IDENTIFICATION OF INDICATOR CILIATES

(CILIOPHORA, ALVEOLATA)

IN WATER QUALITY ASSESSMENT:

AN INTEGRATIVE APPROACH

Master’s Thesis

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ABSTRACT

Globally, ciliates are very abundant in every freshwater system and feed mainly on bacteria and microalgae. Depending on the availability and composition of the food source and abiotic factors, some ciliates are suitable indicator organisms reflecting the trophic status of a freshwater ecosystem. Certain water quality classification systems (e.g. the saprobic system) are based on the occurrence of an indicator organism and therefore, an identification at the species level is required. Nowadays, new molecular methods and bioinformatic tools provide an additional approach beside the former morphologically based identification. Combining these two approaches, a comprehensive description of a specific organism in its environment can be obtained. I collected biofilm from submersed stones, plants or wood debris as well as sediment from different habitats. By microscopy, I (i) identified 22 living ciliates on the species level, (ii) noticed their behavior in respect to food vacuole content, movement, interactions and, (iii) documented the observed ciliates with an image analysis system. After proper identification of a species, single cells were washed in sterile filtrated lake water and starved overnight, before we conducted a single cell PCR followed by a nested PCR for 16 of them. Alternatively, we performed a REPLIg multiple displacement amplification approach on 3 individuals, a method producing many copies of the genome when starting with low biomass, e.g. one single cell. We chose the folded V9 region from the SSU-18S rDNA gene as marker to differentiate ciliates on the species level. This integrative approach provides a basis for future molecular water quality assessment tools applied in freshwater ecosystems, e.g. high throughput sequencing.
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1. Introduction

Ciliates are found globally in different habitats of limnetic and marine ecosystems, soil and as symbionts in animals (Foissner et al. 2009; Berger et al. 1997). These small organisms (15 µm – 300 µm) consist of one cell but display the highest developed cell organization among protists (Beaver & Crisman 1989; Berger et al. 1997). They play a tremendous role in the microbial food web (Müller 1989; Beaver & Crisman 1989; Posch et al. 2015) and- to use modern terminology- they act as “influencer” (Forster et al. 2019). By feeding voraciously on bacteria, phytoplankton, phagotrophic flagellates and other ciliates (Beaver & Crisman 1989; Sommer et al. 2012), they are one of the primary controlling functions of complex food-web interactions (Sonntag et al. 2006). For example, the seasonal succession pattern of ciliate assemblages in oligotrophic alpine lakes can be attributed to the seasonal pattern of the ciliates’ food sources (Sonntag et al. 2006). Due to their fast generation time (hours to days) and their strict dependence on the surrounding nutrient conditions, they can be linked directly to the trophic status of a water body and therefore act as indicators for the current trophic status. Nowadays – thanks to excessive research in the past- comprehensive literature about indicator ciliates and their related autecology is available (Foissner et al. 1991, 1992, 1994, 1995; Berger et al. 1997; Beaver & Crisman 1989).

This gathered knowledge helps to answer ecological questions, shed light on interactions or co-occurrences among different microbes and is practically used, for example, in sewage treatment plants (Curds 1973; Mikhailov et al. 2019). Within the activated-sludge process, ciliates are the central protists and the effluent’s quality correlates significantly to the presence of specific ciliates (Curds 1973; Esteban et al. 1991).

Despite all these benefits, the research community treat them step-motherly and taxonomic education is hardly provided by universities anymore (Friedrich und Herbst 2004; Foissner 2016). Foissner (2016) attributes this lack of interest to the laboriousness that taxonomic work implicates. Nowadays, molecular methods are more and more widely-used for species identification and quantification of protist communities in their natural habitat or to detect and count small protists and cryptic species (for detailed summary see (Stoeck et al. 2014a). Though, molecular approaches are often error-prone, and many biases are common (Stoeck et al. 2014a; Pitsch et al. 2019) e.g., the value of molecular data is strongly correlated with the correct taxonomic identification and the right attributive sequences (Duff et al. 2008; Bittner et al. 2013), otherwise all the obtained data are not relatable.
The knowledge on the importance of ciliates in ecosystems and their value in water quality assessments can get lost. To counter these developments, it is necessary to simplify their taxonomical identification as much as possible. Consequently, Foissner (2016) encouraged to develop a molecular identification kit to resolve this challenge.

Ciliophora belong to the monophyletic taxon Alveolata, which also includes Dinoflagellata and Apicomplexa. Together with Stramenopila Rhizaria, Imbricatae, Endomyxa and Retaria the taxon Alveolata forms the superordinate taxon SAR – an acronym of Stramenopila, Alveolata, Rhizaria (Adl et al. 2019). Even if the species show a great variance in the morphotypes, ciliates all share four identical characteristics (Hausmann 1996):

(i) (many), (short) cilia
(ii) the specific structure of the cortex
(iii) nuclear dualism
(iv) conjugation

The highly differential ciliature serves for locomotion and/or feeding (Hausmann 1996). The somatic cilia cover the cell; the oral cilia are located around the oral apparatus (Hausmann 1996). The cortex structure serves as an identification characteristic, due to its species specificity and consistency within a species (Hausmann 1996). The nuclear apparatus consists of two nuclei (nuclear dualism) and their position and number are used for taxonomic species determination. One or several polyploid macronuclei regulate primarily somatic functions and one or several small diploid micronuclei are responsible for generative functions. Conjugation is a form of sexual reproduction, where two individuals fuse and exchange genetic material through the micronucleus without dividing. Otherwise, ciliates reproduce by division through transverse binary fission (Hausmann 1996).

Additionally, almost every species has one or several contractile vacuoles, which are responsible for osmoregulation (Hausmann 1996). Via excretion pori, the aqueous content and ions are often transported out of the cytoplasm. The number and position of these contractile vacuoles are again species-specific and used for taxonomic identification.

Extrusomes are membrane-bound organelles close to the pellicula which vary in size, morphology and function and are typical characteristics for taxonomical species identification (Corliss 1979; Hausmann 1978). By chemical, mechanical or other stimulation, they are extruded quickly, either for defense or attack. They are named depending on their function, i.e., toxicysts are filled with poison to kill a prey, whereas mucocysts can protect a ciliate from poisoning and the spindel-shaped trichocysts are used for defense (Hausmann & Radek 2014).
The feeding strategies of ciliates are diverse, their nutritional demands vary even within species (Berger et al. 1997). The heterotrophic species are either omnivorous feeding on every available food source- or they are algi- or bacterivorous. Others feed on detritus or small other plankton – even cannibalisms is common (Berger et al. 1997). A specific nutritional strategy is mixotrophy – a combination of heterotrophic and photoautotrophic food intake (Riemann et al. 1995). Therefore, photoautotrophic organisms are used as endosymbionts. The ability of mixotrophy is one reason for the ubiquitous occurrence of protists in almost every habitat on earth (Germond & Nakajima 2016). Kleptochloroplastidy is one possibility for heterotrophic protists to have photosynthetic organelles. Another way of mixotrophy is the symbiosis of free-living endosymbionts and hosts.

According to their different habitats and feeding strategies, ciliates can either be sessile, vagile, free-swimming or attached to a surface in a so-called biofilm (Berger et al. 1997; Hausmann 1996). Biofilm can form on abiotic and biotic surfaces, like minerals, carapaces of dead organisms, or plants, other microbes, and animals, or inside the human body like e.g. dental plaque (Karatan & Watnick 2009; Battin et al. 2016). The thin layer full of life consists of cells enclosed by a self-produced matrix of hydrated extracellular polymeric substances (EPS) (Flemming & Wingender 2010). Biofilms are the most common microbial life form in stream and river ecosystems (Geesey et al. 1978; Lock et al. 1984). Ciliates often dominate in regard of the biomass (Gong et al. 2005) and link riverine organic matter flow (Norf & Weitere 2010).

1.2. Saprobic system

The saprobic system allows an assessment of the biological status of a water body based on indicator species. In 1909, Kolkwitz and Marsson developed the idea of three series-connected lakes with different abilities of self-purification and stages of mineralization: (i) the polysaprobic zone (first lake), (ii) the α – and β- mesosaprobic zone (second lake), and, (iii) the oligosaprobic zone (third lake) (Kolkwitz & Marsson 1909). The polysaprobic zone is marked by an influx of high molecular and putrescible matter, which results in low oxygen levels. In the oligosaprobic zone the mineralization process is mostly finished and typically an oversaturation of oxygen occurs. In between is the asymmetric mesosaprobic zone: the α-mesosaprobic zone - connected to the polysaprobic zone – is characterized by a strong and fast mineralization, which levels off and then declines towards the β-mesosaprobic zone which is connected to the oligosaprobic zone. This classification is also possible for rivers, beginning with the input of putrescible matter and ending, where the natural level is restored (Kolkwitz & Marsson 1909). Each zone shows a different composition of species in quantity and quality. The premise, that the degradable organic nutrients of the saprobic zones have a direct influence on
the organisms inside the zone is the crucial point and was confirmed by several biochemical analyses. The saprobic system is based upon empirical and descriptive data. A simple application without any knowledge or consideration of multiple factors would be disputable and thus useless (Foissner et al. 1994).

From the 1970s on, this saprobic system built the basis of the standard assessment of the biological state of all running waters in Germany (Länderarbeitsgemeinschaft Wasser LAWA Ed.: 2002). Until now, several revisions and a standardization (Deutsches Institut für Normung (DIN) 38 410) were made as well as three maps of the biological water quality (Friedrich 1990; Länderarbeitsgemeinschaft Wasser LAWA Ed.: 2002; Friedrich & Herbst 2004). When the water frame work directive was adopted in October 2000 and set the goal to achieve a “good status” of all water by a set deadline, the biological survey of the water quality was integrated (European Commission 2019; Das Europäische Parlament und der Rat der Europäischen Union 2000). The water quality assessment and monitoring is executed by administrative bodies and concentrates on the following indicator groups: macrozoobenthos, phytoplankton, phytobenthos, macrophytes and fish, regarding diversity and abundance of species communities (Umweltbundesamt Deutschland 2019). The microzoobenthos consisting ciliates is not part of the water quality assessment, despite according to Kolkwitz and Marsson (1909), ciliates are the most important group concerning the assessment of the degree of pollution.

The standardization – the DIN 38410 - consists of 80 taxa of microorganisms, mainly from running water but also from small ponds, lake littorals or waste water treatment plants (Berger et al. 1997). Table 1 shows the species number of each taxon used in DIN 38 410 and highlights the importance of the ciliates in the saprobic classification system.

Table 1: Taxa of the DIN 38 410 and their species number according to Berger et al. (1997):

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schizomycetes</td>
<td>9</td>
</tr>
<tr>
<td>Mycophyta</td>
<td>2</td>
</tr>
<tr>
<td>Rhizopoda</td>
<td>7</td>
</tr>
<tr>
<td>Flagellata</td>
<td>14</td>
</tr>
<tr>
<td>Ciliophora</td>
<td>48</td>
</tr>
</tbody>
</table>
Until now it is impossible to explain a biozone purely by mathematics, but only through approximation procedures. The saprobic index S is calculated according to the DIN 38410-1:2004-10 and the measure of dispersion (Berger et al. 1997). With Table 2 (Berger et al. 1997), the quality classes can be determined according to the saprobic indices. Each quality class has its attributed color.

Table 2: Water quality classes according their attributive parameter, e.g. saprobic index and chemical parameters (BSB5, NH₄N, O₂ Minima) according to (Berger et al. 1997) and modified.

<table>
<thead>
<tr>
<th>Quality class</th>
<th>Colour</th>
<th>Organic Pollution</th>
<th>Saprobity</th>
<th>Saprobic index</th>
<th>BSB5 (mg/L)</th>
<th>NH₄N (mg/L)</th>
<th>O₂ Minima (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>dark blue</td>
<td>clear - extremely low polluted</td>
<td>Oligosaprobity</td>
<td>1.0 &lt;1.5</td>
<td>1</td>
<td>traces</td>
<td>&gt;8</td>
</tr>
<tr>
<td>I-II</td>
<td>light blue</td>
<td>low polluted</td>
<td>Oligosaprobity with β- mesosaprobic impact</td>
<td>1.5 &lt;1.8</td>
<td>1-2</td>
<td>~0.1</td>
<td>&gt;8</td>
</tr>
<tr>
<td>II</td>
<td>green</td>
<td>moderate polluted</td>
<td>balanced β- mesosaprobity β- α- mesosaprobic border zone</td>
<td>1.8 &lt;2.3</td>
<td>2-6</td>
<td>&lt;0.3</td>
<td>&gt;6</td>
</tr>
<tr>
<td>II-III</td>
<td>yellowgreen</td>
<td>critical polluted</td>
<td>balanced β- mesosaprobity β- α- mesosaprobic border zone</td>
<td>2.3 &lt;2.7</td>
<td>5-10</td>
<td>&lt;1</td>
<td>0.5 - much mg/l</td>
</tr>
<tr>
<td>III</td>
<td>yellow</td>
<td>strong polluted extremely strong polluted</td>
<td>strong α- mesosaprobity</td>
<td>2.7 &lt;3.2</td>
<td>7-13</td>
<td>0.5 - much mg/l</td>
<td>&gt;2</td>
</tr>
<tr>
<td>III-IV</td>
<td>orange</td>
<td>excessive polluted</td>
<td>α- mesosaprobity with polysaprobic impact</td>
<td>3.2 &lt;3.5</td>
<td>10-20</td>
<td>much mg/l</td>
<td>&lt;2</td>
</tr>
<tr>
<td>IV</td>
<td>red</td>
<td>excessive polluted</td>
<td>Polysaprobity</td>
<td>3.5 &lt;4.0</td>
<td>&gt;15</td>
<td>much mg/l</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

Ciliates meanly feed on algae and bacteria and react quickly to changing conditions (Šimek et al. 2014; Cairns et al. 1972), so the highest diversity of species can be found in mesosaprobic habitats, while the highest abundance occurs in the α-mesosaprobic to polysaprobic zones. Contrastingly, in mesosaprobic zones, ciliates have low validity on an oligo- or betasaprobic level because of the low food availability (Foissner et al. 1995). In each biotope, specific conditions (e.g. oxygen saturation, food sources, temperature, conductivity) determine the composition of the colonizing communities of protists. Due to this fact, Fjerdingstad (1964) developed the idea of characteristic groups of indicator species and Sladecek (1973) developed well-arranged identification plates. In their taxonomical and ecological revision of ciliates of the saprobic system, Foissner et al. (1994) adapted and updated the community plates. According to their ecological demands and saprobic indices, the indicator ciliates are grouped together to communities. The plates are helpful tools for the water quality evaluation.
Nowadays, the morphological determination and the synecological approach get more and more replaced by genomic approaches to taxon diagnosis (Duff et al. 2008; Stoeck et al. 2006; Countway et al. 2005; Massana et al. 2006; Berdjeb et al. 2018; Pitsch et al. 2019; Stoeck et al. 2014b).

Studies concerning the diversity and/or temporal changes of prokaryotic and eukaryotic microbial communities in both, marine and freshwater ecosystems, are often limited by size, availability or cultivation problems. In contrast, by using (new generation) sequencing techniques, even very small,
low-abundant or cryptic species can be detected and the understanding of microbial diversity globally changed (Countway et al. 2005; Caron et al. 2004; Baldauf 2003; Hebert et al. 2003).

Phylogenetically preserved sequences - such as the small subunit 18S rDNA of the nuclear ribosomal operon – can be targeted with the right primers, amplified by polymerase chain reaction (PCR) and sequenced for taxonomic determination or phylogeny (López-García et al. 2001; Moon-van der Staay, Seung Yeo et al. 2001; Stoeck et al. 2006).

Always being concerned to reduce the cost, next generation or high-throughput sequencing methods have been established (Sogin et al. 2006; Hebert et al. 2003; Kysela et al. 2005), where millions of sequences are produced parallel. In order to simplify the work flow, other taxonomic tools are created such as the idea of barcoding all animals on the basis of a high phylogenetic informative gene, the mitochondrial cytochrome c oxidase I (COI) (Hebert et al. 2003). Zimmermann et al. established a standard laboratory procedure for barcoding diatoms using the V4 subregion on the 18S rRNA gene as valuable marker (Zimmermann et al. 2011). Barcoding ciliates, in contrast, the D1-D2 region of the large subunit ribosomal DNA shows high potential to identify on species level (Stoeck et al. 2014a). Lacking one commonly used gene region to identify the protists’ diversity, the Protist Working Group (PrWG) aims to unify expertise of international experts for establishing one standard protocol (Pawlowski et al. 2012). Initiated by the Consortium for the Barcode of Life (CBOL, http://www.barcodeoflife.org/), the PrWG elaborates a two-step assessment: pre-barcode the V4 18SrDNA to assign the sequence to a major taxonomic group in order to subsequently bar code with group specific markers (Fig.2).
The outcomes are so-called operational taxonomic units (OTUs), clusters of organisms grouped by gene markers or similar DNA sequences identified through searching tools concentrating on sequence similarity of protein and DNA databases, like BLAST (Altschul et al. 1997), FASTA (Pearson 1994) or Bayesian classifiers (Cole et al. 2005).

Undeniably, the advantages are tremendous, but many researchers agree on the biases these approaches arises (Duff et al. 2008; Pitsch et al. 2019). Obviously, the molecular sequencing methods provide huge data sets, but often without deposing any correct taxonomical determination (Saldarriaga et al. 2004).

For ciliates, the most frequently used barcode is COI (e.g. Strüder-Kypke & Lynn 2010; Greczek-Stachura et al. 2012; Hebert et al. 2003), as well as the hyper-variable V4 and V9 regions of SSU-rDNA (e.g. (Stoeck et al. 2010; Amaral-Zettler et al. 2009, Pitsch et al. 2019) or the the D1-D2 region of the LSU-rDNA (Stoeck et al. 2014a). The results of the genetic markers differ among groups, genera or are even species-specific (Santoferrara et al. 2013), thus finding an appropriate genetic markers is still a great concern (Santoferrara et al. 2013).

Figure 2 Two-step protist barcoding pipeline. Individual species are individually picked from the sample, identified morphologically either directly or after culture growth, DNA extracted, and barcoded using a twostep, nested strategy. According to (Pawlowski et al. 2012).
1.3. Aim of the study

The limitations inherent in morphology-based identification systems and the biases occurring in molecular-based surveys of microbial taxa are given but should not diminish the value of each approach. It is important, to avoid blind application and be aware of the limits. By combining the advantages, the best of both approaches can be used to resolve evolutionary, taxonomical and/or ecological questions.

In the thesis, I concentrated on a combination to strengthen the value of ciliates for water quality assessment.

In detail, I conceptualized the following workflow:

(i) Gain expertise of morphological-based taxonomical identification, with special focus on taxa of the saprobic system
(ii) Establish/improve a method to combine morphological and molecular determination on the same specific organisms
(iii) Fill up data bases with correct identified species combined with autecological data
(iv) Propose a standard protocol for the morphological and molecular identification of indicator ciliates
2. Material and Methods

2.1. Investigation Site and Sampling

Samples of ciliates from biofilm on submerged stones and sediment were taken from the shore of Lake Irrsee and Lake Mondsee, different parts of Zeller Ache as well from the shore of Lake Happinger See and the river Schleifenbach.

Lake Mondsee (47°48'02.8"N 13°23'01.5"E, Upper Austria, Austria) is a natural alpine, oligo-mesotrophic and holo-dimictic lake which is situated in a chain of lakes (Fuschlsee and Irrsee upstream, Attersee downstream) connected through the rivers Zeller Ache, Fuschler Ache and Wangauer Ache and the outflow Seeache (Land Oberoesterreich 2007-2009). The samples were evaluated at the Research Department for Limnology Mondsee which is located on the northeastern shore of Lake Mondsee.

The major morphometric data of Lake Mondsee are: altitude = 481 m a.s.l, maximum depth = 68.3 m, mean depth = 35.9 m, area = 13.7 km², volume = 510 million m³, catchment area = 247 km², secchi depth (5-year mean 2011-15) = 4.57 m and theoretical water renewal time = 1.7 years (Ficker et al. 2017). It is one of the warmest lakes in the region with a mean water temperature of 12°C, the ecological state is well/moderate (Land Oberoesterreich 2007-2009; Bundesministerium Nachhaltigkeit und Tourismus 2014-2016).
The major morphometric data of the oligo-mesotrophic, holo-dimictic Lake Irrsee (47°54’02.7"N 13°18’23.0"E) are: altitude = 553 m a.s.l., maximum depth = 32 m, mean depth = 15.3 m, surface area = 347 ha, volume = 53.1 million m³, catchment area = 27.5 km², secchi depth (5-year mean 2011-15) = 4.92 and theoretical water renewal time = 1.7 years (Ficker et al. 2017). The mean water temperature is 14.6 °C and the ecological state is very well. (Land Oberoesterreich 2007-2009; Bundesministerium Nachhaltigkeit und Tourismus 2014-2016).

The river Zeller Ache is 7.4 km long and has a catchment area of 38.3 km² (Land Oberoesterreich 2011).

Additionally, one sample was taken from the River Schleifenbach (47°50’52.7"N 13°20’25.7"E), an inflow of Mondsee located close to the Research Department of Limnology.

Additionally, one sample was taken from the Lake Happinger See in April 2018 after recognizing the ciliate Ophyrydium eutrophicum. Lake Happinger See (47°49’04.8"N 12°07’44.0"E, Upper Bavaria, Germany) is a quarry pond and a popular swimming lake with excellent quality (Landratsamt Rosenheim 2019). The major morphometric data of the oligotrophic Lake Happinger See are: altitude = 446 m.a.s.l., maximum depth = 6 m, no surficial inflow but groundwater influenced and the visible depth (secchi depth) = 1-2m (Landratsamt Rosenheim 2019).

The sampling was conducted during the summer 2018 (April - October) and May 2019 on five main sample points and two additional ones. Based on the DIn 38 410 (Berger et al. 1997), it was standardized for the different niches within the habitats: the biofilm and aufwuchs of four or five stones were brushed down with a clean toothbrush and sediment was collected with clean scoops as well as macrophytes, dead wood or submerged plants. All samples were separated in clean bottles which were filled up two thirds with water from the sample point to leave some air reserves especially at strong polluted lakes/rivers. The samples are stored and transported in cool boxes to avoid heating and direct solar radiation.
Figure 4: Sample points (SP) 1-4. (1) SP1: Lake Mondsee, Slipway Research Department for Limnology, Mondsee 47°50’56.9”N 13°20’32.5”E. (2) SP2: Zeller Ache, Bridge 47°51’04.4”N 13°20’47.8”E. (3) SP3: Zeller Ache, Metallplan Bachmeier 47°51’15.9”N 13°20’14.1”E. (4) SP4: Irrsee, Zeller Ache 47°53’27.2”N 13°18’54.9”E.
Figure 5: Sample points (SP) 5-7. (5) SP5: Mondsee, Influx Zeller Ache 47°50'58.9"N 13°20'51.9"E. (6) additional SP6: Schleifenbach: 47°50'52.7"N 13°20'25.7"E. (7) additional SP7: Happinger See 47°49'07.5"N 12°07'47.6"E. Red arrow marks the position where the sample was taken.
2.2. Live observation and morphological identification of ciliates

The samples in the clean boxes were brought into the laboratory, opened, and the sample water was decanted into clean quadratic plastic boxes. For abundance estimates, three cover glasses (40 x 20 mm) were put on the water surface for about 30 minutes at room temperature so the ciliates which need more oxygen can accumulate on the surface of the cover glass. With a cover glass pipette, they were carefully taken off and placed on a clean slide for counting the species under the microscope. Three drops of the sediment were investigated as well to evaluate the ciliates with less oxygen demand. This cover-glass method is a great tool for getting an overview of the species occurring in the sample even if no abundance estimate is conducted. For morphological identification, the decanted sample was left undisturbed for about 30 minutes to restore the natural situation after the disturbing decanting into the plastic box. With a clean Pasteur pipette a small drop of the surface water was taken off and placed on a clean slide. With the drawn micropipettes, single ciliates were picked from the water drop and placed on a second clean slide within a very small drop of water. The picking was done either under the microscope (small magnification) or under the binocular microscope (Olympus SZ61). To retard the specimens, small drops of vaseline were applied to each corner of a cover slip or directly on the slides through a syringe with a thick needle. Observing constantly the ciliates under the microscope, the vaseline was pressed carefully down with the tip of a thin pencil until the ciliates were squished between the cover glass and the slide to become less mobile and more transparent (Foissner 2014). Thus, the living observation of cell organelles is possible.

![Preparation of slides with vaseline for living observation of ciliates](Foissner & Berger 1996)

Following abiotic parameters were measured on SP4 and SP5 with the multi sonde HQ40d (Hach): pH, dissolved oxygen (mg. l⁻¹), dissolved oxygen (%), conductivity (µS.cm⁻¹) and water temperature (°C).
Morphological determination of the living ciliates was done using an Olympus BX51 with magnifications between 40x and 1000x (oil immersion objective) with differential interference contrast and an integrated camera for photo documenting system. Following literature was used: the taxonomical and ecological revision of the ciliates of the saprobic system, volume 1 to 4 (Foissner et al. 1991, 1992, 1994, 1995) and additional literature for specific ciliates as follows for the species *Uroleptus willii* (Sonntag et al. 2008) and for the species *Furgasomia blochmanni* and *Nassula* sp. (Eisler & Bardele 1986).

With methyl green pyronin, the macronucleus was stained in case of uncertainties regarding position, size or shape (Foissner 1979). Therefore, one drop of the 1% distilled water/methyl green pyronin solution is placed close to the cover glass on the slide following all safety instructions. About one minute later, the dye is passed through the preparation, if not, a filter paper placed closely to the other side of the cover glass will speed up the process. The dye stains following organelles: the macronucleus (blue-green, violet-red), the micronucleus (blue), food vacuoles and the cytoplasm (graded pink); trichocysts (light blue).

![Figure 7: Tools for picking ciliates from the sample: sterile filtrated lake water (1) and cultivation plate (2), syringe filled with lake water and filter (3), drawn micropipette (4), clean slide (5) and syringe filled with vaseline (6).](image)
After morphological identification, two to eight individuals of each species were washed in 3–5 drops of sterile filtered lake water (Sartorius stedim, Minisart, 0.2µm) and pipetted in culture plats filled with sterile filtrated lake water, then stored overnight in the incubator at the same water temperature of the sample point for starving the ciliates to ensure that all food particles are digested. This ensures, that only the ciliates’ DNA is amplified by the following single cell PCR.

2.3. Documentation of ciliate morphology by imaging and dry silver staining

Photo documentation and staining methods enable reliable morphological determination. The cell morphology was documented using the integrated imaging system ProgRes SpeedXT core 5 2.9.0.1.

The dry silver nitrate method is a quick method to provide information on the ciliary and/or silverline pattern. The basic technique by Klein (1926, 1958) was improved by Foissner (1976) and others (e.g. Gelei 1934, Ruzicka 1966) and updated by Foissner 2014. Therefore, a drop of sample water containing ciliates is placed on a clean slide where a thin layer of egg-albumin was spread before and is air-dried. The egg-albumin should be kept in an open flask for at least 20h for the best results. After adding silver nitrate, the slide is exposed for 5-60s to a 40-60 W electric bulb to pre-develop before the developer impregnates the sample. The fixative sodium thiosulfate stops the development and with synthetic neutral medium the air-dried samples are preserved.

Figure 8: Overview of the different stations required for the dry silver nitrate method starting from picture 1 (silver nitrate and electric bulb), picture 2 (developer and fixative) to picture 3 (tap water).
2.4. Single Cell PCR, nested PCR, REPLig

Parts of the 18S rRNA genes including the V4 and V9 region were amplified by single cell PCR directly from the living washed and starved ciliates using the primer pair EAF3 and ITS055R (5’TCGACAATCTGGTGATTCTCGCTCGCAAGGGC’ and 5’CTCCTTGCTACGTGTTTCAAGACGGG3’) according to (Marin et al. 2003). Two to eight cells from the same species were transferred into labeled PCR Tubes together with the master mix for a 50µl PCR reaction using a Qiagen Taq Mastermix Kit. Conditions for PCR were as follows (30 cycles): initial duration (96°C, 5 min), denaturation (96°C, 1 min), annealing (55°C, 2 min), elongation (68°C, 3 min), final elongation (68°C, 10 min), storage (10°C). For control, gel electrophoresis of the PCR products was conducted. After purifying following the manual of the QIAquick® PCR Purification Kit, Qiagen (except step 4: 700 µl Buffer PE), the PCR products are used as templates for nested PCR applying the Primer CilF according to (Lara et al. 2007) (5’ TGGTAGTGTATTGGACWACCA 3’) and ITS055R. Alternatively, the primers N920F (5’ CAAGGCTGAAACTTAAAKGAATTG) (Marin et al. 2003) and ITS055R were used as well as the more universal primer pair E528F/ITS055R. This PCR products were directly cut out from the agarose gel and purified for sequencing with the QIAquick gel extraction kit, Qiagen following the manual.

Additionally, a REPLig approach was performed on three ciliates (Paramecium bursaria, Otertrumia aurea and Stentor roeselii). The ciliates were treated like described before and placed in clean microtubes. With the Qiagen REPLi-g Single Cell Kit the amplification was conducted following the manual provided by the manufactory (Qiagen, Hilden, Germany).

2.5. Sequencing

The sequencing was performed from Eurofins GATC (Cologne, Germany). Therefore, we pipetted 5 µl of each purified PCR product together with 5µl of the primer N920F into the supplied plate.

2.6. Bioinformatics

The sequences were exported from the free DNA sequencing software Chromas in the FASTA format. Using the MEGAX64 software, a known V9 region was aligned with the new sequences. With the Basic Local Alignment Search Tool BLAST (Zhang et al. 2000), we tried to find regions of local similarity between sequences already placed in the data base (NCBI). Afterwards, the V9 region was fold on the mfold web (Zuker 2003).
3. Results

In total, 22 species were found during the sampling period which could be assigned to the communities Stentoretum, Pleuronometum, Colpidietum, Cyrtophoreta, Carchesietosum, Trithigmostometum and Marynetum according to Foissner et al. (1995).

Table 3: Overview of all morphological identified ciliates from the different sample points (SP) from April 2018 till May 2019 and their according communities: Pleuronometum (PLE), Stentoretum (STE), Trithigmostometum (TRI), Cyrtophoreta (CYR), Carchesietosum (CAR), Colpidietum (COL) and Marynetum (MAR). MOO stands for mire influenced, HAS for healthy activated sludge. Saprobity according to Foissner et al. 1991, 1992, 1994, 1995.

<table>
<thead>
<tr>
<th>#</th>
<th>Date</th>
<th>Species</th>
<th>Community</th>
<th>Saprobity</th>
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<td><em>Pleuronema coronatum</em></td>
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</tr>
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<td></td>
<td></td>
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<td>STE, MOO</td>
<td>β-α-meso</td>
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<td></td>
<td></td>
<td><em>Uroleptus willii</em></td>
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<td></td>
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<td><em>Litotodus cf. lamella</em></td>
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<td>α-meso</td>
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<tr>
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<td><em>Loxophyllum meleagris</em></td>
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<td>STE, MOO</td>
<td>β-α-meso</td>
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<tr>
<td></td>
<td></td>
<td><em>Frontonia sp.</em></td>
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<td>Meso</td>
</tr>
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<td></td>
<td><em>Coleps hirtus</em></td>
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<td>β-α-meso</td>
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<td>β-α-meso</td>
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<td><em>Vorticella campanula</em></td>
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<td><em>Vorticella campanula</em></td>
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<td>β-α-meso</td>
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<td><em>Paramecium bursaria</em></td>
<td>STE, MOO</td>
<td>β-α-meso</td>
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<td></td>
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<td><em>Uroleptus sp.</em></td>
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<td></td>
<td></td>
<td><em>Obertrumia aurea</em></td>
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<td><em>Frontonia sp.</em></td>
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<td><em>Euplotes affinis</em></td>
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<td><em>Lacrymia sp.</em></td>
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<td>β-meso</td>
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<td>Date</td>
<td>Species</td>
<td>Community</td>
<td>Saprobity</td>
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<td><em>Vorticella</em> sp.</td>
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<td><em>Litonotus lamella</em></td>
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<td><em>Urotichia</em> sp.</td>
<td>ST E, MOO</td>
<td>β-α-meso</td>
</tr>
<tr>
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<td><em>Cyrotolophosis</em> sp.</td>
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<tr>
<td></td>
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<td><em>Paramecium bursaria</em></td>
<td>STE, MOO</td>
<td>β-α-meso</td>
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<td></td>
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<td><em>Uroleptus</em> sp.</td>
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<td></td>
<td><em>Euplates affinis</em></td>
<td>TRI, STE, CYR, HAS</td>
<td>β-α-meso</td>
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<td></td>
<td></td>
<td><em>Urotichia</em> sp.</td>
<td>HAS</td>
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<td><em>Nassula</em> sp.</td>
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<td><em>Stentor roeselii</em></td>
<td>STE</td>
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<td><em>Ophrydium eutrophicum</em></td>
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16 ciliates have been well documented with the image analysis system: 1 Heterotrichida (*Stentor roeselii*), 4 Hymenostomata (*Lembadion lucens, Paramecium bursaria, Pleuronema coronatum, Tetrahymena-pyriformis-complex*), 2 Hypotrichia (*Euplates affinis, Uroleptus willii*), 4 Nassulida (*Furgasonia blochmanni, Obertrumia aurea, Pseudomicrothorax agilis*), 2 Peritrichia (*Ophrydium eutrophicum, Vorticella campanula*), 1 Pleurostomatida (*Loxophyllum meleagris*) and 1 Prostomatida (*Coleps hirtus*).  

Following abiotic data were measured for SP4: pH = 8.5, dissolved oxygen (mg·L⁻¹) = 8.86, dissolved oxygen (%) = 107.3, conductivity (µS·cm⁻¹) = 229 and water temperature (°C) = 21.7.  

Following abiotic data were measured for SP4: pH = 8.6, dissolved oxygen (mg·L⁻¹) = 9.3, dissolved oxygen (%) = 100, conductivity (µS·cm⁻¹) = 300 and water temperature (°C) = 16.3.
3.1. *Stentor roeselii*

Figure 9 a-i: *Stentor roeselii* in vivo (a-i) and during conjugation (f-i). a: Size measurement of body length and width. b+e: Different shapes of the same specimen: contracted (b) and elongated (e). c: Longitudinal ciliary rows (~80) with enlarging interspace. d: broad oral apparatus (~200µm) and large macronucleus with reaches from the top to the end. f-i: Two conjugating specimen: process lasted around 20 minutes; left specimen was strongly twisted (arrow) and died afterwards. CV – contractile vacuole, MA – macronucleus.
The main morphometric data on *Stentor roeselii* are: body length and width = 420.4 x 189.2 µm, oral apparatus = 154.9 µm, ciliary rows = ~78, n=1.

Following the V9 region of *Stentor roeselii*:

<table>
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<tr>
<th>A</th>
<th>GAU</th>
<th>CG</th>
<th>GAACC</th>
<th>CG</th>
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<tr>
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<td>AAU--</td>
<td>--</td>
<td>AGGGU</td>
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</tbody>
</table>

Following the autecological data on *Stentor roeselii*:

**Saprobic classification:** a-b \((o = 1, b = 4, a = 5, l = 2, Sl = 2.4)\) (SLADECK et al. 1981, WEGL 1983, FOISSNER 1988)

**Community:** Stentoretum

**Distribution:** very frequent in benthic and periphyton

**Locality:** Schleifenbach
3.2. *Lembadion lucens*

![Figure 10 a-h: *Lembadion lucens* in vivo (a-e, g-h) and after dry silver nitrate staining (f). a-b: Size measurement of body length and width (a) and of oral apparatus length and width (b). c: Typical caudal cilia (~10). d: the hump-like spike of the right sight (arrow). e: Longitudinal ciliary rows (~25-35) and pellicula; the first third is striped lengthwise; the second third is square-shaped. f: Ventral view. g-h: Ingested diatoms (arrows), the kidney-like macronucleus and dorsal one contractile vacuole. CC – caudal cilia, CV – contractile vacuole, MA – macronucleus.](image-url)
The main morphometric data on *Lembadion lucens* are: mean body length and width = 55.87 x 40.74 µm (standard deviation (SD) = 4.36 x 2.21), mean oral apparatus length and width = 39.38 x 14.79 µm (SD = 0.11 x 2.93), mean ciliary rows = 26.67 (SD = 1.15), n = 3.

Following the autecological data on *Lembadion lucens*:

**Saprobic classification:** b (b=9, a=1, l=5, Sl=2.1) [SLADECK et al. 1981, WEGL 1983, FOISSNER 1988]

**Community:** Stentoretum

**Distribution:** wide, all-season. Maxima in Autumn.

**Locality:** Zeller Ache, Lake Irsee, Lake Mondsee

### 3.3. *Paramecium bursaria*

Figure 11 a-e: *Paramecium bursaria* in vivo. a+b: Very variable shape: elliptic, egg- or slipper-like. Oral apparatus often indistinct (arrows). c: Numerous spindle-like extrusomes (Trichocystes) under the pellicula. d: Green endosymbionts and food vacuoles. e: Size measurement of body length and width. ES – endosymbionts, FV – food vacuoles.

(Photo (e): Christian Spanner)
The main morphometric data on *Paramecium bursaria* are: body length and width =

Following the folded V9 region of *Paramecium bursaria*:

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<td>CCA</td>
<td>UG</td>
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<td>U</td>
<td>AA</td>
<td>CUUU</td>
<td>AA-</td>
<td>C</td>
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</tbody>
</table>

Following the autecological data on *Paramecium bursaria*:

**Saprobiic classification:** \( b (b = 7, a = 3, l = 4, SI = 2.3) \) [SLADECK et al. 1981, WEGL 1983, FOISSNER 1988]

**Community:** Stentoretum, marsh-influenced

**Distribution:** widespread, Maximum April-June, Aufwuchs and Detritus

**Locality:** Zeller Ache, Lake Mondsee, Lake Irrsee
3.4. *Pleuronema coronatum*

The main morphometric data on *Pleuronema coronatum* are: body length and width = 45.94 x 27.59 µm, ciliary rows = 32, n = 1.

Following the autecological data on *Pleuronema coronatum*:

**Saprobic classification:** b (b = 7, a = 3, l = 4, SI = 2.3) (SLADECK et al. 1981, WEGL 1983, FOISSNER 1988)

**Community:** Pleuronemetum

**Distribution:** cosmopolitan, all-season, low abundance in limnic systems

**Locality:** Lake Mondsee

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Figure 12 a-d: *Pleuronema coronatum* in vivo, stagnant with braced cilia. a: Size measurement of body length and width. b, d: Longitudinal ciliary rows (35-40) and oral apparatus (c), posterior cilia are strongly elongated (d). c: Remarkable undulating membrane (arrow). CC – caudal cilia, MA – macronucleus.
3.5. *Tetrahymena pyriformis*- Complex

The main morphometric data on *Tetrahymena pyriformis*- complex are: mean body length and width = 49.38 x 28.47 µm (SD = 1.63 x 3.07), mean ciliary rows = 19 (SD = 1), n= 3. Following the autecological data on *Tetrahymena pyriformis*- complex:

**Saprobic classification:** p-i (p = 10, l = 5, SI = 4.5E, for limnic systems) (SLADECK et al. 1981, WEGL 1983, FOISSNER 1988)

**Community:** Colpidietum

**Distribution:** wide, all-season, strongly polluted bacteria-rich habitats

**Locality:** Zeller Ache (during dry period in August 2018)
3.6. Euplotes affinis

Figure 14 Euplotes affinis in vivo (a-e, g-h) and after dry silver nitrate staining (f). a: Size measurement of body length and width. b: Lateral view: ellipsoid shape, dorsal side strongly curved. Typical aufwuchs species: specimen grazes on the surface of the cover glass (arrow). c+d: Nine frontoventral cirri (c) and five transversal cirri (d) with lathlike bulges in between. The third from the left is the biggest and longest one (arrow). e: Huge adoral zone of membranelles (~70% of the body length). f: Stained specimen. g+h: Detail view: Excretion of food (diatom) from the cell, probably because of the higher pressure of the cover glass. AZM – adoral zone of membranelle, TC – transversal cirri, V – frontoventral cirri
The major morphometric data on *Euplotes affinis* are: mean body length and width = 97.78 x 62.89 (SD = 54 x 29.89), V = 9, TC = 5, n = 5.

Following the autecological data on *Euplotes affinis*:

**Saprobic classification**: b-a (o = +, b = 6, a = 4, l = 3, Sl = 2.4) (SLADECK et al. 1981, WEGL 1983, FOISSNER 1988)

**Community**: Trithigmostometum, Stentoretum, Cyrtophoresetum, normal activated sludge

**Distribution**: widespread, all-season

**Locality**: Zeller Ache, Lake Mondsee, Lake Irrsee
3.7. *Uroleptus willii*

Figure 15 a-f: *Uroleptus willii* in vivo. a: Size measurement of body length and width, shape fusiform. b: Greenish color because of endosymbionts, brownish on the posterior end because of crystal-like structures (arrow). c+ e: Typical ciliature of the anterior end and big adoral zone (30-45% of body length). d: Marginal rows (arrow) and caudal cilia. f: Two macronuclei in the midline, with micronuclei. Inflexible transverse cirri on the posterior end. AZM – adoral zone of membranelles, CC – caudal cilia, CV – contractile vacuole, MI – micronucleus, MA – macronucleus, TC – transverse cirri.

The main morphometric data on *Uroleptus willii* are: mean body length and width = 87.23 x 33.74 µm (SD = 5.04 x 11.85), n = 3.
3.8. *Furgasonia blochmanni*

Figure 16 a-f: *Furgasonia blochmanni* in vivo. a: Size measurement of body length and width, shape cylindrical. b,d-e: Contractile vacuole with many and huge macronucleus (d). Huge trichocysts (~5μm) are clearly visible. c: Longitudinal ciliary rows (~55-60). b-c, f: Rigid club-shaped cytopharynx consisting out of 16-18 rows (arrow). CV – contractile vacuole, EX – Extrusomes, MA – macronucleus.
The main morphometric data on *Furgasomia blochmanni* are: body length and width = 140.3 x 80.86 µm, longitudinal ciliary rows = 60, trichocysts = 5 µm, macronucleus = 36.04 x 34.62 µm, n =1.

Following the folded V9 region of *Furgasonia blochmanni*:
3.9. *Obertrumia aurea*

The main morphometric on *Obertrumia aurea* are: body length and width = 180.8 x 130.9 µm, longitudinal ciliary rows = 110, n = 1.

Figure 17 a-g: *Obertrumia aurea* in vivo (a-f) and site (g). a: Size measurement of body length and width. b: Cell conspicuously colored (green, yellow, brown, violet) by ingested cyanobacteria and algae. c: Longitudinal ciliary rows (~100-120). d: Oral apparatus: Huge oral basket (arrow). e: central macronucleus. f: Central contractile vacuole surrounded by huge (arrow). g: One site of Obertrumia aurea (Zeller Ache, 08/2018). CV – contractile vacuole, MA – macronucleus.
Following the folded V9 regions of *Obertrumia aurea*: (1) sequenced with Single Cell PCR and nested PCR, (2) sequenced with REPLiG.

Following the autecological data on *Obertrumia aurea:*


**Community:** Marynetum

**Distribution:** wide, all-season

**Locality:** Zeller Ache

**Notes:** Feed mainly on cyanobacteria.
3.10. *Pseudomicrothorax agilis*

Figure 18 a-f: *Pseudomicrothorax agilis* in vivo: a: Size measurement of body length and width. b: Body shape is ellipsoid; ventral side is almost straight, dorsal side is concave. The pellicula pattern is broadly striped. c-d: Different forms of extrusomes: fusiform in the cell (c, arrow) and anchor-shaped after ejection (d). e: Always 12 longitudinal ciliary rows (deep furrows). Mouth subapical (oral basket) f: Macronucleus and contractile vacuole. CV – contractile vacuole, MA – macronucleus.

The main morphometric data on *Pseudomicrothorax agilis* are: mean body length and width = 73.85 x 71.44 (SD = 25.10 x 3.78), mean longitudinal ciliary rows = 12, n = 3.

**Saprobic classification:** b (o = 1, b = 8, a = 1, I = 4, SI = 2.0) (SLADECK et al. 1981, WEGL 1983, FOISSNER 1988, MORAVCOVA 1977)

**Community:** -

**Distribution:** wide, scarce in detritus in presence of cyanobacteria.

**Locality:** Zeller Ache (Detritus).
3.11. *Nassula cf. citrea*

![Image of Nassula sp. in vivo with measurements and annotations]

**Figure 19 a-d:** *Nassula* sp. in vivo. 
- **a:** Size measurement of body length and width.
- **b:** Huge contractile vacuole and macronucleus. Close to the cytostome, yellow pigments are located (arrow).
- **c+d:** Massive cytostome (up to 35 µm).


The main morphometric data on *Nassula cf. citrea* are:
- Body length and width = 62.77 x 44.28 µm,
- Oral apparatus = 20.14 µm, n = 1.

Following the folded V9 region of *Nassula cf. citrea*:

```
A   GAU    C   GA    C---    GGA   U   UU
UCCU CC  UUUGAGUGAU GGGU AC  UUUC  CUGGGUCG C U
//   ||    ||    //    ||    ||    //    ||
AGGA GG  AGAUUCAUCUA UCCA UG  GAAG GGGGUAGGC G /
A   ---   C   AA    AAUU   ---   U  AA
```

*Nassula cf. citrea*
3.12. *Ophrydium eutrophicum*

Figure 20 a-i: *Ophrydium eutrophicum* in vivo. They build colonies if they are undisturbed. All pictures show the motile shape. a: Size measurement of body length and width. b: Typical striped pattern of the pellicula. c: stretched macronucleus and contractile vacuole. d-f, i: Detail view of Peristomkragen: ~ 40-45 µm, bulge-like. Adorale spiral makes ~ 2.5 rounds. g+h: Stretched (g) and contracted (h) specimen. Green color because of Endosymbionts. CV – contractile vacuole, MA – macronucleus.

The main morphometric data on *Ophrydium eutrophicum* are = body length and width = 138.3 x 41.6 µm, body length contracted = 88.94 µm, PERISSTOMKragen = 31.77 µm, adoral spiral = 2.5 rounds, n = 1.
3.13. *Vorticella campanula*

![Image](image_url)

Figure 21 a-i: *Vorticella campanula* in vivo (a-h) and after staining with methyl-green pyronine (i) a,d: Size measurement of body length and width (a) and stalk width (d). b: CV and broad collar (arrow). c: Strong refractive oil drops. d-f: The stalk contracts spirally (e,f) and is ~ 5-12 µm wide (d). g-h: Different shapes of the specimen: globular with spikes in contracted cell (g) and bell/hat-shaped in the stretched cell (h). i: The macronucleus is j-shaped and dark purple because of the staining (arrows). CV – contractile vacuole.

The main morphometric data on *Vorticella campanula* are = mean body length and width = 73.90 x 64.53 µm (SD = 21.96 x 19.20 µm), mean stalk width = 7.27 µm (SD = 1.41), n = 4.
Following the main autecological data on *Vorticella campanula*:

**Saprobic classification: a; b=1, a=9, I=5, SI=2.9** (SLADECK et al. 1981, WEGL 1983, FOISSNER 1988)

**Community:** Stentoretum

**Distribution:** widespread, all-season

**Locality:** Zeller Ache, Lake Mondsee

### 3.14. *Loxophyllum meleagris*

Figure 22 a-d: *Loxophyllum meleagris* in vivo a+d: Shape is lanceolate, the anterior third narrows and bends beak-like (arrow). Remarkable warts (Extrusomes) dorsal, macronucleus consists of 16-31 elliptic nodes. One big contractile vacuole dorsal. b+c: Very flexible and contractile cell. CV – contractile vacuole, EX – Extrusomes, MA – macronucleus.
Saprobic classification: \( b (b = 8, a = 2, l = 4, Si = 2.2) \) (SLADECK et al. 1981, WEGL 1983, FOISSNER 1988)

Community: Pleuronemeta

Distribution: wide, all-season *

Locality: Lake Mondsee

### 3.15. Coleps hirtus

Figure 23 a-g: Coleps hirtus in vivo (a-f) and location (g). a: Size measurement of body length and width. b+d: Typical carapace: windows are pretzel-like (arrow); brownish colour through ingested food. c: Indistinct oral apparatus with undulating membrane (arrow). e: Longitudinal ciliary rows (~12-15). f: Central macronucleus, one contractile vacuole in the back. Green endosymbionts. g: One site of Coleps hirtus (August 2018, Zeller Ache). CV – contractile vacuole, ES – endosymbionts, MA – macronucleus.
The main morphometric data on *Coleps hirtus* are: mean body length and width = 49.83 x 29.96 µm (SD = 1.59 x 3.83), longitudinal ciliary rows = 14, n = 3.

Following the main autecological data on *Coleps hirtus*:

**Saprobic classification:** b-a (b = 5, a = 5, l = 3, Sl = 2.5) (SLADECK et al. 1981, WEGL 1983, FOISSNER 1988)

**Community:** Stentoretum

**Distribution:** wide, all-season

**Locality:** Zeller Ache, Lake Mondsee
4. Discussion

For years, researchers state the tremendous role ciliates play in freshwater ecosystems. For example, Beaver and Crisman (1989) focused on the linking between ciliates and the trophic state of a lake and their nutrition remineralization: taxonomic replacement and changes in morphotypes happen with shifting eutrophication.

We investigated 22 ciliates using a culture independent approach from seven different taxonomic groups either morphologically or both, morphologically and molecularly. According to the saprobity of the indicator ciliates, the trophic status for all sampled water bodies, except Lake Happinger See, can be defined as alpha-beta/betamesosaprob. On Lake Happinger See only *Ophrydium eutrophicum* was sampled, an abundant species without any saprobic classification, but according Foissner et al. (1992) described as betamesosaprob. The most ciliates belong to the communities *Stentoretum* and *Pleononometum* (Tab. 3), typically ciliates assemblages from alpha-beta/beta-alphamesosaprobic water. Both main indicator species (*Stentor roeselli* for *Stentoretum* and *Pleuronema coronatum* for *Pleononometum*) were found. Nevertheless, many subniches were detected in the two Lakes and River Zeller Ache, respectively. SP5, the point where the River Zeller Ache runs into Lake Mondsee, two soil-associated ciliates were sampled (*Furgasonia blochmanni* and *Nassula* cf. *citrea*). *Obertrumia aurea* feeds mainly on cyanobacteria and diatoms and was found in stagnant parts of River Zeller Ache (SP2) and River Schleifenbach (SP6). The attributed community is *Marynetum*.

The sample period was conducted during one of the warmest and driest summer in history (Zentralanstalt für Meteorologie und Geodynamik 2018). Even after multiple sampling and different treatments of the samples, e.g., centrifugation, only once a ciliate of the order Pleurostomatida was found in April 2018 in Lake Mondsee. According to Wilhelm Foissner, species of this order are distributed all season in low abundance and peaks in spring and autumn (Foissner et al. 1995). Yet, there is no knowledge about cysts within the group Pleurostomatids (Foissner et al. 1995), so even in sample water stored in the lab in cooler conditions, no Pleurostomatid developed. Thus, higher temperature might affect the species assemblage by taxonomic replacements.

*Difficulties arising with morphological determination of ciliates*

Cultivation problems figure out as severe obstacle. The first step of the combined approach – the taxonomal identification- is already the crucial point. Many of the specimen we found did not survive the identification process or they died before taxonomical identification was successful. Even if it was possible, finding enough specimen to conduct the single cell PCR often failed. Cultivation could be problem solving, but the most ciliates stay uncultivable until now. Culture-independent molecular
methods (Countway et al. 2005; Massana et al. 2006; Moon-van der Staay, Seung Yeo et al. 2001) circumvent these difficulties, but linking OTUs with known morphospecies depends still on the taxonomical accuracy which is often lacking. To break the circle, we pipetted the ciliates directly from the samples in microtubes filled with RNA later and freeze them. Important characteristics for identification remained visible, single cell PCR was possible as well (Pröschold et al., in prep.).

**Difficulties arising with molecular analysis of ciliates**

Another problem is the small amount of DNA receiving from the single cell PCR. In any case of contamination, e.g. food algae or fungal spores which passed the filter, the following nested PCR amplifies too much of the foreign DNA and hardly evasive treatments can be conducted. More ciliate specific primers prohibit the amplification of foreign DNA, but establishing ciliate specific primers is difficult and an “try and error” – based approach. Additionally, many ciliate specific primers are species or genera specific.

We conducted Single Cell PCR and nested PCR on 16 ciliates from environmental samples, varying the primers. Only three amplification produces suitable sequences, 13 sequences were contaminated and thus useless. With the small amount of DNA the nested PCR produced, no other treatments were possible to conduct.

Starting with low biomass, the whole genome amplification (WGA) can be helpful for generating many copies of the genome (Nature) but is prone to artifact sequences (Ahsanuddin et al. 2017). The REPLI-g multiple displacement amplification – a further development- still has its limitation concerning diversity (Ahsanuddin et al. 2017), but we used the method on already identified species. The handling is easy, and all samples produced workable sequences. Furthermore, one single cell produces enough DNA for different treatments, which simplify the workflow highly. The only limitation can be the high costs per amplification.

**Is the hypervariable V9 region a valuable marker for differentiating ciliates on species level?**

Tanabe et al. (2016) reviewed the three most common hypervariable regions (V1-V3, V4-V5 and V7-V9) used for the investigation of protists’ communities (Tanabe et al. 2016). Even if they conclude that the V1-V3 region will be the most suitable marker to study natural eukaryotic communities, the largest assessment of eukaryotic diversity published to date was conducted on basis of the V9 marker (Vargas et al. 2015). In summary, the following reasons made the V9 region a suitable marker: (i) it is shorter than the V1-V3 and V4-V5 region and conserved in length (Vargas et al. 2015). (ii) It shows the best balance between taxonomic resolution and database coverage (Tanabe et al. 2016). (iii) And finally, it
is frequently used in public reference databases and so the basis for global taxonomical links (Vargas et al. 2015).

*Paramecium bursaria*’s V9 region differs diagnostically from those of its closest neighbor *Paramecium putrinum* (Figure 24 and Figure 25) (Kreutz et al. 2012). Morphologically, a possible likelihood of confusion is given concerning the both green mixotrophic *Paramecium* species *P. bursaria* and *P. chlorelligerum* due to low taxonomical expertise. By comparing the V9 regions, this risk can be excluded. Using rDNA (ITS1-5.8S-ITS2-5LSU) fragments, mitochondrial cytochrome c oxidase subunit I (COI), and H4 gene fragments, recent studies show five syngen in *P. bursaria* (Grecek-Stachura et al. 2012). The V9 marker differs diagnostically among species but not in cryptic species. It can be assumed, that the autecological demands of the cryptic species are the same.

![Diagram](image-url)

Figure 24: Kreutz et al. (2012): This maximum-likelihood (ML) tree showing the phylogenetic placement of *Paramecium* (Viridoparamecium) based on the 18S rRNA gene sequence. Bootstrap values above 50 for the ML (1,000 replicates) and neighbour-joining evolutionary distance (BioNJ, 1,000 replicates) analyses are given at the individual nodes. Large dots at nodes indicate full support from both tree construction methods. Added colored boxes by the author mark the species of interest.
Figure 25: Edited and folded V9 regions of *P. bursaria*, *P. putrinum* and *P. chlorelligerum*. The white boxes show the differences between the three species (hemi compensatory base changes (CBC) and CBCs). The green marked species *P. bursaria* and *P. chlorelligerum* are mixotroph.

According to the phylogeny of Fernandes et al. (2016), the species *Stentor roeselli* and *Stentor muelleri* are very closely related, whereas *Stentor coerulus* and *Stentor olymorpus* build own subgroups (Fernandes et al. 2016) which is represented in the V9 regions, respectively (see Figure 27). Thus, concerning *Stentor*, the V9 region is no valuable marker for differentiation at species level. *S. roeselli* is the most important indicator species of the community *Stentoretum*. The demands concerning food sources and temperatures compared to *S. muelleri* varies slightly: *S. roeselli* is very abundant in stagnant and slow running beta-alphamesosaprobic, strongly polluted water bodies with temperatures from 0-25 °C and feed mainly on bacteria, diatoms, phytoflagellates and other ciliates (Foissner et al. 1992). *S. muelleri* is low abundant in stagnant and slow running beta-alphamesosaprobic water bodies with maxima in May/June and feed mainly on bacteria, phytoflagellates and diatoms (Foissner et al. 1992). Foissner et al. (1994) propose 1994 *S. roeselli* as indicator species for beta- alphamesosaprobic water, as well as *S. muelleri* and *S. polymorphus*. Summarizing, the V9 marker does not differentiate at species level regarding *Stentor*, but finding indicator species in water quality assessment, this differentiation will suffer. The recently published sequence of *S. roeselli* from Lake Zürich (Pitsch et al. 2019), fits 100% with the sequence retrieved from River Schleifenbach.
Figure 26: Fernandes et al. (2016) published the phylogenetic tree inferred from 18S-rDNA sequences based on maximum likelihood (ML). Numbers or letters near the nodes represent the bootstrap values from the ML analysis, the posterior probabilities from Bayesian inference (BI) and bootstrap values from the maximum parsimony (MP) analysis. Dashes (–) reflect disagreement between topologies. Asterisks (∗) indicate full support in all analyses. The scale bar corresponds to 6 substitutions per 100 nucleotide positions. Specimens with newly obtained sequences are in bold. The codes preceding the species names are the GenBank accession numbers. Colored boxes added by the author mark the species of interest.
Figure 27: Edited and folded V9 region of four Stentor species: closely related S. roeselii and S. muelleri, S. coeruleus and S. polymorphus. The white box shows the differences through two CBCs.

The two Nassulida Furgasomia blochmanni and Nassula cf. citrea obliviously differs morphologically (see Figure 16 and Figure 19) whereas BLAST declared them as the same species (F. blochmanni) with 100% identity and 98% identity, respectively. Comparing the V9 region reveals two hemi CBCs, which matches with the differing identity percentage. Thus, the V9 region differs diagnostically group specific.

Another Nassulida, Obertrumia aurea, was morphological identified and well-documented, but BLAST attributed the V9 sequence to O. georgiana (accession number X65149, 100% query and identity). Either the sequence deposited in BLAST is falsely attributed to O. georgiana, but the authors Bernhard et al. (1995) used cultured organisms. It is likely, that the V9 region differs only on genus level. Yet, no data of saprobic classification for O. georgiana is available nor any phylogeny of O. aurea. Comparing the V9 regions of the three nassulid genera, clear differences in the first part as well in the last part are visible (hemi CBCs) (see Figure 29 and Figure 28). Phylogenetically, O. georgiana and F. blochmanni are related closely (see Figure 30).
Figure 28: Edited and folded V9 region of two clearly differing Nassulida: *Furgasonia blochmanni* and *Nassula cf. citrea*. The white boxes show the differences through two hemi CBCs.

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*Furgasonia blochmanni*

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*Nassula cf. citrea*

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Figure 29: Edited and folded V9 region of *Obertrumia aurea* and *O. georgiana*, which differentiate on genera level.

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Bernhard et al. (1995) published the phylogeny of ciliates as inferred from bootstrap analyses. The topology of the tree is computed from the strict consensus of the two most parsimonious PAUP trees (random addition sequence with 10 replicates, tree length 2,549 steps, consistency index 0.51, rescaled consistently index 0.26). Bootstrap values were recorded independently for 1,000 resampling of maximum parsimony (first figure) and neighbor joining (second figure) analyses.
The indispensability to document the morphospecies, illustrates the following example: a specimen found in SP6 was morphological identified as *Obertrumia aurea*. The sequence (amplified with REPLIg) ran in BLAST reveal a 100% query and 100% identity a *Frontonia* sp. Morphologically, the species are unlike to each other, and given to the size of the oral apparatus, it is unlikely, that one ciliate has eaten the other. Confident about the correct identification, no photo documentation was conducted. Thus, the sequence is useless. 

For the applied practical use in water quality surveys or the theoretical taxonomic work, we recommend following combined approach:

- After sampling, investigate the sample with the binocular microscope for overall information, e.g. present of bacteria and other protists.
- Pick the ciliates of interest and transfer them into clean culture plats filled with sterile filtrated lake water (a fast change to medium like Volvic often kills the ciliates).
- 1-3 specimen of each species should be transferred into tubes filled with RNA later (1-2µl) diluted with medium (4-8µl) as back up for both, morphological identification and REPLIg amplification.
- Conduct morphological identification and photo documentation on at least three individuals of each species.
- After morphological identification, the ciliates are to wash 3-5 times in sterile filtrated lake water using clean drawn- micropipettes and slides.
- The ciliates are to be starved over night or at least up to 3h in the temperature conditions of the sample point where they have been found.
- Afterwards, conduct a REPLIg amplification on one specimen and/or the single cell PCR with appropriate primers on up to 8 individuals.
- Test the DNA products with gel electrophoresis and conduct a nested PCR with the purified DNA. Alternatively, the DNA products can be cut out directly from the agarose gel and purified.
- Prepare the samples for sequencing according the manuals.
- Run the edited results in the searching tool BLAST for any already deposited sequences.
- Edit the V9 region as described above.
**Conclusion**

Shifting environmental conditions, such as e.g. temperature can alter the ciliate’s assemblage, but still too less information about the behavior is available for deeper interpretation. Using RNA later to store the ciliates, a new and easy approach for documentation and fixation is given. Complex staining methods, e.g. Quantitative Protragol Stain (QPS) can be simplified in daily routine and only used for specific quests. Amplification of the 18s rDNA through the REPLIg approach figured out to be most promising. Again, the integrative approach was the only way to produce reliable sequences.

New molecular approaches uncovered an underestimated number of protists in the oceans and freshwater systems (Vargas et al. 2015) and still the discovery continues, e.g. in the lake and rivers of Austria, nowadays, still new species are found (Sonntag et al. 2008). Not to mention the lack of knowledge concerning interactions between predators and prey, (endo)symbioses or inter-and intraspecific interactions. But even whole ecosystems like the sediment of lakes are still to discover. Generating reliable results and answers, developing approaches are mandatory just like the comprehensive education in taxonomical skills.
5. Acknowledgments

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6. Literature


DAS EUROPÄISCHE PARLAMENT UND DER RAT DER EUROPÄISCHEN UNION (2000): Amtsblatt_WRRL.


