



University of Cologne

**Ecology and diversity of protists in
sediments and amphibian
associated habitats**

Inaugural – Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Köln, 2023

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Tag der mündlichen Prüfung:	15.12.2023

"Nothing in life is to be feared. It is only to be understood. Now is the time to understand more, so that we may fear less." — Marie Curie."

Acknowledgements

First and foremost, my highest gratitude is owed to the committee members with special emphasis to my supervisor and scientific mentor **Prof. Hartmut Arndt**. For giving me the opportunity to write my thesis in his working group and the myriad of experiences and adventures that came with it, I will always be grateful. The participation of expeditions to the Maldives and Colombia as well as cruises over the Atlantic Ocean and the Baltic Sea were invaluable and thriving experiences for me. Thank you for guidance and support throughout my scientific path.

I would also like to thank **PD Dr. Kathrin Lampert** for being my second assessor. Thank you for your precious and valuable time. I would also like to thank **Prof. Dr. Christine Heim** for being the chairwoman of my disputation

I would also like to express my particular gratitude towards **Prof. Thomas Ziegler** and **Prof. Miguel Vences**, who gave me interesting insights into the world of herpetology and offered me guidance and support during my master studies as well as during following projects.

I would also like to thank **Dr. Frank Nitsche** for helpful advises in and out of the lab and delightful conversations.

For supervision and great organisation within MGF Ostsee, I thank **Prof. Klaus Jürgens** and **Dr. Christina Schmidt**.

Thank you, **Dr. Sabine Schiwitza**, not only for being the best of colleague one could wish for, but also for becoming a close friend, for laughing and crying together, for mental support, shared sorrow, joy and advice.

Thank you **Dr. Manon Dünn**, for endless answered questions regarding NGS analysis, your calm and kind ways, warm friendship, mental support and the best of a conference roommate.

Rosita Bieg I would like to thank for allowing me to take advantage of her experienced advice in the lab but just as much for shared artistic interest and inspiration.

Additionally, I would like to thank **Brigitte Gräfe** for technical support with both light- and electron microscopy and **Bärbel Jendral** and **Anke Pyschny** for keeping paper work an arm-length away from me. I also thank the whole **working group** for help, advice and nice atmosphere and especially **Johannes Werner** for chocolates and camaraderie in the office.

I am also grateful for meeting new friends along my journeys. **Gavin D'Mello** I would like to thank for making quarantine time a lot better. For making the cruises and the work for MGF Ostsee as pleasant as possible, I would like to thank **Tim Henning**, **Dr. Judith Piontek**, **David Clemens** and **Dr. Patricia Roeser** for friendship, advice, hospitality and open arms whenever needed.

Acknowledgements

I would like to thank **Karla Wiechmann, Ruben Rotmann, Malte Burkhard, Svenja Huth, Merle Heilmann, Jacqueline Bruhy** and most important **Julian Wagenhofer** for their support and contribution to this thesis.

I thank my friends for support as well as distraction whenever needed. My special thanks go to **my family, my father and mother** who laid the basis for my interest in biology and for their endless belief in my abilities. But most of all, I thank my partner, my homebase **Oliver Töplitzer** for love and support in every possible way and for believing in me when I wouldn't.

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Abstract

Protists, unicellular eukaryotes, are one of the most diverse (if not the most diverse) group of organisms on the planet and are widely distributed over the eukaryotic tree of life. They exhibit a ubiquitous distribution upon many terrestrial and aquatic ecosystems. Additionally, their ecological roles span across many different trophic modes, from phototrophic primary producers like phytoplankton, to parasites, symbionts and predators, significantly involved into ecological nutrient cycling. In recent years, molecular techniques have unveiled an impressive array of previously unknown protists, changing our perception of their ecological roles and diversity in global ecosystems. The primary aim of the present study was to explore the diversity with regard to taxonomic and functional composition. By combining amplicon sequencing with modern taxonomic methods such as cultivation-based approaches and microscopy, our objective was to expand knowledge of protist diversity in brackish and freshwater habitats. This included exploring the protist communities in their entirety as well as investigating two specific communities closely associated with amphibian species. We selected two regions in the southern Baltic Sea differing in several abiotic factors (Fehmarnbelt and Oderbank), as a model for studying benthic protist communities in brackish-water environments. The study offered novel insights into brackish-water communities, that have not been investigated in this magnitude and might hint at potential correlations between specific protist groups and environmental parameters. This study also has an applied aspect as it was intended to serve as a baseline study to monitor the effect of ground fishing in future. Using a very strictly filtered amplicon sequencing dataset, we found no overlap in taxonomic composition between the two selected regions. While ciliates exhibited remarkable diversity in both regions, marine alveolates belonging to the MALV group displayed the highest read abundances. Previous studies have shown that this group of organisms, with an assumed parasitic life style, is highly abundant in marine environmental datasets, but have not yet been recorded from brackishwater habitats. However, as they are mostly uncultured, not much is known about their ecology. In freshwater environments but also in amphibian hosts, protist parasites and commensals are known to be common. As an example, we wanted to get more insight into the trophic mode of parasitism by investigating the presence of *Batrachochytrium dendrobatidis* (*Bd*) and other amphibian parasites

in the green toad (*Bufo viridis*) and syntopic amphibians. Therefore, we combined DNA metabarcoding with a qPCR survey. The results revealed the presence of *Bd* in green toad populations, highlighting the potential threat of this deadly pathogen to amphibian populations in the investigated region. Additionally, the study identified other parasitic organisms, mainly protists, inhabiting the gut of the amphibians. As an antagonist to the detrimental effects of the presence of protists in connection with amphibians, we were interested in the occurrence of the unicellular alga *Oophila* in German breeding waters of *Rana dalmatina*. The alga is known for its close association with various amphibians, forming a kind of mutualistic relationship. Our study offers valuable insights into the diversity of micro-eukaryotes, with a particular focus on green algae, inhabiting amphibian clutches. Through DNA metabarcoding, we uncovered a wide array of protist taxa colonizing these amphibian egg masses, highlighting the ecological complexity of this specific habitat. Importantly, the study revealed substrate-specific preferences among these protists - clutches are inhabited by a unique protist community. Additionally, we aimed to disentangle parts of the uncertain taxonomy of *Oophila*, showing that a certain clade is exceptionally frequent in amphibian clutches, also in our investigated specimens. These results emphasize how taxonomic studies play a crucial role in enhancing the accuracy and reliability of public databases used in environmental sequencing studies. Modern taxonomy provides essential reference points for researchers to correctly classify and interpret the vast diversity of genetic sequences encountered in various ecosystems. To highlight this aspect further more, we investigated two taxonomic groups in more detail, to revise and/or expand their taxonomy. The genus *Goniomonas* (sensu lato) is not only the single taxon of cryptophytes without a plastid, it also shows a deep genetic divergence between the freshwater and marine strains. Even though it is a very frequently observed heterotrophic flagellate, only few species have been described yet, mainly on the basis of morphologic observations. The objective of our study was to revise the genus *Goniomonas* and offer a suitable Neotype. In the course of the study it became evident that the genus had to be split into several new genera. We based the introduction of *Limnogoniomonas* gen. nov., *Goniomonas* and *Aquagoniomonas* gen. nov., *Neptunogoniomonas* gen. nov., *Marigoniomonas* gen. nov., *Thalassogoniomonas* gen. nov., *Poseidogoniomonas* gen. nov. and *Cosmogoniomonas* gen. nov. on both morphological and phylogenetic

investigations. The taxonomy of the bicosoecid *Cafeteria* has been recently revised. With our study we aimed to resolve the species diversity of the genus furthermore, additionally by adding autecological experiments into an integrative taxonomic approach. In this way, we were able to describe several new species of *Cafeteria* including one from the Baltic Sea. As those sequences are or will be deposited in public databases, this thesis shows the close connection between modern taxonomy and environmental sequencing.

General Introduction

Protists – diversity and ecology

Protists are renowned as the most abundant and diverse group of eukaryotic organisms on our planet, serving as the foundation for all multicellular life. They are widely distributed throughout the eukaryotic tree of life (Adl et al., 2019; Burki et al., 2020; Parfrey et al., 2006, Fig. 1). Within those phylogenetically diverse lineages, protists occupy a wide range of trophic modes covering heterotrophy, phototrophy, saprotrophy, mutualism and parasitism (Worden et al., 2015). Especially heterotrophic protists, feeding on bacteria, are essential in microbial food webs, from marine to terrestrial habitats (Singer et al., 2021; Worden et al., 2015).

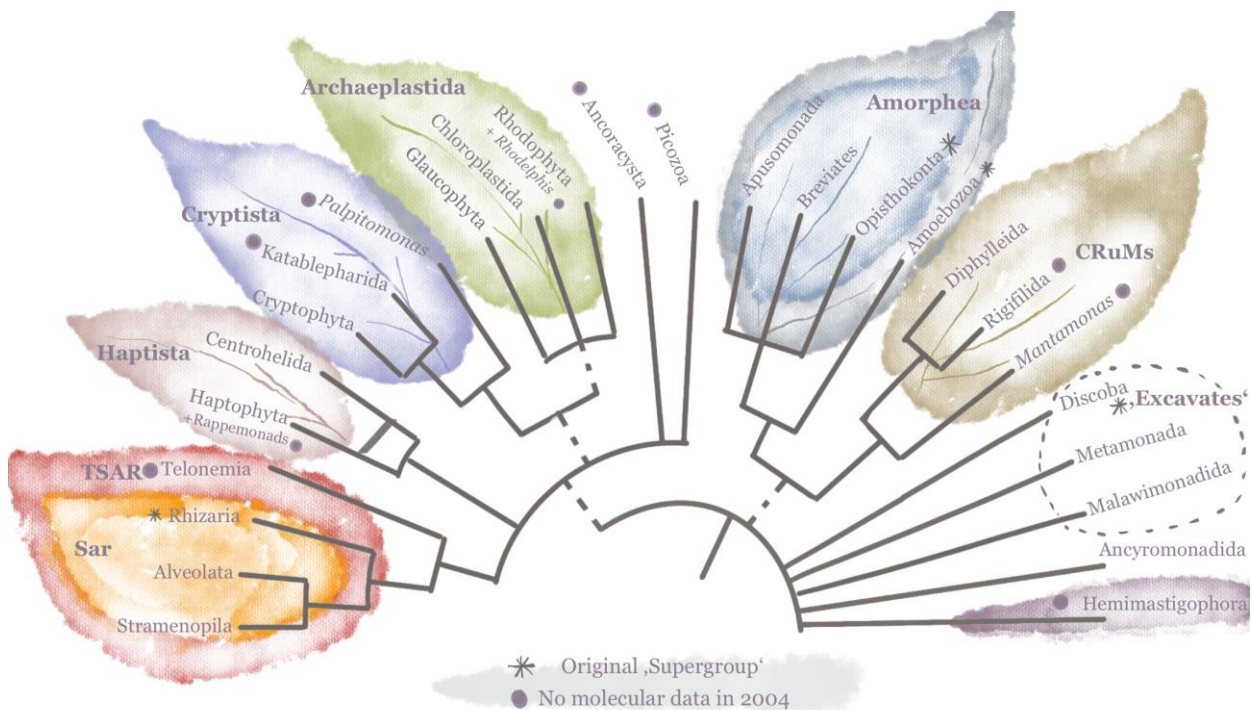


Figure 1 The eukaryotic tree of life after Burki et al. 2020, (modified by the author) based on a consensus of recent phylogenomic studies. While current super groups are highlighted through colors, unresolved branching orders are represented as multifurcations and dashed lines reflect lesser uncertainties about the monophyly of certain highlighted groups. Taxa that that were considered supergroups in earlier versions of the tree are marked with an asterisk, circles show major lineages that had no molecular data when the supergroup model emerged.

Through processes like carbon remineralization (referred to as the microbial loop), they can serve as connections to higher trophic levels, as demonstrated by Azam et

al. (1983). Historically, protists were primarily examined using microscopes, and their, often unassuming, morphology resulted in a significant underestimation of their true diversity. Although the morphogroups previously defined (flagellates, ciliates, and amoeba) are still commonly used as a general categorization, they do not represent the true global diversity, which is currently estimated to be between 26,000-74,000 (Pawlowski et al., 2012) up to 300,000 species (Foissner, 2007). Of this immense number, only a very small fraction is scientifically known or described. As molecular techniques became more and more advanced, immense datasets have been produced through amplicon sequencing. In the marine habitat, however, most studies have focused on expanding inventories of planktonic protist diversity and tended to leave benthic communities untouched or focus on specific environments such as the deep sea and hydrothermal vents (Edgcomb et al., 2002; López-García et al., 2003). But especially the benthic protist communities have important key functions in aquatic ecosystems, as they control the bacterial and microphytobenthic masses and are therefore of great ecological importance. In terms of protist diversity marine sediments furthermore always contain a mixture of the actually active occurring community, free DNA and dormant stages, partly also from planktonic organisms (Marcus and Boero, 1998; Rodríguez-Martínez et al., 2020). Besides the focus on pelagic protist communities, “real” marine environments are much more frequently sampled in terms of protist diversity than e.g. brackish waters. This stands in contrast with the hypothesis that protist species richness is highest in the horohalinicum, meaning a salinity between 5-8 PSU (Telesh et al., 2011a, 2011b). Apart from salinity, also other abiotic factors can have a strong effect on the composition of the protist community. In sediments, physical properties determine the structure of the sediment pores/interstitial. It has been found that grain size seems to have a strong effect e.g. on the ciliate community (Hamels et al., 2005, 2004) and especially on their functional diversity (Xu et al., 2018).

Besides the substrate, oxygen availability is a strong factor shaping the protist community. While some taxa have a large tolerance for changing conditions, some are sensitive for high or low concentrations or even the absence of oxygen (Fenchel and Finlay, 2008; Priya et al., 2019). Especially for ciliates, it is known that some taxa have adapted to anaerobic conditions (Fenchel and Finlay, 1991). Apart from sediment associated taxa, especially gut parasites are adapted to the absence of

oxygen like *Giardia* (Lindmark, 1980), *Entamoeba* or *Trichomonas* (Carvalho-de-Araujo et al., 2023). But also temperature (Liu et al., 2013), direct and indirect physicochemical factors play roles in shaping the composition of protist communities (Bock et al., 2020; Duarte et al., 2000).

Trophic varieties of protists

The large phylogenetic diversity of protists and their ability to adapt to many different environments is connected with a variety of successful trophic modes or feeding types. In the oceans, phototrophy performed by marine protists (algae) serves as a major ecosystem service. Marine algae, in the form of phototrophic protistan phytoplankton account for 50Pg C year⁻¹ (Field et al., 1998). Phototrophic diatoms stand at the basis of the marine food web as a grazing source for micro- and mesozooplankton (Sarhou et al., 2005) and in this way form the foundation of ocean fisheries (Armbrust, 2009)(Fig.2A). Those algae can serve as a food for other protists, as predation is a highly successful and well-studied heterotrophic mode. Micro- and nanozooplankton (<200 µm) can be responsible for the consumption of up to of 62 % of the daily algal production (Schmoker et al., 2013). While some forms such as ciliates, radiolarians and foraminiferans can feed on other heterotrophs, sometimes even including metazoans (Sherr and Sherr, 2002; Swanberg and Caron, 1991), many heterotrophic flagellates are most prominently known as important consumers of bacteria (Massana et al., 2009). As controllers for bacterial biomass, heterotrophic flagellates fulfil an important step within the microbial loop, by

channelling carbon to higher trophic levels (Azam et al., 1983; Fenchel, 2008).

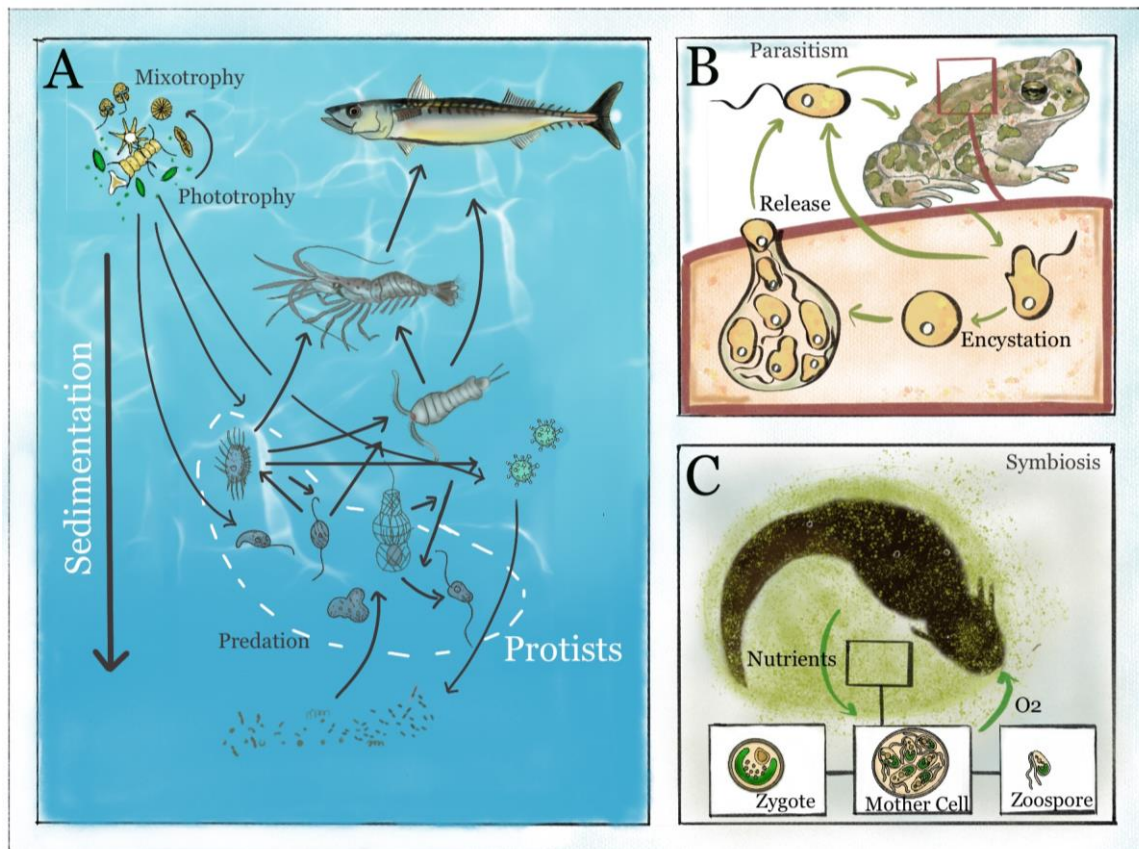


Figure 2 A) Possible trophic modes of protists exemplified in the marine food web, including phototrophy, mixotrophy and predation, B) an example for the parasitism of protists, shown in the life stages of pathogenic chytrid fungi (adapted from Swafford, 2020), C) a case of symbiotic interactions between protists and vertebrates exemplified for the unicellular green alga *Oophila* and amphibian larvae, including the life stages of the algae (after the findings of Kim et al., 2014).

Many heterotrophic protists have also successfully discovered parasitism as a source of nutrition. In marine environments, this role is presumably fulfilled, among others, by marine alveolates, known as MALVs (Guillou et al., 2008). These organisms belong to the dinoflagellate group of Syndinales, of which several forms are known to act as parasites. While many of these remain uncultured and therefore poorly understood, in freshwater ecosystems two aquatic fungi have garnered significant attention in the last decade. They have been identified being responsible for Chytridiomycosis (Fig. 2B), a disease affecting amphibian skin that has resulted in a substantial decline in amphibian biodiversity (Scheele et al., 2019). Not long after the disease was discovered in Australia (Berger et al., 1998), *Batrachochytrium dendrobatidis* was identified as the cause and scientifically described (Longcore, 1999). Around fifteen years later *B. salamandrivorans* was described for being responsible for mass die offs of Dutch fire salamanders (*Salamandra salamandra*) (Martel et al., 2013). Both species were traced to their origin in Asia

(O’Hanlon et al., 2018) from where they were spread globally (Castro Monzon et al., 2020). Apart from those, amphibians can be infected with many other protist parasites like *Balantidium*, *Entamoeba* or *Tritrichomonas* (Baker, 2008), to name only a few. As an antagonist to the parasitic lifestyle, protists can also be involved into mutualistic/symbiotic relationships. Very well known is e.g. the relationship between cnidarians and the endosymbiont *Symbiodinium* (Fransolet et al., 2012; Lampert, 2016) responsible for structuring coral reefs. But protists can also be involved in symbiotic interactions with vertebrates, as known from the unique relationship between the green alga *Oophila amblystomatis* and the North American salamander *Ambystoma maculatum* as well as other amphibians (Kim et al., 2014). While not all aspects of this relationship have undergone comprehensive investigation, there are indications that *Oophila* cells within amphibian egg capsules play a role in elevating partial oxygen pressure (Fig. 2C). They also appear to assimilate some of the embryo's nitrogenous waste, and there is evidence of photosynthate transfer from the algae to the amphibian (Bachmann et al., 1986; Goff and Stein, 1978; Graham et al., 2014; Kerney, 2011; Pinder and Friet, 1994). Those aspects seem to be beneficial for the embryo (Gilbert, 1944, 1942). Some algal cells even seem to enter the host tissue (Kerney et al., 2011), representing a unique form of endosymbiosis. To expand the list of protist abilities, some protists are not limited to either photo- or heterotrophy, but can perform both. Mixotrophy is successful mode for otherwise phototrophic organisms, when e.g. light intensity is low (Flöder et al., 2006).

Assessing protist biodiversity

The idea of DNA barcodes (Hebert et al., 2003), short gene sequences that are distinct enough to identify a single species (Purty and Chatterjee, 2016), has boosted the world of the description of molecular diversity and is one of the the backbones of today’s biodiversity research. Especially for protists, which often show little characteristic morphology, Sanger sequencing of DNA is essential for robust taxonomy.

Next generation sequencing, in form of amplicon sequencing or metabarcoding studies, has revolutionized the view and knowledge that we have on microbial communities in almost all kinds of habitats by revealing a so far unknown taxonomic

diversity (e.g. De Vargas et al., 2015). While many studies aim to reflect the community as a whole (through the use of universal eukaryotic primers, (e.g. Dünn and Arndt, 2023), others focus on specific taxonomic groups (e.g. Fiore-Donno et al., 2019; Singer et al., 2023) depending on research question and habitat. The gene of choice for most protist metabarcoding studies is a fraction of the 18S rDNA, as it offers the largest reference databases (Pawlowski et al., 2012). Over the years, techniques have evolved, making Next Generation Sequencing more and more affordable and accessible, but also regarding data analysis, different methods have been developed. The choice between utilizing OTUs (operational taxonomic units), which involves quality filtering and sequence clustering, and ASVs (amplicon sequence variants), a technique that considers error profiles and claims to produce genuine sequences, has sparked a debate. This discussion has been explored extensively in recent literature (e.g. Chiarello et al., 2022; Jeske and Gallert, 2022), because both approaches offer unique benefits but also introduce their own inherent biases. The method of choice seems to be of personal preference, however, analysing data for ASVs is currently viewed as the state-of-the-art technique.

While environmental sequencing seems to have emancipated biodiversity assessment of protist communities from cultivation-based approaches, the latter remain crucial for accurately identifying taxa. Modern day amplicon sequencing does heavily rely on correct, comprehensive and thus, curated databases. Some studies were able to map faceless sequences to morphology (Schiwitz and Nitsche, 2020) and were able to show a direct connection between both methods. It therefore makes sense to combine both methods for most surveys.

Aims

The main objective of this study was to gain insight into the enormous diversity of protist communities that is represented in environmental metabarcoding datasets and which factors might shape taxonomic and functional composition. As environmental datasets always comprise the community as a whole and combine all sorts of trophic modes, we were additionally interested to gain insight into two communities in detail that are depending on the connection and trophic interaction with other organisms. Furthermore, we wanted to emphasize the importance of taxonomic studies as the backbone of reference databases and contribute to their constant quality improvement by resolving uncertainties in two taxonomic groups. In this way, this thesis addresses the following questions:

- 1) How is the community composition of benthic protists in a brackish water system differing between different geographic regions and what might be the potential factors shaping it?
- 2) Can metabarcoding and cultivation studies serve as tools to assess the diversity of protists living in close association (parasitic/symbiotic) with metazoans?
- 3) How are taxonomic studies connected to metabarcoding and what could be done to further resolve and expand protist taxonomy and reference databases?

In order to address these questions, we conducted sampling under different environmental conditions. To obtain a comprehensive understanding of benthic protist communities in their entirety, we collected samples from two distinct regions of the southern Baltic Sea (Chapter 1) and used an amplicon sequencing approach to capture the full spectrum of protist diversity present. These samples can serve as a representation of naturally existing benthic protist communities, encompassing the diverse range of trophic modes exhibited by protists. To get thorough insight into the trophic mode of parasitism, we tested faecal samples obtained by various amphibians and ambient environments to get an overview of specifically parasitic protist species, again over amplicon sequencing of the 18S rDNA (Chapter 2). As an

antagonist to the parasitic mode, we collected the egg clutches of *Rana dalmatina*, as well as the surrounding spawning waters to gain insight into the occurring micro-eukaryotic community (Chapter 3). In this context the unicellular green algae of the genus *Oophila* is assumed to live in mutualistic connection with the amphibian larvae within the egg capsule. While the taxonomic situation of *Oophila* is not fully resolved, we tried to support previous taxonomic assumptions with our ecological data. Moreover, we wanted to address the fact that cryptic species are highly frequent in metabarcoding datasets, as reference databases often lack comprehensiveness and can only reflect a fraction of the diversity. This is connected to the extensive diversity of protists that in large parts remains taxonomically unresolved. With two additional taxonomic studies we wanted to exemplify how frequent cryptic species occur even in well-known genera such as *Cafeteria* and *Goniomonas*, and we wanted to analyse whether those genera still leave room for expansion. Additionally, we would like to emphasise the importance of modern taxonomical work to refine genera and stock reference databases (Chapter 4, 5).

Summary of Chapters

Part 1 Metabarcoding analyses of protist communities in dependence of environmental factors and special trophic modes

Chapter 1: Benthic heterotrophic protist communities of the southern Baltic analyzed with the help of curated metabarcoding studies

The Baltic Sea represents the largest brackish environment on earth. While several studies have assessed the planktonic protist community in this unique habitat, benthic protist communities have not yet been a topic of larger metabarcoding approaches. Our study, as part of a baseline project evaluating the impact of bottom trawling on the benthic biocoenosis aims to give first insight into benthic protist communities of two regions in the southern Baltic Sea. We achieved this by analyzing metabarcoding data derived from sediment samples. The study revealed substantial differences in community composition between those regions and indicates a potential specificity of brackish water communities; which may require specific measures to protect benthic communities as a whole. *Published in Biology, doi: 10.3390/biology12071010*

Chapter 2: Evidence of *Batrachochytrium dendrobatidis* and other amphibian parasites in the Green toad (*Bufo viridis*), syntopic amphibians and environment in the Cologne Bay, Germany

The chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) are responsible for the most exorbitant loss in amphibian wildlife and diversity worldwide. But also, other unicellular parasites associated with amphibians exist that are far less studied. Within the frame work of a nature conservation project that aims to find the root of the population declines of the Green Toad, *Bufo viridis* in the Cologne area, we analysed skin swaps for *Bd* and *Bsal*. Additionally, we analysed data derived from cloacal swabs and environmental samples to examine possible infections with other protist parasites. We found that *Bd* was present at all sampled locations, with high prevalence and partly with high individual infection load. *Bsal* could not be detected in our samples, but several potentially harmful pathogens were detected in faecal and environmental samples. While no clear clinical infection signs were visible, the influence on the amphibians remains unclear. *Published in Salamandra, 2020, 56 (3)*

Chapter 3: Diversity and substrate-specificity of green algae and other micro-eukaryotes colonizing amphibian clutches in Germany, revealed by DNA metabarcoding.

Apart from the unicellular green algae *Oophila*, that is frequently found in amphibian egg clutches, only few studies tried to capture the occurrence and role of microorganisms within the capsular chamber of amphibian eggs. Through metabarcoding of multiple marker genes, we aimed to depict not only the community composition of micro-eukaryotes within the egg capsules of the agile frog (*Rana dalmatina*) but also of the surrounding environment of the spawning waters including water, sediment and tree leaves. The analysis revealed that the community composition of protists is strongly determined by the substrate. This is especially the case for the egg capsule, that is inhabited by a distinct community of multiple algae, diatoms and ochrophytes. An additional phylogenetic analysis showed that algae assigned to *Oophila* form two distinct clades, of which we assume that only one represents the true *Oophila*. Published in *The Science of Nature*, doi: 10.1007/s00114-021-01734-0

Part 2 Case studies on phylogeny and autecology of protists for two sediment associated taxonomic groups

Chapter 4: Cryptic cryptophytes – revision of the genus *Goniomonas*

As the only plastid lacking cryptomonad, the genus *Goniomonas* has a special phylogenetic position and most likely represents an ancient pre-endosymbiont cryptophyte. Despite this fact, the genus seems not well resolved and under-split from a taxonomical point of view, as earlier studies have already found a deep divergence between freshwater and marine species. Based on morphological and phylogenetic data (18S rDNA) we propose a restructuring of the genus into *Goniomonas* sensu strictu and present a suitable Neotype for the genus, as well as splitting the genus into several new genera which leads to the formation of the freshwater genera *Limnogoniomonas* gen. nov., *Goniomonas* and *Aquagoniomonas* gen. nov. and the marine genera *Neptunogoniomonas* gen. nov., *Marigoniomonas* gen. nov., *Thalassogoniomonas* gen. nov., *Poseidogoniomonas* gen. nov. and *Cosmogoniomonas* gen. nov. Published on bioRxiv, doi: <https://doi.org/10.1101/2024.07.17.603845>

Chapter 5: *Cafeteria* in extreme environments: investigations on *C. burkhardae* and three new species from the Atacama Desert and the deep ocean

To expand the taxonomical and ecological knowledge on the bicosoecid genus *Cafeteria* we isolated and cultivated ten strains from various environments including the deep sea, Baltic Sea and hypersaline waters in the Atacama Desert. Especially, the investigations of the Baltic were part of the present work. Via molecular analyses of the 18S and 28S rDNA we could identify four new strains of *Cafeteria burkhardae* from the Atlantic Ocean, one new species (*Cafeteria baltica* sp. nov.) from the Baltic Sea and two new species from the salt flats of the Atacama Desert (*C. atacamiensis* sp. nov. and *C. paulosalfera* sp. nov.). While *Cafeteria* displays a rather similar morphology for all species, the strains were clearly separated on molecular level. Additionally, we conducted experiments to test the strains for their salt tolerance. Only the marine strains were able to survive higher salinities (up to 150 PSU). *Published in European Journal of Protistology, doi: 10.1016/j.ejop.2022.125905*

Part 1

Metabarcoding analyses of protist communities in dependence of environmental factors and special trophic modes

Chapter 1:

Benthic heterotrophic protist communities of the southern Baltic analyzed with the help of curated metabarcoding studies

Published publication

Sachs, M., Dünn, M., Arndt, H. (2023) Benthic heterotrophic protist communities of the southern Baltic analyzed with the help of curated metabarcoding studies, *Biology*, 12, 1010, <https://doi.org/10.3390/biology12071010>

**Chapter 2:
Evidence of *Batrachochytrium dendrobatidis* and other amphibian parasites in the Green toad (*Bufo viridis*), syntopic amphibians and environment in the Cologne Bay, Germany.**

Published publication

Sachs, M., Schluckebier, R., Poll, K., Schulz, V., Sabino-Pinto, J., Schmidt, E., Simon, K., Künzel, S., Ziegler, T., Arndt, H., Vences, M. (2020) Evidence of *Batrachochytrium dendrobatidis* and other amphibian parasites in the Green toad (*Bufo viridis*), syntopic amphibians and environment in the Cologne Bay, Germany, *Salamandra*, 56,3, pp. 275-284

**Chapter 3:
Diversity and substrate-specificity of green
algae and other micro-eukaryotes
colonizing amphibian clutches in Germany,
revealed by DNA metabarcoding.**

Published publication

Anslan, S., **Sachs, M.**, Rancilhac, L., Brinkmann, H., Petersen, J., Künzel, S., Schwarz, A, Arndt, H., Kerney, R., Vences, M. (2021) Diversity and substrate-specificity of green-algae and other micro-eukaryotes colonizing amphibian clutches in Germany, revealed by DNA metabarcoding, *The Science of Nature*,108 (4) <https://doi.org/10.1007/s00114-021-01734-0>

Part 2

Case studies on phylogeny and autecology of protists for two sediment associated taxonomic groups

Chapter 4: Cryptic cryptophytes – revision of the genus *Goniomonas*

Published publication

Sachs, M., Nitsche, F., Arndt, H. (2024) Cryptic Cryptophytes – revision of the genus *Goniomonas*, bioRxiv 2024.07.17.603845; doi: <https://doi.org/10.1101/2024.07.17.603845>

Chapter 5
***Cafeteria* in extreme environments:
Investigations on *C. burkhardae* and three
new species from the Atacama Desert and
the deep ocean**

Published publication

Schoenle, A., Hohlfeld, M., Rybarski, A., **Sachs, M.**, Freches, E., Wiechmann, K., Nitsche, F., Arndt, H. (2022) *Cafeteria* in extreme environments: Investigations on *C. burkhardae* and three new species from the Atacama Desert and deep ocean. European Journal of Protistology, 85, <https://doi.org/10.1016/j.ejop.2022.125905>

Conclusive Summary and Perspectives

Next Generation Sequencing has truly revolutionized and enlarged the knowledge that we have of protist diversity today. The overarching objective of this thesis was to extend the present knowledge on protist diversity in sediments and amphibian associated habitats. This also includes a special focus on trophic interactions between amphibian and unicellular organisms that can be derived from their cooccurrence in certain habitats. The work includes several novelties on the knowledge of benthic protists in brackish waters, as well as on potential protist parasites in several amphibian populations as well as on the clutch associated protist communities of *Rana dalmatina*. It additionally aimed to resolve some taxonomic uncertainties with regards to the unicellular green algae *Oophila* and the goniomonad cryptophytes as well as enlarges knowledge on species belonging to the genus *Cafeteria*. By adding the latter three studies, we wanted to highlight the direct connection between modern taxonomic and Next Generation Sequencing studies, as diversity can only be reflected correctly and completely with the correct references in databases and a continuous curation of those.

As the accessibility and affordability of high throughput sequencing has been much more facilitated in recent years, techniques of data analysis have changed. OTUs (operational taxonomic units) represent a cluster of sequences, summed by a given identity threshold. ASVs (amplicon sequence variants), that are exact sequence variants, without a clustering method are based on error models that are connected to the sequencing quality. Both methods have their pros and cons. Whereas OTUs reduce the impact of sequencing errors, as erroneous sequences might disappear in the cluster (Eren et al., 2013), ASVs may have a higher sensitivity but a lower specificity (Prodan et al., 2020). Recently, a strong shift towards the analysis via ASVs has been visible, with the DADA2 pipeline found to offer the highest resolution (Prodan et al., 2020). This thesis includes both studies with OTU and ASV datasets, as it expanded over the course of several years, consistently trying to offer state of the art analyses.

Using the Baltic Sea as a model region we wanted to gain insight into benthic protist communities in their entirety (Chapter 1). Two distinct regions in the southern Baltic Sea were chosen to highlight differences and similarities in community composition. The Baltic Sea offers a unique habitat as it is not only fed by the saline

inflows of the North Sea but also by freshwater riverine runoffs. This composition leads to a stratification of waters and changing salinities – both vertically and horizontally. The two chosen sampling regions (Fehmarnbelt-FB and Oderbank – OB) do vary not only in salinity (FB – 19 PSU; OB – 8 PSU) but also in median grain size of the sediment (FB - 55 μm ; OB – 178 μm) and water depth at the sampling stations (FB ~ 23 m; OB ~ 15 m). We employed amplicon sequencing on protists extracted from sediment samples to assess their diversity by targeting the V9 region of the 18S rDNA and, in part, rRNA. Based on RNA we found significant differences in taxonomic composition of the protist communities between the two regions. While those might be connected to the factors water depth, salinity and depth of the sediment layer that the samples originated from also other factors, such as fishing intensity may have an influence on the taxonomic composition of the benthic protist community. As the study itself is part of a larger project investigating the latter, further studies might be able to resolve the reason for those differences further.

While we used strong filtering criteria for our dataset (based on the use of a mock community), it may be assumed that the measured differences are potentially based on those criteria. Individually chosen read thresholds per library preparation are on the one hand an objective measure to reduce the number of noisy ASVs (Dünn and Arndt, 2023) but may on the other hand be responsible for a loss in low abundant ASVs, leaving only the peaks of the community. Hence, we investigated whether adopting a softer ASV table filter would yield comparable outcomes. We applied a method that considered only ASVs with a minimum of three reads (referred to as the three-read filter)(Lennartz et al., 2023; Schoenle et al., 2021). Prior studies have also explored data sets using both strict and soft filtering criteria, (e.g. Dünn and Arndt, 2023; Lennartz et al., 2023) demonstrating the persistence of general trends across various filtering approaches. Our repetitive analysis of RNA samples for comparing sampling stations in the Fehmarnbelt and Oderbank regions, showed only a small overlap of 10% in protist communities (Fig. 2), when applying the three-read filter.

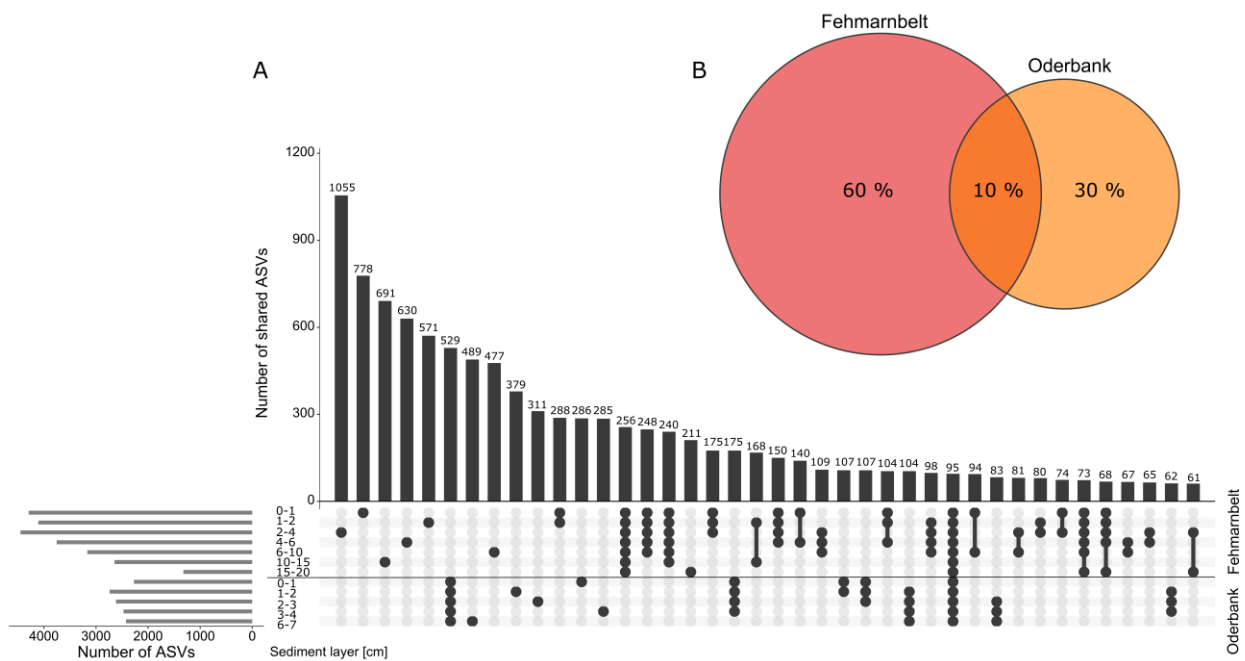


Figure 3 A) Upset plot showing the number of shared and unique ASV per region and sediment depth. Connected dots show ASVs shared between different depth and regions. The bar chart on the left shows total number of ASVs per depth. B) Venn diagram showing the percentage of shared ASVs between the Fehmarnbelt and Oderbank region in total. Both plots originate from a dataset that takes only ASVs into account where at least three reads were recorded (three read filter).

This indicates that even though our filtering criteria may be strict, they still accurately reflect the observed differences.

Regarding the number of ASVs, Ciliophora was the most dominant group in our dataset from the Baltic Sea. Earlier studies have shown, that ciliate species seem to thrive in the brackish environment leading to high species richness (Xu et al., 2018). However, in terms of read abundance, the MALV-I group, a clade of marine Syndiniales, had by far the highest numbers. Organisms of the MALV clade are known to appear in high numbers in marine environmental studies, yet, as most of them are uncultured, they remain rather enigmatic (Guillou et al., 2008). Their dominance may partly be reasoned by the fact that they have high copy numbers of rDNA (Weber and Pawlowski, 2013) but could also be seen as an indication that the parasitic life style is a very successful trophic mode for protists. This assumption is underlined by our study to monitor how frequent amphibians in the Cologne area were infected with the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) as well as with other mainly unicellular parasites or commensals (Chapter 2). This question was integrated into the overall goal of

finding out why the populations of the green toad (*Bufo viridis*) have declined so much despite various conservation measures. In this study, we combined skin swabs analyzed by qPCR with cloacal swabs and environmental samples analyzed over a metabarcoding approach from 5-9 sampling sites in the Cologne area. While *Bsal* could not be detected, the prevalence for *Bd* was high – depending on the sampling site with up to 70 % of the samples. Additionally, we were able to find the OTUs of nine other, potentially harmful organisms in the cloacal swabs. Using a different principle and bioinformatic pipeline for the analysis of the metabarcoding samples, we set a threshold of >500 reads per OTU as an unambiguous identification of the organism. While any set threshold is artificial, we also included findings that were under this detection border. In this way, we found e.g. ~27 % (>500 reads/OTU and sample) resp. ~69 % (<500 reads/OTU and sample) of amphibians to be infected with the metamonad *Tritrichomonas augusta*. How and if both the infection with *Bd* and other parasites may affect the tested hosts, and if those are responsible for the dwindling populations of *Bufo viridis* in specific, remains unclear as the animals appeared to be in good health. Species like the heterotrophic flagellate *Tritrichomonas augusta* can affect the host, if they are present in high densities. Unfortunately, metabarcoding analyses do not allow to draw conclusions on infection loads. Chytridiomycosis (caused by *Bd* and *Bsal*), the disease that is responsible for an unprecedented loss of amphibian diversity worldwide (Scheele et al., 2019), affects the epidermis and leads to a loss of function that can be lethal. We found partially high loads of *Bd* on the skin of the tested animals, but so far, no signs of infection. Still, it may be speculated that all sorts of infections might cause a silent decline over the loss of juvenile individuals that may be more sensible to infections (Pasmans et al., 2018). Meanwhile, no further studies have been published addressing these particular circumstances. However, it has been speculated (personal communication with NABU) that the decline of *Bufo viridis* populations might also be attributed to the influence of global warming and other environmental factors. Several studies have indicated that amphibian fitness can be impacted by mild winters, which in turn disrupts their hibernation period (Griffiths et al., 2010; Reading, 2007). To support conservation measures, the Cologne Zoo has additionally started a successful conservation breeding program for the green toad (Ziegler et al., 2022).

Whereas the first two studies of this thesis refer to individual aspects of either the distribution or the trophic interaction of protists, the third study can be seen as a combination of both or as a transition to the second part of the thesis. Chapter 1 investigated benthic protist diversity and community composition in the southern Baltic Sea, Chapter 2 concentrated on a group of organisms united by a certain trophic mode. Chapter 3 again concentrates on a special community that stands in close connection with other organisms but is also investigating taxonomic matters. Within small pond waterbodies of the Elm, a region near Braunschweig, we sampled three different ponds and different substrates (water, sediment, sunken tree leaves and clutches of *Rana dalmatina*) with the intention to get an overview on the protist community with a special focus on the green microalga *Oophila* and its distribution. *Oophila* is known for its mutualistic relationships. The taxonomic identity of *Oophila* is still unsure. Within the discussion which lineage may represent the “true” *Oophila* basically two opinions can be found in literature. Nema et al. (2019) have created two major clades (A and B) and therefore found *Oophila* to be phylogenetically diverse, claiming that clade A represents the true *Oophila* as they isolated new strains of this clade from amphibian clutches. The majority of other studies consistently considers isolates from clade B as the original *Oophila* (Jurga et al., 2020; Kerney et al., 2019; Kim et al., 2014; Muto et al., 2017) as they all belong to one clade of green algae and were found to be the numerically most abundant algae in egg clutches of two American salamander species (Jurga et al., 2020; Kerney et al., 2019). By isolating samples directly from the clutches of *Rana dalmatina* as well as an additional multigene phylogenetic analysis, we wanted to contribute to the solution of this question. To analyse the protist community with the different substrates, we divided the metabarcoding analysis between the *rbcl* gene and the 18S rDNA gene. Our analyses revealed a unique community within the egg clutches both for *rbcl* and 18S data that contained 16.3% unique OTUs for *rbcl* data and 56.9% unique OTUs for 18S data. Within the *rbcl* data OTUs with the highest read abundance were *Chlamydomonas*, *Nitzschia* and *Oophila*. The substrate had the most significant influence on the community composition in both datasets. But also within the clutch samples, we found significant differences between sampling sites, developmental stage and sampling date, depending on the statistical method used. Other than previous metabarcoding studies targeting the eggs of North American salamander *Ambystoma maculatum* (Jurga et al., 2020), we found that the egg

masses of *Rana dalmatina* were not exclusively colonized by *Oophila*, but also by other algae, which may be due to their looser structure and contamination by taxa from the environment. While the breeding waters of *Rana dalmatina* are rather nutrient rich due to many decaying leaves, they may favour a larger diversity both in algal and protozoological diversity. Still, we found the clutch habitat to be quite distinct from the other sampled pond substrates. Phylogenetically, our analysis of the 18S rDNA of the available *Oophila* sequences revealed, that sequences from *Rana dalmatina* cluster within clade B, which is the first molecular proof that *Oophila* is present in Central Europe respectively globally present in the Holarctic region. We were also able to identify sequences that cluster within clade A from our samples. Still, as those sequences from clade A are scarce both in our and other metabarcoding data sets, we assume that beneficial interactions may rather refer to organisms from clade B. Yet, it was interesting to see, that strains from clade A are also widely geographically distributed, but this fact may also speak against a mutualistic interaction. Overall, our findings support the assumption that the original *Oophila* is much rather assigned to clade B but suggest, that the clade itself might need a more comprehensive taxonomic revision. In conclusion, the study supports our findings, that the surrounding conditions are highly decisive for the structure of the inhabiting protist community (including algae) and that they are also influenced by their complex organismic interactions (Bock et al., 2020). On the other hand, the study also emphasizes the importance of correct taxonomy as a basis of metabarcoding studies. In this case, it is especially relevant since the accurate identification of the taxon is essential to derive information about its ecological and trophic functions. The root of this and similar issues is mostly due to the fact that in earlier times many organisms were described exclusively in morphological terms. Through molecular investigations, especially the inclusion of DNA sequence data and the use of electron microscopy, it became possible to make even the smallest differences visible. However, this underscores that earlier described species usually contain not just one but multiple, cryptic species. Modern taxonomists therefore often pursue an integrative approach and combine morphological, molecular and also ecological information in order to be able to differentiate species as well as possible (Rybarski et al., 2021; Schiwitza et al., 2018; Zivaljic et al., 2020). The genus *Goniomonas* is a well-known representative of the Cryptophyta and is commonly found in marine and freshwater habitats. Despite this and its special position within

the Cryptophyta as the only representative lacking a chloroplast, it is surprising that, only 5 species (*G. truncata*, *G. amphinema*, *G. pacifica*, *G. avonlea* and *G. brasiliensis*) have been scientifically described so far. Earlier studies have found a deep divergence between freshwater and marine species (Kim and Archibald, 2013; Von Der Heyden et al., 2004), but did not make the effort to resolve the genus, eventhough the differences between the species are as high as between certain different genera (Von Der Heyden et al., 2004). Aggravating as in the case of *Oophila* is, that there is no genetic type material. Apart from *G. avonlea*, the most recently described species, no exact genetic reference material is available for all other species, meaning that descriptions are only based on morphology. Especially for *G. amphinema* and *G. pacifica*, their morphology has been used to discriminate them from each other (as both can be found in marine environments). Additionally, the original descriptions for those two are based on light microscopy only. In this way, many sequences have been deposited in GenBank under one of the species names, partly with large genetic differences to each other, e.g. accession numbers LC674566 and AF508277, both under the name *G. pacifica*, but with 2.57 % pairwise distance to each other. Our study has shown, that several other, genetically distinct strains display rather similar morphological traits, which makes a genetic approach for species determination inevitable (Chapter 4). To revise the genus, we therefore proposed a split into several new genera and added and described ten new species from our own culture collection. Within those, and with the help of electron microscopy we were able to determine shared traits within those newly designated genera. To create a new basis for the genus *Goniomonas* sensu strictu we chose a strain isolated from the river rhine as a Neotype for *Goniomonas truncata*, as we assume that the original strain of *G. truncata* was also isolated in Germany. The genus *Goniomonas* would then further on be limited to freshwater environment. The marine species, *G. amphinema* and *G. pacifica* would be newly assigned to the genus *Cosmogoniomonas* gen. nov., taking the elaborate description of former *G. aff. amphinema* (Martin-Cereceda et al., 2010) as a type description for *Cosmogoniomonas amphinema* sp. nov.. *Goniomonas* as well as *Oophila* demonstrate how important it is to designate new type material as all parts of research, even non-taxonomic studies, rely on a correct taxonomic assignment. Similar to *Goniomonas* the bicosoecid *Cafeteria*, especially the species *Cafeteria burkhardae* is a very frequently found heterotrophic flagellate (Atkins et al., 2000;

Massana et al., 2021; Schoenle et al., 2020). *Cafeteria* has been recently revised by Schoenle et al. (2020) revealing that many deposited sequences were wrongly assigned under the name *C. roenbergensis*, which represents the type of this genus. Schoenle et al. (2020) also expanded the genus for several new species, showing that this type of flagellate can occur in marine surface waters but also in deep-sea sediments and seems to represent a rather robust generalist. With our study (Chapter 5) we aimed to resolve the species diversity of the genus furthermore and investigate strains from different, rather extreme habitats as hypersaline salars of the Atacama Desert, deep and surface waters of the North Atlantic Ocean and the brackish waters of the Baltic Sea (Fehmarnbelt area). For an in-depth characterisation of the new strains, we combined morphological observations with a multigene phylogenetic approach and autecological salt tolerance experiments. We were able to describe three new species, two from the Atacama Desert (*C. atacamiensis* sp. nov. and *C. paulosalfera* sp. nov.) and one from the Baltic Sea (*C. baltica* sp. nov.) and obtain four new strains of *C. burkhardae* from the Atlantic Ocean. Similar to *Goniomonas*, *Cafeteria* species show only small morphological differences on basis of high-resolution microscopy. The phylogenetic analysis showed how multigene analyses can refine molecular results. Whereas *C. baltica* sp. nov. and *C. burkhardae* show only minor differences on the 18S and 28S rDNA gene level, they differ highly in the ITS region (18S+ITS: 4.1 % pairwise distance). The strains from the Atacama Desert showed high genetic distance to all other *Cafeteria* species. Regarding salt tolerance, *C. burkhardae* showed highest tolerances, underlining the fact that it is a cosmopolitan species with a highly adaptive character. But also *C. baltica* sp. nov. showed high tolerances, making it possible to survive under a variety of salt concentrations in the Baltic Sea. Additional to our findings, we were able to recover *C. baltica* sp. nov with 100 % identity from our dataset in Chapter 1, from the samples of the Fehmarnbelt area as well as in the dataset from several stations sampled during the Tara Ocean circumglobe expedition (De Vargas et al., 2015). This underlines our hypothesis that *C. baltica* sp. nov is a highly adaptive species, but it also emphasizes the deep connection between modern taxonomy and next generation sequencing approaches. Overall, this thesis gave novel insights into the composition and diversity of protist communities in large environmental datasets as well as in regards to specific trophic

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interactions. The in-depth taxonomic analysis of protist strains showcases how complex and cryptic their diversity can be and how important further studies are.

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<https://doi.org/10.1016/j.ejop.2019.125664>

General References

Subpublications and Records of Achievement

Part 1

Chapter 1: **Sachs, M.**, Dünn, M., Arndt, H. (2023) Benthic heterotrophic protist communities of the southern Baltic analyzed with the help of curated metabarcoding studies. *Biology*, 12, 1010. doi: 10.3390/biology12071010

The study was designed by the author of the present thesis and Prof. Dr. Hartmut Arndt. The author was involved in sampling, performed all laboratory work, bioinformatic analyses and wrote the manuscript.

Chapter 2: **Sachs, M.**, Schluckebier, R., Poll, K., Schulz, V., Sabino-Pinto, J., Schmidt, E., Simon, K., Künzel, S., Ziegler, T., Arndt, H. and Vences, M. (2020) Evidence of *Batrachochytrium dendrobatidis* and other amphibian parasites in the Green toad (*Bufo viridis*), syntopic amphibians and environment in the Cologne Bay, Germany. *Salamandra*, 56, 3, 275-284

The study was planned by the author, Prof. Dr. Miguel Vences and Prof. Dr. Hartmut Arndt. The author was involved in sampling, performed laboratory work for the metabarcoding part, performed bioinformatic and statistical analyses and wrote the manuscript. Sequencing data were obtained during author's master thesis.

Chapter 3: Anslan, S., **Sachs, M.**, Rancilhac, L., Brinkmann, H., Petersen, J. Künzel, S., Schwarz, A., Arndt, H., Kerney, R., Vences, M. (2021) Diversity and substrate-specificity of green algae and other micro-eukaryotes colonizing amphibian clutches in Germany, revealed by DNA metabarcoding. *The Science of Nature*, 108, 29. doi: 10.1007/s00114-021-01734-0

The study was planned by Dr. Sten Anslan and Prof. Dr. Miguel Vences. The author of the present thesis was involved in cultivation and laboratory work and parts of the transcriptome analysis under the supervision of Prof. Dr. Miguel Vences. Parts were contributed to the manuscript.

Part 2

Chapter 4: **Sachs, M.**, Arndt, H., (manuscript) Cryptic cryptophytes – revision of the genus *Goniomonas*.

Conception and writing were conducted by the author of the present thesis under guidance of Prof. Dr. Hartmut Arndt. The author performed phylogenetic and morphologic analyses, was involved in light microscopy and wrote the manuscript.

Chapter 5: Schoenle, S., Hohlfeld, M., Rybarski, A., **Sachs, M.**, Freches, E., Wiechmann, K., Nitsche, F., Arndt, H. (2022) *Cafeteria* in extreme environments: investigations on *C. burkhardae* and three new species from the Atacama Desert and the deep ocean. *European Journal of Protistology*, 85, 125905. doi: 10.1016/j.ejop.2022.125905

The study was conceptualized and written by Dr. Alexandra Schoenle and Prof. Dr. Hartmut Arndt. The author was involved in the cultivation and the salinity experiment of several cultures and contributed illustrations and parts of the manuscript.

Erklärung gemäß § 7 Absatz 8 der Promotionsordnung

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.“

Unterschrift:

Datum: 21.09.2023, Köln

Curriculum vitae

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Education

7/20- 10/2016 – 11/2018	PhD in Biology at University of Cologne, Germany M.Sc. in Biological Sciences at University of Cologne, Germany
10/2013 – 08/2016	B.Sc. in Biology at University of Cologne, Germany
10/2005 – 07/2008	Apprenticeship Florist , Cologne
10/2003 – 08/2004	Diploma Program Geology and Paleontology at University of Cologne, Germany
08/1994 – 06/2003	Ursulinengymnasium Köln – Abitur

Employments

02/2019 – 11/2020	Research assistant University of Cologne, Germany, Institute of Zoology, General Ecology, Working group: Prof. Dr. H. Arndt <ul style="list-style-type: none">• Cultivation of algae from amphibian spawn• Management of climatic chambers• Course management “<i>Introduction to biodiversity</i>”• Assistant course management and student mentor “<i>Ecology and applied biology</i>”
07/2014 – 02/2019	Student/research assistant University of Cologne, Germany, Institute of Zoology, Developmental Biology Working group: Prof. Dr. M. Hammerschmidt

- 06/2008 –
06/2014
 - Animal caretaking of Zebrafish colony
 - Florist**
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 - Operations and sales management
 - Instruction of apprentices
 - Appointed IHK examiner in the department of floristry

- 09/2004 –
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 - Galeria Kaufhof**, temporary worker

Fieldwork experience and external internships

- 06-07/2023
 - Participation Research Cruise „MGF-Salinity “on the Baltic Sea with the research vessel FS Elisabeth Mann-Borgese (EMB320)

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 - Participation Research Cruise AL570 on the Baltic Sea with the research vessel FS Alkor, (**Project MGF-Ostsee**)

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- 04/2019 -
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- 03/2016 –
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 - Haus der Natur, Dormagen**: “*Monitoring of aquatic birds, Fruit meadow maintenance*”

- 09/2015
 - Ecological Field station Rees – Grietherbusch**: “*Comparative studies on gastric contents of gobies in the rivers “Rhine and Lippe”*”

Publications

Sachs, M., Hohlfeld, Arndt, H. (2023) Benthic Heterotrophic Protist Communities of the Southern Baltic Analyzed with the Help of Curated Metabarcoding Studies, *Biology*, 12, 1010, <https://doi.org/10.3390/biology12071010>

Schoenle, A., Hohlfeld, M., Rybarski, A., **Sachs, M.**, Freches, E., Wiechmann, K., Nitsche, F., Arndt, H. (2022) *Cafeteria* in extreme environments: Investigations on *C. burkhardae* and three new species from the Atacama Desert and deep ocean. *European Journal of Protistology*, 85, <https://doi.org/10.1016/j.ejop.2022.125905>

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Conferences

- 07/2022 **41st Meeting of the German Society for Protozoology (DGP) Bergisch Gladbach** Talk: “Baltic Sea project: insights in the benthic protist communities in relation to sediment disturbance”
- 02/2021 **40th Meeting of the German Society for Protozoology (DGP), Online** Poster Präsentation: “Baltic Sea project: Four potentially new species – resisting anoxic conditions in sediment?”
- 03/2020 **39th Meeting of the German Society for Protozoology (DGP), Kaiserslautern** Poster Präsentation: “Unicellular parasites in amphibians and environment in the Cologne Bay, Germany
- 09/2019 **German Society for Limnology (DGL), Münster,**
Attendance
- 02/2019 **38th Meeting of the German Society for Protozoology (DGP), Vienna,** Poster Präsentation: “*Oophila*, an amphibian loving symbiont from Northern Germany”.
Achievements: Poster award 2019, 2nd place
- 02/2019 **37th Meeting of the German Society for Protozoology (DGP), Cologne,** Attendance
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