur Algenflora Lettlands II. *Acta Horti Botanici Universitatis Latviensis*, Latvijas Universitates Botaniska Darza Raksti, XI/XII: 41-169 (+Tab III).	(see Boenigk, 2008)
Monas urceolaris	Perty 1852	 (Perty)	**Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein (cells show theca-like appearance)
Monas uva	Müller 1786 (also see Ehrenberg 1830)	 (Müller, pl. I, 12/13)	**Müller** Otho Fridericus (1786) *Animalcula infusoria fluvia tilia et marina*. Hauniae, p. 8-9 (+Tab I). **Ehrenberg** Ch.G. (1830) Beiträge zur Kenntniss der Organisation der Infusorien und ihrer geographischen Verbreitung, besonders in Sibirien, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische*	Reclassified as: *Polytoma uva* (Müller) Perty 1852 (see Boenigk, 2008)

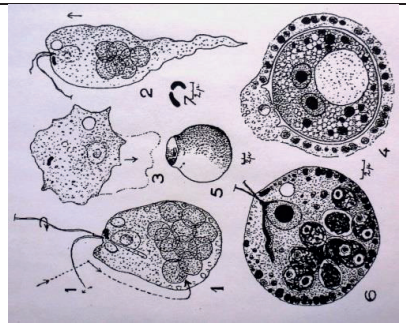
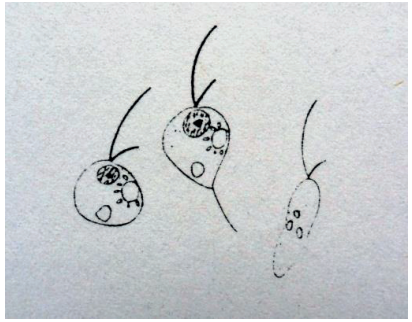
			Klasse: 64.	
Monas varians	Dujardin 1841 without image (image in Perty 1852)	 (Perty)	**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286. **Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Not one of the strains described herein (cells have amorphic structure, cell: 32-40µm)
Monas vestita	Reynolds 1934 → in Stokes 1885/1888 as *Physomonas vestita*	 (Reynolds)  (Stokes)	**Stokes** Alfred C. (1886) Notices of New Fresh-Water Infusoria.-V. *The American Monthly Microscopical Journal*, Vol. VII No.5: 81-86 (incl. fig.). **Stokes** Alfred C. (1888) *Journal of the Trenton Natural History Society 1888*: 81ff (+plate I).	Reclassified as: *Paraphysomonas vestita* (Stokes) De Saedeleer 1930 (see Boenigk, 2008)
Monas vinosa	Ehrenberg 1838		**Ehrenberg** Ch.G. (1838) *Die Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I).	Reclassified as: *Chromatium vinosum* (Ehrenberg) Winogradsky 1888 (see Boenigk, 2008)

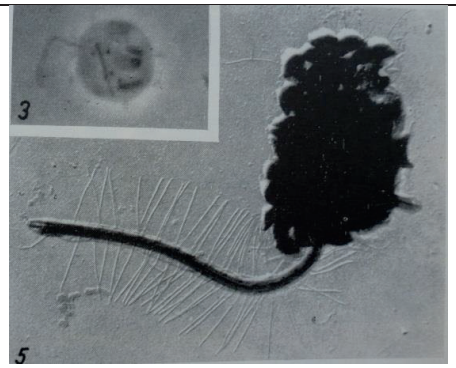
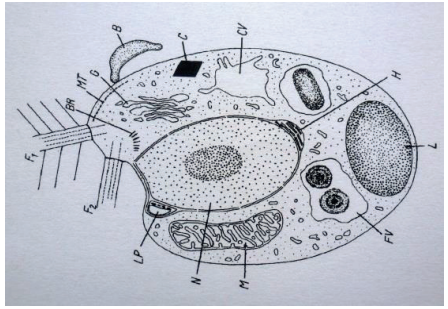
		 (Ehrenberg)		
Monas violacea	Ehrenberg 1836	No image No description	**Ehrenberg** Ch.G. (1836) Vorläufige Mittheilung über die Infusorien der Carlsbader Mineralquellen, *Archiv für Naturgeschichte*, Vol.2: 244.	Invalid
Monas violascens	Diesing 1866 without image → in Perty 1852 as *Chromatium violascens*	 (Perty)	**Diesing** K.M. (1866) Revision der Prothelminthen. Abteilung: Mastigophoren. S*itzungsberichte der Mathematisch-Naturwissenschaftlichen Classe der Kaiserlichen Akademie der Wissenschaften*, Wien. LII. Band, I. Abteilung: 287-401. **Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Reclassified as: *Chromatium violascens*, Perty 1852 (see Boenigk, 2008)
Monas viridis	Dujardin 1841	No image	**Dujardin** Félix (1841) *Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope*, Paris, 279-286.	Not one of the strains described herein as pigmented
Monas vivipara	Ehrenberg 1838		**Ehrenberg** Ch.G. (1838) *Die*	

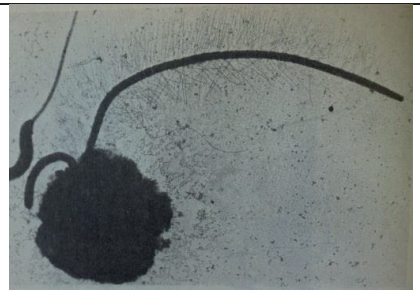
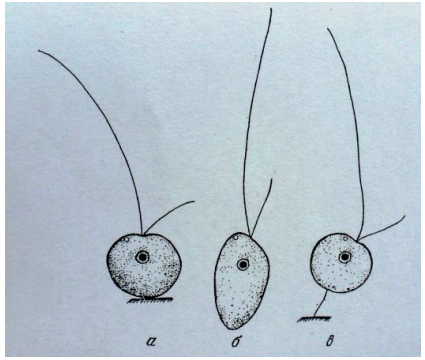
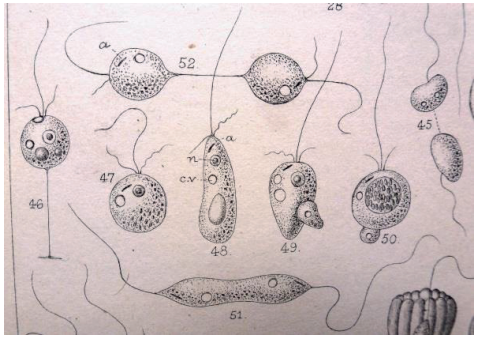
	≠ de Fromentel 1874 and de Seadeleer 1931 (here var. *minima* und var. *ocellata, ocellata* without image)	 (Ehrenberg)  (de Fromentel, pl.XXVI)  (de Seadeleer – var. *minima*, pl. VI)	*Infusionsthierchen als vollkommene Organismen*, Leopold Voss, Leipzig: 6-18 (+Tafel I). **de Fromentel** E. (1874) *Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues*, Paris: 324 (planches XXIII - XXVII). **de Saedeleer** Henri (1931) Nieuwe of weinig bekende Flagellaten. *Natuurwetenschappelijk tijdschrift*: NWT / uitg. Met de steun van de Universitaire Stichting van Belgie en van het Ministerie van Openbaar Onderwijs. Natuur- en Geneeskundige Venootschap, Groningen. 13: 89ff (+plaat VI).	(see Boenigk, 2008) Not one of the strains described herein as feeding on algae
Monas volvox	Ehrenberg 1830	No image No description	**Ehrenberg** Ch.G. (1830) Beiträge zur Kenntniss der Organisation der Infusorien und ihrer geographischen Verbreitung, besonders in Sibirien, *Abhandlungen der königlichen Akademie der Wissenschaften zu Berlin, Physikalisch-mathematische Klasse*: 64.	Invalid
Monas vorax	Dangeard 1934 ≠ Skuja 1939		**Dangeard** P.-A. (1934) Mémoire sur l'*Apistonema submarinum* sp. nov. et considerations generals sur la structure des Protozoaires et des Protophytes, *Le*	Not one of the strains described herein as cells too large (cell: 15µ)

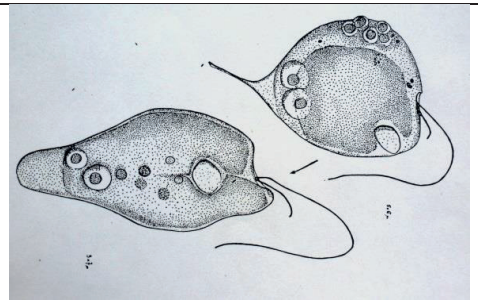
		 (Dangeard)  (Skuja, pl. III)	*Botanist*: 310-318 (+planche XXVIII). **Skuja** H. (1939) Beitrag zur Algenflora Lettlands II. *Acta Horti Botanici Universitatis Latviensis*, Latvijas Universitates Botaniska Darza Raksti, XI/XII: 41-169 (+Tab III).	
Monas vulgaris	Senn 1900 without image (also see Dangeard 1910/1934)	No image (in Senn)  (Dangeard, pl. XX-XXII)	**Senn** G. (1900) Flagellata, in : Engler A., Prantl K.: Die Pflanzenfamilien nebst ihren Gattungen und wichtigeren Arten, I. Teil, Abteilung 1a und 1b, Wilhelm Engelmann, Leipzig. **Dangeard** P.-A. (1903) Observations sur le *Monas vulgaris*, *Le Botanist*: 319-321. **Dangeard** P.-A. (1910) Etudes sur le développement et la structure des organismes inférieurs, avec 33 planches. *Le Botaniste*, Onzième Série, Mai 1910: S.141ff (+ planches XX-XXII). **Dangeard** P.-A. (1934) Mémoire sur l'*Apistonema submarinum* sp. nov. et considerations generals sur la structure des Protozoaires et des Protophytes, *Le*	Recombination from *Spumella vulgaris* (Cienkowsky)

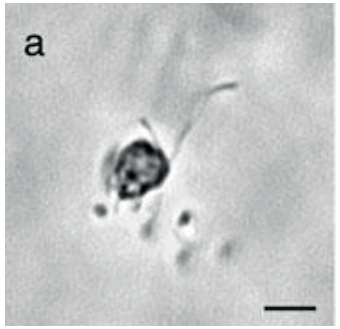
		 (Dangeard, pl.XXVIII)	*Botanist*: 310-318 (+planche XXVIII).	
Monas warmingii	Warming 1876	No image No description	**Warming** Eug. (1875) Om nogle ved Danmarks Kyster levende Bakterier, *Videnskabelige Meddelelser fra den naturhistoriske Forening i Kjöbenhavn*, Utgivne af Selskabets Bestyrelse, Kjöbenhavn, 1875-76: 363-70.	Invalid
Monas weissei	Diesing 1866 without image → in Perty 1852 as *Chromatium weissei*	 (Perty)	**Diesing** K.M. (1866) Revision der Prothelminthen. Abteilung: Mastigophoren. S*itzungsberichte der Mathematisch-Naturwissenschaftlichen Classe der Kaiserlichen Akademie der Wissenschaften*, Wien. LII. Band, I. Abteilung: 287-401. **Perty** M. (1852) *Zur Kenntnis kleinster Lebensformen*, Jent&Reinert, Bern: 172-174 (+Tafeln XIV, XV).	Reclassified as: *Chromatium weissei/weisii*, Perty 1852 (see Boenigk, 2008)
Spumella beauchampii	Silva 1960 comb.nov. (see *Oicomonas beauchampi*, Hovasse 1943)		**Silva** P.C. (1960) Remarks on Algal Nomenclature, III., Taxon, Vol.9: 18-25. **Hovasse** R. (1943) Contribution a l'étude des flagellés libres: *Oicomonas beauchampi* sp. nov. Protomonadine et chrysomonade. *Protistologica*, XCIII: 47-53 (incl. planches).	Not one of the strains described herein as morphology differing (cells of elongated, irregular shape)

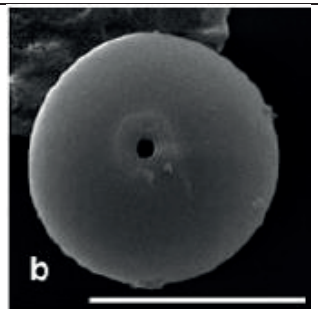
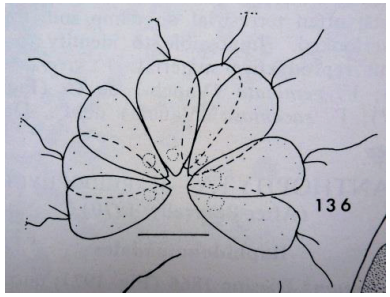
		 (Hovasse)	(Parallelly: *Oicomonas beauchampii*, Hovasse 1943 → *Heterochromonas beauchampii*, Bourrelly 1957 → *Spumella beauchampii*, Bourrelly 1968)	
Spumella danica	in Boenigk 2005 (drawing on *Spumella danica* n. sp. in Bruchmüller (unpublished); in Bruchmüller 1998 as *Spumella* sp. fID; in Ekelund 1996 as *Spumella elongata*)	 (Ekelund, p. 18)	**Boenigk** J. (2005) High diversity oft he ‚*Spumella*-like' flagellates: an investigation based on the SSU rRNA gene sequences of isolates from habitats located in six different geographic regions, *Environmental Microbiology*, 7/5: 685-697. **Bruchmüller** I., Mylnikov A., Juergens K., Weisse T. (unpublished) Phylogenetic analysis of the genus *Spumella* (Chrysophceae) inferred from SSU rDNA Sequences, comparison of ultrastructure, and description of *Spumella danica* sp. nov. **Bruchmüller** I. (1998) *Molekularbiologische Charakterisierung und phylogenetische Einordnung heterotropher Nanoflagellaten und prostomatider Ciliaten des Süßwassers*, Dissertation, Mathem.-Naturw. Fakultät, Christian-Albrecht-Universität Kiel. **Ekelund** F. (1996) Growth kinetics of five common heterotrophic soil flagellates, *Eur. J. Soil Biol*., 32/1: 15-24.	nomen nudum
Spumella elongata	Belcher & Swale 1976		**Belcher** J.H. & **Swale** E.M.F. (1976) *Spumella elongata* (STOKES) nov. comb., a	reclassification technically

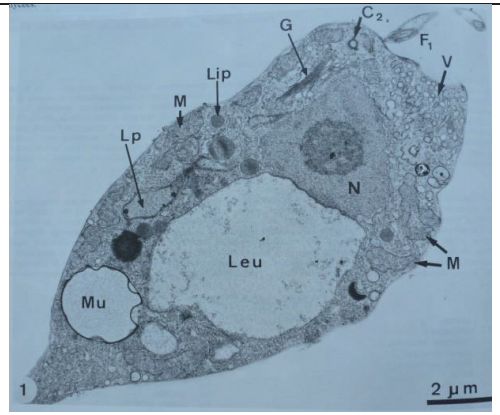
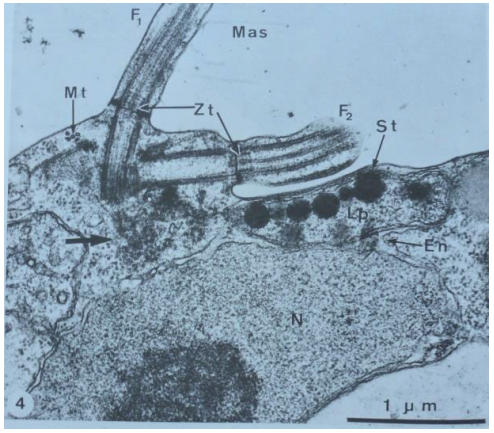
	(see *Physomonas elongata*, Stokes 1888/ see *Monas elongata*, Lemmermann 1910)	 (Belcher and Swale)  (Belcher and Swale)  (Belcher and Swale)	Colourless Flagellate from Soil. *Archiv für Protistenkunde: Protozoen, Algen, Pilze*, 118: 215-220 (+plates 38,39). **Stokes** Alfred C. (1886) Notices of New Fresh-Water Infusoria.-V. *The American Monthly Microscopical Journal*, Vol. VII No.5: 81-86 (incl. fig.). **Stokes** Alfred C. (1888) *Journal of the Trenton Natural History Society 1888*: 81ff (+plate I). **Lemmermann** E. (1910) *Kryptogamenflora der Mark Brandenburg und angrenzender Gebiete*, Gebrüder Borntraeger, Leipzig. Also see: **Mylnikov** A.P. & Mylnikova Z.M. (2005) The morphology of heterothrophic chrysomonads oft he genus *Spumella* (Chrysophyta), *Biology of Inland Waters*, Institute for Biology of Inland Waters, Borok, Russia, 3/05: 57-62.	incorrect: thus invalid, reclassified in this study as: *Pedospumella elongata* (Stokes) nov.comb.
Spumella gregaria	Tanichev 1993		**Tanichev** A.I. (1993) Morphology of the Baikal Chrysomonads, *Spumella termo* and *S. gregaria* sp.n. (Protozoa,	Not one of the strains described herein (flagellum

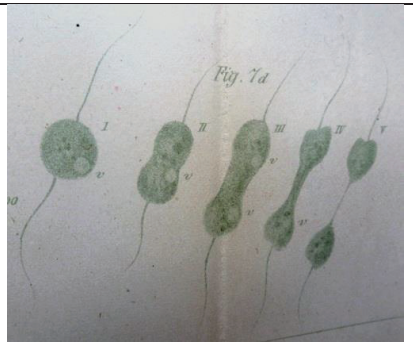
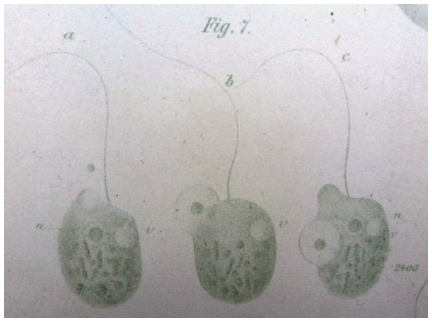
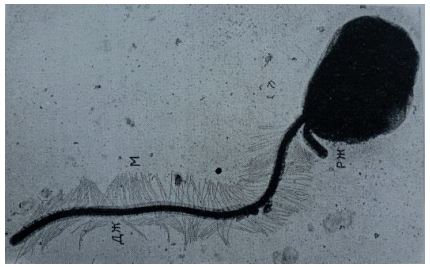
		 (Tanichev)  (Tanichev)	Chrysomonadida*). Zoologicheskij Zhurnal*, Tom 72 (1): 23-29 (incl. plates). Also see: **Mylnikov** A.P., Mylnikova Z.M. (2005) The morphology of heterothrophic chrysomonads oft he genus *Spumella* (Chrysophyta), *Biology of Inland Waters*, Institute for Biology of Inland Waters, Borok, Russia, 3/05: 57-62.	too long)
Spumella guttula	Kent 1881 (see *Monas guttula*, Ehrenberg)	 (Kent, pl. XIV)	**Kent** W. S. (1880/81) *A Manual of the Infusoria: including a description of all known flagellate, ciliate, and tentaculiferous protozoa, British and foreign, and an account of the organization and affinities of the sponges*, London, David Bogue: 305 (plate XIII, XIV). (Identified with both *Monas guttula*, Ehrenberg and *Spumella vulgaris*, Cienkowsky)	Not one of the strains described herein (→ showing caudal filament)
Spumella hovassei	Bourrelly 1968 nov. comb.		**Bourrelly** Pierre (1968) *Les algues d'eau douce, tome II, Les algues jaunes et brunes*,	Not one of the strains

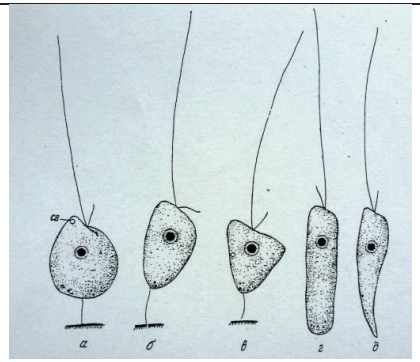
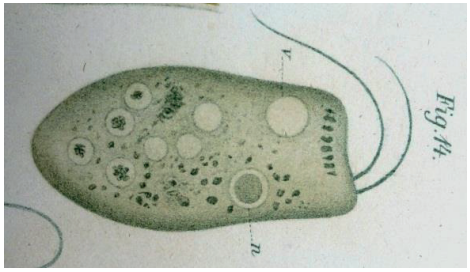
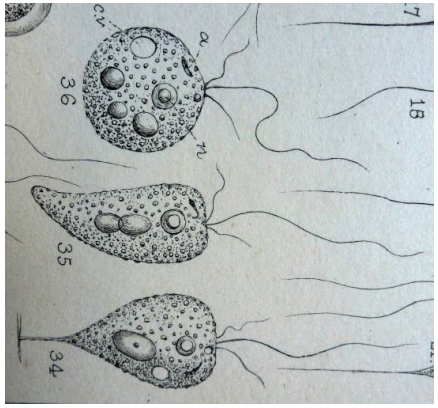
	(see *Hetero-chromonas hovassei* n.sp., Fiatte & Joyon 1965 for image)	 (Fiatte and Joyon)	Boubée, Paris: 78. **Fiatte** M.-C. & **Joyon** L. (1965) *Heterochromonas hovassei* (n.sp.), chrysomonadine décolorée. *Archives de Zoologie Expérimentale et Générale*, 105: 273-283 (incl. plates).	described herein as morphology differing (cells too irregular, cell: 12-15µ)
Spumella neglecta	Bütschli 1878 (see *Monas neglecta*, James-Clark 1866)	No image Mentioned without description/recombination or naming of original description/recombination	**Bütschli** O. (1878) Beiträge zur Kenntnis der Flagellaten und einiger verwandten Organismen. *Zeitschrift für wissenschaftliche Zoologie*, 30: 205-281.	Invalid, Not one of the strains described herein as 1 flagellum
Spumella oblique (obliqua)	in Snelling 2005 (presumably invalidly recombined from *Monas obliqua*, Schewiakoff)	No image Mentioned without description/recombination or naming of original description/recombination	**Snelling** W.J. (2005) Survival of *Campylobacter jejuni* in Waterborne Protozoa, *Applied and Environmental Microbiology*, Vol.71, No.9: 5560-5571. Also see: **Mylnikov** A.P. & Mylnikova Z.M. (2005) The morphology of heterothrophic chrysomonads oft he genus *Spumella* (Chrysophyta), *Biology of Inland Waters*, Institute for Biology of Inland Waters, Borok, Russia, 3/05: 57-62. **Mylnikov** A.P., Mylnikova Z.M., Zolotarev V.A., Kosolapova N.G. (2007) The main cell structure of the small colourless Chrysomonad Spumella oblique (Schewiakoff, 1892) (Ochromonadales, Chrysophyta), *Biology of Inland Waters*, Institute for Biology of Inland Waters,	nomen nudum sequence in NCBI (*Spumella obliqua*) not from type material (*Monas obliqua*), affiliation with type material unclear

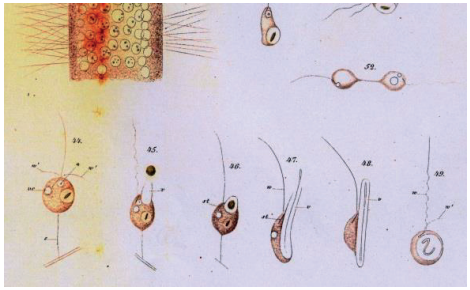
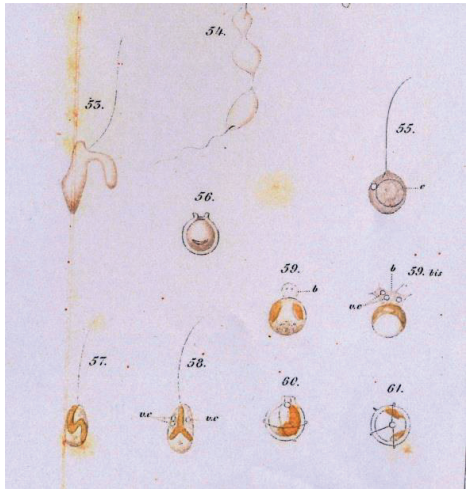
			Borok, Russia, 3/07: 11-16.	
Spumella pudica	Pringsheim 1946 (bzw. 1951 as *Monas pudica*?) --> used in Bruchmüller 1998 and Véra 2001	nom. prov. No image No description, only record of organism in culutre collection → no more available In Bruchmüller and Véra: Without naming of Pringsheim's publication. Strain from Culture Collection of Algae and Protozoa (CCAP), Windemere, UK → no more available	**Pringsheim** E.G. (1946) *Pure culture of algae, their preparation and maintenance*, CUP. **Bruchmüller** I. (1998) *Molekularbiologische Charakterisierung und phylogenetische Einordnung heterotropher Nanoflagellaten und prostomatider Ciliaten des Süßwassers*, Dissertation, Mathem.-Naturw. Fakultät, Christian-Albrecht-Universität Kiel. **Véra** A. (2001) Fatty acid composition of freshwater heterotrophic flagellates: an experimental study, *Aquatic Microbial Ecology*, Vol.25: 271-279.	Invalid
Spumella putida	in Newsham 2007	No image Mentioned without description/recombination or naming of original description/recombination	**Newsham** K.K. (2007) Interactiv effects of warming and species loss on model Antarctic microbial food webs. *Functional Ecology*, 21: 577-584.	Invalid
Spumella rivalis	Findenig & Boenigk 2010 (described from strain AR4A6)	 (Findenig/Boenigk, p. 874)	**Findenig** Barbara M., Chatzinotas Antonis, Boenigk Jens (2010) Taxonomic and ecological characterization of Stomatocysts of *Spumella*-like flagellates (Chrysophyceae). *Journal of Phycology*, 46: 868-881 (incl. plates).	*Spumella rivalis* (Boenigk et Findenig), emended as such in this study

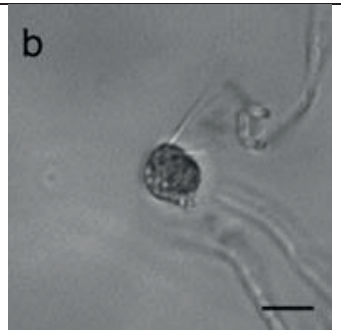
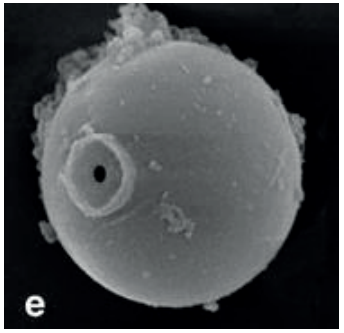
		 (Findenig/Boenigk – cyst, p. 873)		
Spumella sociabilis	Stein 1976 (see *Monas sociabilis*, Meyer)	 (Stein, p. 148)	**Stein** Janet R. (1975) Freshwater algae of British Columbia: the Lower Fraser Valley. *Syesis*, 8: 119-184 (incl. plates). Also see: **Mylnikov** A.P. & Mylnikova Z.M. (2005) The morphology of heterothrophic chrysomonads oft he genus *Spumella* (Chrysophyta), *Biology of Inland Waters*, Institute for Biology of Inland Waters, Borok, Russia, 3/05: 57-62.	Not one of the strains described herein as colonial
Spumella socialis	in Dorothy 2003	No image Mentioned without description/recombination or naming of original description/recombination	**Dorothy** K. Patma (2003) Protozoa associated with leaf litter degradation in Coringa mangrove forest, Kakinada Bay, east coast of India. *Indian Journal of Marine Sciences*, 32 (1): 45-51.	Invalid
Spumella sphaerophora	Mignot 1977 nov. comb. (from *Hetero-chromonas sphaerophora* n.sp. Skuja 1956)		**Mignot** J.-P. (1977) Étude ultrastructurale d'un flagellé du genre *Spumella* Cienk (= *Heterochromonas* Pascher = *Monas* O.F. Muller), Chrysomonadine Leucoplastidée. *Protistologica*, 13: 219-231 (incl. planches). **Skuja** H. (1956) Taxonomische und biologische Studien über das	Not one of the strains described herein as cells too large (cell: 15-20µ)

		 (Mignot)  (Mignot)	Phytoplankton schwedischer Binnengewässer, *Nova Acta Reg. Soc. Sc. Ups.*, Ser.IV, Vol.16, No.3: 313-14 (+Taf. LVI). Also see: **Mylnikov** A.P. & Mylnikova Z.M. (2005) The morphology of heterothrophic chrysomonads of the genus *Spumella* (Chrysophyta), *Biology of Inland Waters*, Institute for Biology of Inland Waters, Borok, Russia, 3/05: 57-62.	
Spumella subterana	Tanichev 1995	No image only abstract from symposion → no paper, no species description	**Tanichev** A.I., Karpov S.A. (1995) The ultrastructural peculiarities of the colourless chrysomonads *Paraphysomonas vestita* and *Spumella subterana* from the Baikal, *European Journal of Protistology*, 31: 118.	Invalid
Spumella termo	Bütschli 1878		**Bütschli** O. (1878) Beiträge zur Kenntnis	

	(from *Monas termo*, James-Clark 1866)	 (Bütschli, pl. XII, fig. 7)  (Bütschli, pl. XII, fig.7)	der Flagellaten und einiger verwandten Organismen. *Zeitschrift für wissenschaftliche Zoologie*, 30: 205-281 (plate XII).	Not one of the strains described herein as only 1 flagellum
Spumella termo	Tanichev 1993 (see *Monas termo* (Müller) Hänel)	 (Tanichev)	**Tanichev** A.I. (1993) Morphology of the Baikal Chrysomonads, *Spumella termo* and *S. gregaria* sp.n. (Protozoa, Chrysomonadida*). Zoologicheskij Zhurnal*, Tom 72 (1): 23-29 (incl. plates). (Already described as *Spumella termo* by Bütschli 1878; but put together as *Monas termo* again by Hänel (using nov. comb.)) Also see: **Mylnikov** A.P. & Mylnikova Z.M. (2005) The morphology of heterothrophic chrysomonads oft he genus *Spumella*	Not one of the strains described herein (→ forms exovacuoles)

		 (Tanichev)	(Chrysophyta), *Biology of Inland Waters*, Institute for Biology of Inland Waters, Borok, Russia, 3/05: 57-62.	
Spumella truncata	Bütschli 1878 (see *Monas truncata*, Fresenius)	 (Bütschli, pl. XIII, fig. 14)	**Bütschli** O. (1878) Beiträge zur Kenntnis der Flagellaten und einiger verwandten Organismen. *Zeitschrift für wissenschaftliche Zoologie*, 30: 205-281 (plate XIII). **Fresenius** G. (1858) Beiträge zur Kenntniss mikroskopischer Organismen, *Abh. Senckenb. Naturf. Ges.*, 2 : 211-242 (227) (+ Tafel X). (nov. comb. without writing it in words)	Reclassified as: *Goniomonas truncata* (Fresenius) Stein 1878 (see Boenigk, 2008)
Spumella vivipara	Kent 1881 (see *Monas vivipara*, Ehrenberg)	 (Kent, pl. XIV)	**Kent** W. S. (1880/81) *A Manual of the Infusoria: including a description of all known flagellate, ciliate, and tentaculiferous protozoa, British and foreign, and an account of the organization and affinities of the sponges*, London, David Bogue: 306 (plate XIII, XIV). (From *Monas vivipara*, Ehrenberg (*Spumella* in Kent only those with 3 flagella!)) (Parallelly: *Heterochromonas vivipara*	Not one of the strains described herein (cell: 25.4-41µm)

			(Ehrenberg) Pascher 1912 (aus *Monas vivipara*!) → type species for *Heterochromonas* for Bourrelly 1957 → to *Spumella vivipara* by Bourrelly 1968)	
Spumella vulgaris	Cienkowsky 1870 (to *Monas vulgaris* by Senn 1900) → Findenig & Boenigk 2010 (epitypified from strain 199hm)	 (Cienkowsky)  (Cienkowsky)	**Cienkowsky** L. (1870) Ueber Palmellaceen und einige Flagellaten, *Archiv für mikroskopische Anatomie*, VI: 421-438 (432-34) (+Tafel XXIV). **Findenig** Barbara M., Chatzinotas Antonis, Boenigk Jens (2010) Taxonomic and ecological characterization of Stomatocysts of *Spumella*-like flagellates (Chrysophyceae). *Journal of Phycology*, 46: 868-881 (incl. plates). (to *Monas vulgaris* by Senn 1900, recombined to *Heterochromonas* by Bourrelly 1957, and back to *Spumella* by Bourrelly 1968)	Epitypified as *Spumella vulgaris* (Cienkowsky) in Findenig et al. 2010

		 (Findenig/Boenigk)  (Findenig/Boenigk)		

3) PROTISTAN COMMUNITY ANALYSIS – KEY FINDINGS OF A LARGE SCALE MOLECULAR SAMPLING

ABSTRACT

Protistan diversity is manifold, yet largely unknown. Deep sequencing technologies bear the opportunity to screen whole habitats in depth and thus allow the detailed comparison of different habitats to illuminate protistan diversity. So far, such comparisons are mostly hampered by a low sampling coverage within single studies and a lack of standardisation between studies. Here, we analysed 232 samples from 10 sampling campaigns in 8 different regions using a standardised PCR protocol and bioinformatics pipeline. We show that protistan community patterns are highly consistent within habitat types and geographic regions, provided that sample processing is standardised. Community profiles are only weakly affected by fluctuations of the abundances of the most abundant taxa and therefore provide a sound basis for habitat comparison beyond random short-term fluctuations in the community composition. Further, we provide evidence that distribution patterns are not solely resulting from random processes. Distinct habitat types and distinct taxonomic groups are dominated by taxa with distinct distribution patterns reflecting their ecology with respect to dispersal and habitat colonisation. However, there is no systematic shift of the distribution pattern with taxon abundance. Rare taxa just show a broader frequency distribution than frequent ones.

3.1 INTRODUCTION

Protists are abundant and diverse in aquatic as well as terrestrial ecosystems and fulfil critical ecosystem functions (Triadó-Margarit & Casamayor 2012; Bates et al. 2013; del Campo & Massana 2011). They do not only strongly contribute to primary production (Field et al. 1998) and bacterial grazing (Boenigk & Arndt 2002; Glücksman et al. 2010), but are also major players in diverse nutrient cycles (Finlay & Esteban 1998; Coleman & Whitman 2004) and are thus, together with bacteria, the basis of aquatic and terrestrial food webs. However, despite their key role in ecosystem functioning, their diversity and distribution on earth is yet largely unknown. Overarching patterns of protistan community structure are still controversial due to limited data (Tringe et al. 2005; Bik et al. 2012).

Following the decade of the exploration of 'unexpected microbial diversity', which was revealed by clone libraries and Sanger sequencing starting in the 1990s, high-throughput sequencing technologies reported even higher estimates of undetected diversity. High-throughput sequencing technologies increasingly opened the possibilities for sufficiently deep community analysis to address the questions of protist and microbial distribution patterns (Stoeck et al. 2009; Caron & Countway 2009; Nolte et al. 2010). With millions of reads, these methods allow for deep-sequencing of microbial communities (Caporaso et al. 2012) having the potential to reveal the hidden diversity and to draw conclusions on the 'true' diversity (Lecroq et al. 2011; Medinger et al. 2010; Degnan & Ochman 2012).

However, most high-throughput studies so far were restricted to few sampling sites (e.g. Medinger et al. 2010; Kammerlander et al. 2015; Lie et al. 2014) and therefore can usually hardly address overarching aspects of protist distribution and the generalisability of these patterns. Meta-analyses based on multiple sequencing studies cannot currently meet this demand for other reasons, either: High-throughput sequencing methods are error prone (Salipante et al. 2014) usually leading to the rejection of a considerable part, often the majority, of the raw sequences during bioinformatics quality filtering (e.g. Nolte et al. 2010). As different sequencing platforms produce different errors (Salipante et al. 2014; Mardis 2013), the results are hardly or not at all comparable between platforms. Even worse, the bioinformatics pipelines themselves are not standardised and slight changes in the filter algorithms result in strong differences on whether sequences are accepted or rejected. In consequence, data can only properly be compared within a single study or between studies which used the same PCR protocol, the same sequencing platform and technology and the same bioinformatics pipeline – which is hardly the case for any two studies. Datasets comprising high numbers of samples covering different habitat types and geographic regions processed with the same molecular and bioinformatics pipelines are therefore urgently required in order to address such overarching aspects as protist distribution.

We here present a so far unequalled dataset of altogether 232 samples from soil as well as fresh, brackish and marine waters - all processed using the same PCR protocol, sequenced with the same 454 deep-sequencing protocol and analysed using the same bioinformatics pipeline to ensure comparability. We thus seek to use the in-depth information gained to illuminate protistan diversity on a broad overarching scale focusing on the questions of protistan community structure and taxa distribution. We specifically address the distribution patterns of taxa as a function of their sequence abundance as well as specific signatures of their distribution with respect to habitat type and location.

3.2 METHODS

3.2.1 Sampling and sample preparation

Sampling sites and sampling procedures of the assembled dataset are summarised in table 3.1. The 10 different sampling campaigns representing 8 differing habitat types are referred to as Alpine spatial, Alpine seasonal, Lake District, Winterpico, Estuarine, Soil, Whale Fall, Borehole, HNF and Biofilm within this paper. Briefly, they comprise an alpine transect of freshwater samples (Austria, 32 lakes at 450-2050 m altitude) and a seasonal sampling of three Austrian lakes (Lake Mondsee region, 9-28 samples respectively, range: April to December), a seasonal sampling in the English Lake District, partially size-fractionated (UK, 7 sites, 2-3 samples respectively) and size-fractionated samples of the same sites in winter (Winterpico), an estuarine gradient (UK, River Colne, 11 sites, partially seasonal), a comparative soil sampling from fallow and re-cultivated fallow fields (UK, 15 sites across England), a sampling on the seabed of and from below a sunken whale (Whale fall) (North Sea, Sweden), a collection of borehole samples (UK, Berkshire), an experimental set-

up of a size-filtered (5 µm) freshwater sample fed with different food bacteria and focusing on heterotrophic nanoflagellates (HNF) (Czech Republic, Římov reservoir) and an experimental set-up of freshwater biofilms treated with the viricide TamiFlu (Southern UK). Due to the different nature of the collected samples, slightly modified sampling protocols and DNA-isolation methods (compare table 3.1) had to be used.

Table 3.1. Sampling campaigns and methods

Sampling site	Nature of sample	Isolation method
Alpine Lakes (spatial and seasonal)	Filtration of 20-200ml water on 0.2 µm polycarbonate filters	Qiagen DNeasy kit
Lake District Lakes and Winterpico	Pre-filtration by 20µm; size fraction filtration of 1-2l water on 0.2 and 2µm polycarbonate filters	Qiagen blood and tissue DNA extraction kit
Estuarine	Sediment samples	phenol-chloroform method as described in Smith et al 2007
Borehole	Filtration of 1-2l water + sediment on 0.5µm polycarbonate filters	MoBio UltraClean DNA extraction kit
Soil	0.5g soil samples	FastDNA® SPIN Kit for Soil (MP Biomedicals LLC, UK)
Whale Fall	Bone scrapes and drills + sediment samples	MoBio UltraClean Soil DNA extraction kit
HNF	Size fractionating by 5µm polycarbonate filters; after the experiment: fitration of 50-200ml water on 0.2 µm polycarbonate filters	extraction as described in Jezberová et al 2010
Biofilm	Scrapings from incubated tiles	CTAB/phenol-chloroform extraction

3.2.2 PCR and pyrosequencing

PCR amplifications of DNA were conducted using primers 1391F (Lane 1991; Stoeck et al. 2010) and Euk B (Medlin et al. 1998; Stoeck et al. 2010) both carrying a 5'-tail for the 454 sequencing (adapter A: GCCTCCCTCGCGCCATCAG, adapter B: GCCTTGCCAGCCCGCTCAG) to amplify a broad spectrum of eukaryotes. The final concentrations in all of the PCR reactions were: 1 μl of DNA template in 20 μl PCR reaction with 0.4 units of Phusion polymerase, primers at 0.25 μM final concentration, and dNTPs at 0.2 mM final concentration, including 4 μl Phusion buffer and 12.2 μl water. The PCR conditions consisted of an initial denaturation at 94°C for 4 min and 35 cycles of: 30 sec at 95°C, annealing for 60 sec at 60°C, elongation for 2 min at 72°C, followed by a final extension step of 10 min at 72°C. Pyrosequencing was carried out using the 454 Genome Sequencer FLX System (454 Life Science Branford, CT, USA).

3.2.3 Bioinformatics and analysis of the sequence dataset

For the bioinformatics analyses, we used a standardised pipeline including (i) quality filtering, (ii) clustering, and (iii) taxonomy annotation. Low quality tails were removed from the reads and the trimmed reads with an average Phred quality score less than 25 were discarded. Additionally, we removed all reads with at least one base with a quality of less than 15. Multiplex Identifiers (MIDs) were used to separate the different samples. Subsequently, the sequences that passed the quality filtering were clustered into Operational Taxonomic Units (OTUs) with UCLUST version 6 (Edgar 2010) at 97% sequence identity for further analyses. For all OTUs, we used BLASTn version 2.2.25+ (Altschul et al. 1990) with database nt and the NCBI Taxonomy Database (http://www.ncbi.nlm.nih.gov/taxonomy) to annotate the OTUs with taxonomic information.

OTUs and their reads from bioinformatic filtering and assembling were used to analyse the community structure and distribution patterns of OTUs (abundant vs rare, habitat-wise, taxon-wise). For analyses on higher taxonomic group level the following taxa were selected: Ciliophora, Dinophyceae, Apicomplexa, Alveolata rest, Bicosoecida, Oomycetes, Chrysophyceae, Synurophyceae, Bacillariophyta, Stramenopiles rest, Cercozoa, Rhizaria rest, Heterolobosea, Euglenida, Kinetoplastida, Euglenozoa rest, Excavata rest, Choanoflagellida, Chytridiomycota, Microsporidia, Ascomycota, Basidiomycota, Glomeromycota, Amoebozoa, Apusozoa, Glaucocystophyceae, Rhodophyta, Viridiplantae without Embryophytes, Cryptophyta, Katablepharidophyta and Haptophyta. OTUs affiliated with metazoa and embryophytes (as multicellular organisms) as well as with Bacteria and Archaea have been excluded before analyses. Thus, only protists were analysed as focus of our investigation. The 'rest' categories within the analysis are meant to collect minor groups and not further affiliated OTUs within Alveolata, Stramenopiles, Rhizaria and Excavata in order to fully show the protistan diversity present in the samples. Eukaryotic OTUs that are neither met by one of the given taxonomic groups nor have been deleted from the dataset by the given reasons are shown as 'others'. Furthermore, OTUs that could not be affiliated to entries of the NCBI database at all are labelled as 'unknown'.

The specific nature of the data matrix that results from deep-sequencing diversity counting is

characterised by a multitude of zero and single counts of OTUs (zeros: 98.35%, single counts: 0.83%, 2x counts: 0.27%, 10x counts: 0.014% a.s.o.), colliding with traditional methods of diversity comparison. We therefore used a 2-step-standardisation of site data: first, rarefaction of the sites by the 'drarefy' function provided by the R-package VEGAN (Oksanen et al. 2011), followed by Hellinger-transformation (Legendre & Gallagher 2001). After application of these procedures, the resulting pre-transformed data matrix of OTUs can be submitted to Ward cluster analysis and further multivariate analyses (see Figs. 3.1 and 3.2) (Borcard et al. 2011; Legendre & Gallagher 2001). Cluster analysis could be calculated on both OTU and meta-group level (for chosen meta-groups see above). The principle component analysis (PCA) based on Hellinger distances shows those taxonomic groups with the most explanatory value for clustering and separation of samples (see Fig. 3.2).

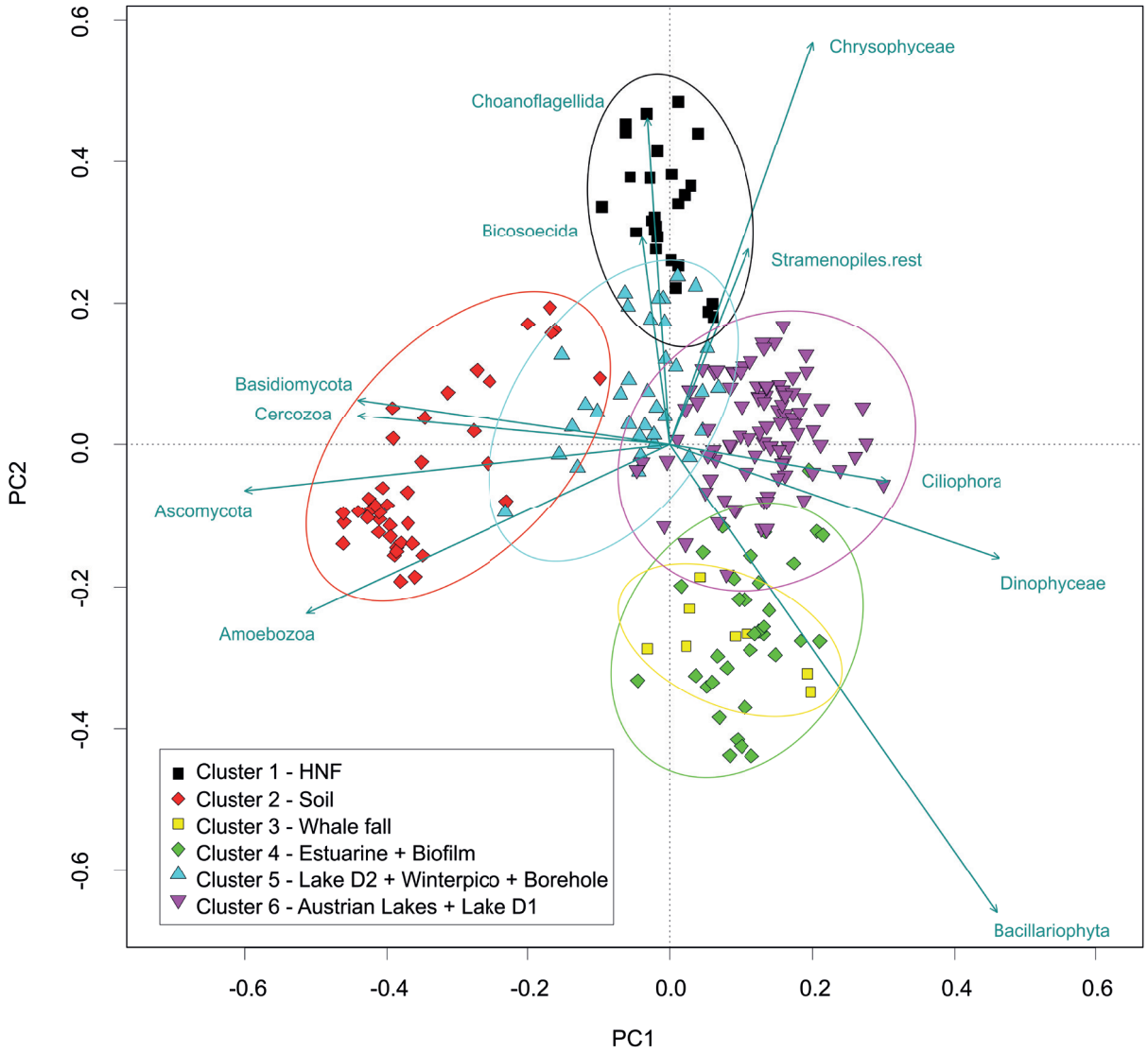


Figure 3.2. Results of cluster analysis of sites: PCA showing clusters on metagroup basis. PCA showing clusters from cluster analysis (encircled) and significant taxonomic groups for sorting of sites (arrows) (pre-treatment of data: drarefy + Hellinger-transformation, see methods); PCA was calculated on the basis of percentage of taxonomic groups within sites.

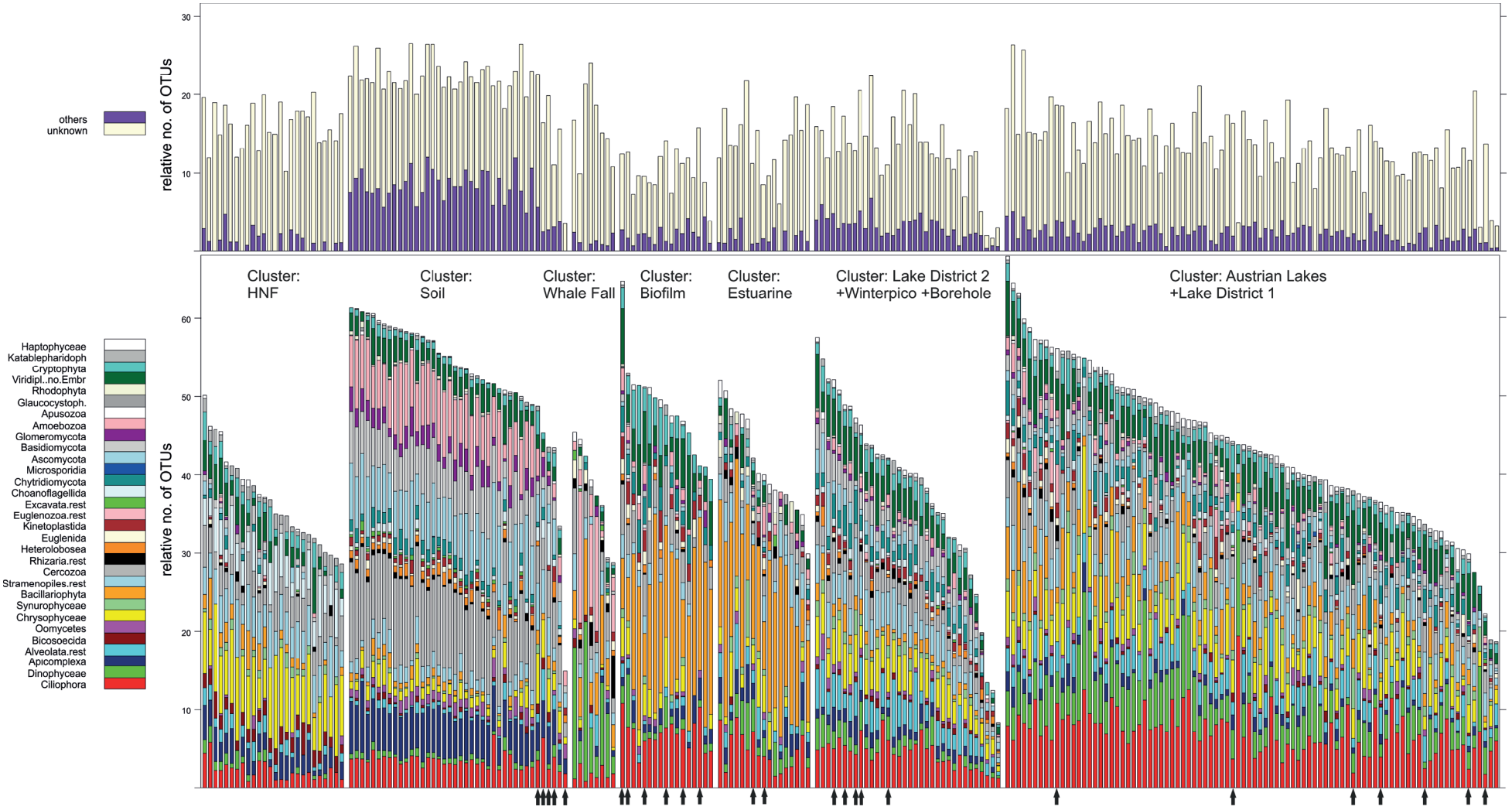


Figure 3.1. Results of cluster analysis of sites: species richness and OTU-composition of sites [rarefaction level = 100 reads]. Bars (left to right) are ordered by clusters from cluster analysis which almost entirely fit the actual sampling campaigns (see cluster names); black arrows at bottom indicate those sites within clusters not identified with the respective sampling campaign; within clusters, order (left to right) is by species richness of identified taxonomic groups respectively; species richness is shown as height of bars out of 100; community composition is shown as colour of bars by identified taxonomic groups (bottom) and merged or unidentified reads (top) (procedure: drarefy, see methods).

As reads per sample differ decisively between samples, rarefaction values at a read level of 100 reads (incl. extrapolation for few samples, see Colwell et al. 2012) were used first to compare species richness and composition of OTUs among samples. For the shown composition within each sample, the 'drarefy' calculation (R-package VEGAN; Oksanen et al. 2011) was used to minimise the bias when counting down OTUs to a common read level. The more classical 'rrarefy' is not suitable here as recently pointed out by McMurdie and Holmes (McMurdie & Holmes 2014). Thus, more exact relations of meta-groups from higher read levels are kept intact (see Fig. 3.1), however possibly resulting in OTU percentages per meta-group of less than 1 at a level of 100. The community structure of the different samples was analysed using the mean relative abundance of meta-group reads per sampling showing significant differences of meta-groups among sampling campaigns (see Fig. 3.3, also compare Table 3.2).

Table 3.2. Percentages of taxonomic groups in sampling campaigns

	Soil	Bore-hole	Biofilm	Whale Fall	Estuarine	Lake District 1	Lake District 2	Austrian Lakes	Winter-pico	HNF
Ciliophora	5.687	11.036	12.621	6.407	11.116	14.922	11.423	18.812	13.326	5.431
Dinophyceae	1.052	3.355	1.369	21.125	4.836	7.747	1.998	12.045	3.301	1.533
Apicomplexa	8.341	4.231	3.378	1.641	5.502	2.395	3.066	1.554	2.657	6.147
Alveolata.rest	0.911	7.155	1.233	2.761	3.822	9.380	11.603	5.972	4.065	2.579
Bicosoecida	0.656	0.169	0.079	0.847	0.117	0.447	0.921	0.566	0.552	4.608
Oomycetes	2.121	3.408	0.625	0.386	0.912	2.036	0.848	0.664	0.273	0.322
Chrysophyceae	3.374	10.212	2.115	2.926	4.468	5.216	8.486	10.612	13.175	20.116
Synurophyceae	0.532	1.262	0.164	0.225	2.180	2.972	1.782	3.082	2.345	2.654
Bacillariophyta	1.593	2.631	36.837	12.626	36.051	2.852	2.115	8.677	3.173	3.270
Stramenopiles.rest	3.519	3.405	1.908	6.811	6.539	8.309	8.408	7.415	6.483	11.746
Cercozoa	14.116	6.046	5.453	7.367	2.347	3.793	4.689	3.595	7.453	8.803
Rhizaria.rest	0.374	0.557	0.046	3.582	0.972	1.104	0.653	0.360	0.246	0.449
Heterolobosea	1.557	4.990	0.573	0.397	0.413	0.158	2.116	0.142	2.822	0.029
Euglenida	0.369	0.153	1.544	2.066	0.915	1.805	0.258	0.635	0.174	0.068
Kinetoplastida	0.676	5.574	2.422	1.302	1.440	0.838	1.874	0.895	3.179	0.072
Euglenozoa.rest	0.020	0.040	0.210	13.277	0.301	0.077	0.171	0.843	0.433	0.031
Excavata.rest	0.565	0.000	0.000	0.721	0.223	0.013	0.114	0.031	0.000	0.007
Choanoflagellida	1.094	0.717	0.046	0.601	0.307	0.995	1.916	1.009	0.688	10.533
Chytridiomycota	1.564	1.350	5.435	0.963	0.897	5.922	5.120	2.460	1.144	0.528
Microsporidia	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.017	0.000	0.000
Ascomycota	12.244	9.565	0.164	0.794	1.061	0.920	4.604	1.281	5.225	0.888
Basidiomycota	8.371	3.452	0.106	0.594	1.479	3.464	3.487	1.139	5.009	1.938
Glomeromycota	2.513	0.361	0.662	0.373	0.710	1.070	1.473	0.371	0.332	0.281
Amoebozoa	9.258	2.706	1.221	2.279	1.462	0.897	1.105	0.644	0.364	0.296
Apusozoa	0.060	1.841	0.182	0.260	0.000	0.020	0.196	0.075	0.573	0.002
Glaucocystoph.	0.031	0.000	0.000	0.214	0.000	0.000	0.005	0.018	0.000	0.000
Rhodophyta	0.298	0.016	0.000	0.629	1.119	0.100	0.102	0.123	0.761	0.098
Viridipl..no.Embr.	3.862	4.637	8.791	2.636	2.959	7.668	8.151	6.071	5.442	6.071
Cryptophyta	0.969	3.741	7.543	1.021	0.920	2.739	3.654	3.655	5.591	4.375
Katablepharidoph.	0.354	0.738	0.000	0.761	0.922	1.139	1.567	1.363	2.056	3.692
Haptophyceae	0.119	0.702	0.637	1.061	2.086	2.246	0.525	1.687	0.349	0.318
others	13.800	5.949	4.635	3.346	3.924	8.731	7.573	4.185	8.810	3.114

Following preliminary tests, the distribution patterns of individual OTUs did neither follow a Poisson distribution ($p<0.05$) nor normal distribution (Ricci 2005). Instead the Weibull distribution among sites (y-axis = no. of sites, x-axis = abundance of OTU in these sites) passed the KS-test (i.e. the H0-hypothesis could not be rejected, $p>0.05$) for almost all OTUs and was consequently used for further analysis. All OTUs passed the KS-test, i.e. the Weibull distribution could not be rejected (all p-values>0.9), when p-values were corrected for multiple testing by using p.adjust (method= 'fdr', R package 'stats'). When corrections for multiple testing were disregarded, the KS-test rejected the Weibull distribution for 10 out of 1249 OTUs. Even though these 10 OTUs passed the test when correctly applied (i.e. corrected for multiple testing) we decided to mark these OTUs in the figures (Figs. 3.4 and 3.5 top). The shape parameter *k* of the Weibull distribution is suitable for comparing the distribution of frequent and less frequent taxa as the frequency primarily affects the scale but not the shape. We therefore focus on the comparative analysis of *k*. For $k= 1$ the two-parameter Weibull distribution reduces to that of the exponential distribution. Shape values *k* of Weibull distributions were compared by Mann-Whitney-U-Tests. All U-tests were calculated with sigmaplot 12.5 (Systat Software Inc.). The control dataset, tested against Bacillariophyta as the closest subgroup dataset within the analysed data, was generated by function 'rnorm' with n=55 (normally distributed), average value and median =1 (Ricci 2005), standard deviation value of Bacillariophyta dataset.

3.3 RESULTS

3.3.1 Taxon diversity

The large scale analysis undertaken herein – carried out as a multiple sample habitat comparison – shows that protistan communities differ decisively. They do so in terms of OTU presences in a distinct sample and habitat as well as in the ratio of higher taxonomic groups (meta-groups) (Fig. 3.1). Taking all samples into account, Ciliophora were dominating in terms of d-rarefied sequence abundance with 13 % followed by Bacillariophyta with 9.7% and Chrysophyceae with 9%.

Despite many OTUs being distributed across different campaigns and sample types, many OTUs were either specific for distinct habitats/campaigns or their relative abundance was considerably deviating from that in other habitats/campaigns. Cluster analysis based on protistan sequence analysis clearly separated different habitat types. Specifically soil, biofilm and brackish sediments (estuarine), and freshwaters were clearly distinguished and show a specific community composition each (Fig. 3.2). Size-fractionated samples such as the HNF data set were also clearly separated by cluster analysis.

This differentiation based on OTUs is also reflected by shifts in the community composition on the level of higher taxonomic groups (Figs. 3.2 and 3.3): Substrate-bound taxa were, as expected, specifically abundant in sediments. However, whereas Amoebozoa and Cercozoa were specifically more abundant in the soil samples (see column A in Fig. 3.3), Heterolobosea, Kinetoplastida,

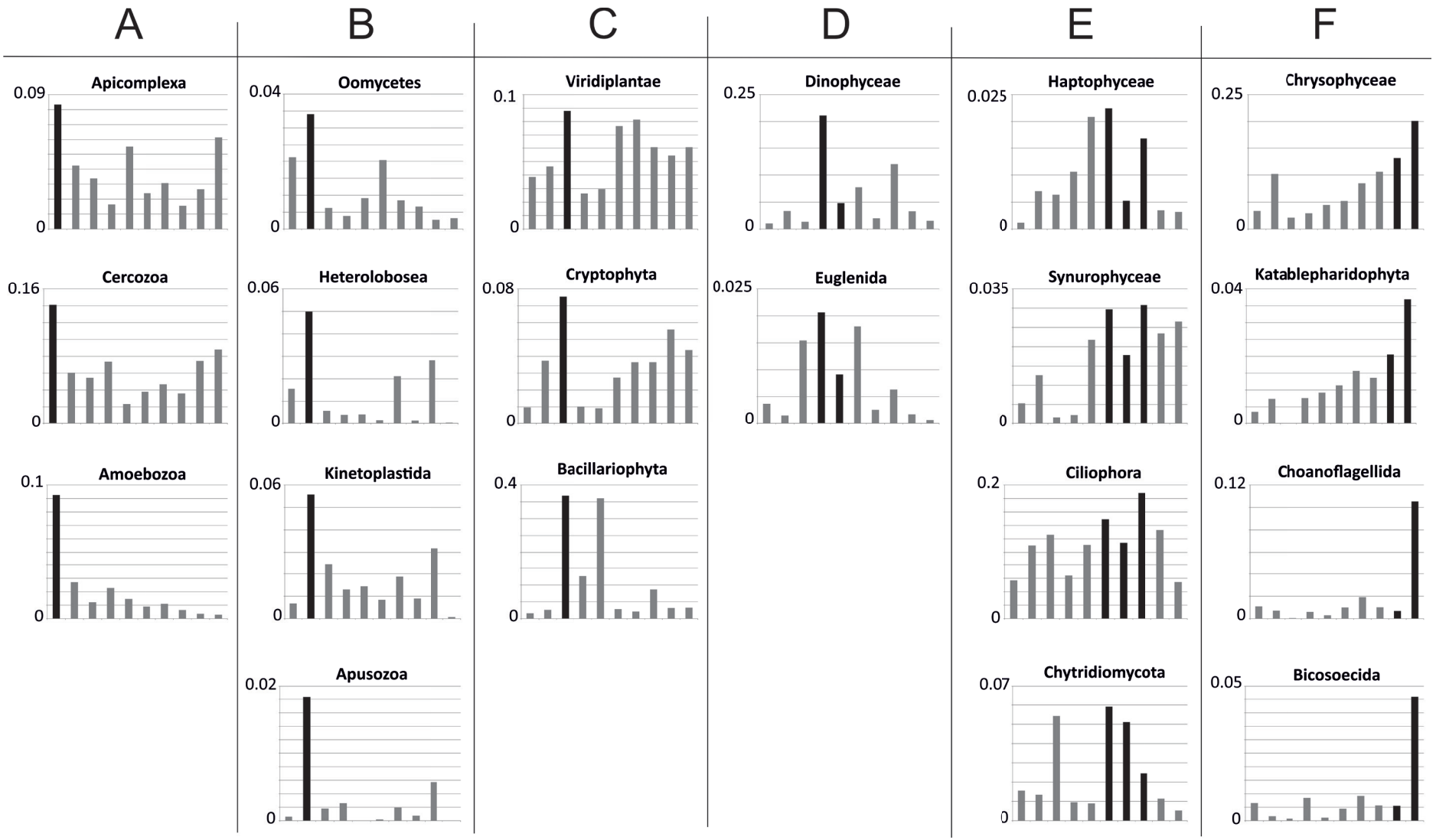


Figure 3.3. Percentage of characteristic taxonomic groups in sites. Bars in individual graphs showing percentage of indicated taxonomic group in sampling campaigns (bars on x-axis = campaigns, respectively from left to right: 1 - Soil, 2 – Borehole, 3 - Biofilm, 4 - Whale fall, 5 - Estuarine, 6 - Lake District 1, 7 - Lake District 2, 8 - Alpine Lakes, 9 - Winterpico, 10 - HNF). Columns (A - F) sorting out taxonomic groups with highest percentage in: A – Soil, B – Borehole, C – Biofilm, D – Whale fall and Estuarine (salt and brackish waters), E – Lake District 1 and 2 and Alpine Lakes (freshwater), F – Winterpico and HNF (size-fractionated samplings).

Apusozoa, and to some extent also Chrysophyceae were specifically abundant in the borehole samples (see column B in Fig. 3.3). The Peridiosporomycota (Oomycetes) were found to be relatively abundant in both campaigns. In the size-fractionated samples, small HNF taxa showed relatively high sequence abundances, specifically Chrysophyceae, Katablepharidiophyceae, Choanomonada, and Bicosoecida (see column F in Fig. 3.3). In our biofilm samples, we found an increased sequence abundance of Chlorophyta and streptophytic algae (labelled as 'Viridiplantae' – without Embryophytes), Cryptophyta, Bacillariophyta and also Chytridiomycota, but interestingly substrate-bound taxa did not have specifically high sequence abundances (see column C in Fig. 3.3).

The marine samples showed comparatively low read counts of Chlorophyta, streptophytic algae, Chytridiomycota, and Cryptophyta, but comparatively high reads of Bacillariophyta (salinity gradient along the Colne estuary) and of Dinophyceae and Euglenida at the whale fall (see column D in Fig. 3.3).

Interestingly, these habitat-specific community profiles were similar for almost all samples of the respective habitat types. Cluster analysis revealed only very few samples which would – based on sequence data – be sorted into the wrong campaign (see arrows in Fig. 3.1).

3.3.2 Differential distribution patterns of protistan taxa

For the analysis of OTU distribution patterns among sites, we analysed the shape parameter *k* of the Weibull distribution (see methods). This analysis did not reveal any principal difference between abundant and less abundant taxa apart from a minor modulation in the shape factor of the Weibull distribution. The mean Weibull shape value *k* is rather similar irrespective of the relative overall abundance of the OTUs (Fig. 3.4 top). Shape values of individual OTUs scatter around a median of 1.67, but there is no distinct change in the median *k* for less abundant OTUs. The median shape value of 1.67 indicates that taxon distribution is not due to unlimited random dispersal (which would favour a value of 1 or lower, i.e. a colonisation from one site with high frequency), but on the contrary that some factors work against invasion and/or colonisation of habitats (significant U-TEST of shape value 1.67 versus 1, $p<0.001$). One might argue that this effect is caused by the different habitat types included in the analysis. However, even when the analysis is restricted to one habitat type within a distinct geographic region, taxon distribution cannot exclusively be explained by unlimited random dispersal (e.g. soil sites UK: shape parameter = 2.099, significant U-Test versus 1, $p<0.001$; e.g. Austrian lakes + Lake District 1: shape parameter = 1.711, significant U-Test versus 1, $p<0.001$; compare methods in this paper and Fig. 3.4 – mid and bottom). In contrast, the shape parameter is even higher when the analysis is restricted to soils, indicating that random dispersal is even less important in soils as compared to aquatic habitats. However, the scatter around the median *k* increases for rare taxa (Fig. 3.5 – top right). Similarly the scatter of sites of occurrence as a function of total read number increases (Fig. 3.5 – top left). In contrast, the most abundant taxa do have very similar distribution patterns indicated by both a very similar shape parameter in the Weibull distribution (Fig. 3.5 – top right) and a narrow range of the ratio of number of sites versus total read abundance (Fig. 3.5 – top left). Both observations indicate a stronger differentiation

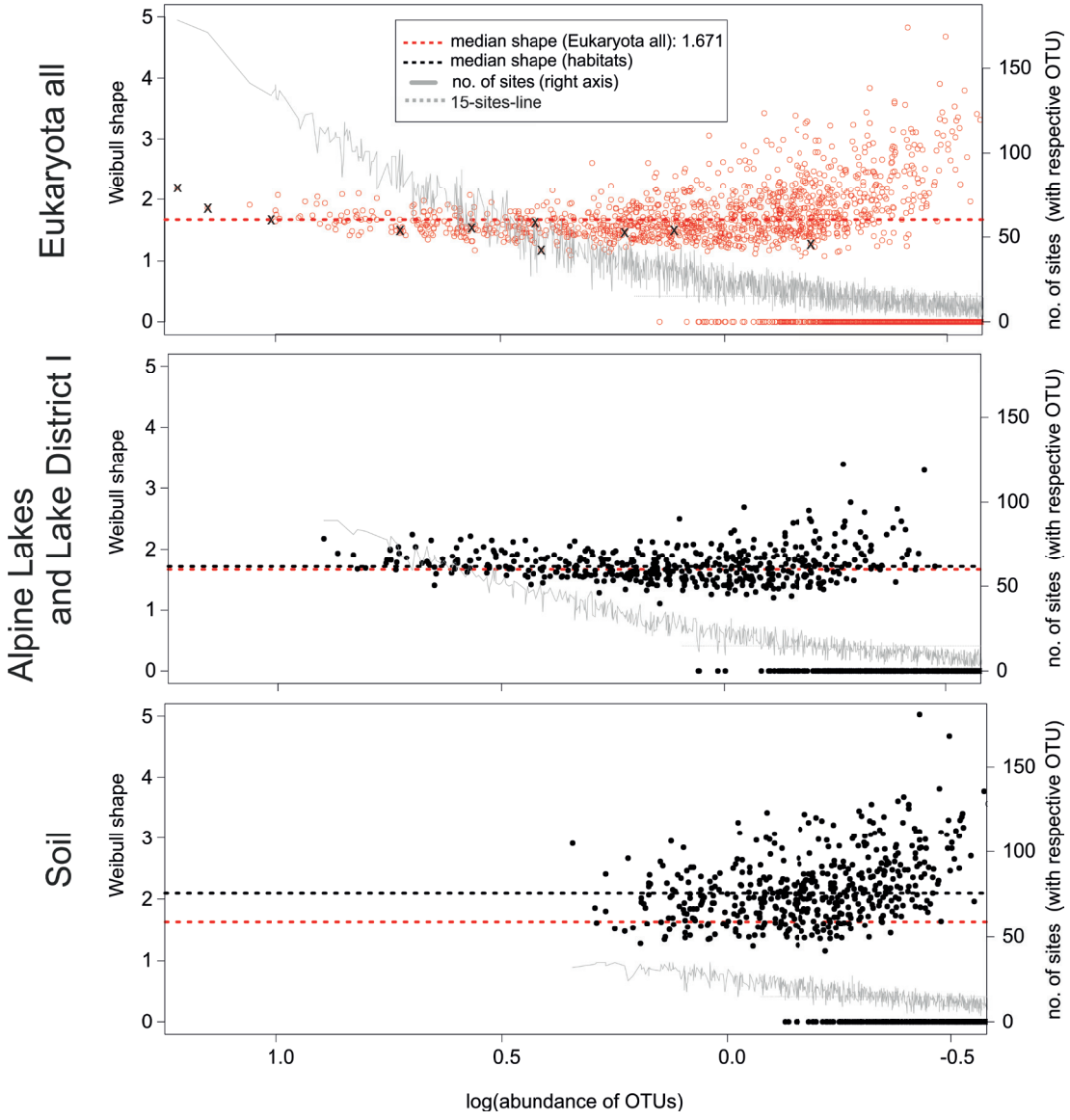


Figure 3.4. Weibull shape by habitats. Distribution patterns as functions of the log abundance of OTUs in the samplings, OTUs are also sorted (left to right) by read abundance (logarithmic scale). The grey line shows the relation between read abundance and the number of sites where the distinct OTU occurs. Weibull shape of individual OTUs (=distribution of reads over sites) as red and black dots for all Eukaryota (top), OTUs occurring in freshwater (middle) and OTUs occurring in soil (bottom); x indicate the 10 OTUs for which Weibull distribution was rejected when corrections for multiple testing were disregarded ($p<0.05$, see methods); for OTUs occurring in less than 15 sites a calculation of Weibull shape is not possible (unreliable), these dots were set to 0 instead of filling the lower right part of the plots; OTUs are sorted (left to right) by read abundance (logarithmic scale); grey line (for values see right y-axis) showing the connection of read abundance and presence in sites; red dotted line showing median shape value of all Eukaryota, black dotted lines showing median shape value of freshwater and soil respectively.

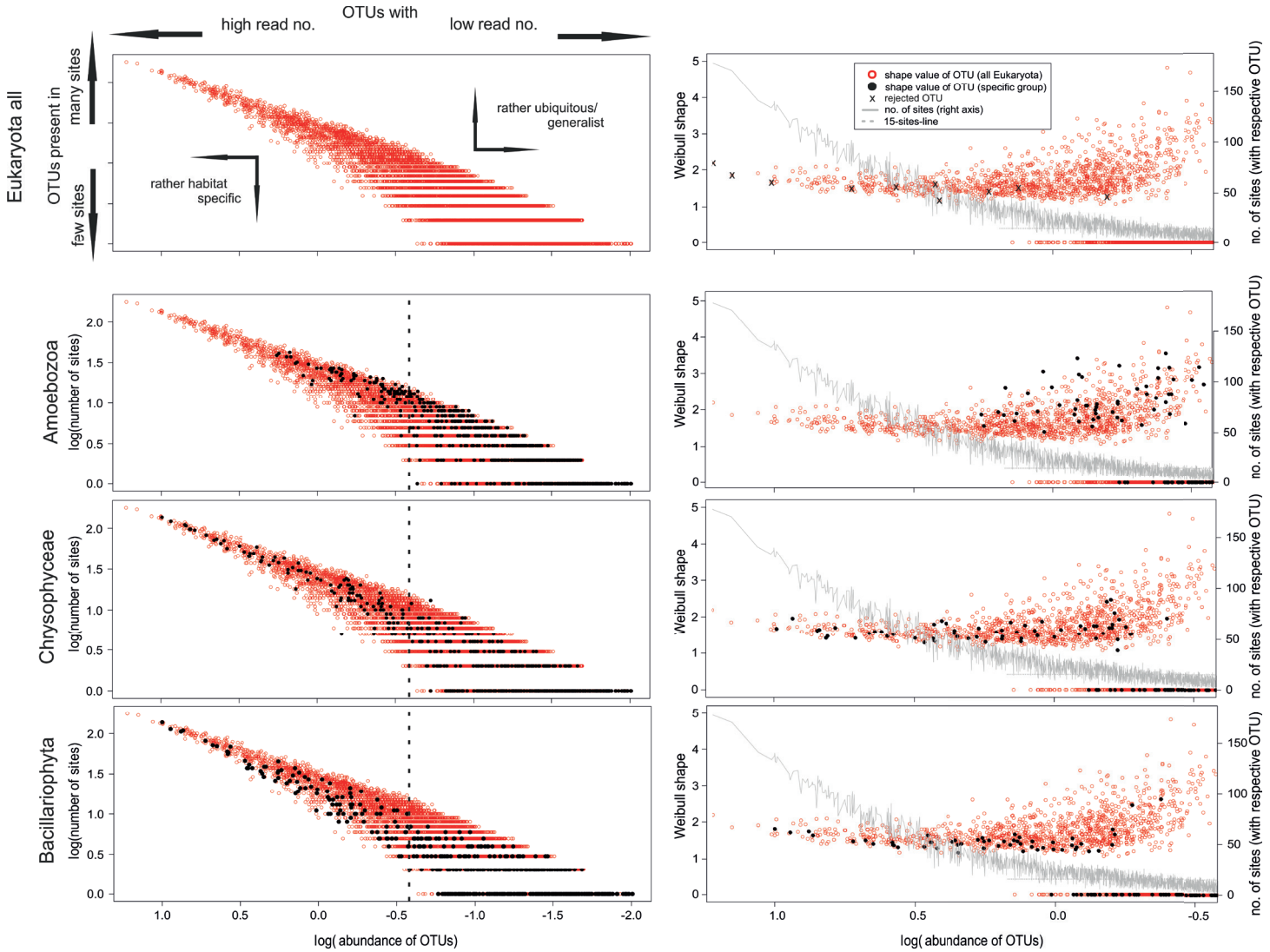


Figure 3.5. Occurence of OTUs in sites and Weibull shape by taxonomic groups. Distribution patterns as functions of the log abundance of OTUs in the samplings, OTUs are also sorted (left to right) by read abundance (logarithmic scale). The grey line shows the relation between read abundance and the number of sites where the distinct OTU occurs. Graphs show OTUs as a function of log of occurrence in sites and log of abundance of reads (left half of figure) and as their Weibull shape value (=distribution of reads over sites) (right half of figure) for all Eukaryota (top – red dots), Amoebozoa (upper middle – black dots), Chrysophyceae (lower middle – black dots) and Bacillariophyta (bottom – black dots); the 10 OTUs for which Weibull distribution was rejected ($p < 0.05$, see methods) are indicated by an x; the dotted black vertical line in left hand graphs indicates the read abundance threshold up to which Weibull shape value calculation (right hand graphs) was carried out (also see x-axis); additionally, for OTUs occurring in less than 15 sites a calculation of Weibull shape was not possible (unreliable), these dots were set to 0 instead of filling the lower right part of the plots.

of distribution patterns in rare taxa whereas abundant taxa show relatively uniform distribution patterns. Within the rare taxa, a differentiation into more generalist taxa (occurring at relatively high numbers of sites) and more specialised taxa (restricted to fewer sites) is thus pronounced.

Similarly, distribution differs between taxonomic groups. Diatom OTUs, as the one extreme, are characterised by a comparatively low *k* and occur at a relatively low number of sites for a given total read count. Amoebozoa, on the other hand, are characterised by comparatively high shape values and occur at a relatively high number of sites for a given total read count. The distribution pattern of Amoebozoa indicates thus a broader (more generalist) distribution whereas the Diatom distribution pattern reflects a narrower (more specialised) distribution (U-test of shape values for Amoebozoa vs Bacillariophyceae: $p<0.001$). The Chrysophyceae are somewhat in between the two strategies. Nevertheless, *k* is significantly larger as would be expected for random distribution for all taxa (Amoebozoa: shape = 2.139, significant U-Test versus 1, $p<0.001$; Bacillariophyceae: shape = 1.449, significant U-Test versus 1, $p<0.001$; Chrysophyceae: shape = 1.595, significant U-Test versus 1, $p=0.009$) (Fig. 3.5 – right and left).

Non-random distribution patterns are also supported by PCA analysis (Fig. 3.2): Based on the molecular diversity, the samples clustered according to habitat types. Nevertheless, within habitat types regional effects were pronounced. Samples from the same sampling site clustered together irrespective of seasonal effects. Further, samples from the same region, e.g. from Austria, clustered largely together.

3.4 DISCUSSION

High-throughput sequencing has vastly sped up environmental surveys of microbial organisms and opened the door for large-scale analyses of microbial distribution patterns. Nevertheless, the adequacy of high-throughput molecular surveys for diversity studies is still questioned as results often deviate from expectations based on microscopic analyses and Sanger sequencing (Medinger et al. 2010). Furthermore, results between different high-throughput sequencing studies also deviate strongly from each other making cross-study comparisons difficult or even impossible. We here address both comparative questions of protistan community structure as well as overarching patterns of protistan distribution.

3.4.1 Habitat specificity of protistan communities

We provide evidence that molecular high-throughput data are very well suited for cross-habitat comparisons and that the molecular data well reflect protistan community signatures, i.e. habitats being characterised by distinct protistan communities. This signatures are unambiguous both on the level of individual OTUs as well as on the level of meta-groups. Soil, freshwater and marine sites can – consistent with microscopic and Sanger sequencing studies (Fenchel 1994; Anderson 2012; Tikhonenkov et al. 2012) – clearly be distinguished based on sequence data. These overarching patterns therefore strongly back up the validity of deep-sequencing data for comparative community

analyses (see Fig. 3.1). Even though these patterns were not necessarily reflected in each individual sample, they are clearly reflected on the level of campaigns, i.e. based on a sufficient number of samples (highlighting the importance of sufficient sample coverage). It is unclear, however, to what extent these patterns are due to a biogeographic separation or solely due to physicochemical similarities between habitats within a certain region as compared to cross-region comparisons.

Soil protist communities are dominated by flagellates, specifically cercomonads, chrysomonads and bodonids, followed by gymnamoebae, testate amoebae and ciliates (Adl & Coleman 2005; Foissner 1991; Adl 2003). Accordingly substrate bound taxa such as Cercozoa and Amoebozoa dominated in our analysed soil samples. Other substrate bound taxa such as Apusozoa, Heterolobosea and Kinetoplastida were specifically abundant in the borehole samples indicating a specific importance of these taxa in aquifers. Again, this is consistent with other studies which report specifically Kinetoplastida (*Bodo* spp.) and amoebae to be abundant in aquifers (Risse-Buhl et al. 2013; Novarino et al. 1997). Apusozoa became only recently into focus and are therefore not reported in older studies whereas the abundance of cercomonads and chrysomonads may differ between aquifers (Novarino et al. 1997).

Likewise, the marine samples showed a high proportion of sequences affiliated with Diatoms (specifically Colne estuary), Dinoflagellates (specifically Whale fall) and Euglenids. Diatoms and Dinoflagellates are generally important marine taxa and their relative importance was therefore to be expected (Ingmanson & Wallace 1995; Graham et al. 2009). In contrast, Synurales, Cryptophytes, Chlorophyta and Chytridiomycota were specifically rare in marine samples, but more prominent in freshwater samples (Graham et al. 2009; Dokulil et al. 2001). The generally high proportion of Alveolate sequences, specifically of Ciliophora, is a well-known artefact in molecular data sets due to a comparatively high ribosomal gene copy number in these taxa (Dyal et al. 1995; Zhu et al. 2005; Medinger et al. 2010). Accordingly, ciliophoran sequences were abundant throughout our samples.

Habitat specificity was also reflected on the level of individual OTUs. PCA as well as cluster analysis revealed similar community profiles for samples originating from similar habitats. These community profiles were highly specific for distinct habitat types. High-throughput sequencing community profiles are therefore strongly consistent as long as the methodology, including PCR, sequencing and bioinformatics pipeline, is kept the same. This finding also strongly emphasises the adequacy of high-throughput sequencing data for purposes of ecosystem quality assessment and monitoring. In contrast to microscopic approaches and clone libraries, even relatively rare taxa are included in the analyses. The analyses are therefore less prone to random shifts in the relative abundance of some dominant taxa. Even seasonal variation of taxa does not blur the habitat-signatures – community profiles based on high-throughput sequencing data seem therefore specifically promising for overarching habitat comparisons as well as for long-term monitoring campaigns. However, such approaches rely on standardised protocols as applied in this study.

3.4.2 Rare/abundant taxa

Distribution patterns of individual protistan taxa have been controversially discussed within the range of 'moderate endemicity' (Foissner 2006 and 2008) to potential worldwide distribution (Fenchel et al. 1997, Finlay & Esteban 1998). Similarly, distribution patterns have been addressed with respect to habitat type, specifically marine versus freshwater, and potential niche width of protistan taxa (Bass et al. 2009; Boenigk et al. 2006). In general, broad niche widths and high ecophysiological tolerances should be reflected by broad distributions of taxa across different samples. In contrast, taxa with more restricted niche widths and/or ecophysiological tolerances should be restricted to fewer samples.

It has been suspected that rare taxa may have systematically different distribution patterns as compared to abundant taxa (Nolte et al. 2010). Based on a large set of samples, we here show that there is no such systematic shift in the general distribution patterns with read abundance. However, whereas the general trend is rather constant, the analysis of individual taxa reveals systematic differences between abundant and rare taxa: for abundant taxa the distribution patterns of individual taxa are relatively uniform. For rare taxa, in contrast, the patterns strongly deviate between individual taxa. Dispersal and success of colonisation seems to be largely comparable for abundant taxa resulting in similar distribution patterns. Within the rare biosphere, however, distribution patterns vary strongly between individual taxa presumably indicating a more selective niche adaptation. We therefore suspect that the rare biosphere offers an even stronger potential for comparative habitat analyses including biological monitoring as compared to the more abundant taxa – which are so far mostly in the focus of such studies.

Furthermore, the different distribution patterns are differentially pronounced between different habitat types and between different taxonomic groups: Aquatic habitats tend to be characterised by a lower *k* as compared to soil habitats indicating a generally broader dispersal of taxa between aquatic sites. In other words, the soil samples are characterised by a high evenness, i.e. a multitude of taxa at higher abundances at the same time. On the contrary, aquatic sites have a comparatively lower evenness with usually only a small number of dominant species reaching higher abundances and many rare taxa at a distinct point in time. Thus, not only species or taxon inventory but also abundance and diversity pattern seem to be characteristic for distinct habitat types.

With respect to taxonomic groups, Amoebozoa are characterised by broader distribution patterns indicating a more generalist strategy whereas the Diatom taxa are characterised by narrower distribution patterns indicating a more specialised strategy. The Chrysophytes, for instance, show intermediate distribution patterns. These differences presumably indicate differential dispersal and proliferation between taxa already on the level of taxonomic groups. It seems plausible that the distribution is pronouncedly different between soil inhabiting and endobenthic taxa which are presumably less prone to random dispersal, such as Amoebozoa, as compared to planktonic or epibenthic taxa. Ecologically, Amoebozoa seem to be more restricted to samples allowing for the growth and build-up of larger populations whereas random components seem to be more

important in explaining distribution patterns of Diatoms. This is likely to be linked to the lifestyle of the respective taxa.

Large scale datasets and parallel investigations made possible by high-throughput methods as presented herein will surely reveal more such patterns and further illuminate the sphere and living of protists on earth and thus broaden our knowledge on so far under-researched organisms such as protists.

REMARKS

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For the bioinformatic processing, I used a pipeline constructed by the Bioinformatics Department of Duisburg-Essen University.

4) PROTISTAN DIVERSITY IN THE ALPS

– MOLECULAR INVESTIGATION OF AN ELEVATION TRANSECT

ABSTRACT

Ecological gradients are one of the main reasons for changes in biodiversity. Whether observations from plants and animals, however, can be verified for protists in the same way, is questionable. In an alpine elevation gradient of 32 freshwater lakes, we investigated ecological drivers of protistan diversity and community structure as well as the explanatory value of species-area-relationship for protistan diversity using a deep sequencing approach. We find that species richness in the investigated protistan communities is not reduced with elevation. Nor is elevation a major factor concerning the observed shifts in community composition. The observed protistan communities differ by many factors with pH and nutrient concentrations being of high explanatory value. Chrysophyceae as a subgroup most precisely reflect the whole protistan community in the observed pattern. By our findings, species-area-relationship alongside the elevation gradient (i.e. less total water in higher elevations) and the strong influence of elevation by co-varying factors on species richness and community composition as known from plants and animals can be rejected for protists. Individual lake size, however, proves to be explanatory for protistan species richness in our investigation.

4.1 INTRODUCTION

Biodiversity forms along ecological and geographical gradients (Adams 2009; Boenigk & Wodniok 2014; Hewitt 2000). Factors such as temperature or pH shape the species inventory of a habitat on land and in water. This holds true for multicellular organisms (metazoans, embryophytes etc.) (Frey & Lösch 2010; Gaston 2000) and is likewise known for protists concerning factors such as nutrient availability (Dziock et al. 2006). Furthermore, species-area-relationship, as e.g. observed in island biogeography, predicts a decreasing number of species with decreasing area (Whittaker et al. 2001), i.e. as less habitats (and with that less niches) are covered.

Along alpine gradients, both environmental parameters as well as species-area-relationship change with elevation and potentially shape protistan communities: environmental parameters gradually change with elevation – community composition is known to change accordingly (Frey & Lösch 2010); habitat size decreases with elevation – beyond a shift in community composition a lower species richness due to reduced space at higher elevation can be expected (Nogués-Bravo et al. 2008; Romdal & Grytnes 2007). For higher plants and animals such mountain biodiversity patterns are not unquestioned (Rahbek 1995), though widely accepted. However, whether and to what extent these general ecological theories hold true for protists, remains unclear (Bryant et al. 2008).

Protistan community composition in freshwater lakes is diverse and shows a strong seasonality due to species-specific ecological optima (Häder et al. 1998; Nolte et al. 2010), nutrient availability (Graham et al. 2009), and specific biotic interactions (Sommer et al. 2010; De Senerpont Domis et al. 2013) with the majority of the year's total community in dormant stages whenever sampled. For lakes in mountainous regions, especially high mountain lakes, usually meso- or oligotrophic conditions apply, favouring those protistan taxa that deal better with low nutrient concentrations such as chrysophytes (Tolotti et al. 2003). Furthermore, high mountain lakes are often comparably small and shallow leading to specific adaptations of the inhabiting organisms, e.g. to UV-light stress (Sommaruga 2001; Sonntag et al. 2011). The remote position of these lakes may favour high degrees of endemicity (Sommaruga 2001; Sonntag et al. 2011). However, protistan diversity along alpine gradients – as in a geographical context in general – is understudied, leaving the communities' variability unrevealed (Weisse 2007). The scarceness of cross-habitat comparisons, thereby, hinders our understanding of protistan diversity and protistan distribution (Tringe et al. 2005; Bik et al. 2012). Deep-sequencing technologies can bridge this gap to a fuller picture of protistan diversity as samples can be analysed both parallel and in depth leading to quantitatively and qualitatively enlarged taxa lists per sample (Triadó-Margarit & Casamayor 2012; Bates et al. 2013). As such, high-throughput sequencing has already shown in numerous cases that protistan diversity was highly underestimated in the past (Stoeck et al. 2010; Nolte et al. 2010; Lecroq et al. 2011). Moreover, such deeper diversity surveys also hold the potential to reveal protistan diversity and distribution patterns among sites and samples that could not be seen so far.

In order to analyse shifts in community composition as well as in species richness with elevation, we used deep sequencing data of 32 freshwater lakes within an alpine transect. Due to the method applied, we expect to find protistan diversity in high resolution. On the basis of such enlarged data, we mean to test general ecological hypotheses neither yet verified nor falsified for protists. Species-area-relationship implies that species richness is reduced with elevation as less total area is available. Other factors changing with elevation, however, imply that species richness rather augments. Habitats in higher elevations (in mountain ranges and on singular peaks) are often disconnected supporting endemicity, lakes being disconnected in a double sense as they form islands of water within land. Moreover, the trend of lower nutrient levels in higher elevations rather implies higher species evenness, and thereby possibly high species numbers.

We therefore hypothesize that species richness of protistan communities is not reduced with elevation, but is rather shaped by factors (or combinations of factors) independent of elevation. Possible effects of species-area-relationship are thus overruled. We further hypothesize that community composition of protists does change with elevation and co-varying factors – as it likewise does along gradients of other environmental factors.

4.2 METHODS

4.2.1 Sampling and sample preparation

To compare organisms on a broad basis, we chose a 454-deep-sequencing amplicon approach targeting the V9-region of the SSU gene. Based on the sequenced OTUs and their reads, we compared species richness and community structure and calculated the similarity of the different sites sampled. We furthermore checked for discriminating environmental features explaining for the similarity and dissimilarity of sites.

Samples were all collected in mid-August 2007 (calendar week 33/34) to ensure comparability of community and diversity data. The 32 lakes sampled form an alpine transect reaching over several mountain ranges from the Salzkammergut area to the Low Tauern (Austria) and cover an elevation gradient from 429 to 2072m asl of diverse water chemistry (Table 4.1). All samples were treated equally in the different processing steps including the sequencing protocol to assure comparability. Samples were taken with a telescope sampling vessel (~3m) from the edge of the water body, filtered on 0.2µm polycarbonate filters (20-200ml), air-dried and stored at -80°C.

Water temperature, pH and conductivity were measured in the field. Alkalinity was determined by Gran - Titration, Orion 960. Total phosphorous (P_{tot}) was measured by the molybdate method according to Vogler (1966). Dissolved organic carbon (DOC) was measured with a Shimadzu TOC - V CPH (Total Organic Carbon Analyzer), dissolved nitrogen (DN) was measured with a Shimadzu TNM - 1 (Total Nitrogen Measuring Unit). The anions chloride (Cl), sulfate (SO4) and nitrate (NO3-N) as well as the cations sodium (Na), ammonium (NH4-N), potassium (K), magnesium (Mg) and calcium (Ca) were measured by means of ion chromatography (Dionex ICS – 1100). Dissolved reactive silicon (DRSi) was measured using the molybdate method (Smith et al. 1981; Skalar, SANplus Segmented Flow Analyser). Total phosphorous and alkalinity were measured from raw water, all other chemical parameters from filtrate (0.6 µm).

Genomic DNA was extracted with the DNeasy Tissue kit (Qiagen). Filters were transferred to a 2ml tube and incubated for 1–3 h in buffer ATL of the DNeasy Tissue kit (Qiagen) supplemented with Proteinase K. DNA was subsequently extracted following the instructions of the supplier. We used HPLCpurified PCR primers, which carry sequences specific for the small subunit (SSU) of the rRNA gene (fw: ATTAGGGTTCGATTCCGGAGAGG, rv: CTGGAATTACCGCGGSTGCTG).

4.2.2 PCR and pyrosequencing

PCR amplifications of DNA were conducted using primers arraying a 5'-tail for the 454 sequencing (Medinger et al. 2010). The final concentrations in all of the PCR reactions were: 1 µl of DNA template in 20 µl PCR reaction with 0.4 units of Phusion polymerase, primers at 0.25 µM final concentration, and dNTPs at 0.2 mM final concentration, including 4 µl Phusion buffer and 12.2 µl water. The PCR conditions consisted of an initial denaturation at 94°C for 4 min and 35 cycles of: 30s at 95°C, annealing for 60 sec at 60°C, elongation for 2 min at 72°C, followed by a final extension step of 10 min at 72°C. Pyrosequencing was carried out using the 454 Genome Sequencer FLX System (454 Life Science Branford, CT, USA).

Table 4.1. Lakes and their environmental variables

Lake	Sample ID	Temp [°C]	Cond [µS/cm]	pH	HCO3	NO3N [µg/L]	SO4 [mg/L]	Cl [mg/L]	NH4N [µg/L]	Na [mg/L]	K [mg/L]	Mg [mg/L]	Ca [mg/L]	DRSi [µg/L]	TP [µg/L]	DOC [µg/L]	DN [µg/L]	TP/DN	DN/DOC	Anions	Cations	ION-SUM	turbidity	altitude [m ü. A.]	area [m2]	species richness in 1000 reads	latitude [°N]	longitude [°E]
Altausseer See	AL33	15.2	127.67	7.7	1560	353	1.25	0.75	19	0.42	0.1	0.82	26.39	217	4.7	2060	428	0.011	0.208	1632.29	1407.03	3039.32	6.04	712	2100000	199.7	47.635	13.783
Augstsee	AU33	11.1	76	8.1	980	88	0.65	0.18	15	0.13	0.11	0.39	18.91	181	10.6	1828	219	0.048	0.120	1005	985.23	1990.23	6.11	1643	23000	183.08	47.663	13.787
Badesee Aich	BA33	18.4	404	7.77	3980	638	13.13	2.54	17	1.89	0.82	20.22	44.14	1257	4.7	1075	697	0.007	0.648	4370.88	3969.97	8340.85	6.14	694	4800	199.61	47.422	13.820
Bodensee (Steirischer)	BD33	13.1	23.67	6.79	219	400	2.94	0.19	8	0.57	0.56	0.44	4.5	397	5.3	1027	439	0.012	0.428	314.19	300.6	614.79	5.74	1157	76900	179.34	47.374	13.824
Fuschlsee	FU33	19	314	8.44	2460	478	5.6	5.36	11	3.24	0.53	14.92	25.63	307	2.9	1954	589	0.005	0.301	2762.02	2661.75	5423.77	6.12	663	2650000	214.32	47.806	13.280
Grafenbergsee	GF33	11.7	192	8.61	2030	45	0.49	0.23	6	0.09	0.05	9.47	22.51	136	7.1	2712	157	0.045	0.058	2049.86	1908.21	3958.07	5.83	1639	15000	219.43	47.467	13.767
Egelsee	EG33	15.1	416.33	7.62	2240	1000	5.43	7.48	89	5.67	2.12	8.36	42.23	2204	81.2	11770	1827	0.044	0.155	2635.39	3102.11	5737.5	9.64	642	8000	118.12	47.959	13.126
Hallstättersee	HA33	16.5	177	8.35	1950	371	5.91	4.08	6	2.64	0.25	2.87	33.25	329	4.1	2264	434	0.009	0.192	2214.89	2016.48	4231.37	6.19	508	8550000	271.9	47.580	13.661
Hüttensee	HT33	11.8	14	6.55	138	248	2.28	0.09	7	0.4	0.43	0.24	3.03	320	1.5	959	280	0.005	0.292	206.2	199.63	405.84	5.63	1519	31416	229.72	47.358	13.815
Imsee	IM33	20.9	291.67	8.3	2290	310	2.18	2.04	13	1.32	0.81	5.67	32.63	454	21.8	5096	643	0.034	0.126	2415.19	2174.26	4589.45	6.63	514	60000	171.63	48.022	13.138
Irrsee	IR33	19.5	296.33	8.5	1920	293	2.98	3.31	17	2.57	0.93	5.26	23.5	405	4.7	2717	456	0.010	0.168	2096.4	1742.15	3838.55	6.22	553	3548700	242.33	47.921	13.301
Krottensee	KR33	16.8	301.33	8.38	2430	964	2.2	1.03	18	0.78	0.22	8.54	31.02	1152	3.5	2865	1075	0.003	0.375	2573.78	2291.19	4864.98	5.66	577	64000	228.88	47.784	13.387
Loibersbacher Teich 1	LO134	19.1	5	5.18	51	0	0.28	0.23	12	0.18	0.28	0.09	0.47	103	47.6	7412	322	0.148	0.043	62.89	52.61	115.49	7.74	1296	600	110.46	47.739	13.302
Loibersbacher Teich 2	LO234	19.6	5	5.24	46	0	0.27	0.26	9	0.06	0.38	0.08	0.33	74	32.4	6587	272	0.119	0.041	58.71	41.87	100.58	7.4	1302	600	106.13	47.740	13.302
Miesbodensee	MI33	20.2	116.33	8.04	1280	7	0.19	0.53	11	0.15	0.09	0.43	24.9	193	3.5	5691	251	0.014	0.044	1299.25	1287.6	2586.85	5.64	1418	9600	171.02	47.491	13.873
Mondsee	MO33	20.2	325	8.54	2220	470	4.56	9.21	11	5.92	1.19	8.46	24.46	513	7.1	2485	586	0.012	0.236	2608.35	2205.83	4814.18	6.24	481	13780000	217.11	47.849	13.362
Nussensee	NU33	17	177.33	8.23	2200	743	1.77	0.63	6	0.42	0.16	5.45	35.73	209	11.2	3685	845	0.013	0.229	2307.75	2253.44	4561.18	6.06	604	100000	178.53	47.705	13.570
Oberer Sonntagskarsee	OS33	8.5	10.33	6.47	72	93	3.15	0.09	7	0.28	0.41	0.19	2.15	196	2.4	548	187	0.013	0.341	147.17	147	294.17	4.19	2062	38013	238.71	47.303	13.832
Obersee	OB33	8.5	17	6.56	148	259	2.53	0.14	3	0.41	0.52	0.25	3.12	294	1.5	704	276	0.005	0.392	223.48	207.37	430.85	5.65	1672	60000	238.71	47.349	13.818
Obertrumersee	OT33	17.3	307	8.22	2120	445	5.23	6.88	33	4.61	1.66	5.02	31.31	611	14.7	3926	701	0.021	0.179	2454.73	2220.59	4675.33	5.97	503	4880000	179.63	47.962	13.076
Oedensee	OE33	13	202	7.61	1930	351	0.94	0.26	3	0.15	0.11	2.38	33.47	357	4.1	5664	509	0.008	0.090	1982.08	1875.49	3857.57	6.06	779	199200	220.44	47.564	13.819
Prebersee	PR33	16.7	86	7.4	890	30	2.37	0.99	24	1.25	1.1	2.3	14.75	1645	6.5	4878	208	0.031	0.043	969.32	1008.92	1978.24	5.96	1514	60000	205.15	47.186	13.856
Riesachsee	RI33	12.02	25	6.64	206	183	3.97	0.2	7	0.42	0.55	0.31	4.79	909	2.4	950	212	0.011	0.223	307.62	298.07	605.69	5.63	1338	170900	232.1	47.331	13.779
Schwarzensee	SC33	17	200	8.51	2470	649	2.77	0.4	22	0.41	0.22	5.96	38.53	532	4.1	3167	739	0.006	0.233	2585.4	2438.05	5023.45	5.73	716	480000	80.26	47.752	13.495
Sommersbergsee	SO33	17.4	244	8.07	3180	4	1.61	0.61	7	0.49	0.27	13.04	37.15	565	10.6	5959	264	0.040	0.044	3231.04	2955.24	6186.28	6.04	856	22000	169.88	47.620	13.738
Unterer Sonntagskarsee	US33	9.9	7	6.29	82	137	2.61	0.1	7	0.26	0.33	0.16	2.01	245	1.2	570	154	0.008	0.270	148.56	134.65	283.22	5.69	1962	31416	185.81	47.307	13.829
Wallersee	WA33	17.6	337.67	8.6	2340	732	4.59	5.68	21	4.05	1.49	7.25	32.08	1502	15.3	4123	959	0.016	0.233	2648.12	2412.9	5061.02	6.48	505	6100000	203.62	47.918	13.165
Wirpitschsee	WI33	11.7	64.33	7.56	700	171	4.29	0.1	12	0.54	0.23	1.51	13.53	1113	1.5	819	195	0.008	0.238	804.42	828.74	1633.16	5.83	1699	76900	199.3	47.235	13.612
Wolfgangsee	WO33	17.2	254.67	8.44	2050	508	3.18	2.36	7	1.69	0.4	5.8	27.46	562	5.3	1881	580	0.009	0.308	2219.14	1931.12	4150.26	6.07	538	12840000	258.83	47.746	13.417

4.2.3 Bioinformatics and analysis of the sequence dataset

For adapter and primer clipping as well as for quality filtering, we used the CANGS software (Pandey et al. 2010; Nolte et al. 2010). Briefly, we performed a pattern search to allow for mutations in homopolymers within the adapter B sequence during trimming, but required the PCR primer sequences to be mutation free, otherwise the read was discarded. Reads for which an indel was identified at the transition between read and PCR primer were removed. Quality filtering included removal of all sequences that did not fit the following criteria: (i) no Ns; (ii) quality score >24, when averaged across the read after clipping adapters and primers; (iii) minimum sequence length of 200 bp (including PCR primers); and (iv) at least two copies of the read present in the entire data set before clipping primers. We blasted all non-redundant sequences against the NCBI database and retrieved the taxonomic classification of the best hit.

As higher taxonomic level of analysis, protistan metagroups (displayed and listed in Fig. 4.2) were chosen to fully reflect protistan biodiversity as well as to meaningfully distinguish the different phylogenetic lineages within the tree of life. Metazoa and embryophytes as well as Bacteria and Archaea were excluded beforehand analyses to diminish the bias within samples and focus on eukaryotic microbial life. OTUs affiliated with other protistan metagroups were summarised as 'others', sequence reads that could not be affiliated with any of the metagroups were treated as 'unknown' (not shown in graphs).

Due to the specific nature of the data matrix (i.e. many zero and single counts), we used a 2-step-standardisation of site data: first, rarefaction of the sites by the 'drarefy' function provided by the R-package VEGAN (Oksanen et al. 2011), followed by Hellinger-transformation (Legendre & Gallagher 2001). After application of these procedures, the resulting pre-transformed data matrix of OTUs can be submitted to Ward cluster analysis and further multivariate analyses (Borcard et al. 2011; Legendre & Gallagher 2001). Cluster analysis was calculated on the basis of OTUs and environmental variables, respectively, as well as on the basis of selected group OTUs only. The principle component analysis (PCA) based on euclidic Hellinger distances shows results of clustering plus significant environmental variables (Figs. 4.4 and 4.5).

As reads per sample differ decisively between samples, rarefaction values at a read level of 1000 reads were used to compare species richness and composition of OTUs among samples. For the shown composition within each sample, the 'drarefy' calculation (R-package VEGAN; Oksanen et al. 2011) was used to minimise the bias when counting down OTUs to a common read level (Fig. 4.2), however possibly resulting in OTU percentages per metagroup of less than 1 at a level of 1000. The community structure of the different samples was analysed using the mean relative abundance (+ standard deviation) of metagroup reads per cluster showing significant differences of metagroups among clusters (Table 4.2).

Table 4.2. Percentages of organismic groups per site

	A1.Ciliophora	A2.Dinophyceae	A3.Apicomplexa	A4.Alveolata.rest	B1.Bicosoecida	B2.Oomycetes	B3.Chrysophyceae	B4.Synurophyceae	B5.Bacillariophyta	B6.Stramenopiles.rest	C1.Cercozoa	C2.Rhizaria.rest	D1.Heterolobosea	D2.Euglenida	D3.Kinetoplastida	D4.Euglenozoa.rest
LO234	32.877	17.808	0	0	0	0	21.918	15.068	5.479	0	0	0	0	0	0	0
LO134	34.177	22.785	0	2.532	0	0	3.797	16.456	0	1.266	2.532	0	0	0	0	0
OS33	12.443	20.778	0.127	9.421	0	0	14.995	4.446	0.127	13.507	2.875	1.353	0	0	0	0
US33	13.471	12.532	0	10.044	0.625	0.339	16.049	4.406	0.294	15.528	3.340	1.174	0	0	0	0
BD33	32.760	3.252	0	2.605	0.497	3.737	17.849	9.642	2.043	7.918	2.000	0.350	0	0	0.350	0
OB33	4.90	32.614	0.097	5.596	0.455	0.097	14.127	7.796	1.578	16.423	1.800	0	0	0	0	0
RI33	12.983	5.921	0.147	5.882	0	1.352	15.716	5.572	4.352	17.609	3.132	0.746	0	0	0.867	0
HT33	6.121	22.857	0.144	6.055	0.266	1.694	21.173	7.405	1.930	11.987	0.988	0.144	0	0.144	0.434	0
SC33	57.928	7.249	0	2.634	0	0	13.476	0.698	3.188	5.417	0.698	0	0	0	0	0
GF33	10.480	54.404	0.566	1.914	0.241	0.241	9.731	1.397	0.241	3.697	0.241	0	0	0	0	0
NU33	39.115	3.266	0	2.956	0.053	0.106	19.086	3.048	3.146	9.158	1.320	0	0	0.053	0.618	0
MI33	7.910	16.513	0	4.676	0.579	0.503	23.548	6.850	1.024	15.376	1.297	0	0	0.341	0.503	0
SO33	41.696	25.133	0	6.151	0	0	8.509	1.187	1.935	5.126	0.428	0	0	0	0	0
AL33	24.513	18.325	0.354	4.852	0	0.177	12.725	5.525	2.443	13.300	2.026	0	0.177	0.816	0	0
IM33	13.680	13.976	0.357	3.587	0.425	0.626	15.591	2.793	6.744	7.007	0.892	0.271	0	0	0.693	0
AU33	41.137	5.424	0.331	6.758	0.077	0	22.291	0.491	0.386	5.098	0.742	0	0	0	0.077	0
BA33	17.412	6.510	0.189	4.485	0.103	0	17.586	4.568	10.230	15.788	1.734	0.506	0	0	0	0
WI33	25.758	6.571	0.594	4.884	0.074	0.222	11.821	3.799	8.152	13.548	1.627	0	0	0.576	0.222	0
WA33	36.257	8.026	1.034	9.219	0.169	0.085	13.966	4.274	1.896	5.043	1.543	0.085	0	0.169	0	0.156
PR33	17.416	8.790	1.282	4.215	0.477	1.327	12.023	6.648	1.876	12.689	1.713	0	0	0.192	0	0
MO33	28.209	7.804	1.570	8.992	0	0.149	13.218	5.221	3.381	9.023	3.736	0	0	0	0.149	0.349
KR33	24.268	5.719	0	2.562	0.234	0.975	19.410	4.949	6.402	11.034	1.395	0	0.082	0.165	0.586	0.342
OT33	42.944	3.651	0.186	6.430	0.704	0.371	5.029	4.523	2.605	1.802	2.623	0	0	0.490	0.567	0
EG33	33.177	8.245	0.242	3.644	0.484	0.382	5.200	4.800	3.541	6.247	1.313	0	0	0.382	0.496	0
FU33	13.775	20.680	0.851	6.508	0	0.378	16.425	2.534	10.058	9.125	1.149	0.437	0	0.221	0.221	0
OE33	27.061	13.361	0.325	6.167	0.118	0.235	14.384	6.121	0.746	9.838	1.332	0	0	0	0	0
IR33	27.600	9.152	0.899	7.538	0.080	0.588	18.993	3.428	4.483	6.953	3.325	0	0.080	0	0.473	0.080
WO33	20.835	18.694	0.587	6.338	0.134	0.124	13.615	4.830	6.836	8.181	0.951	0.067	0	0	0.124	0.067
HA33	22.357	11.342	0.522	6.774	0.126	1.482	10.127	4.164	7.156	8.022	4.541	0	0.205	0.778	0.961	0.174

		D5.Excavata.rest	E1.Choanoflagellida	E2.Chytridiomycota	E3.Microsporidia	E4.Ascomycota	E5.Basidiomycota	E6.Glomeromycota	E7.Amoebozoa	E8.Apusozoa	F2.Rhodophyta	F3.Viridipl..no.Embr.	G1.Cryptophyta	G2.Katablepharidoph.	H1.Haptophyceae	others
LO234		0	0	2.740	0	0	0	0	0	0	0	1.370	0	0	1.370	1.370
LO134		1.266	2.532	0	0	1.266	1.266	0	0	0	0	3.797	1.266	1.266	2.532	1.266
OS33		0	0	0.994	0	2.771	0.692	0.127	0	0	6.097	3.814	0.748	2.091	0.356	2.238
US33		0	0.926	0.849	0	2.691	2.048	0.294	0.294	0	3.880	6.227	0.339	2.121	0.169	2.359
BD33		0	1.100	2.002	0	3.714	2.742	0	0	0	0.350	2.454	1.746	0	0.934	1.957
OB33		0	0.330	0.505	0.097	0.932	1.942	0.238	0	0	0.097	7.694	0.272	0.825	0.097	2.199
RI33		0	0.642	2.641	0.250	3.271	1.789	0.410	0.294	0	1.481	7.297	1.470	0.785	1.261	4.129
HT33		0	1.322	1.976	0.072	2.231	1.806	0	0	0	0	6.722	1.038	0.144	0.749	2.596
SC33		0	0	0	0	0.349	1.047	0	0	0	0	1.778	1.661	1.396	1.520	0.963
GF33		0	0	6.756	0	0	0	0	0	0	0	1.489	3.916	4.262	0.425	0
NU33		0	0	1.051	0	0.515	1.334	0	0.159	0	0	0.455	9.168	2.033	1.790	1.569
MI33		0	0	2.173	0	3.241	3.451	0	0	0	0	3.466	4.681	1.149	0	2.720
SO33		0	0	0	0	0	1.083	0	0	0	0	1.489	2.818	0.843	3.062	0.541
AL33		0	0.962	1.061	0	1.366	0.835	0	0.609	0	0	2.760	3.356	1.382	1.955	0.481
IM33		0	0	3.236	0	0.734	0.309	0	0	0	0	9.641	14.449	1.236	1.717	2.037
AU33		0	0	0.434	0	0.154	0.424	0	0	0	0	4.149	2.514	2.289	6.332	0.889
BA33		0	0.189	1.316	0	1.580	1.516	0	0.103	0	0	4.202	5.013	1.694	2.284	2.992
WI33		0.243	0.360	1.510	0	1.331	1.257	0	0	0	0	3.640	1.953	1.091	7.382	3.384
WA33		0	0.648	1.623	0	0.972	0	0	0	0	0	3.427	5.201	1.811	3.497	0.898
PR33		0	0	2.762	0.096	4.185	3.662	0	0	0	0.096	5.244	9.931	1.481	2.461	1.434
MO33		0	1.257	1.461	0	1.905	0.432	0	0	0	0	2.541	5.664	1.814	1.374	1.752
KR33		0	0.468	1.060	0	3.062	1.461	0	0.165	0.082	0	3.547	6.260	1.133	2.368	2.271
OT33		0	1.623	1.968	0	2.050	1.106	0	0.186	0	0	2.465	9.006	2.927	5.759	0.985
EG33		0	0.280	1.609	0	0.382	0	0	0	0	0.140	7.194	9.200	8.242	2.785	2.017
FU33		0	0.541	2.519	0	1.107	0.126	0	0	0	0	2.118	3.036	3.243	1.636	3.314
OE33		0	0.728	1.210	0	2.161	1.237	0	0	0	0.118	1.820	9.822	1.842	0.442	0.934
IR33		0	0.426	2.369	0	2.944	0.747	0	0.080	0	0	2.310	2.226	2.944	1.212	1.072
WO33		0	1.409	1.536	0	0.966	0	0	0	0	0.405	1.977	3.535	2.907	4.046	1.835
HA33		0.216	0.437	0.535	0	1.383	2.181	0	0.493	0.068	0	4.455	7.076	1.251	1.863	1.312

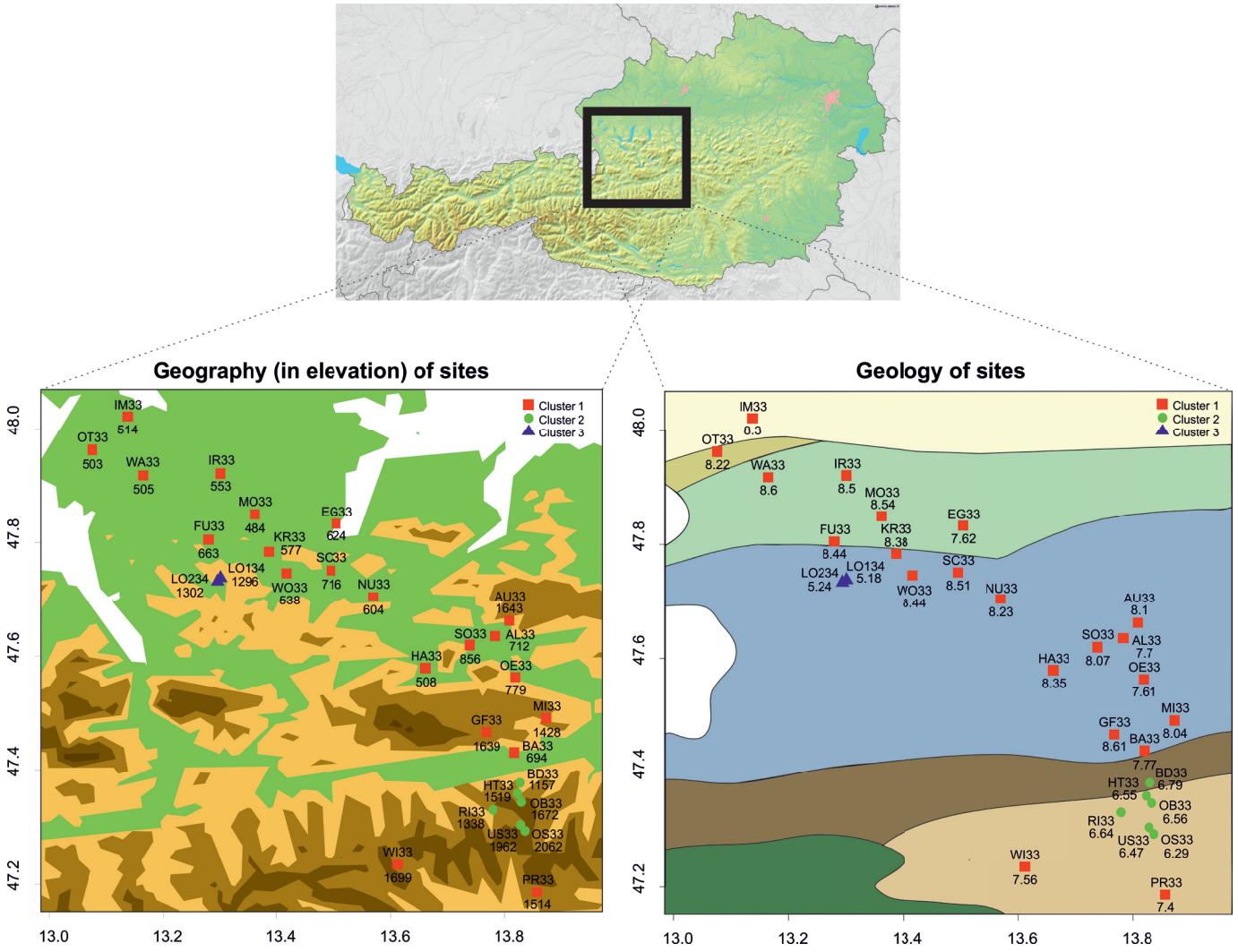

Figure 4.1. Position and cluster sorting (Ward-clustering) of sites within the sampling area. Site coordinates are plotted on an elevation profile + elevation of individual lakes in numbers (left), and on a geological profile + pH of individual lakes in numbers (right). The main geological formations shown are (from top to bottom): Molasse (pale brown), Rhenodanubian Flysch (light green), Northern limestone Alps (blue), Austroalpine Crystalline (brown colours).

4.3 RESULTS

Three of the sampled lakes (Ahornsee, Bad Heratingersee, Holzoestersee – not shown in figures and tables) were represented by too few sequences after bioinformatic quality filtering and were discarded from further analyses. The remaining 29 lakes formed three clusters in cluster analysis on the basis of OTUs, two smaller clusters of respectively two and six lakes (subsequently: Cluster 1 and Cluster 2) and one larger cluster of 21 lakes (subsequently Cluster 3). These clusters are shown in geographic maps in Figure 4.1 and in graphs of combined cluster/PC-analysis in Figures 4.4 and 4.5. The geographical mapping of Figure 4.1 reveals that the sampling sites of the three clusters are situated in differing geology. Cluster 2 (6 sites) is situated in siliceous geology, Clusters 3 (21 sites) and 1 (2 sites) are situated in calcareous geology. This is in general correspondence with measured pH-values of the lakes. Only Cluster 1 (two boggish ponds) with a low pH within calcareous geology must be interpreted as a local phenomenon. The two smaller clusters (2 and 1) are moreover locally restricted. A connection of elevation with the three clusters is not pronounced.

4.3.1 Species richness

Species richness in the sampled lakes varied between 80 and 270 different OTUs for 1000 reads. Species richness did not correlate with elevation (Pearson cor: 0.0078), it was, however, positively correlated with log of lake surface area (Pearson cor: 0.5196, p-value = 0.0038) and negatively correlated with TP (Pearson cor: -0.6028, p-value = 0.0005). Elevation and TP were not correlated in our dataset (Pearson cor: -0.1727). The two lakes affiliated with Cluster 1 both had a comparatively low species richness. The largest cluster (Cluster 3) showed a broad variation of species richness values (Fig. 4.2).

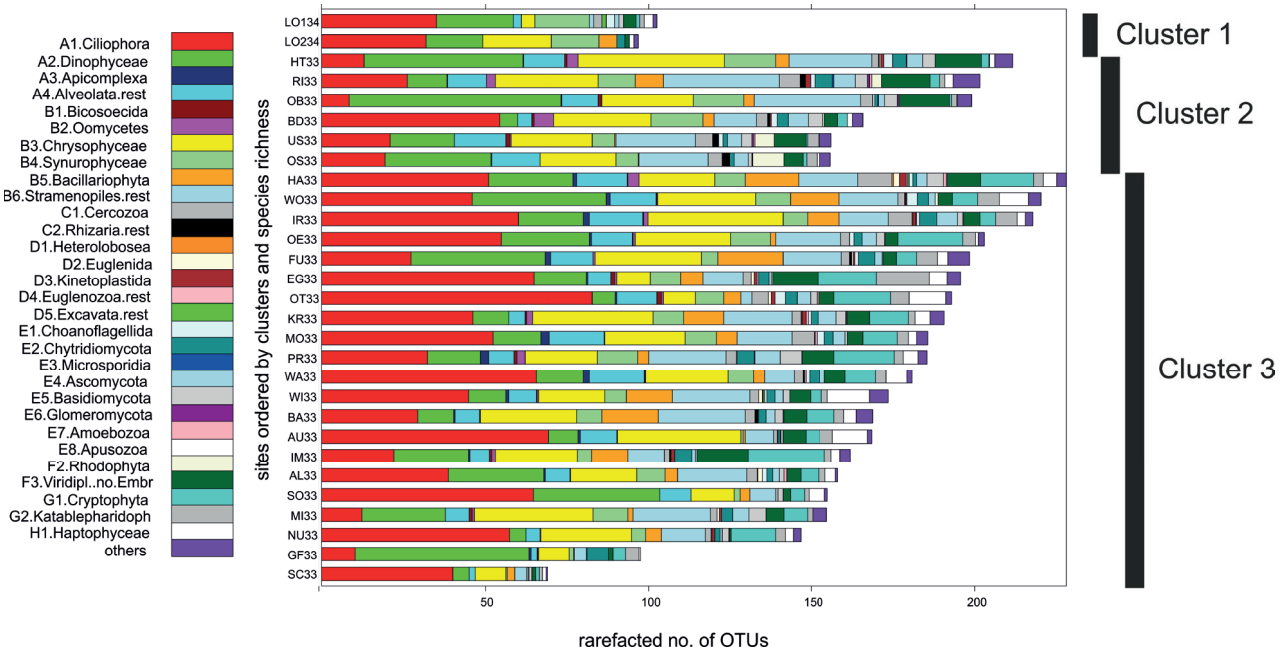

Figure 4.2. Species richness and OTU-composition of taxonomic groups [rarefaction level = 1000]. Bars (top to bottom) are ordered by clusters from cluster analysis and by species richness (high to low), respectively in clusters. Species richness is show as hight of bars in rarefacted no. of OTUs; community composition is shown as colour of bars by identified taxonomic groups (see legend).

4.3.2 Community composition and OTU distribution

Community composition analysis revealed typical freshwater communities of meso- to oligotrophic lakes with high proportions of Ciliophora (23.9%), Chrysophyceae (14.6%), Dinophyceae (13.5%), other Stramenopiles (Stramenopiles.rest) (9.6%), other Alveolates (Alveolata.rest) (5.6%), Synurophyceae (5.1%) and Cryptophyta (4.6%) for all sites. Alveolates showed generally high read numbers due to high gene copy numbers (Fig. 4.2).

Generally, differences between communities (calculated on OTU basis) were high, ranging from Bray-Curtis distances of 0.5 to 0.99 and mostly being higher than 0.8 mainly due to the high number of singleton sequences present in one site only. Differences between sites within clusters were generally lower than differences between sites among clusters (Table 4.3). For those OTUs that occurred in more than one site, OTU distribution was not strongly affected by elevation. Tested in a direct comparison of OTUs that occur in more than three sites (1670 out of 4612 total), only 20.24% were restricted to either elevations below 700m or elevations above 1300m whereas 62.22% of OTUs occurred in both the highest and lowest lakes sampled (Fig. 4.3).

On the level of metagroups, the high dissimilarity between sites does not show likewise. Smaller differences here include common trends of single groups within Cluster 2 as compared with the large Cluster 3 (Dunnett-T3 Post-hoc test), i.e., for example, a relatively low fraction of Cryptophyta (p-value<0.001) in Cluster 2. The two lakes of Cluster 1 show a comparably high fraction of Alveolates and Stramenopiles (together 83.1%, p-values: Ciliophora = 0.016, Synurophyceae < 0.001, Stramenopiles.rest <0.001) reducing fractions of all other taxonomic groups.

Table 4.3. Bray Curtis distances between sites

Bray Curtis Unähnlichkeit, WienAlpineSpatialEuksort, rrarefy_putback_1000 (Minimum: 0.53, HT33 gegen OB33)

```
> print(spe.BC)
       AL33  AU33  BA33  BD33  FU33  GF33  EG33  HA33  HT33  IM33  IR33  KR33 LO134 LO234  MI33  MO33  NU33  OE33  OB33  OT33  OE33  PR33  RI33  SC33  SO33  US33  WA33  WI33
AU33  0.935
BA33  0.913 0.946
BD33  0.970 0.970 0.955
FU33  0.894 0.945 0.900 0.950
GF33  0.974 0.972 0.942 0.995 0.816
EG33  0.929 0.934 0.922 0.970 0.893 0.959
HA33  0.864 0.866 0.908 0.921 0.873 0.941 0.904
HT33  0.932 0.941 0.950 0.774 0.924 0.986 0.965 0.930
IM33  0.955 0.943 0.927 0.984 0.904 0.970 0.841 0.950 0.975
IR33  0.895 0.762 0.901 0.944 0.786 0.947 0.837 0.809 0.928 0.900
KR33  0.916 0.932 0.842 0.932 0.835 0.970 0.901 0.846 0.880 0.855 0.773
LO134 0.994 0.986 0.990 0.996 0.974 0.990 0.985 0.993 0.996 0.992 0.985 0.989
LO234 0.992 0.988 0.990 0.994 0.981 0.999 0.996 0.995 0.996 0.993 0.991 0.996 0.763
MI33  0.924 0.974 0.878 0.924 0.891 0.959 0.931 0.891 0.926 0.900 0.904 0.867 0.993 0.995
MO33  0.828 0.939 0.856 0.944 0.807 0.963 0.848 0.818 0.934 0.907 0.711 0.800 0.989 0.993 0.914
NU33  0.898 0.942 0.841 0.971 0.867 0.933 0.876 0.882 0.937 0.822 0.824 0.538 0.992 0.991 0.888 0.808
OE33  0.964 0.970 0.950 0.924 0.933 0.959 0.961 0.956 0.877 0.969 0.931 0.938 0.994 0.999 0.954 0.947 0.950
OB33  0.929 0.952 0.949 0.830 0.938 0.989 0.975 0.937 0.530 0.976 0.942 0.911 0.996 0.997 0.940 0.943 0.969 0.887
OT33  0.844 0.893 0.886 0.957 0.831 0.951 0.769 0.831 0.950 0.918 0.804 0.846 0.981 0.989 0.924 0.790 0.880 0.944 0.965
OE33  0.879 0.925 0.881 0.946 0.848 0.914 0.899 0.900 0.901 0.797 0.852 0.808 0.986 0.994 0.830 0.864 0.776 0.961 0.922 0.914
PR33  0.914 0.938 0.869 0.938 0.898 0.956 0.912 0.856 0.920 0.939 0.863 0.860 0.981 0.989 0.898 0.860 0.888 0.949 0.925 0.888 0.861
RI33  0.953 0.977 0.949 0.878 0.936 0.987 0.972 0.943 0.776 0.974 0.921 0.908 0.992 0.997 0.940 0.939 0.958 0.797 0.813 0.950 0.928 0.934
SC33  0.958 0.968 0.982 0.989 0.953 0.993 0.973 0.967 0.974 0.974 0.935 0.960 0.987 0.980 0.990 0.953 0.958 0.993 0.981 0.940 0.973 0.947 0.974
SO33  0.956 0.916 0.875 0.960 0.846 0.860 0.967 0.891 0.985 0.947 0.898 0.811 0.961 0.962 0.939 0.903 0.841 0.975 0.990 0.859 0.952 0.911 0.981 0.930
US33  0.919 0.951 0.956 0.933 0.914 0.968 0.948 0.932 0.853 0.959 0.936 0.925 0.995 0.994 0.953 0.936 0.947 0.597 0.825 0.869 0.944 0.951 0.788 0.985 0.983
WA33  0.895 0.939 0.838 0.938 0.843 0.951 0.852 0.811 0.964 0.791 0.855 0.868 0.985 0.987 0.918 0.812 0.862 0.975 0.963 0.788 0.897 0.857 0.969 0.968 0.872 0.958
WI33  0.904 0.948 0.890 0.949 0.850 0.971 0.927 0.898 0.869 0.945 0.867 0.851 0.992 0.989 0.928 0.881 0.915 0.919 0.896 0.891 0.916 0.891 0.779 0.960 0.953 0.925 0.861
WO33  0.848 0.907 0.866 0.940 0.605 0.846 0.860 0.790 0.889 0.898 0.681 0.754 0.986 0.990 0.909 0.743 0.833 0.903 0.925 0.778 0.873 0.874 0.917 0.944 0.844 0.879 0.840 0.837
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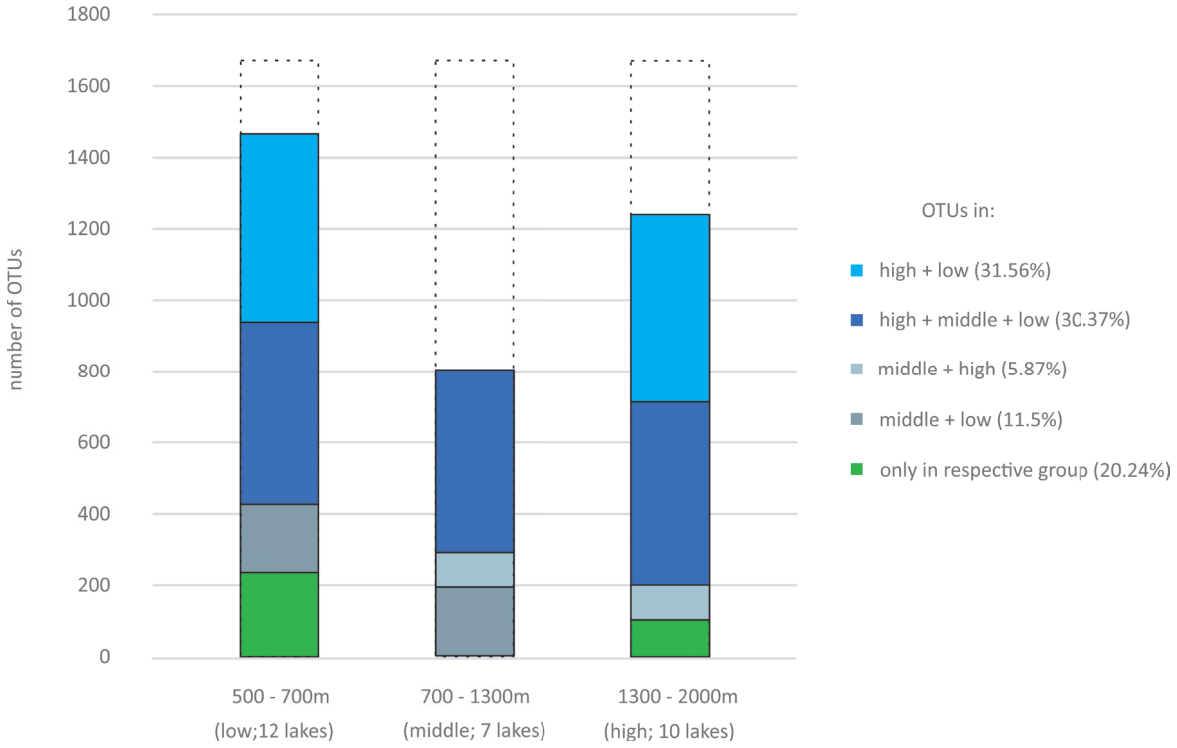

Figure 4.3. Occurence of OTUs > 3 sites (sum = 1670 from 4612 total) by altitudinal groups. Graph showing direct comparison of OTUs >3 sites by elevation. The three bars (= three altitudinal levels: low, middle, high) show all OTUs out of a total 1670 present in the respective elevation level sorted into groups as indicated in legend (green = only present in high or low – 20.24% 0f OTUs, blue = present in high and low – 62.22% of OTUs).

4.3.3 Principle component analyses

In the PCA plots, the three clusters were clearly separated, Cluster 3 (the largest cluster) being quite scattered within itself. Most of the measured environmental variables were significant (at a significance level of 0.001) by vector fitting. However, in forward selection of variables in RDA – which reduces variables to a necessary minimum – conductivity, TP (or TP/DN), pH and temperature remained as explanatory factors, together explaining for 88.4% of the clustering of sites. In the plots, especially pH (and covariant factors, i.e. HCO_3, Na, Ca, etc.) and TP appear as independent of each other and therefore in combination have high explanatory value. Cluster 1 is characterised by low pH (around 5.2) and low calcium, but high phosphate and DOC concentrations. Cluster 2 is likewise characterised by low pH (6.3-6.8) and low calcium (around 3µg/l), but also low values of phosphate and DOC. Cluster 3 in contrast covers a wide range of values, however throughout with higher pH (7.4-8.6) and calcium (14-44 µg/l). Temperature could not be substituted by elevation in the calculation, ruling out elevation as a factor of high explanatory value for community analysis (Fig. 4.4 and Table 4.1). Nor was lake size (applied as lake surface area) relevant for cluster sorting.

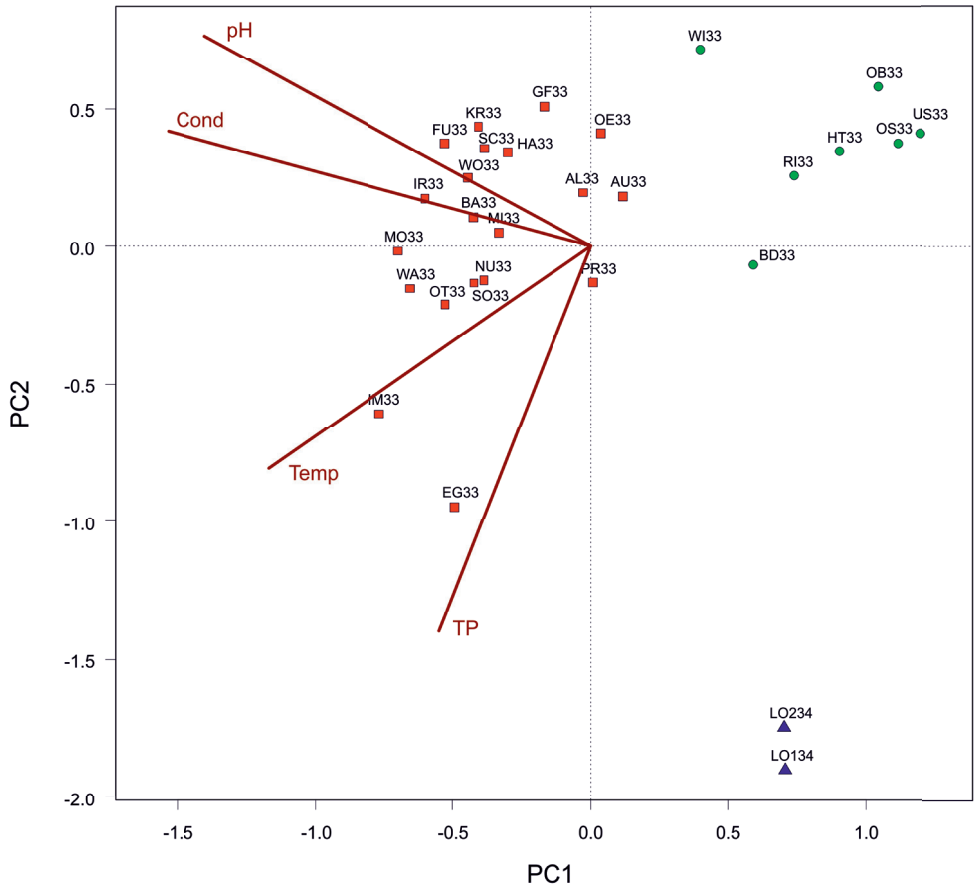

Figure 4.4. Principle component analysis showing relevant environmental variables for cluster sorting. After forward selection of variables in RDA, Conductivity (Cond), pH, TP (total phosphate) and Temperature (Temp) remain as explaining sorting of clusters without further variables.

PCA also pronounces the observed trend that the dissimilarity of sites mainly shows on the OTU-level. On the metagroup level, in contrast, relevance for cluster sorting is stongly blurred. Only few metagroups appear as significant for cluster sorting whereas OTUs clearly separate the three clusters from each other.

Combined cluster/PC-analysis based on environmental variables on the one hand and based on OTUs on the other hand revealed almost identical PCA plots. The applied environmental variables, therefore, co-varied with OTU inventory. pH (plus conductivity), TP, and also temperature seem to be a good proxy to predict protistan community similarity among (alpine) lakes. This also held true when the analysis was restricted to distinct taxonomic groups, namely Chrysophyceae and Ciliophora (Ciliophora not shown). The PCA based exclusively on chrysophycean OTUs even showed higher cluster consistency than the two above mentioned. In contrast, when the analysis was restricted to Dinophyta, clusters were indistinct (Fig. 4.5).

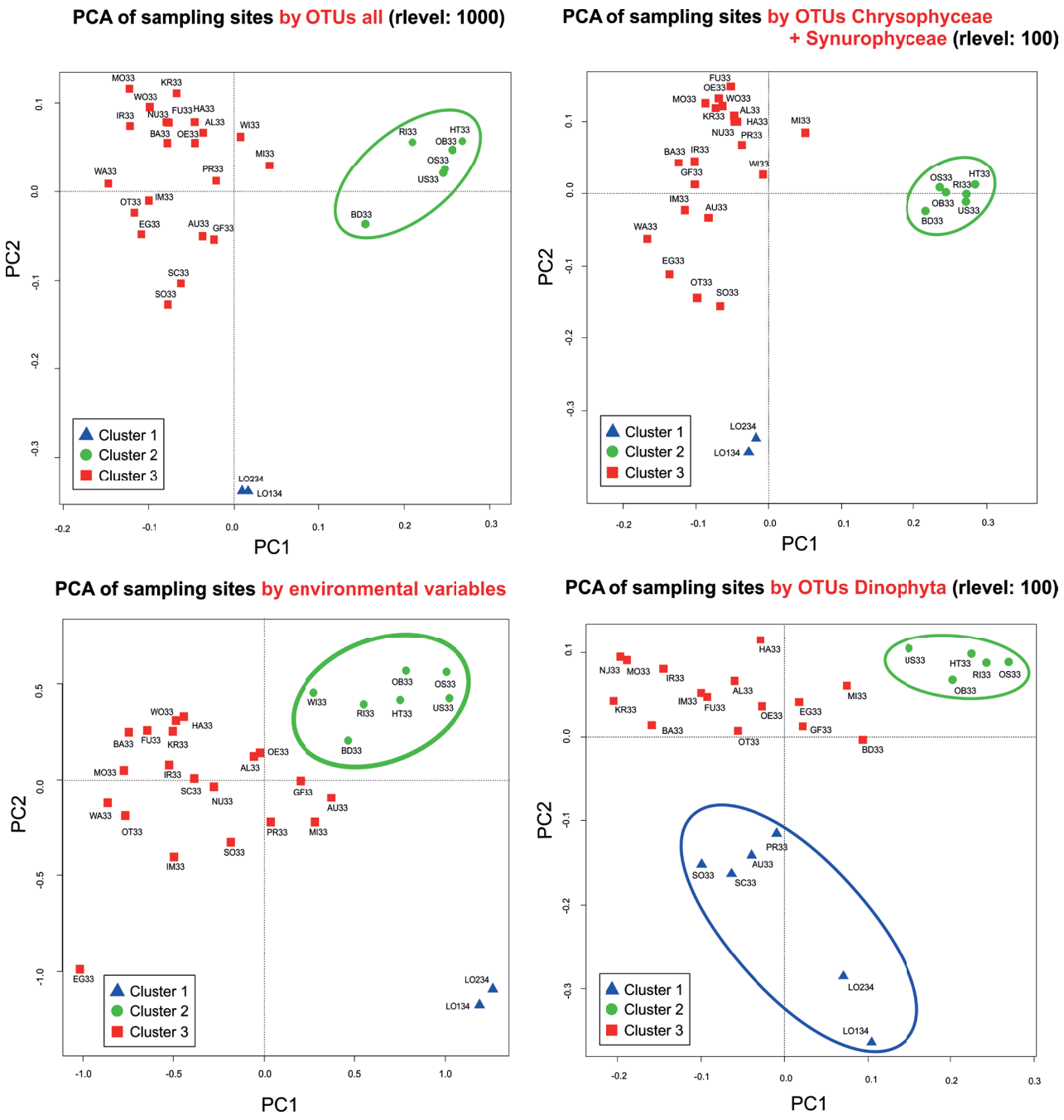

Figure 4.5. Comparative principle component analyses showing clusters of sampling sites. Graphs give results of cluster analysis (Ward-clustering) on the basis of all OTUs (top left), of environmental variables (bottom left), of OTUs of Chrysophyceae + Synurophyceae (top right) and of OTUs of Dinophyta (bottom right). Cluster 2 and enlarged Cluster 1 are encircled for reasons of comparison. OTU-data for cluster analysis are Hellinger-transformed.

4.4 DISCUSSION

Ecological and biogeographical theories are largely derived from metazoans and embryophytes – the applicability to other organisms, specifically microbial organisms, remains, however, unclear. Species-area-relationship hypotheses predict lower species numbers in smaller habitats. In an elevation gradient, land area as well as the number and mean size of freshwaters decreases gradually when ascending to higher elevation. Likewise, other factors such as temperature change alongside an elevation gradient. We, here, investigated alpine freshwater lakes covering an elevation gradient of about 1500m of elevation (429m to 2072m asl.) and differing in ecological parameters for patterns of protistan biodiversity.

4.4.1 Species richness in the elevation gradient and species-area-relationship

Based on our analyses, we can reject a general reduction of diversity (in terms of species richness) of freshwater protists with elevation. On the contrary, although the most diverse lakes proved to be the larger lakes at lower elevation, lakes at an elevation of 1500 and 1600 m asl. Also were among the more diverse within the tested 29 lakes. Reduced area in terms of the total available freshwater in higher elevations did not affect species richness of protists. Species-area-relationship in this respect can, therefore, be rejected. However, independent of the elevation gradient, species richness was positively correlated with individual lake size. Our results, therefore, demonstrate that individual habitat size matters for protist species richness as it is predicted from species-area-relationship hypotheses. As protists seem to follow the general rule of higher species numbers in larger area size, species richness in mountain lakes of higher elevation has to be upheld by other factors than habitat size. Endemism in mountain protists due to special adaptations in high mountain regions as well as due to the remoteness of high mountain habitats has been described (Sommaruga 2001; Sonntag et al. 2011) and is likely to contribute to higher species numbers. The special climatic, geological and ecological conditions in mountain ranges might as well produce diversification of habitats and niches – at least on the scale of single cell organisms. Thus, taking into account the small size of protists as compared to multicellular organisms, a direct comparison in one and the same habitat might be inappropriate. In terms of the scale ratio of organism and habitat, a small mountain lake can hold diverse niches for single cell protists, but hardly any for larger organisms. Another feature of high mountain lakes is a general low nutrient level. As species richness was negatively correlated with nutrient availability in our study, nutrient scarceness also qualifies to produce higher species evenness and with that possibly higher species numbers in high mountain lakes. So, whereas species richness of animals and plants decreases with elevation, protistan species richness is stable or can even increase (as shown in our study) for reasons yet to be investigated in subsequent studies.

4.4.2 Community composition in the elevation gradient

Community composition in our study was multiple factor driven. Elevation and co-varying factors explained for some, even though a minor part, of the variance. Only 20% of the OTUs occurring at three or more sites were restricted to either high or low elevation. The observed changes in

community composition were, therefore, mainly independent of elevation. Also, cluster analysis formed clusters of lakes not separated by elevation levels, but strongly deviating in e.g. pH and nutrient level. However, elevation generally co-varies with nutrient concentrations, i.e. lakes at a higher elevation contain lower nutrient concentrations. Community shifts associated with nutrient concentration have been demonstrated also on higher taxonomic levels: for instance, Jeppesen et al. 2005 reported a community shift towards diatoms, chrysophyceans, cryptophytes and dinophytes with decreasing nutrient load in freshwater lakes. In our study, we did not observe such decisive changes in protistan community structure on metagroup level, probably due to a comparably low nutrient status of all sampled lakes, i.e. all investigated lakes were oligotrophic or mesotrophic. Nor did we find a pronounced correlation of elevation and nutrients within our dataset. On the level of individual observed OTUs, differences in nutrient level were, however, large enough to change community composition decisively. Besides nutrient level, pH and conductivity, temperature also explained significantly for variation in protistan community composition. It was, however, not directly connected to the elevation gradient. As such, the influence of temperature in our dataset is not in accordance with studies on embryophytes and metazoa for which temperature clearly correlates with the elevation gradient and distribution patterns in mountainous regions on a large scale (Livingston & Dokulil 2001). The multifactorial drivers of protistan community composition in our study most likely reflect a combination of respective local habitat characteristics of the different sites. This is in accordance with other studies of protistan mountain lake communities (Tolotti et al. 2005; Triadó-Margarit & Casamayor 2012) as well as with studies on bacterial distribution patterns in lakes at high elevation (Sommaruga & Casamayor 2009). The comparative combined cluster/PC-analysis on both the basis of environmental variables as well as on the basis of OTUs, as carried out with our dataset, further underlines the connection of multifactorial ecological conditions and changing community composition on the level of OTUs. A resolution on metagroup level can reflect overarching ecological trends among habitats as for example in comparisons between soil and freshwater ecosystems. For a more precise and deep resolution of similar habitats, as with the mountain lakes of this study, an analysis on OTU-level is necessary. In that, Chrysophyceae prove to be a promising candidate to most precisely reflect the total community within an elevation gradient and qualify as bio-indicators in an alpine context. Due to their sensitivity to environmental changes (e.g. climate, pH, nutrients, seasonality) (Lotter et al. 1997; Pla & Catalan 2005) as well as to stressors (e.g. heavy-metal pollution) (Kamenik et al. 2005), Chrysophyceae are an established tool in palaeolimnological studies (Facher & Schmidt 1996). By our findings in the diversity of Alpine freshwater protists, we propose to draw on Chrysophyceae also as a model taxon in studies of current diversity.

CONCLUSION

As our investigation of protistan communities in an alpine elevation gradient implies, protistan species richness is not reduced with elevation. Nor are elevation and co-varying factors main drivers of protistan community composition in an alpine context. Protistan diversity patterns might, thus, well be different from those of higher organisms and testing of theories derived from the latter is meaningful and possibly reveals patterns yet unknown and much singular for single cell organisms.

REMARKS

The data from the sampling was provided by Jens Boenigk (formerly Institute of Lake Ecology, Mondsee)

For the bioinformatic processing, I used a pipeline constructed by the Institute of Population Genetics, Vienna.

5) EFFECTS OF SILVER NITRATE AND SILVER NANOPARTICLES ON A PLANCTONIC COMMUNITY: GENERAL TRENDS AFTER SHORT-TERM EXPOSURE

ABSTRACT

Among metal pollutants silver ions are one of the most toxic forms, and have thus been assigned to the highest toxicity class. Its toxicity to a wide range of microorganisms combined with its low toxicity to humans lead to the development of a wealth of silver-based products in many bactericidal applications accounting to more than 1000 nano-technology-based consumer products. Accordingly, silver is a widely distributed metal in the environment originating from its different forms of application as metal, salt and nanoparticle. A realistic assessment of silver nanoparticle toxicity in natural waters is, however, problematic and needs to be linked to experimental approaches. Here we apply metatranscriptome sequencing allowing for elucidating reactions of whole communities present in a water sample to stressors. We compared the toxicity of ionic silver and ligand-free silver nanoparticles by short term exposure on a natural community of aquatic microorganisms. We analyzed the effects of the treatments on metabolic pathways and species composition on the eukaryote metatranscriptome level in order to describe immediate molecular responses of organisms using a community approach. We found significant differences between the samples treated with 5 µg/L $AgNO_3$ compared to the controls, but no significant differences in the samples treated with AgNP compared to the control samples. Statistical analysis yielded 126 genes (KO-IDs) with significant differential expression with a false discovery rate (FDR) < 0.05 between the control (KO) and $AgNO_3$ (NO3) groups. A KEGG pathway enrichment analysis showed significant results with a FDR below 0.05 for pathways related to photosynthesis. Our study therefore supports the view that ionic silver rather than silver nanoparticles are responsible for silver toxicity. Nevertheless, our results highlight the strength of metatranscriptome approaches for assessing metal toxicity on aquatic communities.

5.1 INTRODUCTION

Engineered silver nanoparticles (AgNP) are used in a wide variety of applications, for example as antimicrobial additives in textiles, as household products and in medical applications. The recent upward trend in production (estimated 500 t/a worldwide) (Müller & Nowack 2008) and application resulted in an increasing release of AgNP as well as of ionic silver into the environment as can be seen from elevated levels of Ag in the aquatic environment (Batley et al. 2012; Fabrega et al. 2011; Kahru & Dubourguier 2010; Scown et al. 2010). So far, the impact of AgNP, as well as of ionic silver species on aquatic organisms has been studied mostly in laboratory experiments using single test species, sometimes even clonal cultures (e.g. *Chlamydomonas* sp.) (Bondarenko et al. 2013; Navarro et al. 2008; Piccapietra et al. 2012; Shi et al. 2012). As a general trend it appears that toxicity of silver is due to ionic silver as the molecular toxicant (Xiu et al. 2012; Chernousova

& Epple 2013). Nevertheless, toxicity of AgNP is still relevant as particles represent a source from which Ag^+ can be formed continuously with subsequent toxic effects (Navarro et al. 2008).

Realistic assessment of nanoparticle toxicity (mediated by their ionic forms) in natural waters is difficult due to the interaction of nanoparticles and ions with other inorganic and organic molecules (Batley et al. 2012). Accordingly, it is necessary to transfer laboratory results to field conditions. Also, the use of single species as test organisms as well as analyses of single parameters such as cell numbers or chlorophyll content will be insufficient if community effects and functional diversity of ecosystems are of interest (Cadotte et al. 2011). In this context, a metatranscriptome sequencing approach is able to elucidate reactions of whole communities present in a water sample to stressors like toxic substances (Chen et al. 2012). Differential transcription of genes related to various metabolic pathways (e.g. photosynthesis, fatty acid biosynthesis or glycolysis) is not only linked to single organisms, but shows the ecological functionality of certain groups of taxa in a sample (Osborn & Hook 2012; Simon et al. 2013; Wang et al. 2009). Therefore, this method allows detection of possible environmental hazards in a realistic approach, taking into account the species community as a whole.

To the best of our knowledge, no information exists on the effects of silver nitrate ($AgNO_3$) as compared to AgNP on aquatic communities to date. Accordingly, we compared the toxicity of ionic silver and AgNP by short-term exposure of a natural community of aquatic microorganisms in a laboratory exposure experiment. Since the activity of AgNP is influenced by the ligands, ligand-free nanoparticles are especially suitable for such comparisons (Grade et al. 2012). Effects of the treatments on metabolic pathways and species composition were analyzed on the eukaryote metatranscriptome level in order to describe immediate molecular responses of organisms using a community approach.

5.2 METHODS

5.2.1 General experimental set-up

A one-day exposure experiment was conducted in June 2013 in a climate chamber at 16°C with homogenously distributed artificial day light. The intensity of the light was 60-78 µE m^{-2} s^{-1} with a 16h/8h light-dark-cycle. Approximately 150 L of water containing a natural plankton community from a eutrophic pond at the campus Essen of the University Duisburg-Essen, Germany, were transferred to a 200 L glass tank. The next day, 10 L of pond water from the glass tank were filled to nine 20 L plastic tanks respectively and aerated by aquarium pumps. The nine tanks were divided into three experimental groups (control, $AgNO_3$ and AgNP) with three replicate tanks each.

Silver exposure was performed using a Ag-standard solution (ICP-Standard Silber, 1g Ag/L, Bernd Kraft GmbH, Duisburg, Germany) for the $AgNO_3$-group and a freshly laser generated silver nanoparticle suspension for the AgNP-group. For each treatment, silver was added to the water resulting at a nominal Ag concentration of 5 µg/L, which was shown to be sublethal in pre-test

experiments (see supplementary material, Figure S5.1). Monitoring of silver concentrations during exposure was performed by Ag analyses of 10 ml water samples taken from each tank 30 min and 24 h after the start of exposure; from the silver exposed groups one additional water sample was drawn after 5 h following exposure start. Half of the water samples were filtered (0.2 µm, cellulose acetate single use filter, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) to remove organisms and organic particles. These samples were considered to reflect the concentration of dissolved silver. All water samples were acidified with 10 µl HNO_3 (subboiled from 65% HNO_3, p.a., Bernd Kraft GmbH, Duisburg, Germany) and were analyzed on the same day. The experiment was terminated after 24 h. Before exposure to silver, samples were taken for the metatranscriptomic sequence analysis (2.5 L of water containing the native plankton community) and for determination of water characteristics (1 L) from the 200 L glass tank. After 24 h of exposure the same sample volumes were taken from each treatment group. Additionally, temperature, pH, conductivity and O_2-concentration were measured twice during the exposure period in every tank.

5.2.2 Preparation of AgNP, metal analyses and water chemistry

Silver nanoparticles were generated via laser ablation of a silver target in liquid aqueous medium according to Barcikowski & Compagnini (Barcikowski & Compagnini 2013) and Zeng et al. (Zeng et al. 2012). To this end, a silver foil (Goodfellow, 99.99 %) was ablated with a Nd:YAG nanosecond pulsed laser (Rofin PowerLine 20E) at λ = 1064 nm with a repetition rate of 10 kHz and a pulse energy of 0.3 mJ. The ablation process was carried out in a flow-through chamber with a volume of 1.8 ml, while the target was constantly covered with a liquid layer of 5.5 mm (Rehbock et al. 2013). The carrier stream contained sodium phosphate buffer (pH 7) at an ionic strength of 50 µM and was continuously pumped through the ablation chamber using a peristaltic pump (Ismatec ISM321C) at a constant flow rate of 11.5 mL/min. To avoid the inhibition of silver ion release, which was shown by Grade et al. (Grade et al. 2012), the generation of silver colloid was carried out without additional stabilizer. Characterization of the sample was done by UV-Vis spectroscopy (Thermo Scientific Evaluation 201), recording spectra from 200 – 900 nm in a quartz cuvette (volume 3.5 ml, path length 10 mm). A significant plasmon resonance of silver nanoparticles (Fig. 5.1a) was detected via UV-Vis measurement. A narrow surface Plasmon resonance peak at λ=393 nm was found, which indicates the formation of small spherical nanoparticles, while no agglomerates (scattering in the NIR regime) were detected (Menéndez-Manjon et al. 2013). Particle size and particle size distributions were analyzed via analytical disc centrifugation (CPS Instruments Disc Centrifuge DC24000) and TEM (Philips CM12). TEM micrographs confirmed the formation of spherical nanoparticles with mean particle diameters of 6 nm (distribution PDI 0.33, Fig. 5.1b).

Metal analyses of the water samples were carried out by electrothermal atomic absorption spectrometry (ET-AAS) using a Perkin-Elmer model 4110ZL atomic absorption spectrometer equipped with a Zeeman effect background correction system (Perkin-Elmer, Massachusetts, USA). Twenty microlitres of the samples were injected without addition of a modifier in a pyrolytic graphite furnace tube with L'vov platform by the autosampler AS 70 and run under optimized operating parameters with pyrolysis at 600°C and atomization at 1700°C. Calibration was performed by matrix

adapted calibration using water of the control group which was spiked with increasing amounts of Ag. Concentrations of Ag were calculated by fitting linear regression lines to the points defined by the spiked concentration values and the corresponding integrated peak areas in each sample. Correlation coefficients were always >0.99.

The concentration of ammonium, dissolved phosphate, nitrate and nitrite were determined using Spectroquant® test kits (Merck KGaA, Darmstadt, Germany). For the determination of the chloride concentration and the carbonate and total hardness titrimetric tests (Merck KGaA, Darmstadt, Germany) were used. The determination of chlorophyll a was performed according to DIN 38412-16:1985-12 (Deutsches Institut für Normung 1985). All analyses were performed before and after exposure.

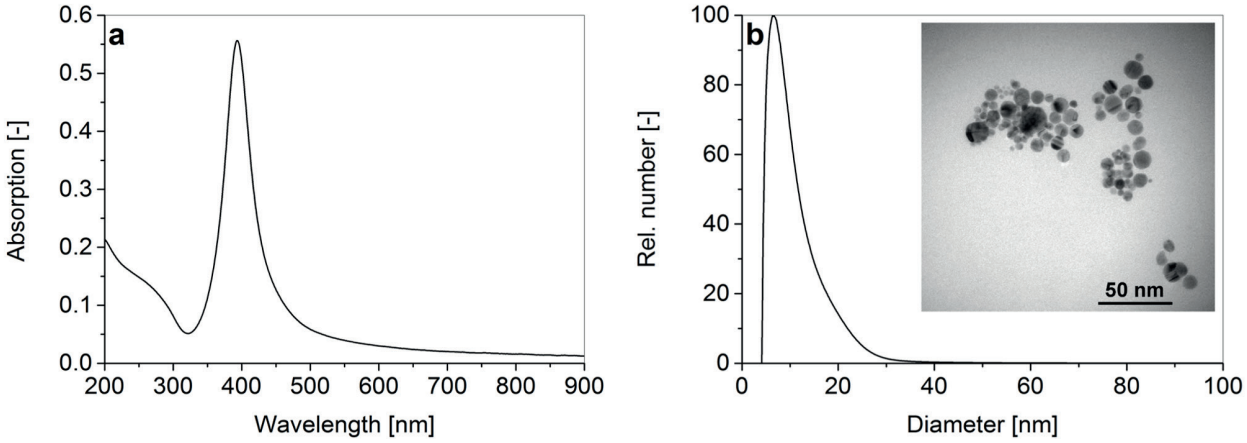

Figure 5.1. a) Absorption spectrum and b) size distribution data from analytical disc centrifugation of laser-generated silver nanoparticles with an exemplary TEM micrograph (insert).

5.2.3 Microscopic analyses

For the visual analysis of exposure effects, aliquots of the plankton samples were monitored by light microscopy. Therefore, protist communities were analyzed from lugol-fixed samples following established protocols by using Sedgewick Rafter chambers and Utermöhl chambers (Auinger et al. 2008; Medinger et al. 2010; Jost et al. 2010). Protists were analyzed at 200x magnification under an inverted microscope (Nikon Eclipse Ti); for small taxa 400x magnification was applied. Bacteria were counted from formaldehyde-preserved subsamples by means of epifluorescence microscopy (Nikon Eclipse 80i) after DAPI (4',6-diamidino-2-phenylindole) staining at 1000x magnification.

5.2.4 Metatranscriptome analyses

Following exposure, RNA was extracted from 0.2 µm polycarbonate filters using TRIzol (Life Technologies, Paisley, Scotland - modified). For lysis and homogenization, the cells were ground in liquid nitrogen in a mortar and pestle and incubated for 15 min with TRIzol. RNA, DNA, proteins

and lipids were separated in phases by adding chloroform and subsequent centrifugation. The RNA containing aqueous phase was transferred to a clean reaction tube and precipitated with isopropanol. The RNA pellet was washed three times with 75% ethanol and afterwards resuspended in DEPC water. Preparation of the cDNA library as well as sequencing was carried out using an Illumina HiSeq platform via a commercial service (Eurofins MWG GmbH, Ebersberg, Germany). After quality control, one amplified short insert cDNA library (poly-A enriched) with an insert size of 150-400bp was prepared per sample, individually indexed for sequencing on HiSeq 2000 and sequenced using the paired-end module. In the following steps the preprocessing of raw reads was performed and the trimmed and filtered reads were subsequently mapped to the Uniprot database (Magrane 2011) for annotation. Transcript quantification and differential gene expression analysis was conducted thereupon.

The quality control tool FastQC (http://www.bioinformatics.babraham.ac.uk/projects/ fastqc/) was used to analyze the quality distribution of the raw reads. Adapter sequences at the ends of the reads were removed using the cutadapt software (Martin 2011). Cutadapt was also used to trim bad quality bases with a quality score below 20 and discard reads with a length below 30 bp after trimming. The amount of rRNA in the samples was determined by mapping the reads to the SILVA rRNA database (Quast et al. 2013) using Bowtie2 (Langmead & Salzberg 2012), a short read aligner that maps sequencing reads efficiently by using a Burrows-Wheeler transformed index. The index was built from the downloaded SILVA database release 111. All quality trimmed reads were mapped as single-end reads against the index to determine the amount of remaining rRNA in each sample. Only the unmapped reads were used for further metatranscriptomic analysis.

All remaining reads were mapped to the UniProt Knowledgebase (Magrane 2011) at the amino acid level using RAPSearch2 (Zhao et al. 2011). RAPSearch2 uses a reduced amino acid alphabet for a very fast protein similarity search. We built the RAPSearch2 index from the downloaded UniProtKB (version May 2012) and mapped each single read of a read pair against the index. For each pair, the hit with the highest score was chosen as protein annotation. The mapping from UniProt IDs to KEGG Orthology IDs (KO-IDs) (Kanehisa & Goto 2000) is provided by the UniProt database, and the corresponding KO-IDs were assigned to the reads. Mapping results were summarized as a count matrix of 14100 KO-IDs x 12 samples with the number of counts for each gene (KO-ID) in each sample.

The count matrix was normalized using the “weighted trimmed mean of M-values” (TMM) method from the R package EdgeR (Robinson et al. 2009). This transforms the raw counts into counts per million (CPM) by normalizing for different sample sizes.

5.2.5 Statistical analysis

To explore sources of variation in the normalized count matrix, we used correspondence analysis (CA), as implemented in the R package vegan (Oksanen et al. 2013). CA is a technique that maps high-dimensional data onto a low-dimensional space (here two-dimensional plot) by singular value decomposition of the correspondence matrix. Each axis reveals relations between groups

of samples and data points. Samples and data points having high similarity with respect to this relation have similar coordinates in the plot. For reasons of clarity, only the samples were depicted in the plots.

For testing differential expression of genes between sample groups, the R package EdgeR (Robinson et al. 2009) was used. EdgeR models count data as negative binomial distributed. The gene-wise dispersion is estimated by conditional maximum likelihood, and an empirical Bayes procedure is used to shrink the dispersion by borrowing information between genes. An exact test is used to test for differential expression between groups with a model-based normalization. Generalized linear model (GLM) likelihood ratio tests are used to determine differential expression in complex experiments with multiple factors. The GLM likelihood ratio test was applied to the metatranscriptome count data for each gene to account for all identified sources of variation, yielding a p-value for each KO-ID and treatment group.

To visualize significant differences between groups, the p-values obtained from the statistical tests were plotted as a histogram. If no significant effect is present, the p-values follow a uniform distribution by definition. According to Pounds and Morris (Pounds & Morris 2003), a p-value distribution can be modeled by a beta-uniform mixture model, where the signal component is represented by the beta distribution and the null component by the uniform distribution. Thus a beta-uniform mixture model was fitted to the p-value distribution using the R package BioNet (Beisser et al. 2010).

The significantly differentially expressed genes (KO-IDs) from the GLM test were used subsequently in an enrichment analysis. Methods from the R package iSubpathwayMiner (Chunquan 2012) and own implementations were used to perform a hypergeometric test for each KEGG pathway. All mappings of genes to KEGG pathways and pathways with a significant enrichment were reported.

Table 5.1. Water parameters (mean ± SD)

	Sample date	pH[1]	Temp.[1] [°C]	Conductivity[1] [µS/cm]	Oxygen[1] [mg/L]	Carbonate hardness[2] [mmol/L]	Total hardness[2] [mg/L]	Chloride[2] [mg/L]	Nitrate[2] [mg/L]	Chlorophyll a[2] [µg/L]
Stock	09.07.2013	8.4	17.5	550	9.9	2.5 ± **0.1**	103 ± **6**	103 ± **1**	4.4 ± **1.4**	89 ± **8**
Control	10.07.2013	8.4 ± **0.1**	17.0 ± **0.1**	548 ± **4**	9.4 ± **0.2**	2.4 ± **0.1**	105 ± **5**	102 ± **3**	3.6 ± **1.1**	60 ± **3**
AgNP	10.07.2013	8.4 ± **0.1**	17.2 ± **0.1**	549 ± **4**	9.3 ± **0.1**	2.3 ± **0.1**	100 ± **5**	100 ± **3**	4.5 ± **1.2**	62 ± **10**
$AgNO_3$	10.07.2013	8.4 ± **0.1**	17.0 ± **0.1**	548 ± **3**	9.2 ± **0.1**	2.1 ± **0.1**	107 ± **6**	101 ± **3**	3.0 ± **0.3**	68 ± **13**

[1]: two measurements in three replicate tanks, n=6; except stock, n=2) [2]: three replicate tanks, n=3; except stock, n=3 of the same tank

5.3 RESULTS

5.3.1 Metal analyses and water chemistry

The Ag concentrations in the tank water of the different experimental groups were lower in the filtrated water samples compared to the corresponding unfiltrated samples (Fig. 5.2). In all tanks the Ag concentrations remained constant after 5 h of exposure, except for the filtrated AgNP samples which showed a slight decrease at the end of the exposure period. No differences were detected for physical and chemical water parameters between the experimental groups and their replicates. Mean ± SD values for each experimental group are summarized in table 5.1.

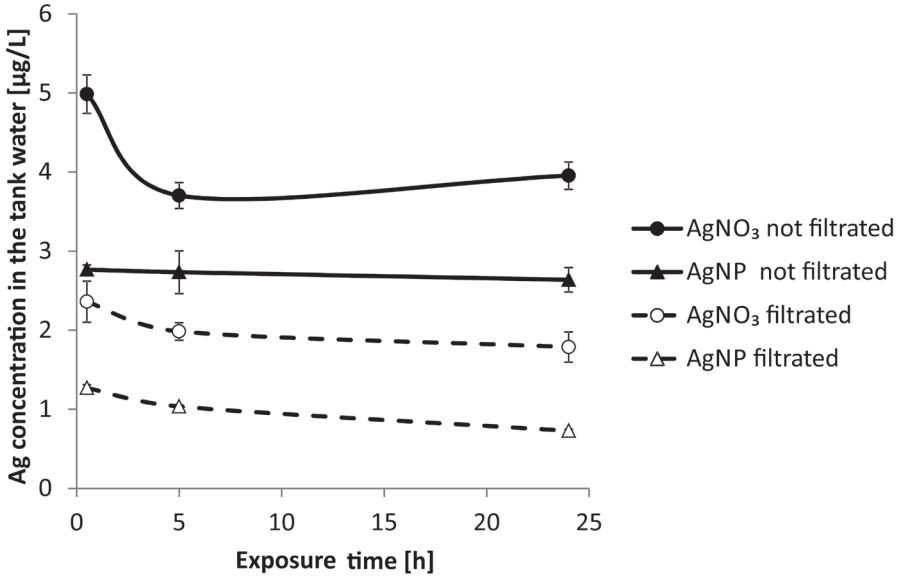

Figure 5.2. Ag concentrations in the tank water (mean ± SD of three replicate tanks) as determined by ET-AAS. Initially added Ag concentration was 5 µg/L. Ag concentrations in the control were below detection limit.

5.3.2 Organismic changes

Microscopic analyses revealed qualitative and quantitative changes within the plankton community (Fig. 5.3; Table S5.1). The phototrophic prokaryotes (mainly consisting of *Microcystis wesenbergii*) showed no significant changes between the two silver treatments (Fig. 5.3A). The cell counts of phototrophic prokaryotes were slightly higher in the silver treatments even though this was not significant. Nevertheless, this may indicate a similar effect of silver as observed for the eukaryotic phototrophs (see below). Discrepancies between the two silver exposure groups were documented in the cell counts of heterotrophic prokaryotes. The control and the AgNP treatment nearly had the same mean cell count ($11.2*10^6$ cells/ml and $10.8*10^6$ cells/ml respectively) whereas the mean cell count of $AgNO_3$ was only $7.5*10^6$ cells/ml (Fig. 5.3B). The dominating phototrophic eukaryotes in all treatments were Chlorophyta, mainly consisting of the groups *Scenedesmus/Desmodesmus* and *Pediastrum/Sorastrum* (see Table S5.1). Both key groups showed an increase in the mean cell counts in the two silver treatments (Fig. 5.3C). One taxon which was highly influenced by $AgNO_3$ is *Ceratium* sp. The cell counts showed only 13 individuals in the $AgNO_3$ treatment in comparison to 53 in the control group (Fig. 5.3E).

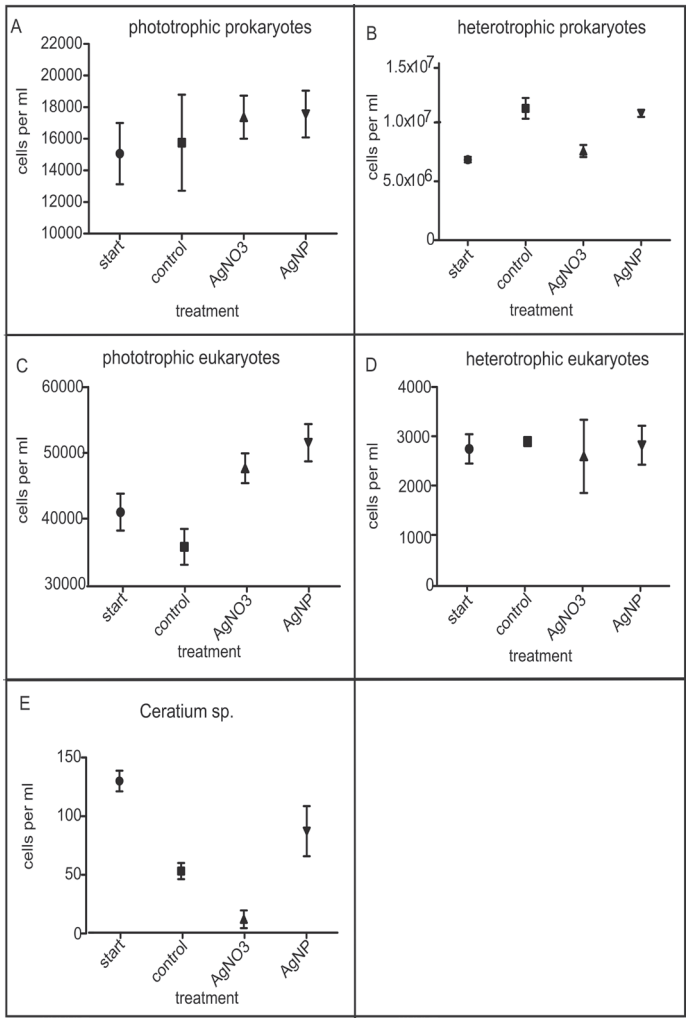

Figure 5.3. Results of the morphological cell counts. A) phototrophic prokaryotes, B) heterotrophic prokaryotes, C) phototrophic eukaryotes, D) heterotrophic eukaryotes, E) *Ceratium* sp.

5.3.3 Metatranscriptome sequencing results

From the 12 environmental RNA samples, between 5.6 and 15.2 million read pairs of 2x100 bp were obtained with good mean quality values of 35. After preprocessing (removal of sequencing adapters, low-quality parts and rRNA reads), we obtained 81% to 92% high-quality reads (Table 5.2), which were used for the metatranscriptome analysis.

Each remaining read was assigned a UniProt ID and a KEGG Orthology ID (KO-ID). The KO-ID converts the species-specific protein annotation from the UniProt database into ortholog groups for all proteins and functional RNAs present in the metatranscriptome samples, independent of the species. This resulted in a gene count matrix of 14100 KO-IDs × 12 samples, where each entry in the table corresponds to the number of times this KO-ID was identified in the sample.

Table 5.2. Summary of sequencing results for each sample. Summary of the yield in Mbp, the number of raw read pairs, the percentage of reads with a quality value larger than 30 (%Q30), the mean quality value, the number of reads remaining after trimming in forward (R1) and backward (R2) direction, and the number and percentage of remaining high-quality (HQ) read pairs after rRNA removal.

Sample	Yield (Mbp)	#Reads	%Q30	Mean Q	#Reads R1 trimmed	#Reads R2 trimmed	#Reads mRNA	%HQ mRNA
RAW-1	2,588	12,939,060	90.91	35,06	12,580,021	12,191,810	11,200,104	86.56
RAW-2	2,596	12,982,356	90.76	35,03	12,636,705	12,207,824	11,850,143	91.28
RAW-3	3,039	15,196,013	91.15	35,17	14,984,485	14,462,616	14,047,866	92.44
KO-1	2,541	12,703,316	90.85	35,10	12,279,020	11,915,989	11,642,172	91.65
KO-2	1,986	9,931,991	89.56	34,64	9,508,355	9,105,627	8,729,591	87.89
KO-3	2,087	10,432,882	91.00	35,17	10,101,010	9,785,979	9,566,241	91.69
NO3-1	2,277	11,386,953	91.09	35,16	11,046,068	10,717,279	10,463,186	91.89
NO3-2	2,227	11,136,125	89.81	34,72	10,660,890	10,287,130	9,788,102	87.90
NO3-3	1,873	9,365,969	90.25	34,88	8,914,752	8,654,549	8,440,827	90.12
NP-1	1,813	9,066,148	91.17	35,24	8,900,984	8,559,029	8,313,082	91.69
NP-2	2,911	14,554,562	91.32	35,26	14,229,864	13,764,018	13,174,110	90.52
NP-3	1,125	5,623,772	84.55	32,92	4,818,956	4,660,164	4,565,203	81.18

5.3.4 Statistical analysis results

We analyzed and compared normalized counts between the four sample groups (raw samples "RAW", control "KO", after treatment with silver nanoparticles "NP", and with silver ions "NO_3") and between the three replicate groups (suffixes "-1", "-2", "-3"). Figure 5.4 shows a clustering of samples from a correspondence analysis (CA) of the normalized count matrix. On the first two principal axes (CA1, CA2), a clustering due to replicate groups is predominantly visible (Fig. 5.4A). The four different treatment groups cluster according to the second and third axis (CA2, CA3; Fig. 5.4B).

After statistical testing for gene-wise differential expression between groups, accounting for the batch effect using a GLM with a multifactor design, we obtain p-values for each gene (KO-ID) and each pair of treatment versus control groups. Figure 5.5 depicts the obtained p-value distributions. For the test between control and $AgNO_3$ groups (Fig. 5.5A) we observed a deviation from the uniform p-value distribution induced by the null hypothesis. The deviation emerges from an enrichment of significant p-values outlined by the beta component in a fitted beta-uniform mixture model (BUM model). The beta component of a BUM model describes the signal in the p-value distribution, the amount of significant differences between the two groups, whereas the uniform component arises from the null hypothesis. In contrast, the test between control and AgNP showed no significant results (Fig. 5.5B). The p-value distribution contained no signal in the NP vs. control tests, as seen by the lack of a beta component in the BUM model. Thus, there are significant differences between the samples treated with $AgNO_3$ compared to the controls, but no significant differences in the samples treated with AgNP compared to the control samples.

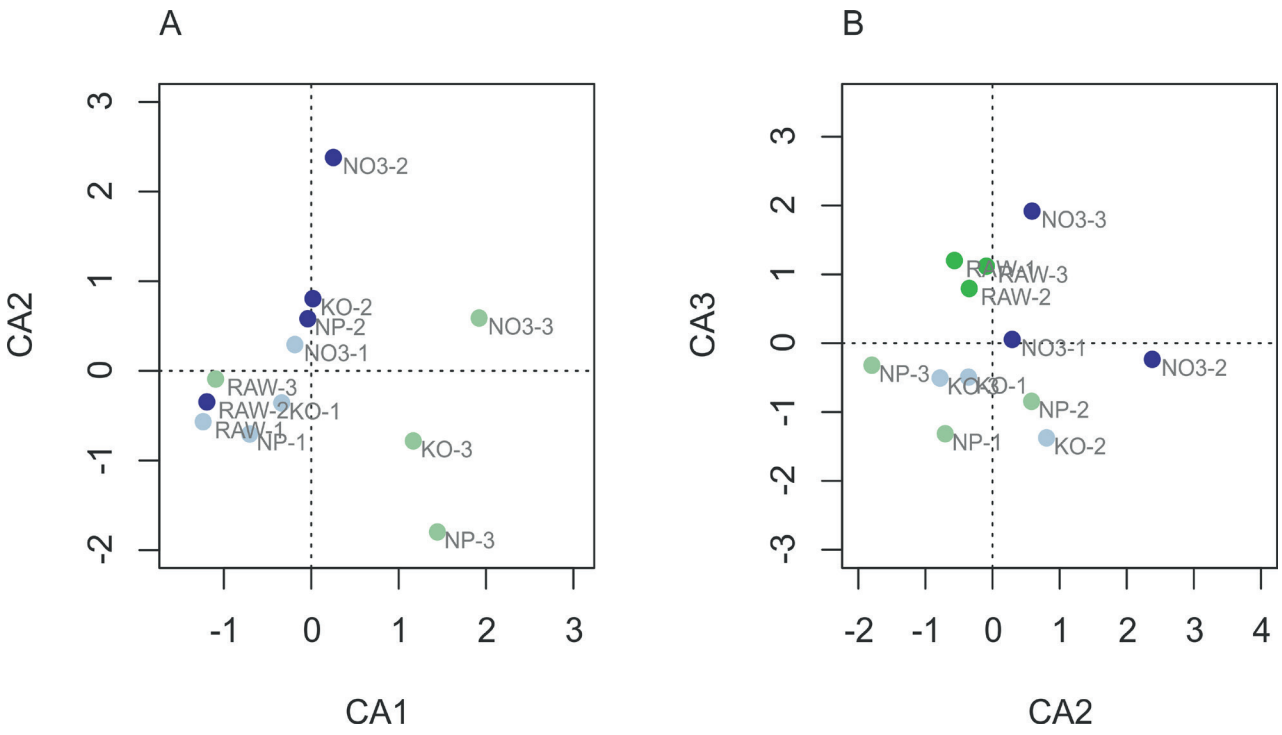

Figure 5.4. Correspondence analysis of normalized count matrix. Location of samples (RAW, KO, NP, NO3), each in three replicates (-1, -2, -3), is shown after correspondence analysis on the first two principal axes (A: axes CA1, CA2) and on the second and third principal axis (B: axes CA2, CA3). Roughly, the first two axes cluster according to replicate group, while the second and third axes cluster according to treatment.

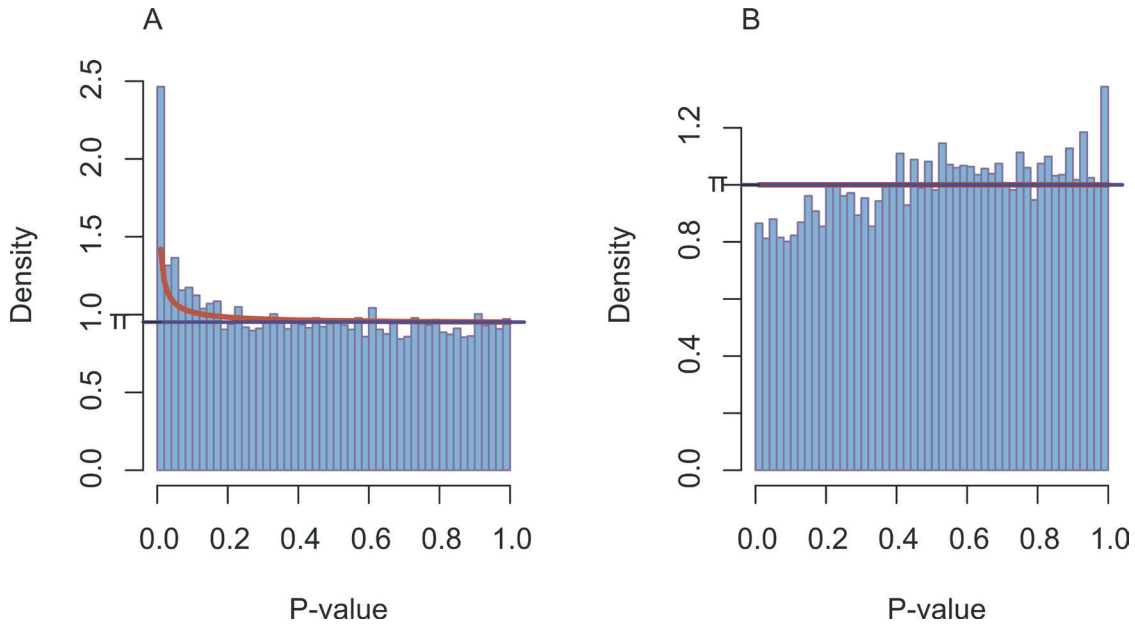

Figure 5.5. Histogram of p-values after gene-wise tests for differential expression between control vs. $AgNO_3$ (NO3) group (A) and between control vs. silver nanoparticle (NP) group. A beta-uniform mixture model is fitted to the p-value distribution, where the uniform distribution (blue) describes the null component and the beta distribution the enrichment of low p-values (red).

Statistical analysis yielded 126 genes (KO-IDs) with significant differential expression with a false discovery rate (FDR) < 0.05 between the control (KO) and $AgNO_3$ (NO3) groups. The normalized count matrix for the significant genes is visualized as a bubbleplot in Figure 5.6, where the counts for each gene are displayed as filled circles. Figure 5.6A depicts all genes that cannot be annotated to a KEGG pathway, while in Figure 5.6B up to two pathways are specified for each gene. The 126 significant genes were used for a KEGG pathway enrichment analysis. Three pathways showed significant results with a FDR below 0.05. The enriched pathways were: photosynthesis - antenna proteins, carbon fixation in photosynthetic organisms and photosynthesis (Figure 5.6 and Table 5.3).

Data Availability

The raw sequence data in FASTQ format will be made available at the Short Read Archive (SRA) under NCBI accession number SRP040767.

Table 5.3. Significantly enriched KEGG pathways. Pathways are shown along with their pathway ID, pathway name, the ratio of genes mapped to the pathway from all selected genes and the ratio of genes belonging to the pathway from all genes in all pathways, p-value for the enrichment and FDR.

pathwayId	pathwayName	annMoleculeRatio	annBgRatio	PValue	FDR
path:00196	Photosynthesis - antenna proteins	6/126	28/14039	1,49E-07	4,46E-05
path:00710	Carbon fixation in photosynthetic organisms	5/126	35/14039	1,41E-05	1,72E-03
path:00195	Photosynthesis	6/126	61/14039	1,72E-05	1,72E-03

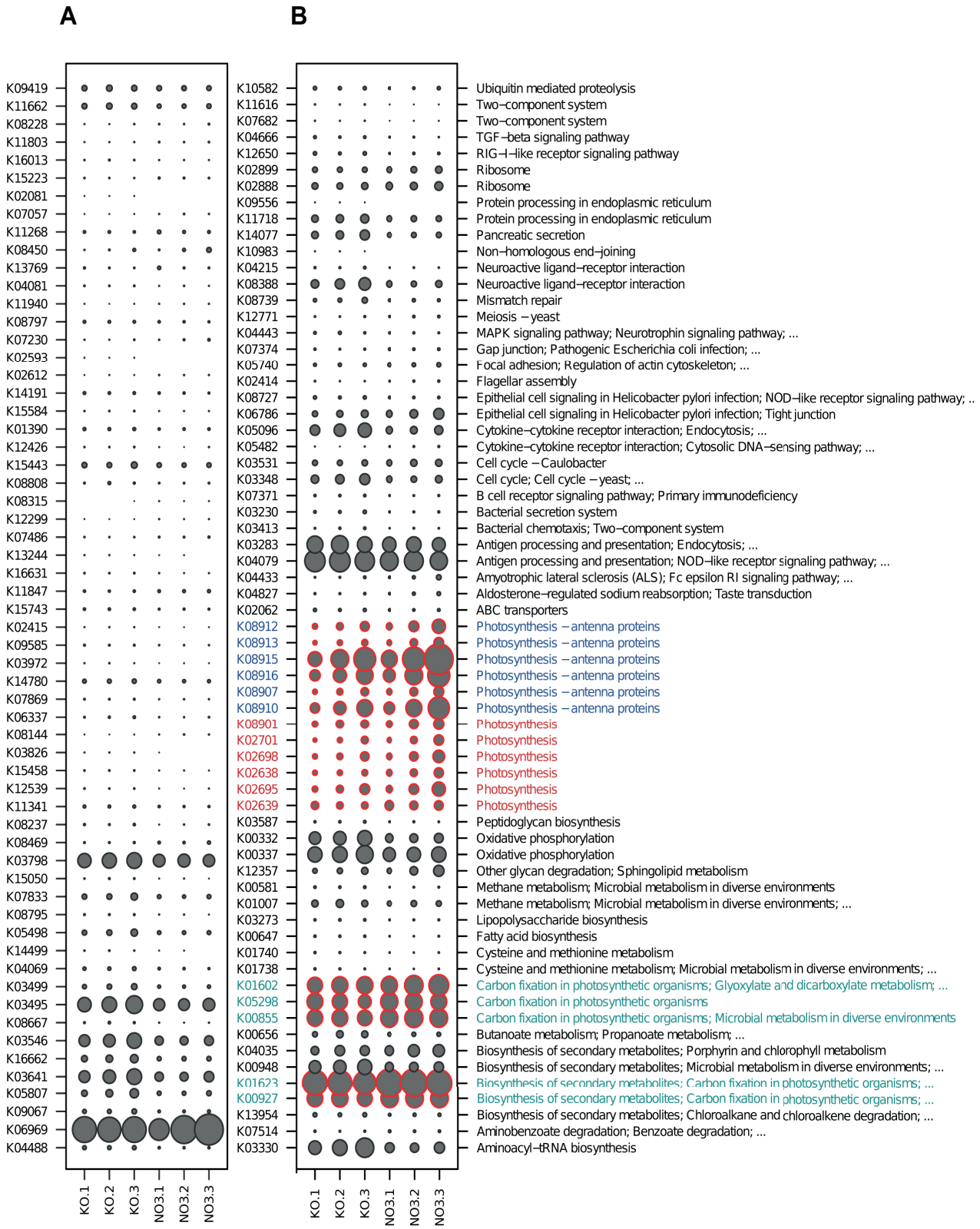

Figure 5.6. Bubbleplot of count matrix for significant genes. A: genes that do not have an annotated KEGG pathway; B: genes with annotated pathways. Enriched pathways (Table 3) are colored: photosynthesis - antenna proteins: blue, carbon fixation in photosynthetic organisms: turquoise; photosynthesis: red).

5.4 DISCUSSION

In the present study, we analyzed effects of silver applied as $AgNO_3$ and AgNP on the composition and metatranscriptome of a natural community of aquatic protists from a eutrophic pond in a 24 h laboratory exposure. To our knowledge, this is the first approach assessing the impact of silver on ecological functions and shifts in species composition on transcriptome level. The silver exposure concentration applied in this experiment was 5 µg/L as either $AgNO_3$ or AgNP. According to pre-trials, higher concentrations were found to alter the planktonic community in a way that was visible microscopically. For example, all eukaryotic organisms were dead after 24 h exposure to a silver concentration of 100 µg/L using $AgNO_3$. Even at a silver concentration of 10 µg/L ($AgNO_3$) fewer flagellates and dinoflagellates survived a one-day exposure compared to unexposed controls. Thus, we opted for a silver concentration of 5 µg/L to guarantee survival of most protist taxa, but similarly to induce effects in the community. This concentration is close to environmentally relevant data which range between 0.01 to 65 µg/L for freshwater ecosystems in Germany (Hund-Rinke et al. 2008). At all concentrations during the pre-trials AgNP exposure resulted in less pronounced effects than ionic silver, which is in line with the assumption that the toxicity and antimicrobial effect of AgNP is considered to be caused by bioavailable Ag^+ ions released from nanoparticles by oxidation (Kumar et al. 2005). Ion leaching from silver nanoparticles is a process which is influenced by factors such as storage conditions (Kittler et al. 2010), electrochemistry (influence of ions) (Hahn et al. 2011), medium additives (like proteins) (Grade et al. 2012), and chemicals (Tiedemann et al. 2014; Grade et al. 2013). Simultaneous interactions of these factors, which cannot be analyzed under controlled analytical conditions at once, provide complex kinetics of ion release. In the present study at least the role of biomolecules cannot be excluded completely. This means that not all released ions are bioavailable or toxic. For example, in the case of Ni-alloyed nanoparticles it was shown by Hahn et al. that high ion release from nanoparticles does not correlate with higher toxicity if the ions are bound in complexes by albumin or citrate molecules (Hahn et al. 2012). Further, it was shown that citrate deactivates silver ion release more than albumin (Grade et al. 2013). Since all experiments in the present study were conducted in the same way and we have not registered any effects of the nanoparticles used, no detailed silver leaching experiments from Ag nanoparticles were performed. Generally, toxicity of $AgNO_3$ is higher compared to AgNP (Chernousova & Epple 2013; Fabrega et al. 2011) with toxic concentrations and inhibitory concentrations being in a range of 0.1 to 20 mg/L Ag^+ for prokaryotes. For eukaryotic cells toxic concentrations of silver ions range between 1 to 10 mg/L and for silver nanoparticles between 10 to 100 mg/L (Chernousova & Epple 2013). However, it has to be stressed that only little research has been conducted on toxicity of AgNPs to planktonic taxa (Fabrega et al. 2011). Recently, Ribeiro et al. (Ribeiro et al. 2014) described impaired reproduction of *Daphnia magna* at 1 µg AgNP/L and 0.5 µg/L (Ag^+) after 21 d of exposure, and feeding rates being affected at 10 µg/L AgNP and 2 µg/L Ag^+ after 24 h exposure. Nevertheless, AgNP can have toxic effects that are higher than expected according to the concentration of dissolved ionic silver (Navarro et al. 2008), probably due to additional effects of particles and agglomerations on cell membranes (Lapresta-Fernández et al. 2012), depending on various factors like media used (Oukarroum et al. 2012), organic molecules, light conditions and particle size or NP coating (Shi

et al. 2012; Liu & Hurt 2010; Liu et al. 2010). In the present experiment we detected a toxic effect on protists based on cell counts for the $AgNO_3$ treatments (5 µg/L) and an effect on their gene regulation which is in accordance with the findings of other studies (Piccapietra et al. 2012). Effects seem to be taxon-specific, e.g. the presence of a cell wall in a protist species seems to buffer the negative effects of both AgNP and $AgNO_3$ in comparison to those protists lacking one (Piccapietra et al. 2012; Oukarroum et al. 2012). In our study the effects were also clearly species-specific with some taxa such as *Ceratium* being affected stronger than others. However, on the community level we did not observe significant shifts, i.e. the community composition based on higher taxonomic ranks (orders, phyla) remained the same after one-day exposure. This may change following longer exposure periods as it could be shown in a study using a marine mesocosm where bioconcentration and trophic transfer of silver occurred among different taxonomic groups (Cleveland et al. 2012).

The AgNP treatments also showed no significant differences in transcriptomic response compared to the control. It may be assumed that only low concentrations of Ag^+ were available to cause effects within the exposure period. On the other hand, the same concentration of $AgNO_3$ showed significant differences in transcriptomic response compared to the control. To avoid a bias in the functional analysis due to the overexpression of a single gene, a misannotation or a false positive significance, we analyzed the changes between the silver treated samples and controls on pathway level. Pathways were reported which were significantly enriched in deregulated genes and therefore the effect of a single gene was compensated. Three pathways were significantly deregulated in the presence of ionic silver: photosynthesis - antenna proteins, carbon fixation in photosynthetic organisms and photosynthesis. The effects of Ag^+ ions on several organisms based on differences in transcribed genes were analyzed in previous studies (Simon et al. 2013) where photosynthesis was also amongst the most strongly affected pathways. However, genes with photosynthesis-related function were mostly down-regulated after exposure to silver in previous studies (Simon et al. 2013), but up-regulated in our study. In our experiment all samples were dominated by a bloom of Chlorophytes and no significant change of the Chlorophytes and heterotrophic organisms in the different treatments was observed. In our view, the most likely explanation for the deviating results concerning photosynthesis-related genes are the different concentrations of silver applied in the respective studies. For example, Simon et al. (Simon et al. 2013) exposed *Chlamydomonas reinhardtii* to a AgNP concentration of 1 mg/L which is 200 times the concentration of our experiment. Silver ions are probably taken up through the cell membrane via the Cu (I) transporter (Lee et al. 2005), due to similar properties of both metals. Therefore, Cu^+ and Ag^+ might cause a similar cellular response as the cells might not discriminate between the effects of these two metals. Copper ions are known to bind strongly to chlorophyll (Küpper et al. 2002; Scotter et al. 2005) and thus the efficiency of photosynthesis should decrease in the presence of Cu ions. To compensate such an effect, expression of genes relevant for photosynthesis will be up-regulated as we found for $AgNO_3$ in our experiments. Further evidence for such silver effects in the literature are scarce, but Watanabe & Kobayashi (Watanabe & Kobayashi 1988) reported a similarly strong binding of silver to chlorophyll, which may cause an up-regulation of genes related to photosynthesis in the presence of sublethal levels of silver. At higher silver concentrations direct toxic effects on

metabolic pathways may be the dominating effects which thus decrease the photosynthesis rate. Such a reduction in photosynthetic yield was reported by Navarro et al. (Navarro et al. 2008) in *Chlamydomonas reinhardtii* exposed to $AgNO_3$ (EC50: 188nM after 1 h) and AgNP (EC50: 3300M after 1 h) and by Wang et al. (Wang et al. 2012) in *Raphidocelis subcapitata* exposed to $AgNO_3$ (EC 50: 290 nM) and AgNP (EC50: 1112.63 µM) after 4.5 h.

REMARKS

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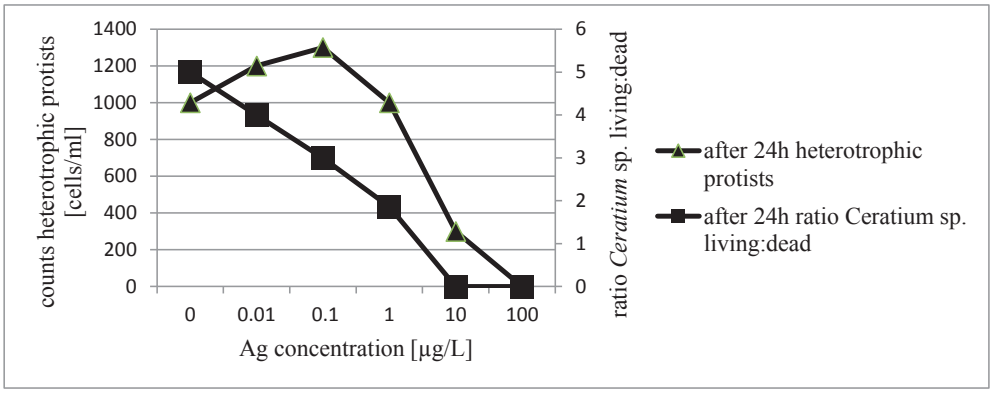
Boenigk, J., Beisser, D., Zimmermann, S., Bock, C., Jakobi, J., Grabner, D., Großmann, L., Rahmann, S., Barcikowski, S., Sures, B. 2014. Effects of silver nitrate and silver nanoparticles on a planktonic community: general trends after short-term exposure. *PLoSONE*, 9:e107092.

As co-author, I was involved in the conceptual planning of the experiment, the carrying out of the experiment and the analysis of the results. I conducted the RNA-isolation and wrote parts of the introduction, methods and discussion of the paper.

Supplementary figure S5.1. Results of the pre-test experiments. In a preliminary set of experiments we exposed a plankton community from a eutrophic pond at the campus Essen of the University Duisburg-Essen, Germany, to different concentrations of $AgNO_3$, as a basis for selection of silver concentrations to be used in the main experiment. For the pre-tests, approximately 30 mL of pond water were transferred into cell culture flasks and exposed to $AgNO_3$ (0 µg/L, 0.01 µg/L, 0.1µg/L, 1 µg/L, 10 µg/L, 100 µg/L) under the same experimental settings as described for the main experiment. After 24h sub-samples were taken from the cell culture flasks and checked for the occurrence of living cells under the light microscope. Since most dead protist cells lyse within minutes to hours, only the living cells were counted. The pretest focused on the abundance of heterotrophic protists. In addition we used one phototrophic dinoflagellate, i.e. *Ceratium* sp.: *Ceratium* sp. seemed to be a sensitive indicator organism when exposed to silver. Further, for this species the enumeration of living and dead cells was possible. Therefore, the ratio of living to dead *Ceratium* sp. individuals were counted as well. Analysis of survival of heterotrophic protists as well as of the ratio living: dead *Ceratium* sp. showed EC_{50}-values ranging between 1 to 10 µg/L. Accordingly, we have decided to apply an Ag concentration of 5 µg/L.



Supplementary table S5.1. Microscopic cell counts of planktonic organisms

	(cells/ml)	start (repl. 1)	(repl.2)	(repl.3)	control (repl.1)	(repl.2)	(repl.3)	AgNO3 (repl.1)	(repl.2)	(repl.3)	AgNP (repl.1)	(repl.2)	(repl.3)
Amoebozoa		**0,00**	**0,00**	**0,00**	**0,00**	**2,86**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**
(heterotrophic)	Amoeba	0,00	0,00	0,00	0,00	2,86	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Bacilariophyta		**533,33**	**540,00**	**410,00**	**372,50**	**371,43**	**430,00**	**633,33**	**488,57**	**596,67**	**471,04**	**653,33**	**565,71**
(phototrophic)	Amphora sp.	73,33	93,33	63,33	2,50	8,57	23,33	10,00	8,57	10,00	27,03	20,00	25,71
	Asterionella sp.	80,00	40,00	90,00	67,50	65,71	83,33	146,67	45,71	43,33	30,89	66,67	45,71
	Cyclotella sp.	310,00	316,67	210,00	202,50	191,43	223,33	350,00	322,86	323,33	247,10	340,00	365,71
	Melosira sp.	23,33	40,00	20,00	52,50	48,57	33,33	40,00	54,29	133,33	54,05	80,00	57,14
	Nitschia sp.	46,67	50,00	26,67	47,50	57,14	66,67	86,67	57,14	86,67	111,97	146,67	71,43
Charophyta		**73,33**	**120,00**	**40,00**	**57,50**	**48,57**	**106,67**	**73,33**	**102,86**	**136,67**	**104,25**	**173,33**	**120,00**
(phototrophic)	Staurastrum sp.	73,33	120,00	40,00	57,50	48,57	106,67	73,33	102,86	136,67	104,25	173,33	120,00
Chlorophyta		**36895,00**	**40404,17**	**42580,83**	**32467,50**	**34590,00**	**37722,50**	**48976,67**	**46650,71**	**44481,67**	**52825,82**	**47097,50**	**51334,29**
(phototrophic)	Scenedesmus/ Desmodesmus	13575,00	13087,50	12737,50	9005,00	7750,00	13137,50	13100,00	12630,71	12178,33	14137,50	13444,17	14650,00
	Pediastrum/ Sorastrum	21833,33	26016,67	28283,33	22350,00	26100,00	23725,00	34700,00	32500,00	31566,67	37225,00	31250,00	35750,00
	Pandorina sp.	853,33	840,00	1180,00	735,00	428,57	466,67	726,67	937,14	170,00	814,67	1593,33	500,00
	Coelastrum sp.	276,67	180,00	66,67	112,50	102,86	106,67	13,33	142,86	120,00	239,38	236,67	114,29
	Oocystaceae	80,00	40,00	40,00	35,00	11,43	80,00	53,33	97,14	106,67	61,78	146,67	45,71
	Pteromonas sp.	210,00	180,00	193,33	130,00	117,14	166,67	183,33	160,00	180,00	146,72	176,67	205,71
	Tetratsrum sp.	66,67	40,00	80,00	100,00	80,00	40,00	200,00	182,86	160,00	200,77	250,00	68,57
	Ankistrodesmus sp.	0,00	20,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Ciliophora		**68,00**	**136,00**	**90,00**	**96,33**	**90,29**	**111,67**	**106,00**	**83,10**	**115,67**	**186,08**	**152,33**	**90,29**
(heterotrophic)	undetermined ciliat 1	53,33	116,67	73,33	65,00	74,29	93,33	80,00	68,57	96,67	142,86	133,33	74,29
	undetermined ciliat 2	0,00	6,67	6,67	20,00	0,00	3,33	10,00	2,86	6,67	30,89	3,33	0,00
	Vorticella sp.	14,67	12,67	10,00	11,33	16,00	15,00	16,00	11,67	12,33	12,33	15,67	16,00
Crustacea		**0,00**	**0,00**	**0,00**	**0,00**	**0,00**	**3,33**	**0,00**	**2,86**	**0,00**	**0,00**	**0,00**	**2,86**
(heterotrophic)	Bosnia sp.	0,00	0,00	0,00	0,00	0,00	3,33	0,00	2,86	0,00	0,00	0,00	2,86
Cryptophyta		**80,00**	**43,33**	**56,67**	**52,50**	**42,86**	**30,00**	**20,00**	**5,71**	**10,00**	**84,94**	**80,00**	**34,29**
(phototrophic)	Cryptomonas sp.	80,00	43,33	56,67	52,50	42,86	30,00	20,00	5,71	10,00	84,94	80,00	34,29
Cyanobacteria		**13800,00**	**17300,00**	**14100,00**	**16625,00**	**12375,00**	**18266,67**	**17850,00**	**18425,00**	**15833,33**	**17433,33**	**19100,00**	**16150,00**
(phototrophic)	Microcystis wesenbergii	13800,00	17300,00	14100,00	16625,00	12375,00	18266,67	17850,00	18425,00	15833,33	17433,33	19100,00	16150,00
Dinophyta		**300,00**	**326,67**	**296,67**	**170,00**	**180,00**	**236,67**	**233,33**	**200,00**	**175,00**	**277,99**	**310,00**	**308,57**
(mixotrophic)	Gymnodinium sp.	163,33	206,67	163,33	125,00	122,86	180,00	213,33	188,57	170,00	173,75	246,67	214,29
(phototrophic)	Ceratium sp.	136,67	120,00	133,33	45,00	57,14	56,67	20,00	11,43	5,00	104,25	63,33	94,29
Euglenophyta		**60,00**	**53,33**	**106,67**	**55,00**	**71,43**	**70,00**	**43,33**	**48,57**	**53,33**	**61,78**	**30,00**	**37,14**
(phototrophic)	Euglena sp.	6,67	3,33	16,67	12,50	2,86	6,67	6,67	11,43	6,67	7,72	10,00	2,86
	Monomorphina sp.	3,33	0,00	13,33	2,50	2,86	3,33	10,00	5,71	10,00	3,86	3,33	2,86
	Phacus sp.	0,00	0,00	3,33	2,50	5,71	0,00	6,67	0,00	3,33	23,17	10,00	0,00
	Trachelomonas	50,00	50,00	73,33	37,50	60,00	60,00	20,00	31,43	33,33	27,03	6,67	31,43
Rotifera		**4,00**	**3,33**	**4,33**	**7,00**	**4,67**	**4,67**	**4,33**	**7,86**	**3,33**	**4,67**	**6,00**	**4,67**
(heterotrophic)	Keratella sp.	4,00	3,33	4,33	7,00	4,67	4,67	4,33	5,00	3,33	4,67	6,00	4,67
	Undetermined Rotifera	0,00	0,00	0,00	0,00	0,00	0,00	0,00	2,86	0,00	0,00	0,00	0,00
heterotrophic Flagellates		**2985,06**	**2327,85**	**2592,37**	**2878,18**	**2748,15**	**2715,82**	**2078,43**	**3353,38**	**2008,18**	**2418,78**	**3089,63**	**2463,95**
	heterotrophic Flagellates	2985,06	2327,85	2592,37	2878,18	2748,15	2715,82	2078,43	3353,38	2008,18	2418,78	3089,63	2463,95
Sum		**54798,73**	**61254,68**	**60277,54**	**52781,51**	**50525,25**	**59697,99**	**70018,77**	**69368,62**	**63413,84**	**73868,68**	**70692,13**	**71111,76**

6) TRADE-OFF BETWEEN TAXON DIVERSITY AND FUNCTIONAL DIVERSITY IN EUROPEAN LAKE ECOSYSTEMS

ABSTRACT

Inferring ecosystem functioning and ecosystem services through inspections of the species inventory is a major aspect of ecological field studies. However, ecosystem functions are often stable despite considerable species turn-over. Based on metatranscriptome analyses we analysed a so far unequalled freshwater dataset comprising 21 mainland European freshwater lakes from the Sierra Nevada (Spain) to the Carpathian Mountains (Romania) and from northern Germany to the Apennines (Italy) covering an altitudinal range from 38 m above sea level (asl) to 3110m asl. The dominant taxa were Chlorophyta and streptophytic algae, Ciliophora, Bacillariophyta and Chrysophyta. Beyond shifts in community composition, the metatranscriptomic analyses provided insights into the regulation of metabolic pathways and the pathway-specific share of distinct taxa on the ecosystem level. Ecosystem functioning in terms of active metabolic pathways was surprisingly stable, in contrast to strong shifts in taxon inventory. The dominant metabolic pathways in terms of the fraction of expressed sequences in the cDNA libraries were affiliated with primary metabolism, specifically oxidative phosphorylation, photosynthesis and the TCA cycle. Our analyses indicate that community composition is a good first proxy for the analysis of ecosystem functions. However, differential gene regulation modifies the relative importance of taxa in distinct pathways. Whereas taxon composition varies considerably between lakes, the relative importance of distinct metabolic pathways is much more stable indicating that ecosystem functioning is buffered against shifts in taxon inventory through a functional redundancy of taxa.

6.1 INTRODUCTION

One major aim of ecological studies is to infer ecosystem function and ecosystem health from the organisms present in an ecosystem. Usually the species inventory, at least the species inventory of distinct target groups, is used as an indicator of ecosystem health status and in quality assessments. Among widely used applications of species inventories is the estimation of trophic state in lakes and of saprobic state in streams and flood plains (Dziock et al. 2006; Kahn & Ansari 2005). Trophic state denotes the extent of primary production within a system whereas saprobic state denotes the extent of respiration / decomposition within a system. Species inventory is one of the most commonly used indicators to estimate these factors. Beyond these examples, species inventory and community composition is widely used to infer functional aspects of ecosystems. Aspects of eutrophication, acidification, organic pollution, self-purification and resource consumption are just few selected examples (Schmidt-Kloiber & Hering 2015; Cardinale et al. 2002).

Currently, we are facing a dramatic decline in global biodiversity (Boenigk et al. 2015; Cardinale et al. 2012) which is regarded as the presumable onset of the sixth major mass extinction on

planet Earth within the Phanerozoic eon (Barnosky et al. 2011; Palfy 2005). However, ecosystems are – despite strong species turn-over – often surprisingly stable with respect to their function – presumably due to a functional redundancy of taxa (Cardinale et al. 2007). As ecosystem functions and element cycling are largely driven by microbes (van der Heijden et al. 2008) the predominant stability has been attributed to a high functional redundancy between different microbial taxa (Allison & Martiny 2008) and the ease of dispersal of small-sized organisms as proposed by the 'everything is everywhere hypothesis' (Bass & Boenigk 2011 and references therein). Nevertheless, biodiversity loss has mainly been studied for macroorganisms (Flynn et al. 2011; Fugère et al. 2012; Jabiol et al. 2013) whereas the extent of biodiversity loss is still largely unknown for microbial taxa. We are just beginning to understand the effect of changes in biodiversity on ecosystem functioning: A relatively high species richness in, at first glance, uniform or homogeneous environments such as the pelagic realm of the ocean and lakes have stimulated scientific curiosity since early on (Margalef 1978). This high species richness in planktonic systems was formulated as 'the plankton paradox' (Hutchinson 1961) highlighting the conflict between the theoretical conceptual basis of species richness, basically the niche concept, and the observed high diversity despite lacking habitat differentiation into 'niches'. The same phenomenon is now reconsidered in the light of functional redundancy (Allison & Martiny 2008; van der Putten et al. 2007; Fonseca & Ganada 2001): Different microbial taxa with overlapping strategies occurring in the same habitat may take over specific functions under different environmental conditions and thereby help stabilising ecosystem functions in changing environments.

Proteomics, transcriptomics and genomics now allow for a more direct approach to such functional aspects (Daniel 2005; Muller et al. 2014; Aylward et al. 2015). Specifically the advent of high-throughput sequencing technologies makes the analysis on the level of complex biological communities possible.

Community analyses on the one hand, specifically analyses of microbial communities, rely increasingly on molecular approaches (Head et al. 1998; Franzosa et al. 2015). The most commonly used marker gene in such diversity studies is the SSU rRNA gene. Recent studies increasingly addressed diversity based on the rRNA itself rather than on the rRNA gene (Trousselier et al. 2002; Logares et al. 2014). The idea behind analysing RNA in contrast to DNA (the rRNA gene) is based on the different stability of RNA and DNA. Whereas DNA is relatively stable, RNA is easily degraded by hydrolytic cleavage of the RNA chain due to the presence of a hydroxyl-group at the 2' position of the ribose. The comparatively stable DNA is therefore an indicator of the actual or recent presence of taxa in the system whereas RNA is a more direct measure of presence and activity of specific organisms. Thus, rRNA analyses based on cDNA library sequence analysis have been used in order to identify the metabolically active fraction of microbial communities (Mills et al. 2012; Chambouvet et al. 2014; Lejzerowicz et al. 2013).

On the other hand – beyond the potential of rRNA analyses which indicate the relative importance of the metabolically active cells – metatranscriptome analyses (mRNA analyses) provide insights into the regulation of protein-coding genes (Boenigk et al. 2014; Frias-Lopez et al. 2008; McCarren

et al. 2010). Such analyses, focusing on functional genes and metabolic pathways, offer a more direct approach to ecosystem functions. Taxonomic assignment of these mRNA sequences further allows for the identification of key taxa or key taxonomic groups expressing the respective genes. Metatranscriptome analyses therefore have, in principle, the potential for addressing shifts in ecosystem functions as well as for indicating key taxa behind the distinct functions. Sequencing costs and taxon representation in sequence databases so far hindered the exploitation of the full potential of metatranscriptomic approaches in the field. With the ongoing reduction of sequencing costs and the strong increase of reference genomes and transcriptomes in databases these restrictions are currently eroding rapidly (Cooper et al. 2014).

The relative contribution of distinct genes from distinct organisms in expression profiles largely depends on two factors: On the one hand, shifts in the abundance of organisms and their general activity may increase or decrease the relative fraction of genes in the library. On the other hand, an up- or down-regulation of distinct genes or pathways affects gene abundance in mRNA libraries. Both factors are likely involved, but their relative contribution is largely unknown. In consequence, it remains unclear to what extent the presence and general activity of distinct organisms is a suitable proxy for the expression of distinct genes and metabolic pathways. This aspect can, however, be tackled by comparative analysis of rRNA profiles and mRNA profiles.

In a joint analysis of protistan species richness and community function in lake ecosystems, we analysed rRNA and mRNA profiles from 21 lakes across Europe covering a trophic gradient from oligotrophic to eutrophic and an altitudinal gradient from 38m to 3110m above sea level (asl.). Quantitative shifts in taxon composition as well as taxon-specific differential gene expression should affect overall expression profiles. Here, we investigated to what extent these two factors contribute to overall gene expression across the different lakes. Deviations between the patterns of taxon abundance and taxon-specific gene expression would indicate differential regulation of distinct genes and pathways within taxa. As different taxa or taxonomic groups may play a differential role in different systems, we hypothesize that the concerted analysis of taxon diversity and functional diversity will either reveal patterns of functional redundancy of taxa across different systems or distinctly different gene expression profiles.

6.2 METHODS

6.2.1 Sampling and sample preparation

We sampled 21 mainland European freshwater lakes from the Sierra Nevada (Spain) to the Carpathian Mountains (Romania) and from northern Germany to the Apennines (Italy) covering an altitudinal range from 38 m asl to 3110m asl (compare Fig. 6.1 and Table 6.1). All sampling took place in August 2012.

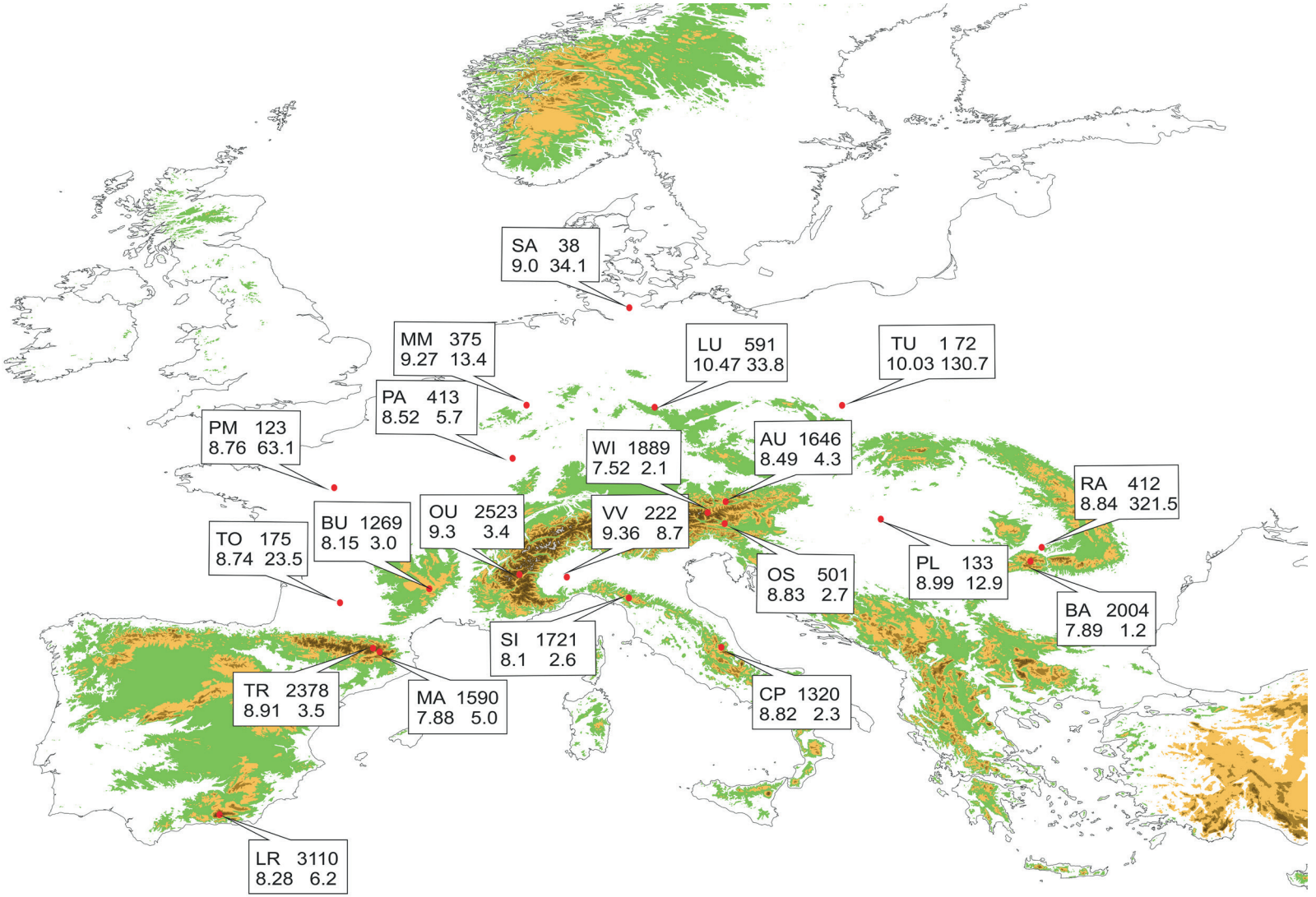


Figure 6.1. Location of the 21 sampled lakes (red dots) within mainland Europe's elevation profile. Boxes give lake ID (top left), elevation in m asl. (top right), pH (bottom left) and measured TP (total phosphate) in µg/L (bottom right).

Table 6.1. Lakes and their environmental variables

Lake	Country	Code	ID	elevation	Coordinates (N)	Coordinates (E)	pH	Cond	Temp	NO3-N	SO4	Cl	NH4-N	Na	K	Mg	Ca	TP	DP	DOC	DN	DRSi
				[m]				[µS/cm]	[°C]	[µg/l]	[mg/l]	[mg/l]	[µg/l]	[mg/l]	[mg/l]	[mg/l]	[mg/l]	[µg/l]	[µg/l]	[µg/l]	[µg/l]	[µg/l]
Réservoir de Panthier	F	S022PA	PA	413	47.238072°	4.631336°	8.52	300	23.9	673	23.369	14.151	56	8.774	2.469	3.998	52.522	5.7	5.1	3976	1086	996
Lac du Bouchet	F	S031BU	BU	1269	44.906383°	3.792808°	8.15	27.66	19.7	0	2.466	1.266	0	1.009	0.493	1.642	2.508	3	2.1	3253	215	104
Laguna d. l. Aquas Verdes	E	S102LR	LR	3110	37.048097°	3.368362° (W)	8.18	35.66	16.3	8	3.101	0.245	0	0.702	0.091	1.459	4.713	6.2	5	1627	58	822
Estany de Trebens	F	S153TR	TR	2378	42.577055°	1.962192°	8.88	6.33	17	10	1.791	0.239	0	0.982	0.168	0.356	1.923	3.5	3	1086	64	851
Lac de Matemale	F	S171MA	MA	1590	42.573792°	2.111855°	7.88	55.33	18.4	8	3.935	4.431	0	3.414	0.603	1.132	7.286	5	2.1	3387	146	211
Lac du Tondre	F	S271TO	TO	175	44.0228°	1.459363°	8.74	350	23.5	7	34.319	34.691	0	17.126	4.562	11.485	43.557	23.5	9.3	6175	494	613
Retenue de Pincemaille	F	S281PM	PM	123	47.462362°	0.221633°	8.75	361.33	22.4	6	24.611	25.549	0	12.121	4.404	4.538	55.932	63.1	14.9	7648	615	5829
Meerfelder Maar	D	S301MM	MM	375	50.100403°	6.76335°	9.27	298.33	21.3	13	9.909	15	0	24.891	5.003	14.354	17.523	13.4	4.9	4986	333	828
Augstsee	A	A111AU	AU	1646	47.663431°	13.787581°	8.51	101.66	14.7	79	0.609	0.238	10	0.206	0.106	0.463	22.099	4.3	2.3	1650	162	263
Ossiacher See	A	A132OS	OS	501	46.652044°	13.904869°	8.82	109.66	24.8	25	13.418	5.733	0	4.625	1.616	7.787	30.417	2.7	2.4	3121	211	297
Windebensee	A	A152WI	WI	1889	46.888563°	13.804230°	7.49	68	15.3	9	3.111	0.616	12	0.851	0.286	1.375	11.823	2.1	1.8	2078	115	768
Lago Compostoto	I	Z042CP	CP	1320	42.52795°	13.370268°	8.81	198	22.6	6	9.935	2.386	0	3.375	0.808	8.504	26.375	2.3	1.7	1387	104	275
Lago Sillara	I	Z071SI	SI	1721	44.36448°	10.07026°	8.06	16	18.4	9	1.149	1.75	0	1.286	0.169	0.274	2.001	2.6	2	1559	81	294
Lago Viverone	I	Z111VV	VV	222	45.4175°	8.048519°	9.35	205	29.3	0	19.437	5.779	0	4.173	1.889	11.204	21.067	8.7	4.6	4805	346	287
Lac l'Ouillette	F	Z122OU	OU	2523	45.429787°	6.99512°	9.29	153	18.1	0	30.954	0.167	14	0.299	0.066	3.512	25.5	3.4	1.1	733	36	134
Sankelmarker See	D	N121SA	SA	38	54.711812°	9.433274°	9.03	365.33	20.5	1347	34.834	30.037	0	16.523	5.683	5.113	67.059	34.1	8.2	10899	1825	691
Lütschetalsperre	D	N261LU	LU	591	50.733924°	10.756962°	10.47	206.66	20.2	55	17.832	43.181	0	23.393	6.366	2.136	10.676	33.8	10.7	3193	237	1426
Jezioro Turawskie	Pl	O022TU	TU	172	50.712830°	18.104304°	10.03	274	29.0	8	50.118	24.586	0	15.414	6.271	6.572	35.748	130.7	38.1	7378	480	1580
Lacul Bâlea	Ro	O111BA	BA	2004	45.603303°	24.615456°	7.89	89.66	13.7	37	4.563	0.36	22	0.437	0.846	2.611	17.708	1.2	0.9	812	97	399
Lacul Raura	Ro	O121RA	RA	412	45.9281°	24.052967°	8.83	743.33	22.9	7	145.662	50.773	0	104.412	25.778	20.122	31.766	321.5	1.8	15294	1356	2868
Balaton (dt. Plattensee)	H	O241PL	PL	133	46.933963°	18.117123°	8.99	777.33	24.9	9	174.403	51.091	0	53.351	9.934	81.102	28.577	12.9	11.7	8413	778	6188

Samples were taken with a telescope sampling vessel (~3m) from the edge of the water body, filtered on 0.2µm polycarbonate filters (until filters were blocked), air-dried, stabilised in RNA stabilisation solution (LifeGuard Soil Preservation Solution, MoBio Laboratories Inc., Carlsbad, CA) and stored at below -80°C. The cooling chain until Essen University laboratories was ensured by transportation in Cryo Shipper cooling vessels (Chart/MVE, Ball Ground, USA).

Water temperature, pH and conductivity were measured in the field. Alkalinity was determined by Gran – Titration with an Orion 960 pH electrode. Total phosphorous (P_{tot}) and total dissolved phosphorous (P_{dis}) were measured by the molybdate-method according to Vogler (1966). Dissolved organic carbon (DOC) was measured with a Shimadzu TOC - V CPH (Total Organic Carbon Analyzer) and dissolved nitrogen (DN) was measured with a Shimadzu TNM - 1 (Total Nitrogen Measuring Unit). The anions chloride (Cl), sulfate (SO4) and nitrate (NO3-N) as well as the cations sodium (Na), ammonium (NH4-N), potassium (K), magnesium (Mg) and calcium (Ca) were measured by means of ion chromatography (Dionex ICS – 1100). Dissolved reactive silicon (DRSi) was measured using the molybdate method (Smith et al. 1981; Skalar, SANplus Segmented Flow Analyser). Total phosphorous and alkalinity were measured from raw water, all other chemical parameters from filtrate (0.6 µm).

RNA was extracted using the TRIzol method. Briefly, RNA extraction was carried out under sterile conditions using TRIzol (Life Technologies, Paisley, Scotland – protocol modified). Filters were ground in liquid nitrogen and incubated for 15min in TRIzol. Chloroform was added and the mixture was centrifuged to achieve separation of phases. The aqueous phase was transferred to a new reaction tube and RNA was precipitated using isopropanol (incubation for 1h at -20°C and centrifugation). The RNA pellet was washed three times in 75% ethanol and resuspended in DEPC water.

6.2.2 Sequencing and preprocessing of sequencing data

Preparation of cDNA libraries as well as sequencing was carried out using an Illumina HiSeq platform (Eurofins MW G GmbH, Ebersberg, Germany). Two amplified short insert cDNA libraries (poly-A enriched mRNA and total RNA) with an insert size of 150-400 bp were prepared per sample, individually indexed for sequencing on HiSeq 2000, sequenced using the paired-end module (v3 chemistry) and finally demultiplexed.

The quality control tool FastQC (http://www.bioinformatics.babraham.ac.uk/projects/ fastqc/) was used to analyse the quality distribution of the raw reads. Adapter sequences at the ends of the reads were removed using the cutadapt software v1.3 (Martin, 2011). Cutadapt was also used to trim bad quality bases with a quality score below 20 and to discard reads with a length below 30 bp after trimming.

The amount of rRNA in the mRNA samples was determined by mapping the reads to the SILVA rRNA database (Quast et al. 2011; Yilmaz et al 2014) using Bowtie2 v2.0.2 (Langmead et al. 2009; Langmead & Salzberg 2012), a short read aligner that maps sequencing reads efficiently by using a Burrows-Wheeler transformed index. The index was built from the downloaded SILVA database

release 111. Only unmapped reads were used for further metatranscriptomic analysis.

6.2.3 Taxonomic assignment of total RNA and mRNA and functional annotation

After preprocessing, the sequences were mapped to the SILVA SSU database (v119) to determine the taxonomic composition of the samples from the rRNA sequences. rRNA reads were mapped to the SILVA SSU database using Bowtie2. Hits on species level were summarised to predefined sets of taxonomic groups. Count matrices reflecting the number of reads per taxonomic group and sample were created.

To specifically examine transcription in certain taxonomic groups, the mRNA reads were mapped to an inhouse transcript database. This database includes transcripts of 140 species belonging to 27 higher eukaryotic taxonomic ranks (Ciliophora, Dinophyceae, Apicomplexa, Chrysophyceae, Bacillariophyta, Oomycetes, other Stramenopiles, Cercozoa, other Rhizaria, Heterolobosea, Kinetoplastida, Euglenida, other Euglenozoa, other Excavata, Choanoflagellida, Amoebozoa, Chytridiomycota, Ascomycota, Basidiomycota, Glomeromycota, Metazoa, Glaucocystophyceae, Rhodophyta, Chlorophyta + streptophytic algae, Embryophyta, Cryptophyta, Haptophyceae) as well as to Archaea and Bacteria as outgroups. It further includes 19 unpublished chrysophyte transcriptome assemblies. Because of partly short contigs, the read pairs were mapped separately using SHRiMP v2.2.3 (Rumble et al. 2009; David et al. 2011) and the read with the best scores was chosen as a match. Only high-scoring reads with at least half of the maximal score were kept as a high-quality dataset to reduce false positive assignments.

All reads unmapped to sequences in the SILVA database were mapped to the UniProt Knowledgebase (Consortium Uniprot 2015) to assign a functional annotation to each read. The assignment was performed on the amino acid level using RAPSearch2 (Zhao et al. 2012; Ye et al. 2011). RAPSearch2 uses a reduced amino acid alphabet for a very fast protein similarity search. We built the RAPSearch2 index from the downloaded UniProtKB (version May 2012) and mapped each single read of a read pair against the index. For each pair, the hit with the highest score was chosen as protein annotation. To produce a high-quality dataset, only reads with a log10 E-value below -2 and an alignment length of at least 20 amino acids were kept. The mapping from UniProt IDs to KEGG Orthology IDs (KO-IDs) (Kanehisa & Goto 2000; Kanehisa et al. 2014) is provided by the UniProt database, and the corresponding KO-IDs and KEGG pathways were assigned to the reads. Mapping results were summarised as a count matrix containing the number of reads per KEGG Orthology ID and sample and a matrix containing the number of reads per KEGG pathway and sample.

6.2.4 Statistical analysis

Statistical analyses were performed using R (R Core Team 2015). To compare biodiversity based on sequence counts across samples, the raw counts were normalised by using proportions (counts were divided by total library size) or transformed to a common scale by multiplying the proportions with the minimal library size. To account for heteroscedasticity the *varianceStabilizingTransformation* function from the DESeq2 package (Love et al. 2014) was used before ordination and calculation

of correlations. Normalised pathway and gene counts were correlated to selected environmental variables using Pearson and Spearman correlation, namely elevation, pH, HCO_3, conductivity, Ca, P_{tot}, P_{dis}, DOC and latitude (GPS coordinate north). In addition, unconstrained (PCA, CA) and constrained ordination (RDA, CCA) were performed using the R package vegan (Oksanen et al. 2015).

The count matrices of taxonomic assignments per sample were correlated with the above mentioned environmental variables using linear correlation (Pearson) and rank correlation (Spearman) on logarithmic scale.

All figures were produced using R and the ggplot2 package (Wickham 2009).

The different steps of the bioinformatics workflow are summarized in Fig. 6.2.

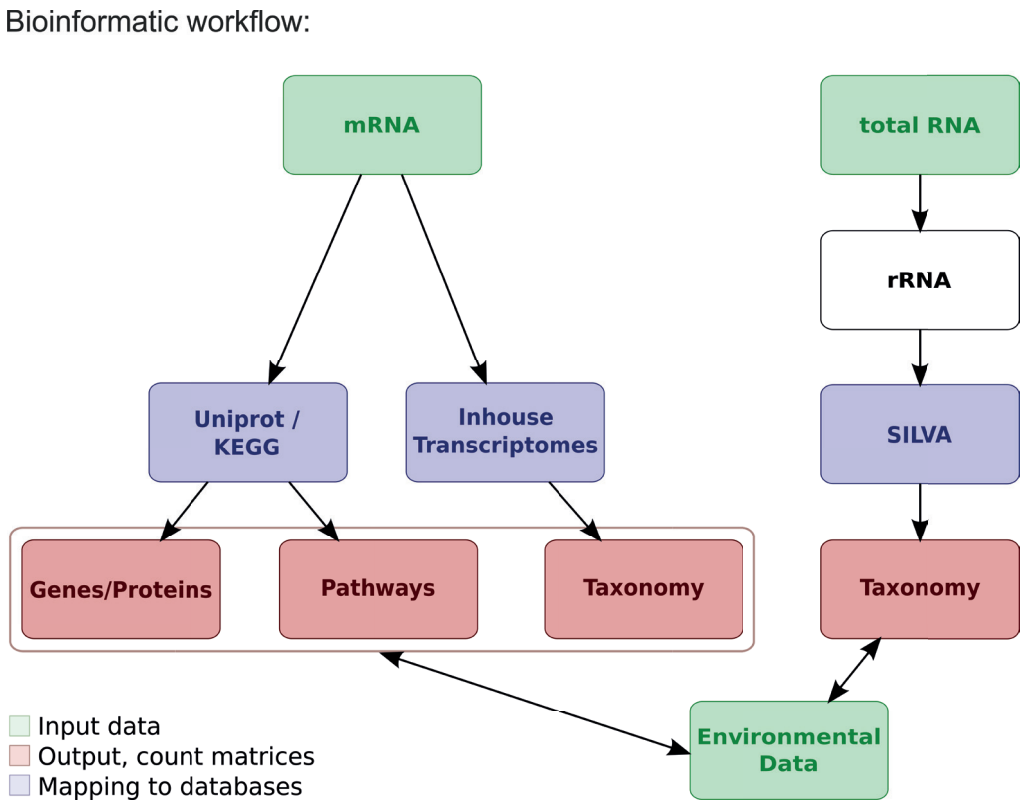


Figure 6.2. Bioinformatic workflow showing processing of both mRNA and rRNA sequences. Taxon and gene/pathway assignment is carried out with the help of reference databases (Uniprot, KEGG, SILVA).

6.3 RESULTS

6.3.1 Community composition based on rRNA

The total RNA data comprised 2 178 109 to 14 719 630 read pairs with on average 38% SSU-mapped rRNA reads affiliated with protist taxa which were used for the diversity analysis. Depths of coverage for the mapped and filtered rRNA reads ranged from 66 914 to 2 424 231 per lake. Based on the normalised reads, the molecular diversity (i.e. ribosomal sequence reads) in all investigated lakes was high with the dominant groups being Chlorophyta and streptophytic algae (avg. 22.82%), Ciliophora (avg. 10.82%), Bacillariophyta (avg. 10.49%), Chrysophyceae (avg. 10.04%) and other Stramenopiles ('Stramenopiles.Rest') (avg. 13.27%), in terms of read abundance (Fig. 6.4). Despite similar taxonomic groups dominating the plankton in the different lakes, the community composition differed considerably between lakes (Fig. 6.3). For instance, the relative abundance of Chlorophyta and streptophytic algae tended to increase with total phosphate concentration whereas the relative abundance of Chrysophyta showed the opposite trend (Fig. 6.4).

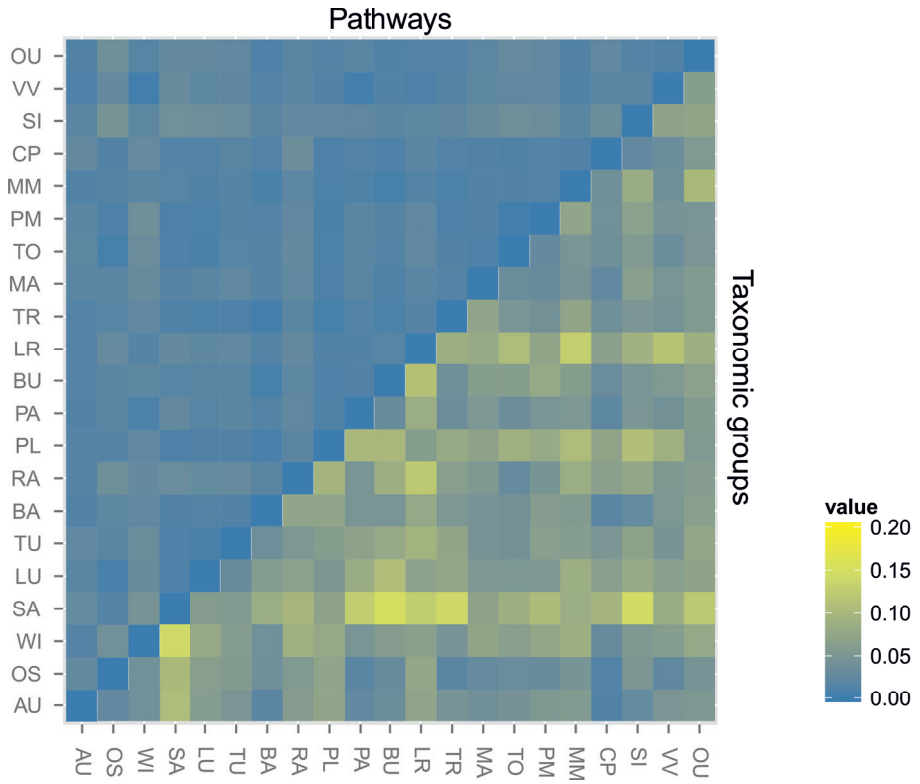


Figure 6.3. Heatmap showing pairwise comparisons of the investigated lakes. Pairwise comparison is based on correlation distances (individual squares) of sites on the basis of both pathways (top left half) and taxonomic groups (bottom right half); yellowish colours indicate stronger difference in comparison to blueish colours (compare value legend).

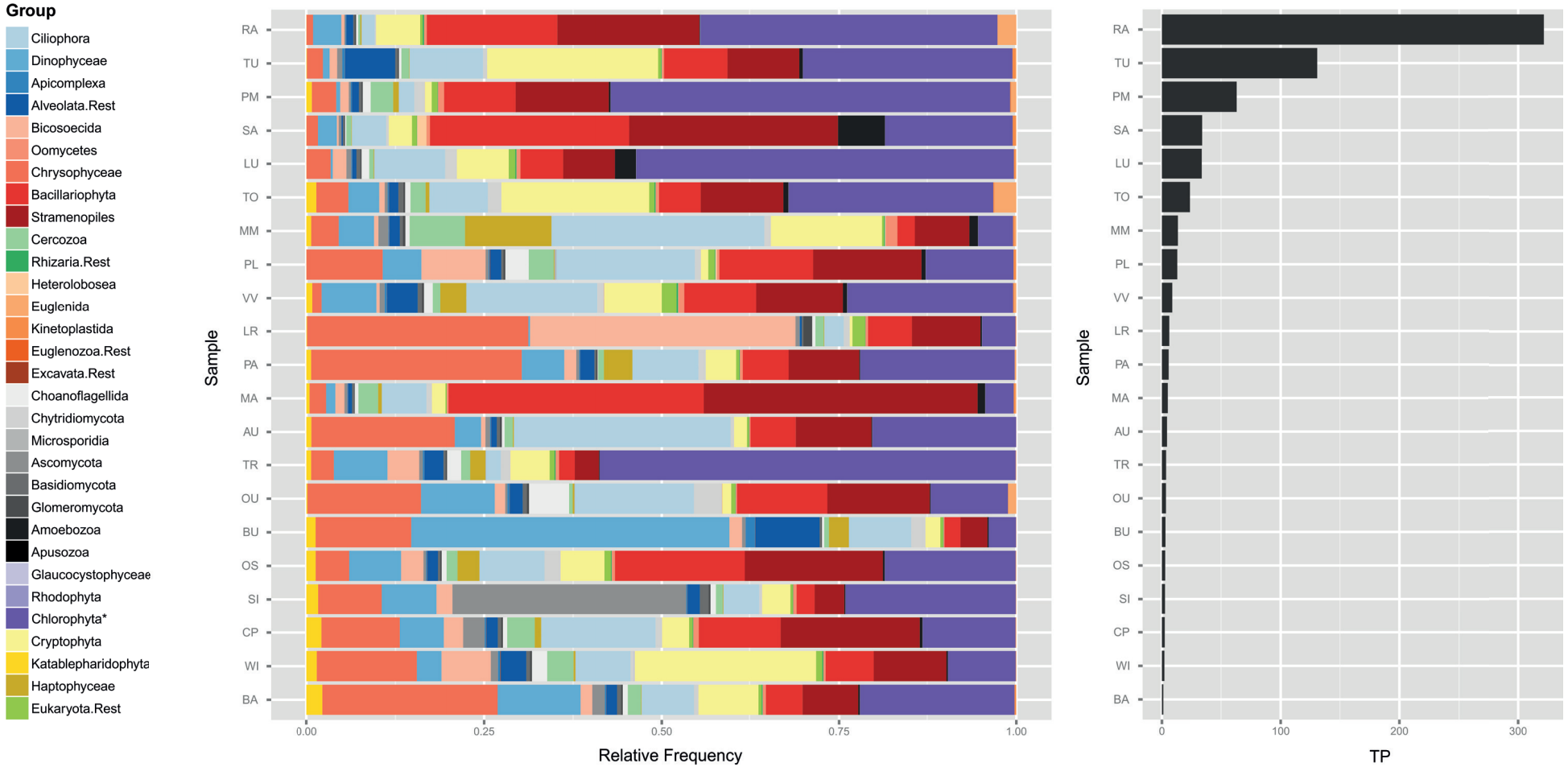


Figure 6.4. Barplots of relative frequencies of taxonomic groups in sites. Relative frequencies of taxonomic groups (=colours in plot) differ decisively (also compare high dissimilarity values in pairwise comparison – Figure 6.2) The most abundant groups are: *Chlorophyta and streptophytic algae (avg. 22.82%, purple), Ciliophora (avg. 10.82%, light blue), Bacillariophyta (avg. 10.49%, light red), Chrysophyceae (avg. 10.04%, orange), Cryptophyta (avg. 7.27%, light yellow), Dinophyceae (6.71%, blue) and other Stramenopiles ('Stramenopiles.Rest') (avg. 13.27%, dark red). Bars of barplots are ordered by TP (total phosphate) from highest to lowest measured value (top to bottom).

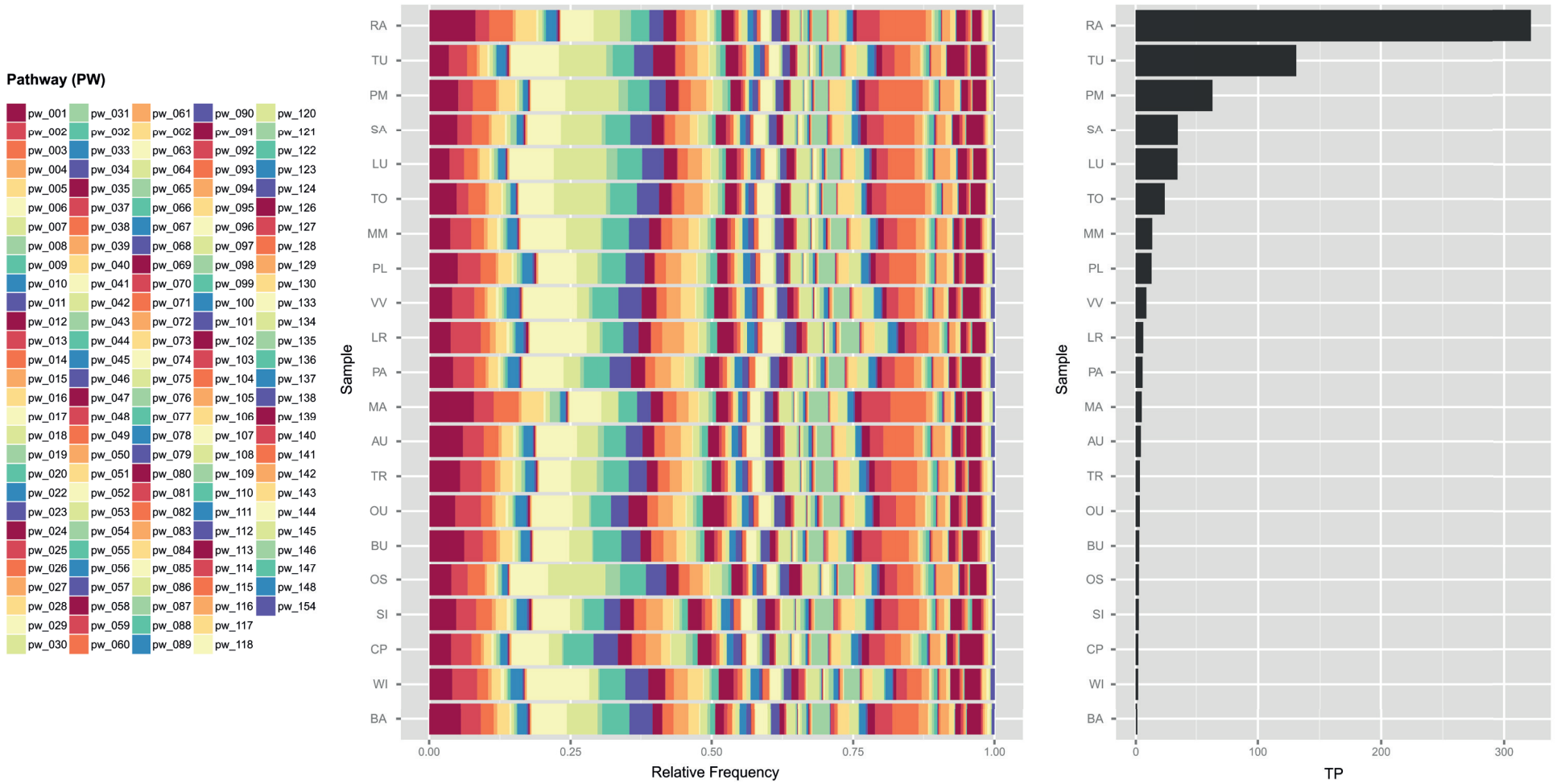


Figure 6.5. Barplots of relative frequencies of pathways in sites. Relative frequencies of pathways (=colours in plot) are much uniform with 'oxidative phosphorylation' (avg. 7.17%, pale yellow), 'photosynthesis' (avg. 5.03%, light green) and 'glycolysis/gluconeogenesis' (avg. 4.95%, dark red) being the highest expressed. Bars of barplots are ordered by TP (total phosphate) from highest to lowest measured value (top to bottom). Compare Table 2 for the key of pathway names by pathway numbers.

Table 6.2. Key to pathway names by pathway numbers

pw1	Glycolysis / Gluconeogenesis	pw78	Glycerophospholipid metabolism
pw2	Citrate cycle (TCA cycle)	pw79	Ether lipid metabolism
pw3	Pentose phosphate pathway	pw80	Arachidonic acid metabolism
pw4	Pentose and glucuronate interconversions	pw81	Linoleic acid metabolism
pw5	Fructose and mannose metabolism	pw82	alpha-Linolenic acid metabolism
pw6	Galactose metabolism	pw83	Sphingolipid metabolism
pw7	Ascorbate and aldarate metabolism	pw84	Glycosphingolipid biosynthesis - lacto and neolacto series
pw8	Fatty acid biosynthesis	pw85	Glycosphingolipid biosynthesis - globo series
pw9	Fatty acid elongation in mitochondria	pw86	Glycosphingolipid biosynthesis - ganglio series
pw10	Fatty acid metabolism	pw87	Pyruvate metabolism
pw11	Synthesis and degradation of ketone bodies	pw88	Dioxin degradation
pw12	Steroid biosynthesis	pw89	Xylene degradation
pw13	Primary bile acid biosynthesis	pw90	Toluene degradation
pw14	Secondary bile acid biosynthesis	pw91	Polycyclic aromatic hydrocarbon degradation
pw15	Ubiquinone and other terpenoid-quinone biosynthesis	pw92	Chloroalkane and chloroalkene degradation
pw16	Steroid hormone biosynthesis	pw93	Naphthalene degradation
pw17	Oxidative phosphorylation	pw94	Aminobenzoate degradation
pw18	Photosynthesis	pw95	Glyoxylate and dicarboxylate metabolism
pw19	Photosynthesis - antenna proteins	pw96	Nitrotoluene degradation
pw20	Purine metabolism	pw97	Propanoate metabolism
pw21	Puromycin biosynthesis	pw98	Ethylbenzene degradation
pw22	Caffeine metabolism	pw99	Styrene degradation
pw23	Pyrimidine metabolism	pw100	Butanoate metabolism
pw24	Alanine, aspartate and glutamate metabolism	pw101	C5-Branched dibasic acid metabolism
pw25	Tetracycline biosynthesis	pw102	One carbon pool by folate
pw26	Glycine, serine and threonine metabolism	pw103	Methane metabolism
pw27	Cysteine and methionine metabolism	pw104	Carbon fixation in photosynthetic organisms
pw28	Valine, leucine and isoleucine degradation	pw105	Reductive carboxylate cycle (CO2 fixation)
pw29	Geraniol degradation	pw106	Thiamine metabolism
pw30	Valine, leucine and isoleucine biosynthesis	pw107	Riboflavin metabolism
pw31	Lysine biosynthesis	pw108	Vitamin B6 metabolism
pw32	Lysine degradation	pw109	Nicotinate and nicotinamide metabolism
pw33	Penicillin and cephalosporin biosynthesis	pw110	Pantothenate and CoA biosynthesis
pw34	beta-Lactam resistance	pw111	Biotin metabolism
pw35	Arginine and proline metabolism	pw112	Lipoic acid metabolism
pw36	Clavulanic acid biosynthesis	pw113	Folate biosynthesis
pw37	Histidine metabolism	pw114	Atrazine degradation
pw38	Tyrosine metabolism	pw115	Retinol metabolism
pw39	DDT degradation	pw116	Porphyrin and chlorophyll metabolism
pw40	Phenylalanine metabolism	pw117	Terpenoid backbone biosynthesis
pw41	Chlorocyclohexane and chlorobenzene degradation	pw118	Indole alkaloid biosynthesis
pw42	Benzoate degradation	pw119	Monoterpenoid biosynthesis
pw43	Bisphenol degradation	pw120	Limonene and pinene degradation
pw44	Fluorobenzoate degradation	pw121	Diterpenoid biosynthesis
pw45	Tryptophan metabolism	pw122	Brassinosteroid biosynthesis
pw46	Phenylalanine, tyrosine and tryptophan biosynthesis	pw123	Carotenoid biosynthesis
pw47	Novobiocin biosynthesis	pw124	Zeatin biosynthesis
pw48	Benzoxazinoid biosynthesis	pw125	Sesquiterpenoid biosynthesis
pw49	beta-Alanine metabolism	pw126	Nitrogen metabolism
pw50	Taurine and hypotaurine metabolism	pw127	Sulfur metabolism
pw51	Phosphonate and phosphinate metabolism	pw128	Caprolactam degradation
pw52	Selenoamino acid metabolism	pw129	Phenylpropanoid biosynthesis
pw53	Cyanoamino acid metabolism	pw130	Flavonoid biosynthesis
pw54	D-Glutamine and D-glutamate metabolism	pw131	Anthocyanin biosynthesis
pw55	D-Arginine and D-ornithine metabolism	pw132	Isoflavonoid biosynthesis
pw56	D-Alanine metabolism	pw133	Flavone and flavonol biosynthesis
pw57	Glutathione metabolism	pw134	Stilbenoid, diarylheptanoid and gingerol biosynthesis
pw58	Starch and sucrose metabolism	pw135	Isoquinoline alkaloid biosynthesis
pw59	N-Glycan biosynthesis	pw136	Tropane, piperidine and pyridine alkaloid biosynthesis
pw60	Other glycan degradation	pw137	Betalain biosynthesis
pw61	O-Glycan biosynthesis	pw138	Glucosinolate biosynthesis
pw62	High-mannose type N-glycan biosynthesis	pw139	Aminoacyl-tRNA biosynthesis
pw63	Other types of O-glycan biosynthesis	pw140	Metabolism of xenobiotics by cytochrome P450
pw64	Amino sugar and nucleotide sugar metabolism	pw141	Insect hormone biosynthesis
pw65	Streptomycin biosynthesis	pw142	Drug metabolism - cytochrome P450
pw66	Biosynthesis of 12-, 14- and 16-membered macrolides	pw143	Drug metabolism - other enzymes
pw67	Polyketide sugar unit biosynthesis	pw144	Biosynthesis of unsaturated fatty acids
pw68	Butirosin and neomycin biosynthesis	pw145	Biosynthesis of ansamycins
pw69	Glycosaminoglycan degradation	pw146	Biosynthesis of siderophore group nonribosomal peptides
pw70	Glycosaminoglycan biosynthesis - chondroitin sulfate	pw147	Biosynthesis of vancomycin group antibiotics
pw71	Glycosaminoglycan biosynthesis - keratan sulfate	pw148	Biosynthesis of type II polyketide backbone
pw72	Glycosaminoglycan biosynthesis - heparan sulfate	pw149	Biosynthesis of type II polyketide products
pw73	Lipopolysaccharide biosynthesis	pw150	Acridone alkaloid biosynthesis
pw74	Peptidoglycan biosynthesis	pw151	Metabolic pathways
pw75	Glycerolipid metabolism	pw152	Biosynthesis of secondary metabolites
pw76	Inositol phosphate metabolism	pw153	Microbial metabolism in diverse environments
pw77	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	pw154	Phosphatidylinositol signaling system

6.3.2 Functional diversity based on mRNA

The poly-A selected dataset comprised 1 514 373 to 5 058 120 read pairs. 384 584 to 2 555 921 read pairs (49.60-60.90% of the preprocessed reads) matched known sequences in the Uniprot database. Out of these, 218 573 to 1 434 237 read pairs (66 385 to 578 216 with high quality) were affiliated with KEGG Orthology genes. Regarding the sequence reads which could be affiliated with known proteins, the most highly expressed genes were affiliated with elongation factor 1-alpha (EEF1A, K03231) avg. 1.13%, followed by molecular chaperone HtpG (htpG, HSP90A, K04079) avg. 0.89%, tubulin alpha (TUBA, K07374) avg. 0.82%, large subunit ribosomal protein L3e (RP-L3e, RPL3, K02925) avg. 0.80% and chaperonin GroEL (groEL, HSPD1, K04077) avg. 0.71%. Whereas community composition differed considerably, functional diversity based on relative read abundance per site affiliated with the different metabolic pathways hardly differed between lakes (Fig. 6.3). Most of the reads were affiliated with “oxidative phosphorylation” (avg. 7.17%), “photosynthesis” (avg. 5.03%) and “glycolysis / gluconeogenesis” (avg. 4.95%). In general, pathways associated with the basic primary metabolism, specifically the catabolic pathways “carbon fixation in photosynthetic organisms”, “terpenoid backbone biosynthesis”, “photosynthesis”, “photosynthesis - antenna proteins” and the anabolic pathways “fatty acid metabolism”, “glycolysis / gluconeogenesis”, “glyoxylate and dicarboxylate metabolism”, “citrate cycle (TCA cycle)” and “oxidative phosphorylation”, accounted for roughly 30 % of the reads (Fig. 6.5 + Table 6.2 and Fig. 6.6). Whereas the contribution of glycolysis (4.95 ± 1.30%) and TCA cycle (3.47 ± 0.78%) to the overall expressed sequences were very similar between lakes, the contribution of photosynthesis (5.03 ± 2.86%) was more variable.

6.3.3 Correlation of community composition and functional diversity with environmental variables

Although the differentiation between habitats was more pronounced for taxon diversity as compared to functional diversity (Fig. 6.3), phosphate (P_{tot}) and elevation had the highest explanatory power for the observed differentiation in both RDA analyses (Fig. 6.7). The automatic stepwise model building for constrained ordination methods in the analysis of metabolic pathways (mRNA) yielded a significant model containing phosphate and elevation (p-value 0.002). Here, phosphate and elevation explained for 21.58% of the variation. In the community analysis (rRNA) phosphate and elevation, but also DOC and pH explained for differences in RDA analyses. The stepwise model selection yielded a significant model containing these factors (p-value 0.001), which explained for 36.72% of the variation (Fig. 6.7). Chrysophyceae, for instance, were negatively correlated with phosphate concentration (Spearman correlation coefficient: -0.59, p = 0.00515) whereas other taxa such as Chlorophyta and streptophytic algae (Spearman correlation coefficient: 0.46, p = 0.0366) increased with increasing phosphate.

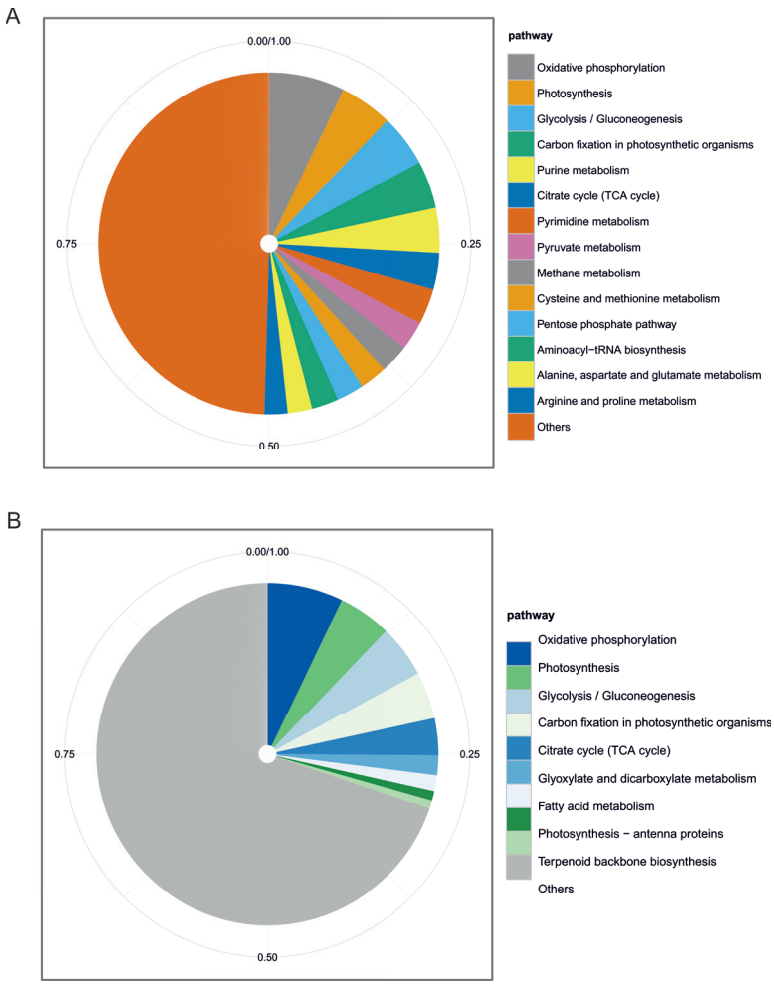


Figure 6.6. Piecharts showing percentages of pathways in the analysis. Percentages are given out of a 100, respectively of A) the 50% pathways with highest frequencies and B) all anabolic and katabolic pathways.

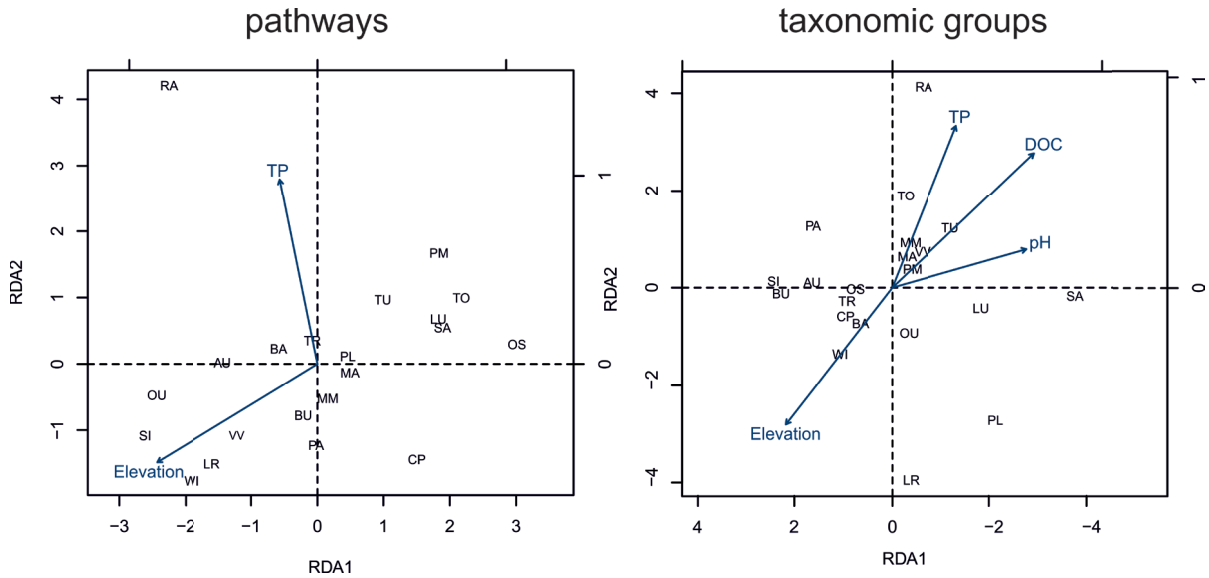


Figure 6.7. Redundancy analysis (RDA) on the basis of both pathways (left) and taxonomic groups (right). RDA shows the significant abiotic factors phosphate and elevation (p-values 0.0002) and phosphate, elevation, DOC and pH (p-value 0.001) correlated with the respective distribution of sites in the matrix (non-significant factors not shown).

6.3.4 Contribution of distinct taxa to overall mRNA libraries

Even though the contribution of distinct metabolic pathways to the overall expressed sequences varied only slightly (Fig. 6.5), the contribution of distinct taxonomic groups to overall expressed genes varied greatly. In general, the read abundance of genes affiliated with a distinct taxonomic group co-varied with their abundance as indicated by rRNA gene copy number in the total RNA dataset (compare Figs. 6.8 and 6.9).

For instance, the contribution of distinct taxonomic groups to mRNA reads associated with glycolysis and TCA cycle largely reflected the general activity of the taxa as indicated by the rRNA community profiles (Fig. 6.9). Taxon-specific regulation of these pathways certainly contributed to the observed variance, but the co-variation of mRNA profiles and rRNA profiles suggests that such taxon-specific regulations of pathways play a minor role. For photosynthesis, this was more differentiated. For Dinoflagellates, Diatoms and Chlorophytes + streptophytic algae, the general activity of the groups largely co-varied with their share in overall mRNA reads associated with photosynthesis. In contrast, the expression of genes involved in photosynthesis in Chrysophytes did hardly co-vary with rRNA profiles, but was strongly modified by taxon-specific up- or down-regulation of this pathway between the lakes (Fig. 6.8).

For instance, genes coding for proteins involved in photosynthesis pathways made up on average 5.92 % (range 1.92 – 12.78 %). The fraction of chrysophycean rRNA sequences generally decreased with increasing phosphate concentrations (Figs. 6.4, 6.9 and 6.8) reflecting the decreasing importance of Chrysophytes with increasing phosphate concentration. However, the contribution of chrysophyte photosynthesis genes to the overall expressed photosynthesis genes did not co-vary with chrysophyte rRNA abundance. In contrast, a taxon-specific regulation of this pathway, i.e. the relative importance of photosynthesis in the expressed chrysophyte sequences, largely co-varied with the overall share of Chrysophytes to overall expressed photosynthesis genes (Fig. 6.8).

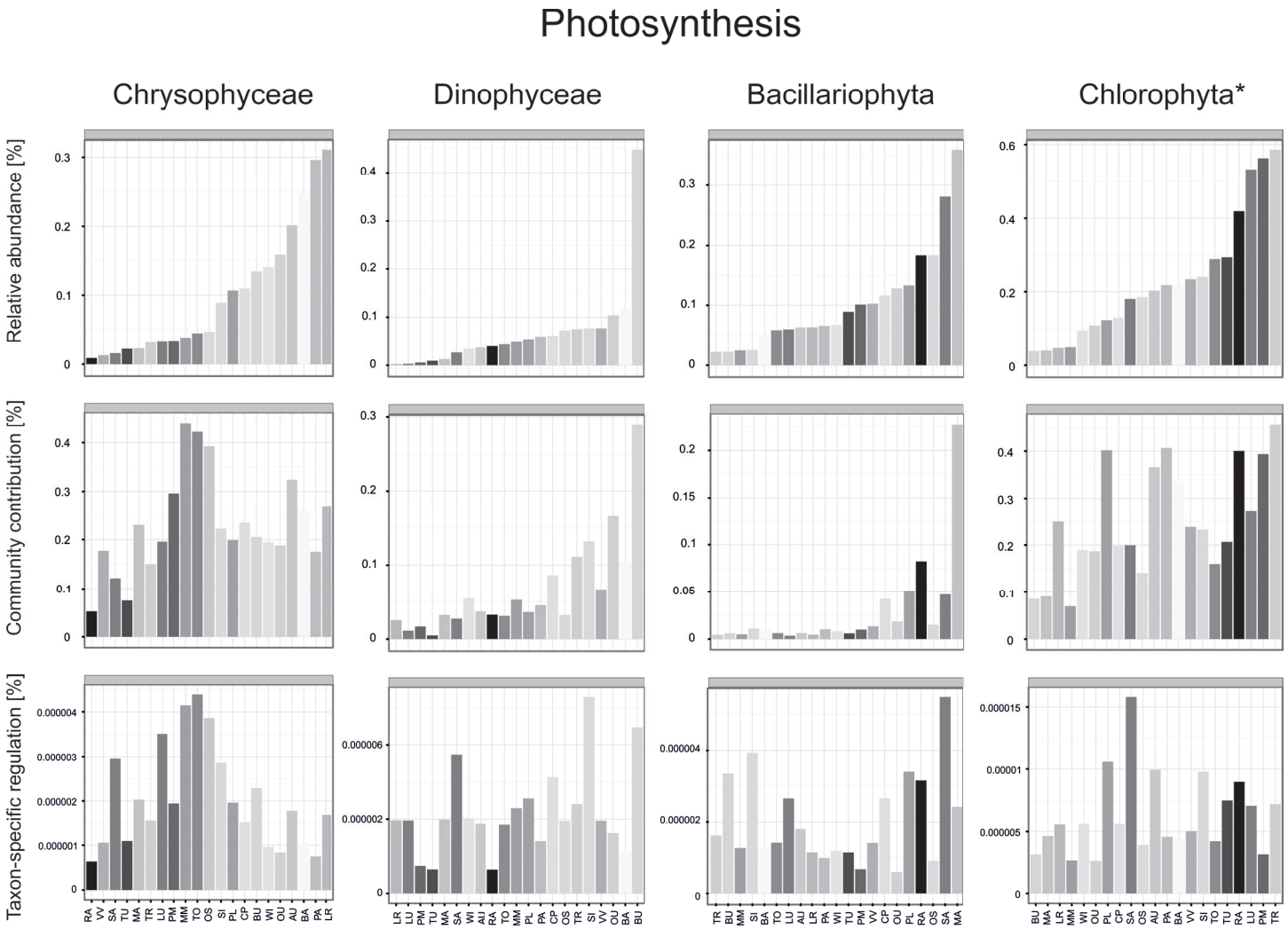


Figure 6.8. Metatranscriptomic profile of photosynthesis genes. Comparison of the relative abundance of distinct taxa based on rRNA sequence abundance (upper row), the contribution of these taxa to a distinct pathways based on mRNA reads (middle row) and the contribution of these pathways to the expressed mRNAs of the respective taxon (lower row). Sequence abundances are exemplarily shown for Chrysophyceae, Dinophyceae, Bacillariophyta, and Chlorophyta* (Chlorophyta and streptophytic algae). All plots are ordered according to the rRNA sequence abundance of the respective taxon. Colouring of bars additionally shows log value of TP (total phosphate) of sites, black = highest value within sites, white = lowest value within sites.

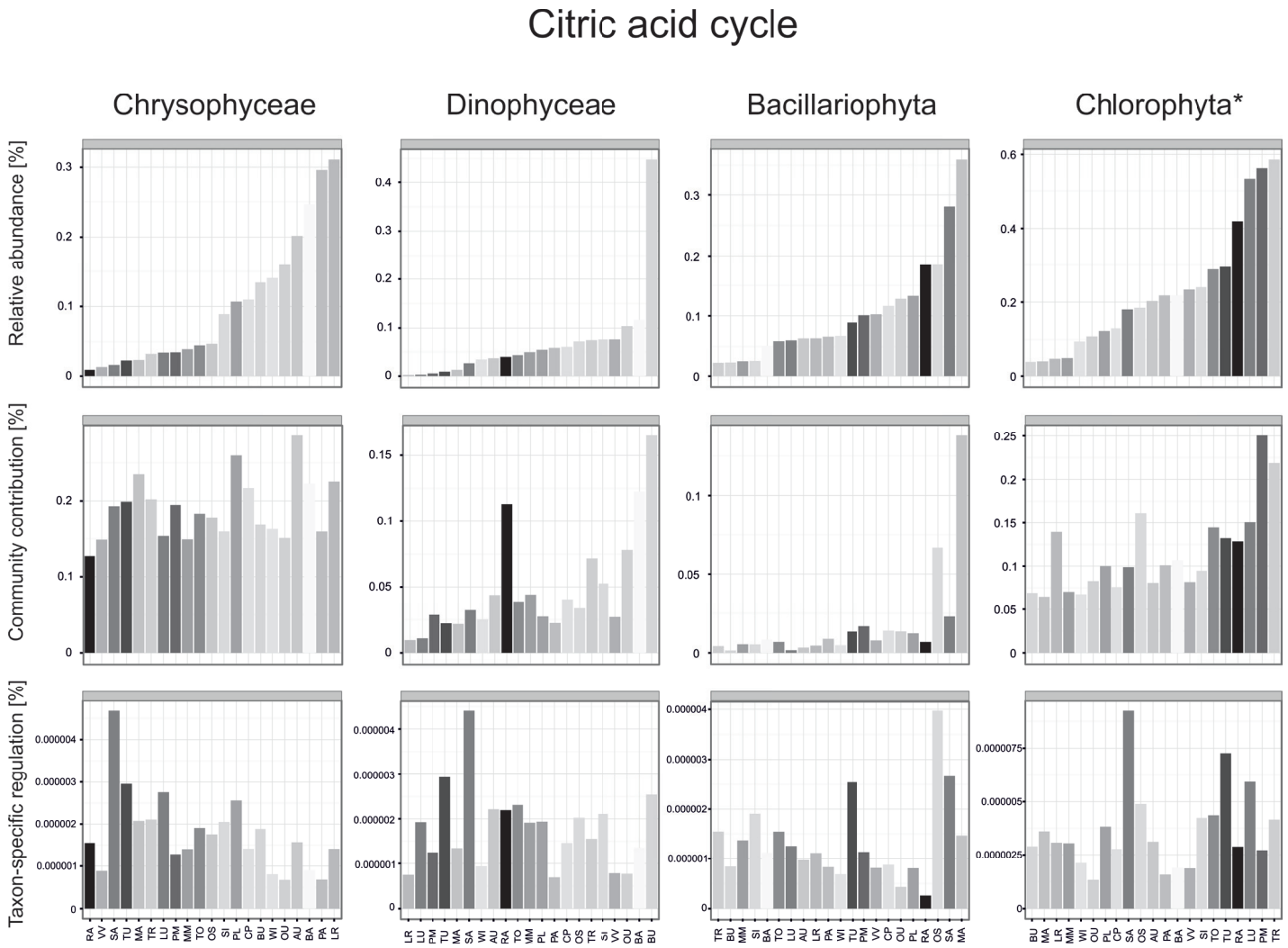


Figure 6.9. Metatranscriptomic profile of citric acid cycle genes. Comparison of the relative abundance of distinct taxa based on rRNA sequence abundance (upper row), the contribution of these taxa to a distinct pathways based on mRNA reads (middle row) and the contribution of these pathways to the expressed mRNAs of the respective taxon (lower row). Sequence abundances are exemplarily shown for Chrysophyceae, Dinophyceae, Bacillariophyta, and Chlorophyta* (Chlorophyta and streptophytic algae). All plots are ordered according to the rRNA sequence abundance of the respective taxon. Colouring of bars additionally shows log value of TP (total phosphate) of sites, black = highest value within sites, white = lowest value within sites.

6.4 DISCUSSION

Here, we show that functional redundancy between protistan taxa contributes to ecosystem stability. Consequently, ecosystem functioning is buffered against shifts in taxon inventory. Such analyses rely on the concurrent analyses of taxon diversity and functional diversity. It is only now that the recent high-throughput sequencing technologies allow for the analysis of taxon composition as well as of genetic diversity of functional genes and metabolic pathways on the ecosystem scale (Goffredi et al. 2015). These techniques have, therefore, the potential to revolutionise ecosystem analyses. Taxonomic assignment of mRNA reads is currently restricted to higher taxonomic levels due to the patchy taxonomic coverage within public databases (Keeling et al. 2014). Despite the technological challenges, microbial metatranscriptomics have already elucidated the functional diversity of the free-living and intestinal eukaryotic microorganisms (Goffredi et al. 2015; Damon et al. 2012; Qi et al. 2011), microbial responses to organic and inorganic pollution (Mason et al. 2012; Boenigk et al. 2014) and symbiotic relationships between taxa (Balzano et al. 2015; Mayfield et al. 2014).

For the here presented dataset of 21 freshwater lakes, we applied a standardised processing – from sampling to bioinformatics. Furthermore, as all samplings were carried out during summer stagnation, seasonal effects should be negligible. The focus of our analysis was the metabolic diversity and differentiation of lake ecosystems on a European scale, specifically the effect of environmental factors on community composition and functional diversity as well as the implications of deviating patterns between these two aspects of biodiversity. We found a functional redundancy between taxa and taxonomic groups, i.e. a relative stability of ecosystem functioning despite shifts in taxon composition.

6.4.1 Taxonomic composition

The molecular community profiles correspond with the expectations: lake phytoplankton is generally dominated by Bacillariophyta and Dinophyceae (Bellinger & Sigee 2010); Cryptophyta, Chlorophyta and Chrysophyceae usually also contribute significantly to lake phytoplankton (Dokulil et al. 2001; Graham et al. 2009). Other algal groups are present, but usually less important in terms of biomass and production (Belliger & Sigee 2010). The most abundant heterotrophic taxa in the pelagial of lakes are heterotrophic Chrysophytes, Bicosoecids, Choanoflagellates, Katablepharids and some further heterotrophic nanoflagellate taxa (Boenigk & Arndt 2002). Even though Ciliophora and heterotrophic Dinophyta are important in terms of biomass, these groups are less dominating in terms of cell abundance. In our study Ciliophora (10.82% on average), Chrysophyceae (10.04%), Dinophyceae (6.71%), Bicosoecida (3.88%), Ascomycota (2.27%), Cercozoa (1.94%), Chytridiomycota (1.14%) and Choanoflagellida (1.1%) were the dominating heterotrophic taxa. The high share of ciliate and dinophyte sequence reads seems to be in contrast to the generally low cell numbers of these taxa. However, these taxa have a relatively high copy number of the ribosomal operon in their genomes and are therefore usually overrepresented in molecular diversity data sets (Medinger et al. 2010).

6.4.2 Expressed sequences and metabolic pathway analysis

We expected genes and pathways involved in primary metabolism to be dominant in the mRNA libraries (Boenigk et al. 2014; Mayfield et al. 2014). Accordingly, the dominant metabolic pathways in terms of the fraction of expressed sequences in the cDNA libraries were oxidative phosphorylation, photosynthesis and the TCA cycle. Even though phosphate concentration and elevation explained for some of the variance between the lakes, none of the distinct pathways was significantly correlated to the measured environmental parameters. At a first glance, this finding was surprising as the lakes differed considerably with respect to geographical location, elevation and trophic status. Even though species inventory as indicated by rRNA profiles differed substantially between lakes, the relative contribution of distinct metabolic pathways to the overall mRNA libraries was rather similar for all lakes. Our mRNA analyses did not aim at absolute abundances of sequence tags, but at their relative contribution to the environmental mRNA pool. From an ecosystem perspective it seems convincing that distinct metabolic pathways have similar relative importance across a variety of ecosystems. This applies specifically for pathways affiliated with the primary metabolism, which were dominating the sequence libraries, as these pathways can be expected to be pivotal for the metabolism of organisms and, consequently, for ecosystem functions.

6.4.3 Contribution of distinct taxonomic groups to overall gene expression profiles

Taken together, molecular diversity and functional diversity provide deep insights into the molecular basis of lake ecology: On the one hand, taxon composition considerably differed between lakes, consistent with various microscopic and molecular studies (Reynolds 1998; Eiler et al. 2013; Medinger et al. 2010). On the other hand, the overall contribution of distinct metabolic pathways as indicated by metatranscriptome analysis hardly differed between lakes. Thus, even though distinct ecosystem functions and metabolic pathways do hardly change, the organisms behind these functions did change considerably. This is confirmed by the observed shifts in taxon composition between lakes as well as by shifts in the taxon assignment of the expressed sequences for distinct pathways (Fig. 6.4 and Figs. 6.8 and 6.9). We identified both shifts in taxon composition and activity as well as shifts in the taxon-specific regulation of the expression of genes and pathways as factors shaping community mRNA profiles. Interestingly, the intensity of these two factors contributed differently to the share of mRNA sequences depending on both the taxonomic group and the metabolic pathway. Our analyses indicate that community composition is a good first proxy for the analysis of ecosystem functions. However, differential gene regulation obviously modifies the relative importance of taxa in distinct metabolic and ecologically relevant pathways. Beyond the limitations of amplicon analyses, metatranscriptome analyses resolve the functional diversity of ecosystems on a taxon- and pathway-specific level. Beyond shifts in community composition, such analyses provide insights into the regulation of metabolic pathways and the pathway-specific share of distinct taxa to the ecosystem metabolism.

We further conclude that ecosystem stability, specifically the stable relative importance of different metabolic pathways on the ecosystem level as indicated by mRNA read abundance, is maintained

through a functional redundancy of distinct taxa (Allison & Martiny 2008; van der Putten et al. 2007; Fonseca & Ganada 2001). This functional redundancy presumably works as a buffer saving ecosystems from losing essential functionalities when the species inventory changes. On the ecosystem level, distinct functions can be taken over by different taxa or taxonomic groups providing the ecosystem with a certain flexibility in the case of environmental changes. Ecosystem functioning depends on the sustainability of distinct (metabolic) functions rather than on a distinct species inventory. Diversity, in turn, is crucial to maintaining ecosystem functioning and prevents a collapse of the system under environmental change. Our results demonstrate that, indeed, ecosystem functioning as indicated by mRNA read abundance is buffered against shifts in taxon inventory.

REMARKS

I submitted chapter 6 as first author to the ISME Journal.

For the bioinformatic processing, I worked in cooperation with Dr. Daniela Beisser (formerly Genome Informatics, Institute of Human Genetics, University Hospital Essen).

7) CONCLUSION

Protistan diversity can be observed and investigated in many respects. There is a huge phylogenetic evolutionary diversity in protists as is represented in the tree of life by many and distant individual lineages. Protists, therefore, also hold an immense functional diversity being manifested in their physiological traits, in the biomolecules they produce and in the way they interact with their environment and with each other. Morphologically, however, protistan diversity seems to be limited. There, indeed, is a great amount of morphological characters in protists, but still even larger diversity is hidden away by indistinct morphology as molecular surveys reveal. Cryptic taxa look alike, but differ in terms of phylogeny, physiology and ecology. It is, therefore, worth to take a second look with the help of molecular tools and encounter such hidden diversity.

In this study, I could show cryptic diversity in small heterotrophic colourless chrysophytes. Lumped together as '*Spumella*-like flagellates', this phylogenetic as well as ecophysiological diversity is hidden away in environmental surveys. I, here, pave the way to recognize these organisms properly in the environment and learn about their functioning in ecosystems. On an evolutionary level, the found polyphyly in small heterotrophic colourless chrysophytes reveals a pattern of diversified feeding behaviour much in accordance with the many cases of mixotrophy in the group. The loss of chloroplasts appears as a successful strategy to encounter new niches thus having taken place in multiple cases in chrysophytes.

As for the whole of protistan diversity, much is unknown about the occurrence and distribution of protists on earth and in different habitats. With the help of a huge combined dataset of terrestrial, aquatic and experimental protistan communities, I could prove that molecular screening successfully and precisely reflects protistan community structure as known from detailed single habitat investigations. On that basis, the large and diverse scaling of the survey was able to reveal distribution patterns both on metagroup and habitat level. I could observe higher taxa evenness in soil as opposed to aquatic habitats and likewise rather generalist strategies and broader distribution in soil-inhabiting taxonomic groups such as Amoebozoa. This fits with the presumed less random dispersal of terrestrial organisms. Chrysophytes, by my findings, perform an intermediate strategy in these respects.

In terms of the distribution of protistan taxa in Alpine lakes, Chrysophytes also show an intermediate distribution pattern reflecting trends of the whole protistan community. In testing theories from multicellular organisms for protists in an Alpine elevation gradient, I could show that protistan communities do not automatically fit the ecological rules of animals and plants. Elevation does neither largely affect protistan species richness nor community composition. In that, species-area-relationship that in general applies for protists is most probably countered by effects of endemism and specialised adaptation so that small high mountain lakes are surprisingly species rich in protists.

Not only focusing on the occurrence of protists in ecosystems, but also on their functionality, I used metatranscriptomics to observe the organsims' activity in the system. In an ecotoxicological experiment that proved the general applicability of metatranscriptomics in protists, silver heavy-metal stress was shown to have a direct effect on planktonic communities. The metatranscriptomes revealed that especially the photosynthetic fraction of the community is affected as silver blocks the photosynthetic apparatus. The respective protists react with an up-regulation of photosynthesis related genes to replace the damage. The investigation, thereby, shows that silver as an environmental toxin (silver is increasingly found in the environment for its growing use in consumer products) is underestimated and may well cause severe damage in aquatic protistan communities.

Metatranscriptomics, however, also hold the potential to resolve the functional contribution of individual taxa to the community's activity. With the help of a Europe-wide dataset, I compared aquatic protistan communities in their taxonomic and functional diversity and could show functional redundancy among taxa. Thereby, community dissimilarity between sites was much higher than functional dissimilarity, the relative similarity of the functional profiles being produced by two different strategies. I could show, that on the one hand functions were taken over by different taxonomic groups, on the other hand taxonomic groups showed strong shifts in the regulation of certain pathways. These two strategies were differently performed by different taxa, Chrysophytes, for example, showing strong regulation of pathways within the taxon. The general observed functional redundancy of taxa can be interpreted in the light of ecosystem functioning. Biodiversity, thereby, serves as a buffer against changes in the environment as ecosystem functioning is upheld by different taxa taking over the functions in different conditions. Vice versa, an ecosystem is more vulnerable the less biodiversity, e.g. in protistan communities, it holds.

As the investigations undertaken in this work show, protistan diversity and protistan diversity patterns are observable. They reveal strategies and relationships on the ecosystem level and prove the importance of protists in their habitats. Molecular methods do a great deal to successfully investigate such patterns. Especially, new advances in high-throughput technologies such as metatranscriptomics allow insights into protistan communities that lead to deeper knowledge of ecosystems and their inhabitants. But also rather classical methods are needed to illuminate diversity on a much smaller scale as in the observed heterotrophic chrysophytes. Only such detailed investigations make work on the ecosystem level possible and successful. The method, therefore, is only a tool, but the knowledge gained makes it valuable and justifies its use.

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Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. (2) f) der Promotionsordnung der Fakultäten für Biologie, Chemie und Mathematik zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Diversity of Protists with Special Emphasis on Chrysomonads: Morphological and Molecular Diversity, Distribution Patterns and Functional Differentiation" zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Lars Großmann befürworte und die Betreuung auch im Falle eines Weggangs, wenn nicht wichtige Gründe dem entgegenstehen, weiterführen werde.

Essen, den 7.9.15 _____

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. (2) c) + e) der Promotionsordnung der Fakultäten für Biologie, Chemie und Mathematik zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den 7/9/2015 _____

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultäten für Biologie, Chemie und Mathematik zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuch in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereich abgelehnt worden ist.

Essen, den 7/9/2015 _____

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. (2) f) der Promotionsordnung der Fakultäten für Biologie, Chemie und Mathematik zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Taxonomic affiliation of and species concept for ‚problematic' chrysophyte genera" zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Herrn Lars Großmann befürworte und die Betreuung auch im Falle eines Weggangs, wenn nicht wichtige Gründe dem entgegenstehen, weiterführen werde.

Essen, den 13.9.2011

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