Taking a look from different angles:
A multifaceted approach for identifying planktonic protists in sunlit zones and deep pelagic redoxclines of the Baltic Sea

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Abstract

Free-living protists – single-celled eukaryotes – are an integral part of microbial food webs in aquatic environments. Here they play multiple ecological roles with high relevance for aquatic carbon cycling. They are phylogenetically diverse and versatile in their trophic modes. Protists of the Baltic Sea are assumed to require various adaptations due to high spatial and temporal fluctuations in environmental parameters such as oxygen and salinity. The aim of this thesis was to identify dominant protists and their functional roles in the Baltic Sea, which is – regarding its protist fauna – rather understudied. To balance the shortcomings of a single method approach, a combination of different techniques was applied: e.g., microscopy, gene sequencing, enrichment and cultivation. Microscopy and gene sequencing applied in concert and in high-resolution along depth profiles in the stratified central basins revealed a characteristic and stable structuring of the protist community over years. In addition, novel species and lineages have been detected using cultivation and cultivation-independent techniques. With respect to the nutritional modes of protists, experimental manipulations revealed otherwise masked trophic functions of hitherto uncultured protist taxa and could be linked with environmental diversity data. Concluding, the present thesis, in its methodological multiplicity, sheds considerable light on the diversity and functions of protist assemblages in the Baltic Sea.

Kurzfassung

Freilebende Protisten – einzellige Eukaryoten – sind ein wesentlicher Bestandteil mikrobieller Nahrungsnetze in aquatischen Habitaten. Dort erfüllen sie eine Vielzahl an Funktionen mit hoher Relevanz für den aquatischen Kohlenstoffkreislauf. Sie sind stammesgeschichtlich sehr divers und vielseitig hinsichtlich ihrer ökologischen Kapazitäten. In der Ostsee lebende Protisten benötigen vermutlich verschiedenartige Anpassungen hinsichtlich der starken räumlichen und zeitlichen Schwankungen von Umweltparametern wie Sauerstoff und Salzgehalt. Das Ziel dieser Arbeit war es, dominante Protisten sowie deren Funktionen in der Ostsee zu identifizieren, die hinsichtlich der Protistenfauna kaum untersucht ist. Um die Schwächen einzelner Methoden auszubalancieren, wurde hierfür eine Kombination verschiedener Ansätze angewandt: mikroskopische, Sequenz-basierte, Anreicherungs- und Kultivierungsmethoden. Mikroskopie kombiniert mit Gensequenzierung entlang hochauflösender Tiefenprofile ermittelte eine charakteristische und über mehrere Jahre stabile Strukturierung der Protistengemeinschaften in den geschichteten Tiefenbecken. Mit Kultivierungs- und Kultur-unabhängigen Methoden konnten zudem mehrere neue Arten und

Abstammungslinien detektiert werden. Außerdem gelang es mithilfe experimenteller Manipulationen, trophische Funktionen bisher unkultivierter Taxa aufzudecken und mit Diversitätsdaten zu verknüpfen. Abschließend kann gesagt werden, dass die vorliegende Arbeit in ihrer methodischen Vielfältigkeit wichtige Erkenntnisse über die Diversität und Funktion von Protistengemeinschaften der Ostsee erbrachte.

Summary

State of the Art

Significance and ecological role of protists in aquatic ecosystems

Protists (unicellular eukaryotic microbes) are extremely versatile with regard to their trophic functions and play multiple ecological roles in aquatic environments. While plastid-bearing protists are the most important photosynthesizers, predatory protists act as the smallest predators in the oceans, and mixotrophs even can do both at the same time. Saprotrophic protists are involved in the decomposition and remineralization of organic matter. Others form complex symbiotic interactions mostly with other microbes, whereas parasitic protists infect organisms of all trophic levels (Fig.1).

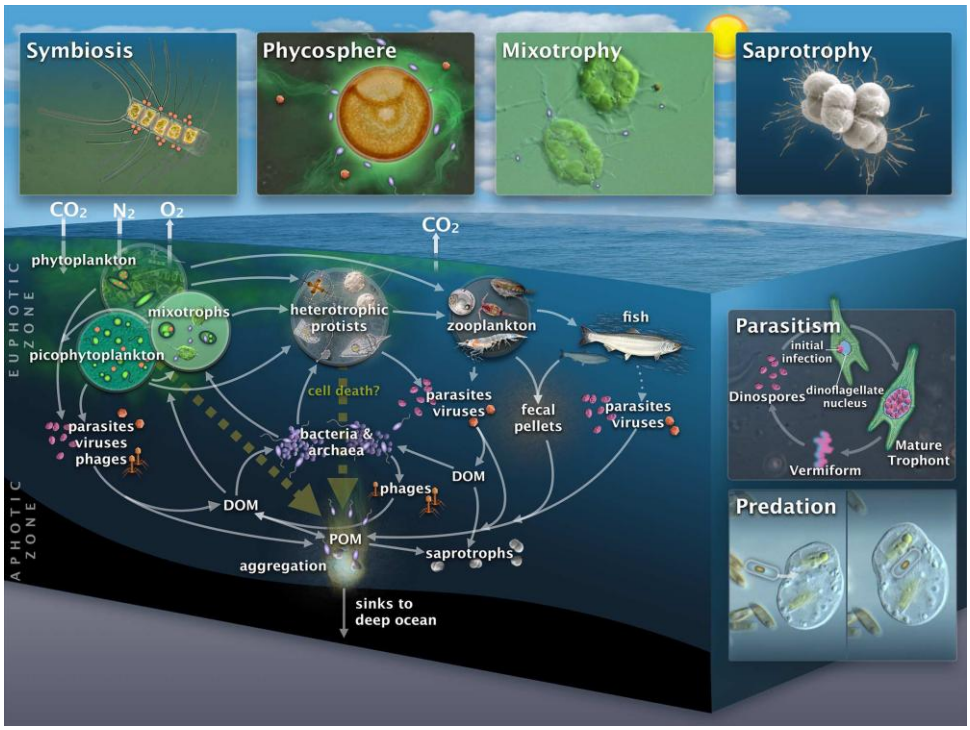

Figure1. Functional diversity of eukaryotic microbes and their interactions with other organisms in the marine microbial food web (Worden et al. 2015).

Phototrophy

A landmark event in the history of eukaryotic life was the acquisition of phototrophic activity, as an evolutionary consequence of serial endosymbiosis. The plastid progenitor, cyanobacterial in nature, was engulfed by a eukaryotic host perhaps 1.5 billion years ago (Archibald 2015) and evolved into primary chloroplasts that are shared today by three algal groups, the glaucophytes, red algae, and green algae (Dorrell & Smith 2011). Subsequently, photosynthesis has spread from archaeplastid algae to unrelated eukaryotes by secondary (e.g., heterokonts and haptophytes) and tertiary endosymbiosis events (e.g., dinoflagellates) (Worden & Not 2008, Archibald 2015).

In the modern ocean, marine algae account for a primary production of 50 billion tonnes C per year, rivaling that of land plants (Falkowski 2012). The most abundant phytoplankters are cyanobacteria such as *Synechococcus* and *Prochlorococcus*. Nevertheless, in terms of biomass photosynthetic picoeukaryotes often dominate phytoplanktonic assemblages and are responsible for around three quarters of the total primary production (Li 1994, 1995, Worden et al. 2004). In marine systems, chlorophytes, heterokonts and haptophytes are considered to be the most important phyla among picoeukaryotic photosynthesizers (Worden & Not 2008). Finally, phototrophic protists are an integral part of the biological carbon pump and form the base of the ocean´s food chain (Falkowski 2012, Worden et al. 2015).

Heterotrophy

Phagocytosis, the ability to engulf larger particles, is thought to be central to the genesis of the eukaryotic cell and its acquisition of endosymbionts which later evolved into mitochondria and chloroplasts (Cavalier-Smith 2002, Yutin et al. 2009). Therefore, phagotrophy represents one of the most ancient modes of nutrition, and is shared by virtually all lineages of the protistan phylogenetic tree (Cavalier-Smith 2002, Sherr & Sherr 2002).

Albeit high bacterial growth rates in aquatic systems, the bacterioplankton´s standing stocks remain remarkably constant, which implies the existence of efficient loss factors (Jürgens & Massana 2008, Kirchman 2012). Inter-ecosystem comparisons ranging from the euphotic zones of fresh- and marine waters to the deep ocean revealed strong correlations of bacterial numbers and the abundance of phagotrophic protists, indicating their tight trophic relationship (Sanders et al. 1992, Pernice et al. 2014). The major removal agents for bacteria are small (<5 µm) heterotrophic nanoflagellates (HNF) (Jürgens & Massana 2008). These typically dominate the bacterivore assemblages in freshwater and marine systems (Sherr & Sherr 2000), with heterokont taxa (mainly chrysomonads and bicosoecids), choanoflagellates and kathablepharids constituting the bulk of HNF biomass in the pelagial (Boenigk & Arndt 2002). Additionally, uncultured lineages of bacterivorous

flagellates, e.g., marine stramenopiles (MAST) form a substantial fraction of total HNF abundance (Massana et al. 2002). The grazing activity of HNF has been estimated to be responsible for 45-87% of the bacterial biomass consumption (Christaki 2001). Moreover, selective predation acts a shaping force for the phenotypic and genotypic composition of planktonic bacteria (Jürgens & Matz 2002, Pernthaler 2005), thereby affecting the biogeochemical transformations, such as nitrification rates, mediated by the prey (Lavrentyev et al. 1997).

Although, large heterotrophic flagellates (>15-200µm, LHF) represent a significant portion of the total protistan biomass (Arndt et al. 2000), their contribution to total bacterivory is rather unknown (Pernthaler 2005). Among ciliates, which graze less selectively the most important bacterivors are small oligotrichs, peritrichs and scuticociliates (Pernthaler 2005). Apart from bacterivory, the majority of phagotrophic protists, including heterotrophic flagellates, dinoflagellates and ciliates, have evolved a wide variety of feeding strategies enabling access to a broadened food spectrum (omnivory) of different trophic levels (Boenigk & Arndt 2002, Sherr & Sherr 2002). Especially, herbivory by protists that use pico-sized algae as prey seems to be at least equally important compared to bacterivory (Sherr & Sherr 1994) and expanded the concept of carbon flow in the microbial food web (Arndt et al. 2000, Sherr & Sherr 2002).

Additionally, phagotrophic protists serve as an important food source for metazooplankton and thus they channel the production of bacteria and small phytoplankton to higher trophic levels (Sherr & Sherr 2002, Zöllner et al. 2009). Further, the excretions of protists are the major source of regenerated nutrients (Sherr & Sherr 2002) such as particulate and dissolved organic matter (POM, DOM), trace metals, nitrogen and phosphorus compounds which in turn promote heterotrophic and photoautotrophic growth of further prey (Nagata 2000, Sherr & Sherr 2002, Pernthaler 2005).

Mixotrophy

Beyond the traditional “black-and-white” view that characterizes protists as being either phototrophic or phagotrophic, there is an increasing attention on the dual capability of many protists to engage both trophic modes in a single cell, known as mixotrophy (Mitra et al. 2014). Mixotrophy is not a feature restricted to phagotrophic phytoplankton that are able to ingest particles (e.g., chrysophytes), but also frequently occurs among mainly heterotrophic species (e.g., ciliates, dinoflagellates) that acquired the photosynthetic activity by kleptoplastidy or the enslavement of bacterial or eukaryal algal cells (Esteban et al. 2010, Worden et al. 2015). Therefore, mixotrophy is a common phenomenon shared by most of the protist lineages (Mitra et al. 2014). Even among green algae (*Micromonas*, chlorophytes), that constitute the smallest and most abundant picoeukaryotic primary producers, bacterivory has been demonstrated (González et al. 1993, McKie-Krisberg & Sanders 2014). Currently, estimates that suggest that 40-90 % of bacterivory in euphotic layers is

exerted by mixotrophs (Unrein & Massana 2007, Zubkov & Tarran 2008), seem to change our paradigm of food web dynamics including carbon flow and nutrient recycling (Mitra et al. 2014). Nevertheless, the underlying mechanisms such as environmental factors as well as the genetic regulations, that influence the varying proportions of phototrophic and heterotrophic activities within mixotrophs are only marginally understood (Matantseva & Skarlato 2013, Worden et al. 2015).

Saprotrophy and osmotrophy

Another nutritional mode of heterotrophic protists is the uptake of organic matter from the external environment (osmotrophy) often combined with previous excretion of enzymes that mediate extracellular degradation of complex macromolecules (saprotrophy) (Worden et al. 2015). The incorporation of dissolved organic matter has been observed in mixotrophic algae that obviously supplement their nutritional needs beyond photosynthesis by osmotrophy (Vila-Costa et al. 2006).

Within the stramenopiles ubiquitous labyrinthulids and thraustochytrids are well known osmotrophs, with the latter group becoming most abundant during post-bloom phytoplankton degradation (Raghukumar et al. 2001, Massana 2011). Further, a member of the widespread and abundant Picozoa (former picobiliphytes), a largely uncultured lineage with puzzling trophic functions, seems to feed on colloid-sized particles by a novel fluid-phase, bulk flow uptake mechanism (Cuvelier et al. 2008, Seenivasan et al. 2013).

Symbiosis and Parasitism

Most recently, sequencing data from the circumglobal *Tara* Oceans expedition pointed out that most of the eukaryotic plankton biodiversity (including small metazoans) is comprised by protistan groups that are parasites or symbiotic host (de Vargas et al. 2015).

Concerning marine protistan parasites, the most striking finding in recent years was the discovery of widespread novel marine alveolates (MALVs) by various environmental sequencing surveys (Guillou et al. 2008, Not et al. 2009). These uncultured alveolates are represented by five main groups that are suggested to occur exclusively in marine systems (Guillou et al. 2008). Here they usually constitute 10-50% of all eukaryotes in genetic libraries (Chambouvet et al. 2008, Massana 2011) with MALV group I thriving preferentially under anoxic and suboxic conditions whereas the groups II-V seem to inhabit the euphotic realm of the oceans only (Guillou et al. 2008). MALVs correspond to syndiniales, a parasitic order within the *Dinophyceae* (Guillou et al. 2008). Some groups have a high host specificity for certain photosynthetic dinoflagellates (e.g., MALV II) whereas others seem to infect a wide range of hosts, extending from other protists to a variety of metazoans (e.g., MALV I) (Chambouvet et al. 2008, Massana 2011). Members of syndiniales but also

perkinsozoa (alveolata) and chytridiomycota (fungi) have been shown to be able to control the community structure and population dynamics of phytoplankton blooms (Coats & Bockstahler 1994, Chambouvet et al. 2008, Jephcott et al. 2015). Likewise to viral attacks, eukaryotic parasitoids cause the release of POM and DOM by the induced death of their hosts (Worden et al. 2015). Estimates suggest that nearly half of the host's biomass gets rerouted into the carbon pool of the marine food web (Jephcott et al. 2015), an issue that is to date largely neglected by biogeochemical models of carbon fluxes (Siano et al. 2011, Worden et al. 2015). Further eukaryotic parasites, especially the ones with a wide host spectrum, may affect the marine food web at multiple trophic levels including primary producers (e.g., picoalgae), intermediate consumers (e.g., zooplankters) and even top predators (e.g., fish).

Many protists form versatile symbiotic relationships (defined here as mutualism) with prokaryotes, other protists or other eukaryotes as permanent or temporary associations (Gast et al. 2009). Either as ecto- or endosymbionts, the mutualistic partners endow the protistan hosts with new metabolic capabilities, with access to refractory nutrient pools, with defence mechanisms to repel grazers and parasites or the ability to explore otherwise hostile environments (Gast et al. 2009, Dziallas et al. 2012). In the photic zone, the main function that protists acquire by endosymbiotic eukaryotes is photosynthesis, with the associations among plastidic cryptophytes and ciliates (e.g., *Mesodinium rubrum*) or dinoflagellates as hosts (e.g., *Gymnodinium aeruginosum*), as prominent examples (Nowack & Melkonian 2010). Extreme habitats such as anoxic systems seem to be hot spots for symbiotic relationships between protists and prokaryotes (Bernhard et al. 2000). Here, up to 90 % of the ciliates act as hosts for epibiontic prokaryotes (Edgcomb et al. 2011) which often cover an individual's cortex in their thousands (Epstein et al. 1998). Further, many anaerobic ciliates benefit from higher growth rates through endobiotic methanogenic archaea, which function as scavengers of hydrogen that is generated by fermentation in the host cell´s hydrogenosomes (Fenchel & Finlay 1995, Yamada et al. 1997).

Thus far, symbiotic interactions involving protists have been largely overlooked in microbial food web dynamics (Worden et al. 2015) even though they can significantly influence ecological processes and strengthen the metabolic potential of microbial assemblages as a whole.

What is known on the Baltic Sea protist communities?

The Baltic Sea, as one of the world´s largest semi-enclosed brackish water bodies, has formed after the last glaciation and represents a geologically young ecosystem (Lass & Matthäus 2008). Its estuarine circulation and the resulting brackish water conditions as well as latitudinal and vertical steep physico-chemical gradients are the major hydrological characteristics of the Baltic Sea. A positive water balance from freshwater supply (riverine runoff, precipitation) that exceeds

evaporation, results in a net outflow of surface water which is partly compensated by saline water inflows from the North Sea (Reissmann et al. 2009). Nevertheless, the narrow and shallow connection of the Baltic Sea with the North Sea, through belts and the Øresund restricts a continuous exchange of water, resulting in a latitudinal salinity gradient throughout the Baltic Sea (Reissmann et al. 2009). The salinity regime range from limnic to nearly marine conditions from north to south, whereas the largest part of the Baltic Sea (Baltic proper) is characterized by brackish water conditions, with average surface-water salinities of 5-8 (Lass & Matthäus 2008, Telesh et al. 2011a).

Other events that influence the Baltic salinity budget and the ventilation of the deep basins are wind driven major Baltic inflows of marine and well oxygenated water from the North Sea (Krauss et al. 2001). These occur in rather random intervals, such as in 1983, 1993, 2003 and just lately in 2014 with the tendency to happen less frequently in the past two decades (Meier et al. 2006, Mohrholz et al. 2015). Between these perturbations, the water column is relatively stable stratified and a permanent halocline separates surface water of lower and deep water of higher salinity (Krauss et al. 2001). During these stagnation periods, restricted vertical mixing leads to oxygen depletion by biological respiration and the production of hydrogen sulfide in the deep basins (Reissmann et al. 2009).

Further, due to the geographic extension of the Baltic Sea (54°–66°N), the climate ranges from temperate in the south to sub-arctic conditions in the north, which is usually ice covered for 6 month of the year (Krauss et al. 2001). This also implies strong seasonal variations as well as latitudinal differences in surface water temperature (Samuelsson et al. 2006) and in the annual solar radiation cycle (Andersson et al. in press) accompanied by a north-south primary production gradient (Samuelsson et al. 2006, Dahlgren et al. 2010).

Another peculiarity of the Baltic Sea as a geological and ecological youngtimer is the ongoing change in the community composition (e.g., phytoplankton) due to niche occupation of freshwater and marine species, speciation processes and invasions of nonindigenous species (Pereyra et al. 2009, Ojaveer et al. 2010, Olli et al. 2011, Telesh et al. 2011a). Hence, the Baltic Sea can be considered as a sea of environmental gradients with severe consequences for its organisms, their diversity, distribution and abundance. Especially, the salinity gradient is thought to be the main environmental factor to define the structural and functional characteristics of aquatic biota in brackish water habitats (Telesh & Khlebovich 2010, Telesh et al. 2015). On account of macrozoobenthos data, brackish water conditions were found to be a physico-chemical barrier (salinity of 5-8, defined as horohalinicum, (Kinne 1971)) for species of marine and freshwater origin and to harbor a low number of genuine brackish water species (Remane 1958). This biodiversity pattern coined by Remane as the “species minimum model” was often erroneously generalized to argue that the Baltic Sea is a species poor environment (Telesh et al. 2011a). On the contrary, newer

investigations challenge the validity of Remane´s concept for planktonic organisms, especially when the smallest size fractions such as bacteria and protists are considered (Telesh et al. 2011a, b, 2015, Herlemann et al. 2011). Telesh and colleagues discovered protists to have their species maximum in the horohalinicum and estimated the Baltic Sea to harbor almost 3500 formally described protistan taxa, with the majority belonging to heterokonts (Hällfors et al. 2004, Telesh et al. 2011a, b).

The vertical structuring of protists along the pelagic redox gradients of the Baltic Sea was first recognized on account of ciliates that show a bimodal distribution in the water column, with abundance peaks in the euphotic zone and around the oxic-anoxic boundary layer (Mamaeva 1988). Within the redoxcline, *Mesodinium* species, oligotrichous ciliates (*Strombidium*, *Strombilidium*) and very large unidentified ciliates have been encountered in various studies (Setälä 1991, Setälä & Kivi 2003, Anderson et al. 2012, 2013). A similar distribution pattern was found with regard to phototrophic and heterotrophic pico- and nanoflagellates (Detmer et al. 1993). Nevertheless, the bulk of the protistan biomass is constituted by ciliates, and grazing estimates suggest that 50 to 100% of the prokaryal standing stocks are consumed per day in these strata (Setälä & Kivi 2003, Anderson et al. 2012). The construction of 18S rRNA clone libraries in the Gotland Deep revealed a high protistan diversity in oxygen deficient and anoxic waters, with a majority of phylotypes that were distantly related to described species (Stock et al. 2009). Moreover, a changing community structure from suboxic depth, where ciliate and fungi related phylotypes dominated, to sulfidic conditions, where jakobids predominated, was found (Stock et al. 2009). Another molecular study, using RNA-Stable Isotope Probing (RNA-SIP), identified five active redoxcline protists (two ciliates, two stramenopiles and a cercozoan) that graze on the abundant chemolithoautotrophic bacterial keyplayer *Sulfurimonas gotlandica* (Anderson et al. 2013). Further, cultivation attempts with redoxcline samples from the central Baltic Sea succeeded in obtaining cultures of two new choanoflagellate species from the genus *Codosiga* (*C. balthica*, *C. minima*) which possess cellular peculiarities, such as atypical mitochondrial cristae and endosymbiotic bacteria (in *C. balthica*), apparently as an adaption to hypoxic conditions (Wylezich et al. 2012).

The high seasonal and spatial variability of biotic and abiotic factors in the Baltic Sea represent additional shaping forces that govern the distribution and community structure of pelagic protists. Across the geographic extension of the Baltic Sea a primary production gradient exists with a 5 to 10-fold higher production in its southern part (Samuelsson et al. 2006, Dahlgren et al. 2010). Likewise, the abundances, cell size and biomass of protists increases with the increasing primary production from north to south (Samuelsson et al. 2006). The seasonal succession of the protist community seems to follow a general pattern: choanoflagellates, large flagellates and ciliates show peaks during spring and autumn, while small bacterivorous nanoflagellates peak during summer. The latter are mainly regulated by changes in temperature and bacterial numbers in the yearly cycle

(Samuelsson et al. 2006) explained by the high correlation between temperature and protist feeding rates (Vaqué et al. 1994). Among small heterotrophic flagellates, chrysophytes are common and dominate in the Bothnian Bay, whereas they contributes less than 10% of the total HNF biomass in the Baltic proper (Andersson et al. in press). Especially the two genera *Paraphysomonas* and *Spumella* are frequently detected throughout the Baltic Sea (Samuelsson et al. 2006). Large seasonal variations often result in short-lived blooms of small flagellate groups (Samuelsson et al. 2006). These have been observed, for example, in open water samples of the Baltic proper with *Goniomonas* sp. (crytophyta) (Andersson et al. in press) and in coastal regions with uncultured representatives within the pedinellales, MAST, cercozoa and syndiniales (Piwosz & Pernthaler 2010).

The variations in abundance of larger protists seem to be additionally controlled by phytoplankton as a food resource and metazooplankton as predators (Samuelsson et al. 2006). Larger flagellates, such as the two cryptophytes *Leucocryptos marina*, mostly found in the Baltic proper, and *Katablepharis* cf. *remigera*, occurring in all basins, have their maxima during spring blooms (Andersson et al. in press), likely as a consequence of elevated resource availability. Heterotrophic dinoflagellates are especially important in nutrient-rich coastal areas like the Gulf of Gdańsk where they typically account for around 30% of the total protistan biomass (Rychert 2011, Rychert et al. 2013). An increase of choanoflagellate abundance in the Bothnian Sea has been observed to coincide with diatom blooms in spring and high concentrations of detritus in late autumn, probably as a consequence that both provide suitable surface-attachment sites required by choanoflagellates (Samuelsson et al. 2006).

The Baltic Sea´s ciliate inventory seems to be well studied and currently includes about 800 described species (Mironova et al. 2009, Telesh et al. 2011a) of which only a minority (160 species) are typical planktonic species (Mironova et al. 2009). Small pelagic oligotrichous ciliates (*Strombidium, Strobilidium*, and *Lohmaniella*) are the most important ciliate groups in the Baltic Sea (Mironova et al. 2009), often accounting for more than 50% of total ciliate biomass (Johansson 2004). Nevertheless, over the course of the seasons, considerable shifts in the planktonic ciliate communities take place and the dominating species change (Mironova et al. 2009). The ciliate *Mesodinium rubrum* (synonym *Myrionecta rubra*) occurs from north to south, often as the dominant member in the protistan and phytoplankton communities (Andersson et al. 1996) and is even present in Baltic Sea ice (Majaneva et al. 2011). In the south-eastern part of the Gdańsk Basin, *M. rubrum* makes up to 10% of total primary production (Witek 1998) and its mass development has been interpreted as an evidence of eutrophication (Andersson et al. in press). Further, *M. rubrum* has a typical vertical migration pattern and often shows overlapping population maxima with *Dinophysis* spp. and *Teleaulax* spp. in thin layers in the water column, indicating a tight interdependency of these three species (Sjöqvist & Lindholm 2011).

In general, three different types of protist-related studies that have been conducted in the Baltic Sea can be distinguished (Fig. 2). First, a variety of morphological and taxonomic approaches with a wide coverage of sampling sites across the whole Baltic Sea. These studies primarily targeted the microscopical identification of protists that posses conspicuous morphological features such as ciliates and larger flagellates, while neglecting the identity of the smaller size fractions (e.g., pico- and nanoflagellates). Likewise, ecological approaches have been also performed throughout the Baltic Sea, but treated protists mostly as functional units or guilds (e.g., HNF, heterotrophic ciliates, phytoplankton), and therefore do not provide comprehensive information on the taxonomic composition of the Baltic Sea´s protist assemblages. At the beginning of this thesis, there were only two punctual studies that used 18S rRNA gene sequences to identify protists (including nano-sized eukaryotes) in the euphotic zone of a coastal site and in anoxic depths of the central Baltic Sea (Fig. 2). Consequently, the spatial and temporal resolution of these few molecular data is rather low and does not allow to draw a general picture of the Baltic Sea´s protist community composition on the basis of 18S rRNA marker genes.

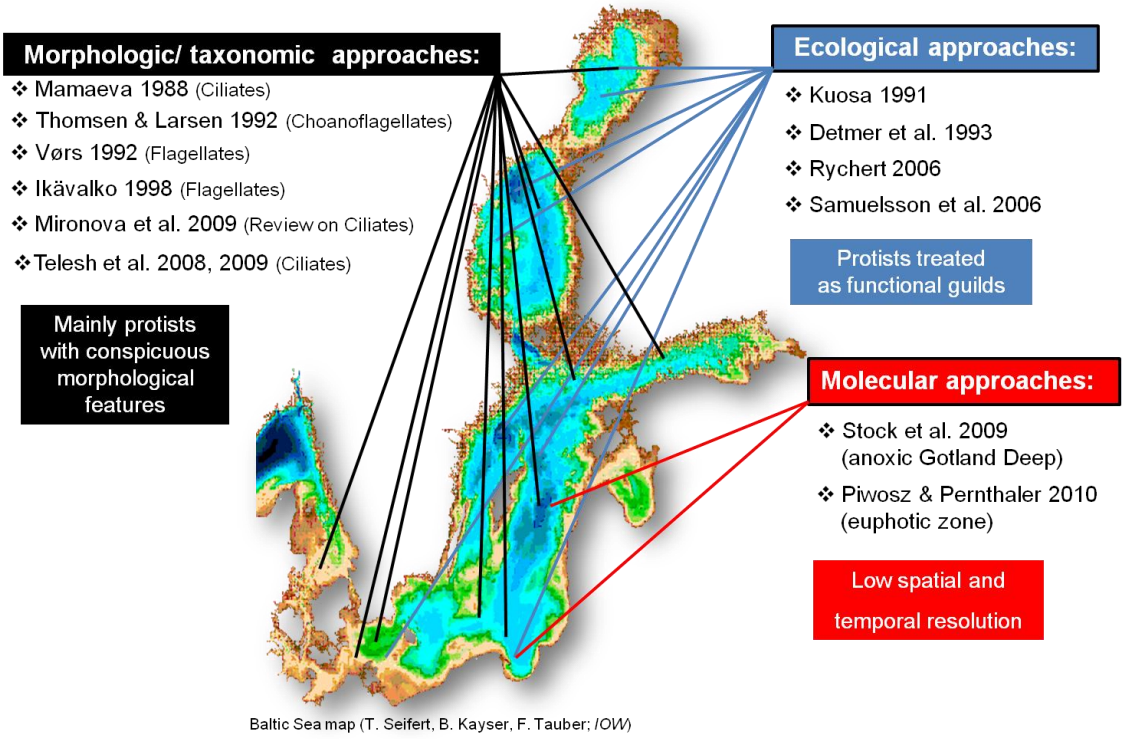

Figure 2. Geographic distribution of different protist related studies in the Baltic Sea, differentiated for morphological/ taxonomic approaches (black), ecological approaches (blue) and molecular approaches (red). The sampling site locations of the respective studies (indicated by black, blue and red lines) were derived from the depicted literature.

Since the Baltic Sea, in comparison with the open ocean, has been rated to be vastly understudied with regard to its taxonomic composition of the bacterioplankton (Andersson et al. in press), it can

be claimed here that such statements even more apply to our knowledge on the protistan assemblages of the Baltic Sea.

Methods to study protists in ecology — advantages and hurdles

Protists have been studied using a variety of different methods to address their phylogenetic diversity, distribution and abundance or functional diversity. An overview is given in Fig. 3.

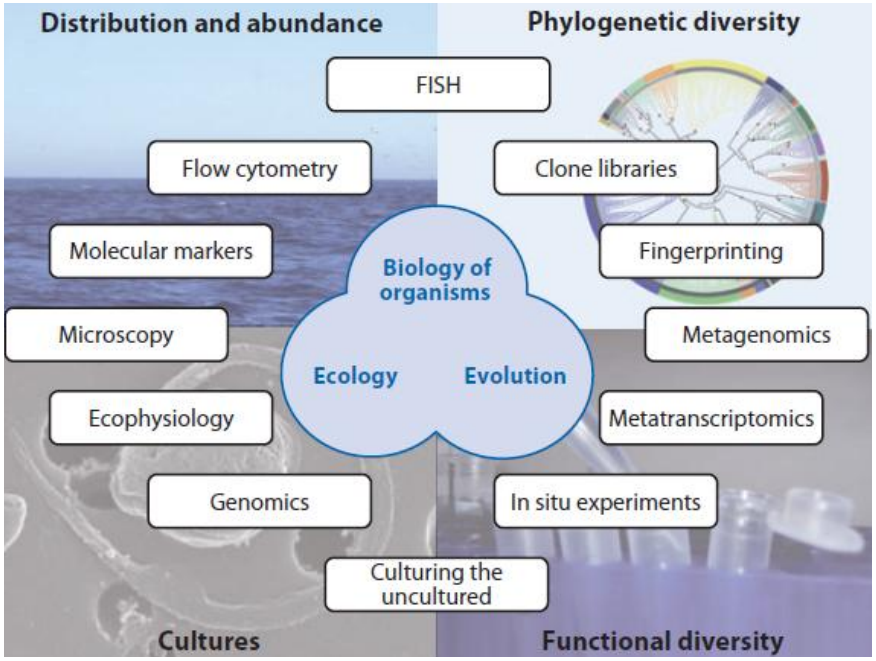

Figure 3. Overview of approaches to investigate cell biology,ecology, and evolution of marine picoeukaryotes (and microorganisms in general), treating four main study areas: abundance, phylogenetic diversity, functional diversity, and culture studies. Abbreviation: FISH, fluorescence in situ hybridization (Massana 2011).

The isolation of single protist cells and their cultivation as pure cultures is one of the most traditional approaches in protistological research, and dates back to the middle of the18th century (Hausmann et al. 2003). Till the present day, laboratory studies on cultured strains established our fundamental knowledge on the general biology (morphology, taxonomy, physiology) and ecology (trophic function, activity, behavior) of protists, and still represent the gold standard for full species descriptions (del Campo, Balagué, et al. 2013). Nevertheless, similar to the phenomenon of the "great plate count anomaly" reported for prokaryotes (Staley & Konopka 1985), quantification attempts including cultivation steps, such as the Most Probable Number (MPN), also yielded underestimates of microbial eukaryote abundance by several orders of magnitude, when compared to direct microscopical counts (Caron et al. 1989). The strongest line of evidence for a strong culturing bias in protist studies was derived from cultivation-independent molecular approaches, which recovered unknown phylotypes that are different from those obtained by cultivation-based approaches (Massana et al. 2004, 2013, Shi et al. 2009). The latter seems to retrieve mainly the same pool of "easy to culture species" underscoring the selective nature of cultivation (Jürgens & Massana 2008). Therefore, the relevance of these cultured strains as model organisms for the dominant

protists in planktonic environments has been questioned (del Campo, Balagué, et al. 2013). Nevertheless, now that protistological research has reached the age of sequence-based surveys, cultures of ecological relevance are needed more than ever, in order to act as a reference database for our interpretations of high-throughput sequencing and meta-omics data, likewise to sequences of cultures that represent the backbone of the eukaryotic phylogenetic tree (del Campo et al. 2014, Keeling et al. 2014).

The first glimpse into the microscopic world of protists was reserved for Antoni van Leeuwenhoek with his home-made light microscope, more than 350 years ago (Caron et al. 2009). Today, a variety of microscopical techniques with differing suitability to study the abundance, distribution and morphological and ultrastructural diversity of protists, are available. In ecological studies, epifluorescence microscopy and inverted microscopy (Utermöhl technique) of fixed and concentrated cell material are commonly used. Contrary to live observations with the light microscope, these methods allow the storage of samples and are supposed to yield more accurate and reproducible cell counts (Caron 1983). Whereas inverted microscopy tends to underestimate nanoplankton concentrations (Booth et al. 1982) presumably due to slow settling rates of small cells (Caron 1983), epifluorescence microscopy is inferior in the quantification of larger protists (e.g., LHF) since these are often disrupted by fixation and membrane filtration (Arndt et al. 2000).

Epifluorescence microscopy visualizes just a few morphological features (cell size, general shape, presence of flagella or stems) that restrict proper classifications of nano and pico-sized eukaryotes, even into high-ranking taxonomic groups (Massana 2011). The major advantage of this technique is the differentiation of phototrophic and heterotrophic protists by the presence or absence of autofluorescing pigments (Caron 1983). The taxonomic resolution for larger protists (dinoflagellates, ciliates) is generally higher with inverted microscopy (Massana 2011). Both methods provide basic information (size and general shape of cells) necessary for biomass estimates. Nevertheless, the use of fixatives has been demonstrated to cause morphology distortions, cell shrinkage or simply loss of cells and complicates the identification, enumeration and biomass calculations of protists (Choi & Stoecker 1989, Sherr & Sherr 1993). The highest taxonomic resolution for ultrastructural studies can be achieved by electron microscopy, but it is costly, time consuming and suffers from cell loss (Caron 1983, Vørs et al. 1995, Jürgens & Massana 2008).

Other obstacles that complicate morphology based taxonomic assignments are crypticism and polymorphism in some protist groups. For instance, some small flagellates are considered as “naked”, thus their cell surface is not covered by scales that form loricae or thecae. Examples are species of the chrysophyte genus *Spumella* (Mylnikov et al. 2008) and many flagellates with a resemblance to that morphology were lumped together as *Spumella*-like flagellates (Boenigk et al. 2005). Indeed, 18S rRNA gene sequences revealed this morphology to be shared by a wide range of

flagellates which showed high sequence divergence (up to 10%) and different placements in the phylogenetic tree of chrysophytes (Boenigk et al. 2005). A similar phenomenon was observed with the homogeneous morphology within the parasitic dinoflagellate genus *Amoebophrya* which masked an extensive genetic diversity (Gunderson et al. 2002). Thus, the existence of cryptic species might cause considerable underestimations of protist diversity. In return, the phenomenon of intraspecific polymorphism, e.g., changing shape during different life-history stages or changing environmental conditions, results in overestimations of diversity (Pizay et al. 2009, Dolan 2015). An extreme example for polymorphism is that of the dinoflagellate *Ceratium ranipes* which exposes a remarkable variability between its night and day morphologies (Pizay et al. 2009). Further, morphology based taxonomy, although mostly performed by experts, is not immune against a certain personal bias and taxonomic errors. For example, synonyms in species lists resulting from repeated description of the same form can considerably inflate global species estimates (Dolan 2015). Similar problems occur with so called “oncers”, species that have never been observed again since their original description (Thessen et al. 2012). Often these do not represent a real taxon, but rather a poorly described species of ambiguous identity, such as the example of the genus *Gymnodinium* which comprises 268 described species of which nearly 40 % represent “oncers” (Thessen et al. 2012). Altogether, crypticism, polymorphism and taxonomic errors can considerably obscure taxonomic work on protists and estimates of species richness, and therefore would take enormous benefit from the inclusion of molecular tools (Dolan 2015).

Due to inherent limitations of the above mentioned methods, microbial eukaryotes especially of the smallest size fractions were treated for decades as a black box of difficult access (Massana 2011). The application of 18S rRNA gene based molecular approaches has revolutionized our understanding of protistan diversity and of their evolutionary relationships, and revealed the existence of many previously undetected protist lineages, some of them globally distributed in the world´s oceans (Stoeck & Epstein 2003, Caron et al. 2004, Caron 2009, Massana et al. 2013). Today fingerprinting techniques, the construction of genetic libraries and high throughput sequencing (HTS) are routinely applied in ecological studies to investigate natural protistan community compositions, single-cell characterizations and the biogeography of certain taxa (e.g., Díez et al. 2001, Stoeck & Epstein 2003, Stepanauskas 2012, Logares et al. 2014). Nevertheless, incomplete nucleic acid extraction, variations in gene amplification efficiencies during polymerase chain reaction (PCR), and the choice of PCR primers may favor or discriminate the analysis of certain phylogenetic groups, resulting in a skewed view of the composition of natural protist assemblages (Stoeck et al. 2006, Caron 2009, Bass et al. 2012, Kermarrec et al. 2013). Additionally, extreme variations in rDNA copy numbers (Zhu et al. 2005) and rRNA expression levels among different protists lineages (Weber & Pawlowski 2013) considerably influence our interpretations of certain taxa´s relative contribution to

the whole protist community and therefore disqualify 18S rRNA gene and transcript based approaches from being of real quantitative nature (Stoeck et al. 2014). Further, sample processing via PCR and sequencing may produce artifactual data, such as sequencing errors or the formation of chimeric sequences which bear the risk to generate spurious estimates of microbial diversity (Berney et al. 2004). Other problems occur with the *in silico* processing of high amounts of sequence data.

Due to the incompleteness of molecular databases for known or described eukaryotes many environmental phylotypes are falsely judged as novel eukaryotic groups (Berney et al. 2004). Some well-known protist groups contain fast-evolving members whose sequences might be misplaced in phylogenetic tree reconstructions by long-branch attraction and suggest them to represent novel protist groups (Philippe et al. 2005). Modern deep-sequencing technologies allow massive gatherings of environmental sequences, but the use of mock communities to evaluate the accuracy of HTS approaches revealed that, depending on the parameters used for analysis of the dataset (denoising of sequencing artifacts, sequence clustering), species richness can be overestimated by several orders of magnitude (Bachy et al. 2013, Wylezich unpubl.). Apart from these technical problems, the major drawback of 18S rRNA based approaches is that they cannot provide valuable information on the morphology and general biology. In fact, different trophic modes or lifestyles (e.g., heterotrophy, phototrophy) seem to be intermingled among unrelated protist lineages (Fig. 4) and therefore cannot be brought directly into accordance with the phylogenetic positioning of protist taxa. This challenges to derive functional predictions from molecular diversity data, especially for the bulk of environmental phylotypes that are distantly related to already described organisms (Worden & Not 2008). However, possibilities exist to bridge the gap between environmental diversity data and according functional properties, such as fluorescence *in situ* hybridization (FISH) for certain taxa or metagenomics and metatranscriptomics when it comes to the community level (Massana 2011). With FISH probes, specific protist taxa can be visualized with respect to their general morphology (cell size, shape) and quantified in mixed natural assemblages. Further, to some extent, basic ecological features (e.g., plastidic, aplastidic, bacterivorous) can be assigned to members of uncultured clades (Massana, Terrado, et al. 2006). Currently, massive environmental DNA and RNA sequencing to either reveal the metabolic potential (metagenomics) (Cuvelier et al. 2010) or the realized functions (metatranscriptomics) (Lin et al. 2010) will offer new avenues to functional analysis of protistan communities as a whole, but is still in its infancy (Worden et al. 2015). However, these environmental sequence-based approaches generally use a comparative approach, and therefore require suitable reference data of ecologically relevant cultures, which are an extremely limited resource when it comes to microbial eukaryotes (Worden & Allen 2010, del Campo et al. 2014, Keeling et al. 2014).

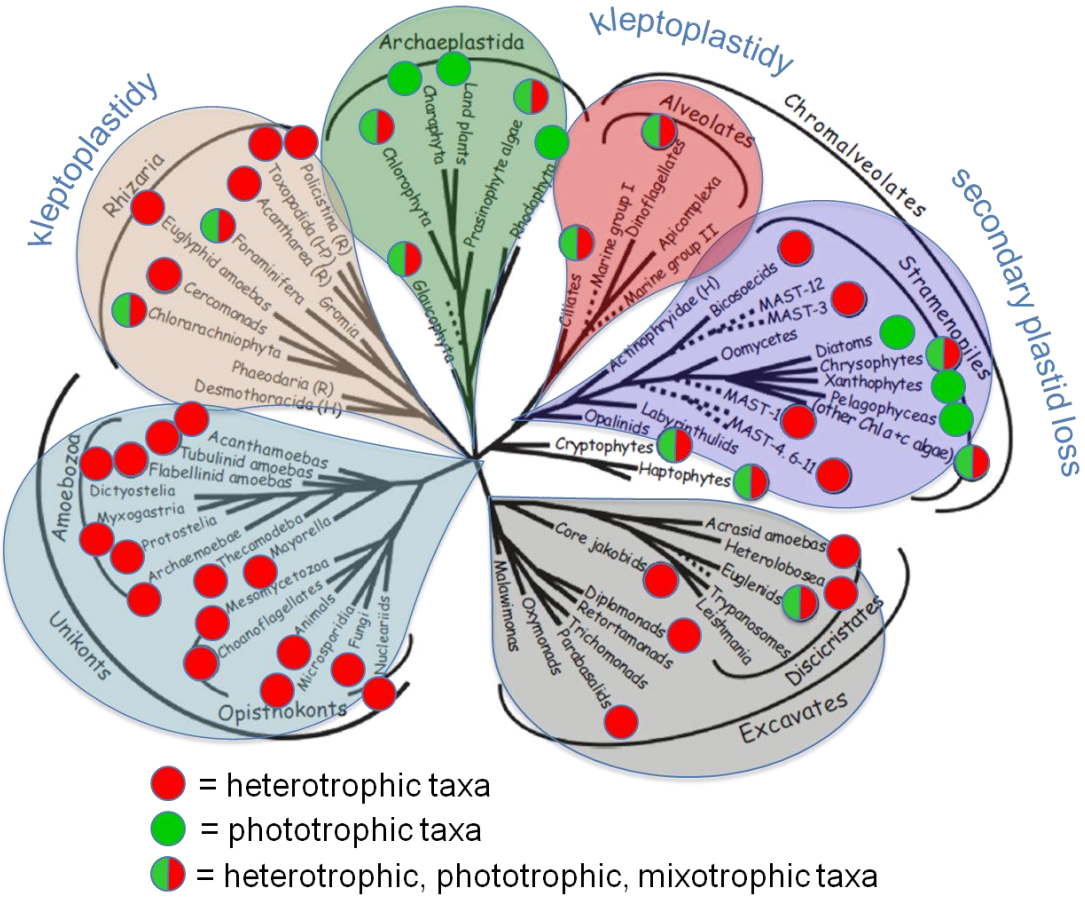

Figure 4. Occurrence of trophic functions within the six major eukaryotic supergroups. Modified from Jürgens & Massana (2008).

Objectives

Manifold techniques are available to study the diversity and ecology of protists, with each showing its specific advantages and hurdles. The application of a single method mostly provides information only on certain aspects e.g., the diversity or functions of protists. In this thesis a multitude of methods was applied in the field and in laboratory experiments in order to reveal important insights into the diversity, ecological functions, distribution and abundance of protists in the Baltic Sea. To achieve this goal, specific objectives were defined according to the individual chapters of this thesis:

1) The Morphological vs. the molecular approach – do both approaches resolve the vertical structuring of the protist community along the central Baltic Seas redox gradient?

2) Culturing the uncultured – is it still worth to follow traditional cultivation attempts to identify new protist taxa of ecological relevance?

3) Alternating lifestyles – what can we learn from *Massisteria voersi* sp. nov., a rare species isolated from coastal waters of the Baltic Sea?

4) The artifice to link protist diversity data with trophic functions – is it possible, starting from natural assemblages, to selectively enrich the heterotrophic fraction of protists for further diversity analysis, while avoiding culturing bias?

Perspective) Putting a face to uncultured protists by developing phylogenetic stains –
demonstrated for two uncultured clades of chrysophytes, the whole cycle 18S rRNA approach was closed via newly designed oligonucleotide probes for fluorescence *in situ* hybridization. Is this the clue to link phylogenetic and autecological information on protists?

Synthesis

The Morphological vs. the molecular approach

A recent symposium article invited the scientific community to a resurgence in protistological field research and argued that the combination of molecular and microscopical methods offer the most promising path towards, e.g., the discovery of new protistan lineages, the untangling of ecological interactions and functions, and their roles in larger ecosystem processes (Heger et al. 2014). Following this strategy, a multi-annual microscopy-based study was conducted, combined in one year with 18S rRNA gene and transcript-based DGGE fingerprints to examine the vertical structuring of dominant protist taxa along pelagic redox gradients of the central Baltic Sea. Both approaches, applied in unprecedented high vertical resolution, demonstrated strong stratifications of the protist community composition in the whole water column as well as in redoxcline profiles.

Cell numbers for protist functional groups (HNF, dinoflagellates and ciliates) showed a typical bimodal distribution in the water column with highest abundances in the euphotic zone and a second, smaller peak around the oxic-anoxic boundary layer. Biomass estimates of the respective groups revealed a major change in protist composition, with HNFs constituting the bulk of total protistan biomass at the surface, and ciliates dominating at redoxcline depth. This implies a fundamentally different structure of the microbial food web, with flagellates as the dominant bacterial consumers in surface waters and ciliates as the major bacterivores within the redox zone (Fig. 5). At this point an open question for future studies arises: what is the fate of protistan biomass in the anoxic-aphotic zone, where predatory metazoans are generally absent? Some possibilities might be transient copepod invasions from the upper zones, lysis of protists induced by specific virus infections, or apoptosis (Fig. 5).

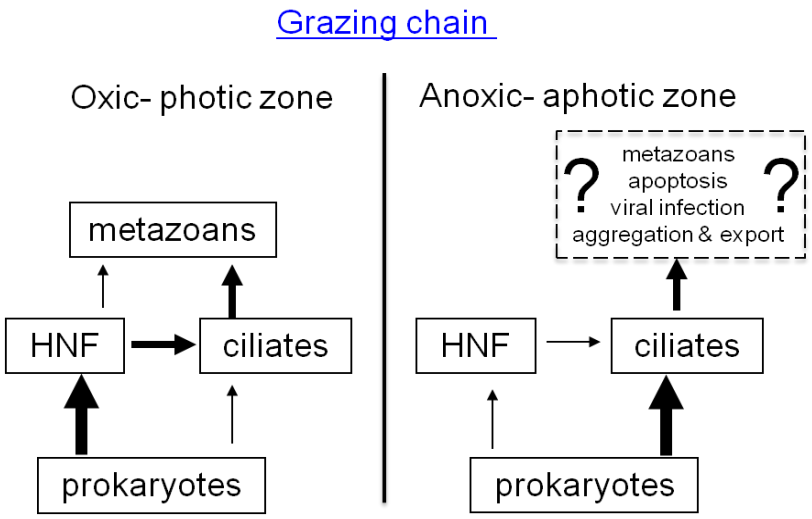

Figure 5. Schematic representation of the major trophic interactions within the microbial food web in oxic-photic zones and anoxic-aphotic zones.

Within the redoxcline, three different zones (suboxic, oxic-anoxic interface and sulfidic zone) could be clearly distinguished by their composition of dominant ciliate morphotypes (*Strombidium* spp., *Mesodinium* spp., and *Metacystis* spp.), based on DGGE cluster analysis and phylotype distribution. Although the application of microscopy and DGGE in concert revealed coinciding results on the vertical distribution of the genus *Strombidium* and dinoflagellates taxa (Fig. 6), the overall congruence of taxa detected by both approaches was unexpectedly low. Comparable results have been obtained by Savin et al. (2004), which highlights the great need for such combined approaches to cover a large and comprehensive portion of taxa in natural protistan assemblages.

Indeed, during our study the simultaneous use of microscopy and molecular tools turned out to compensate for shortcomings related to the application of just a single method. For instance, the semi-quantitative nature of molecular techniques was outbalanced by obtaining cell counts and biomass estimates of certain functional and taxonomic protist groups. In general, microscopy was superior in the detection of larger ciliates like *Metacystis* and *Mesodinium*, which represent major components of the redoxcline protist community, but remained undetected by the molecular

approach. *Metacystis* species were often described as sessile or as benthic dwellers (e.g., Aladro-Lubel & Martinez-Murillo 1999). However, the Baltic Sea seems to be the first aquatic system where *Metacystis* species are described as an important component of the pelagic protist community. Here this genus was represented with a minimum of five different species, mainly large forms (70 to 100 µm) of which at least one is a novel species. *Metacystis* was preferentially detected at the oxic-anoxic interface and the upper sulfidic zone (Fig. 6) and dominated the protistan assemblage with up to 80% of total biomass and thus likely represents the dominant grazers of prokaryotes in these strata. Further FISH analysis suggests that *Metacystis* cells maintain partnerships with large numbers of endosymbiotic *Eubacteria* and ectosymbiotic *Gammaproteobacteria*, which argues for multiple functions mediated by this key component of the Baltic Sea redoxclines. Unfortunately, *Metacystis* lacks any 18S rRNA sequences entry in GenBank, which precludes the assignment of environmental sequencing data to this genus. Consequently, the phylogenetic affiliation remains unsolved, and therefore *Metacystis* species can be identified exclusively by their distinctive morphological criteria at present.

Another limitation of 18S rRNA based approaches is the so-called primer bias (Stoeck et al. 2006). In our study, this became evident by the fact that the employed primer set applicable with DGGE discriminates the genus *Mesodinium* from amplification. If the molecular approach would not have been complemented by microscopical observations, this important member with highest abundance at the oxic-anoxic boundary layer would have remained undetected. Otherwise, 18S rRNA sequences represent the best proxy for unambiguous taxonomic identifications which is especially important for amorphous protists with few diagnostic features (e.g. small flagellates).

The most prominent flagellates that were detected by strong DNA- and RNA-derived signatures in DGGE gels belonged to uncultured representatives within jakobids and symbiontids (euglenozoa). Further, the high resolution sampling effort along whole water column and redoxcline profiles demonstrated that these two phylotypes are important components of the protist community in the entire sulfidic zone (ca. 100 m extension) in terms of presence (DNA) and activity (RNA). Another phylotype was closely related to the recently described choanoflagellate *Codosiga balthica* (Wylezich et al. 2012). This species was isolated during our cultivation survey (Chapter 2) and was additionally detected by two other cultivation-independent approaches on the basis of 18S rRNA genes (Stock et al. 2009, Anderson et al. 2013), indicating *C. balthica* as a permanent member of the Baltic Sea redoxcline community. Other prominent residents in oxygen-deficient habitats are members of parasitic syndiniales group 1, which were suggested to be exclusively marine (Guillou et al. 2008), but our findings in the brackish Baltic Sea indicate this group to have a wider salinity tolerance.

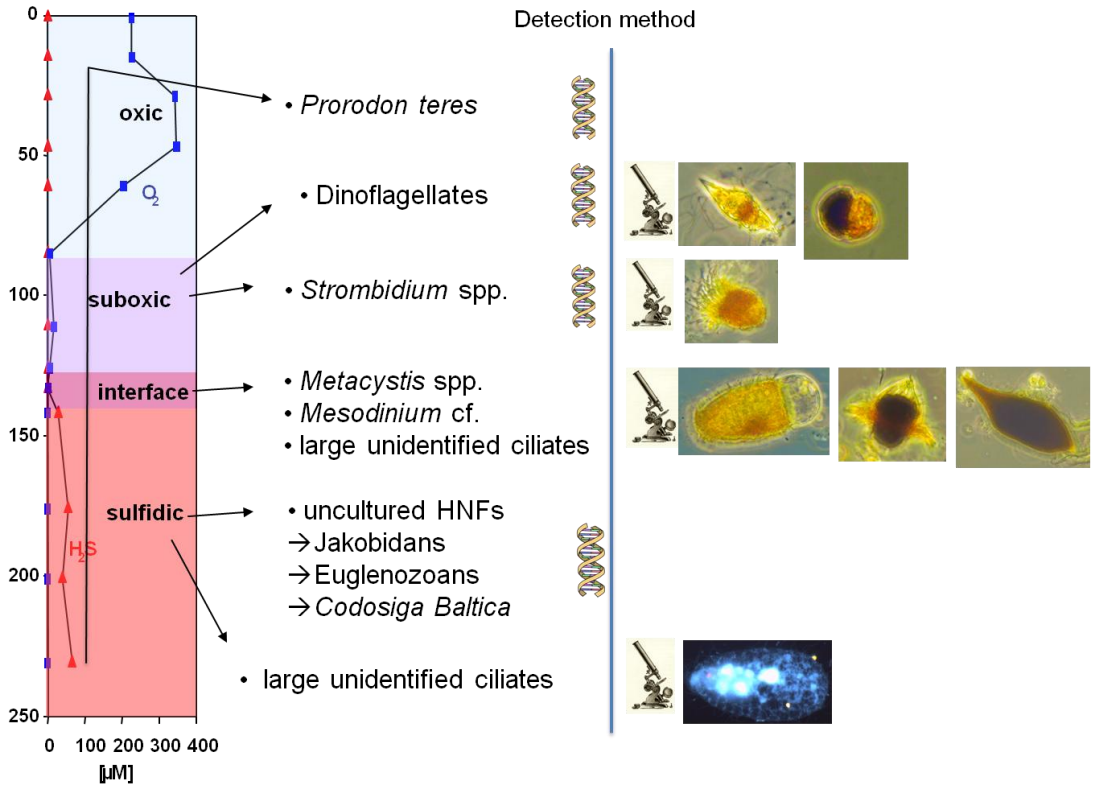

Figure 6. Vertical structuring of dominant protist groups or taxa along the central Baltic Sea's redox gradient identified by microscopy and 18S rRNA gene and transcript sequencing.

Finally, our study indicates that the pelagic redoxclines of the central Baltic Sea are inhabited by a stable and characteristic protist community. Further, fine-scaled assessments of dominant protists in oxygen depleted environments represent crucial steps in understanding their impact and interactions with the prokaryotic world and the biogeochemical processes in these zones.

Culturing the uncultured

The scarcity of well characterized protist cultures, that mirror the dominant and relevant taxa in the environment, represents a bottleneck for ecosystem studies (Heger et al. 2014). Currently, a call in the scientific community arises to increase cultivation efforts in order to obtain protist lineages that help to evaluate ecosystem processes and to establish biogeochemical models (del Campo et al. 2014, Worden et al. 2015). Facing these obstacles, we conducted an extensive traditional cultivation survey (Chapter2), yielding 273 monoclonal protist cultures from a coastal monitoring station (Heiligendamm) and from suboxic to anoxic depths in the central Baltic Sea. Both culture collections (coastal and central Baltic Sea) consisted mainly of tiny amorphous flagellates, and were thus identified based on their 18S rRNA gene sequences. After applying an armada of primer sets we obtained high quality partial and complete sequences from 128 cultures.

The majority of isolated strains were affiliated to already cultured and described taxa (Fig. 7), mainly belonging to chrysophytes (e.g., *Paraphysomonas*, *Spumella*) and bodonids (e.g.

Procryptobia). Those taxa are notorious as opportunistic species that get selectively enriched under altered *in vitro* conditions and rich culture media (Jürgens & Massana 2008, del Campo, Balagué, et al. 2013) and likely indicate a culturing bias. This might be as well the explanation for the considerable share of species in both culture collections, which is astonishing, given the fact that both sampling stations differ fundamentally in their environmental conditions (euphotic, coastal versus aphotic, anoxic). On the other hand, this result provides some information on these taxas` high tolerance to abiotic factors, at least towards varying oxygen conditions. Nevertheless, *Paraphysomonas* and *Spumella* species have been frequently detected throughout the Baltic Sea by microscopical observations (Samuelsson et al. 2006), and *Paraphysomonas* related sequences were found in clone libraries conducted from the sampling station in Heiligendamm (Chapter 4), parallel to our cultivation attempts. Consequently, some of these "easy to culture" flagellates might be relevant representatives of the Baltic Sea protistan community, at least under certain conditions.

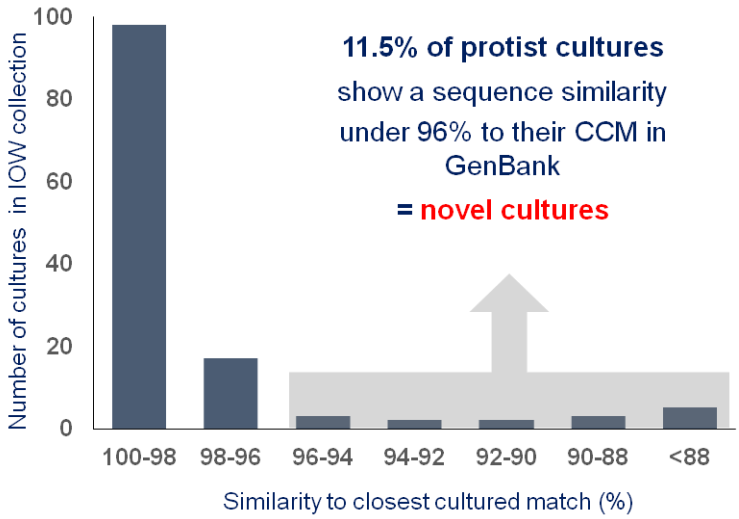

Figure 7. Distribution of 18S rRNA gene sequence similarities of IOW cultures to their closest cultured match (CCM) in GenBank. Cultures with a similarity less than 96% to their CCM are considered as novel cultures.

Apart from that it was surprising that around 11% of the isolates in our culture collection were characterized by highly divergent (<88-96% sequence similarity) 18S rRNA gene sequences compared to known organisms (Fig. 7), and phylogenetic analysis suggested these to represent novel taxa at species or even genus level.

Among chrysophytes, some strains were affiliated to three different environmental clades (clade C, F1, J) while one of them might represent a novel protistan genus (clade J). Interestingly, members from two of these clades (J and C) have been found at the same sampling station (Heiligendamm) by the use of a cultivation-independent technique (Chapter 4). Within cercozoans, a potential novel genus related to the Ventricleftida was found as well as *Massisteria* related strains that were used for the formal description of the new species *M. voersi* (Chapter 3). A further candidate for a novel genus was affiliated within the glaucophytes, and two novel species with the genus *Rhynchomonas* and the marine *Goniomonas* cluster were found. Finally, on two of the

choanoflagellate cultures, the novel species *Codosiga minima* and *Codosiga balthica* were erected (Wylezich et al. 2012). Environmental sequencing revealed the latter to be a dominant member of the protistan community in the central Baltic Sea's redoxcline (Chapter 1).

Indeed, the cultivation of protists represents a laborious task and implies a high failure rate. Nevertheless, our study shows that even traditional cultivation techniques have the potential to recover novel protistan lineages that are of ecological relevance. Therefore we encourage other researchers to follow such approaches, or to develop more inventive strategies to increase the eligibility of cultivation. Pure protist cultures are certainly an important biological resource and represent the gold standard to further study their trophic modes, their interactions with other microbes and their ecological roles.

Alternating lifestyles

The phenomenon of intraspecific polymorphism, e.g., changing shape during different life-history stages or in response to changing environmental conditions, can considerably obscure taxonomic work on protists and potentially causes overestimations of species richness (Pizay et al. 2009, Dolan 2015). Polymorphism also occurs among the smallest protist size fractions (nanoflagellates), and is here even more challenging to detect compared to larger taxa (e.g., ciliates). Examples are the genera *Massisteria* and *Minimassisteria* (Cercozoa, Leucodictyida) that alternate between amoeboid and flagellate stages (Fig. 8) and – in case of *Minimassisteria* – an additional crawling state (Howe et al. 2011).

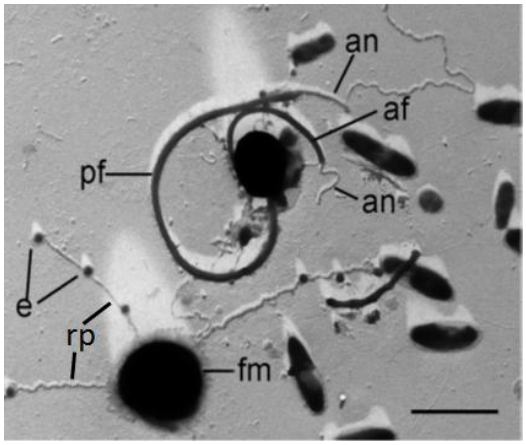

Figure 8. Transmission electron microscopy of amoeboid and flagellar cells of *Massisteria voersi*. Abbreviations: an, acroneme; af, anterior flagellum; e, extusomes; fm, fibrous material; pf, posterior flagellum; rp, rhizopodia. Scale bars: 2 µm.

For many years, *Massisteria* comprised only the species *M. marina* (Larsen & Patterson 1990). With this study, we described a second species of this genus, *M. voersi* from the Baltic Sea (Chapter 3). This example proves that also culturing of protists has the potential to expand the tree of eukaryotes (see Chapter 2). The comparison of both species provides important insights into the morphological

and genetic diversity within the genus *Massisteria*. Microscopical and ultrastructural investigations strongly justify the erection of the new species. *Massisteria voersi* differs from *M. marina* by smaller cell size (2.3–3 µm vs. 2.5–9 µm), the absence of fused motile cells, and of a paranuclear body and the parallel arrangement of the kinetosomes versus acutely angled ones in *M. marina*. Additionally, both species are quite distantly related regarding their 18S rRNA gene sequences (< 94% sequence similarity). Surprisingly, no closely related environmental sequences to *M. voersi* were found in Genbank, which could suggest that this new species is rarely represented in the environment. Indeed, first quantification attempts using a newly designed and evaluated (based on *M. voersi* cultures) FISH probe (Mv_1431) did not detect *M. voersi* at the sampling site where it was isolated.

For various reasons the observed biphasic life cycle of *M.voersi* might imply some stumbling blocks for the detection and quantification of these cells in the environment. Likewise to *M. marina,* rapid shifts from the amoeboid to the flagellate stage probably allow *M. voersi* to actively migrate between the sediment and pelagial. Probably at the time of sampling for our FISH analysis, the abundance of swimming flagellate stages of *M. voersi* in the pelagial was extremely low. Further, in long-term cultures *M. voersi* showed a tendency to colonize detritus particles. The particle attached fraction was largely excluded by prefiltration (200 µm) of water samples prior to FISH counts. However, the use of prefiltered water samples is a common practice in protistological field experiments. This circumstance, together with the postulated migrational behavior of *M. voersi,* might be a reasonable explanation for the absence of *Massisteria*-related 18S rRNA gene sequences in clone libraries (Chapter 4) performed parallel to the isolation of this strain and for the lack of closely related GenBank counterparts. Therefore, proof of the abundance and distribution of *M. voersi* in the environment requires further studies (e.g. FISH or CARD-FISH analyses) that include analyses of sediment samples and detritus particles.

For further investigations on the alternating attached and free-living lifestyles of the genus *Massisteria* it is necessary to reveal the ecological consequences of their ability to exploit different habitats (pelagial, benthos, particles) and resource pools (free living vs. attached bacteria). In fact, attached protists are more efficient in collecting suspended bacteria, and larger particles might represent microhabitats and a refuge from zooplankton predation (Pernthaler 2005).

Finally, without reliable genetic or ultrastructural data, the polymorphic species of the genus *Massisteria* cannot be properly identified. Additionally, the detection via cultivation independent techniques (environmental sequencing, FISH) might be affected by the biphasic life cycle or just by rareness in the environment. This highlights the necessity of studies on cultured organisms in order to improve our understanding of the morphological variety in protists that is often accompanied by a different behavior in the environment, since a single species might occupy multiple ecological niches during its life-history stages.

The artifice to link protist diversity data with trophic functions

Environmental 18S rRNA gene sequencing represents a powerful approach to investigate the diversity and community structure of natural protistan assemblages. However, these approaches suffer the fundamental drawback that relevant information on the ecological roles and trophic modes of the found taxa remain unknown. With the intention to overcome this problem, unamended dark incubations of Baltic Sea water samples were combined with 18S rRNA sequencing, and aimed to link molecular diversity data of protists with a heterotrophic and bacterivorous lifestyle. The greatest venture during these experiments was to avoid the mass growth of typical easy to culture protists, a phenomenon defined as culturing bias. Therefore, and in contrast to our cultivation survey (Chapter 2), these bottle incubations were not supplemented by allochthonous nutrient additions or nonindigenous food bacteria (unamended). Predatory metazoans and large ciliates were mainly excluded by pre-filtration of water samples. The incubation in the dark induced first a drastic decline in algae, probably accompanied by their degradation and organic matter release, followed by a peak of naturally occurring heterotrophic bacteria and a moderate enrichment in heterotrophic, presumably bacterivorous flagellates (HF). This general microbial succession, monitored by daily microscopical cell counts and flow cytometry, was judged to be comparable to productive periods in the Baltic Sea, such as phytoplankton post-bloom situations. Apart from cellular abundance, consistent shifts from a protistan community dominated by phototrophs to one in which heterotrophs predominated were additionally confirmed by 18S rRNA sequences derived from fingerprint analysis (DGGE) and clone library construction at the beginning and the end of the incubations. This was recognized by assigning reported trophic functions (phototroph or heterotroph) of well-known organisms to those phylotypes that were closely related to cultured representatives in GenBank (based on sequence similarity over 98%). All other sequences not complying with the similarity criterion were classified as unassigned in their trophic function, except the ones belonging to choanoflagellates or cercozoans (excluding chlorarachneans) as obligate heterotrophic groups. As a result, sequences related to phototrophs decreased from 64% to 3% during the incubation, whereas sequences related to heterotrophs and those that were unknown in their trophic function increased from 11% to 44% and 25% to 53% of all clones, respectively. However, from the tightly coupled succession of bacteria and HF during the incubations and the observed relative increase (at amplicon level) of previously trophically unassignable phylotypes in the dark, one can conclude that most of them can now be attributed with the ability to grow heterotrophically. Phylogenetic analyses revealed that the most important phylotypes, seemingly able to grow in the dark, belonged to uncultured marine environmental clades within the

chrysophytes (belonging to ochrophytes), other ochrophytes, picobiliphytes, choanoflagellates and cercozoans (Fig. 9).

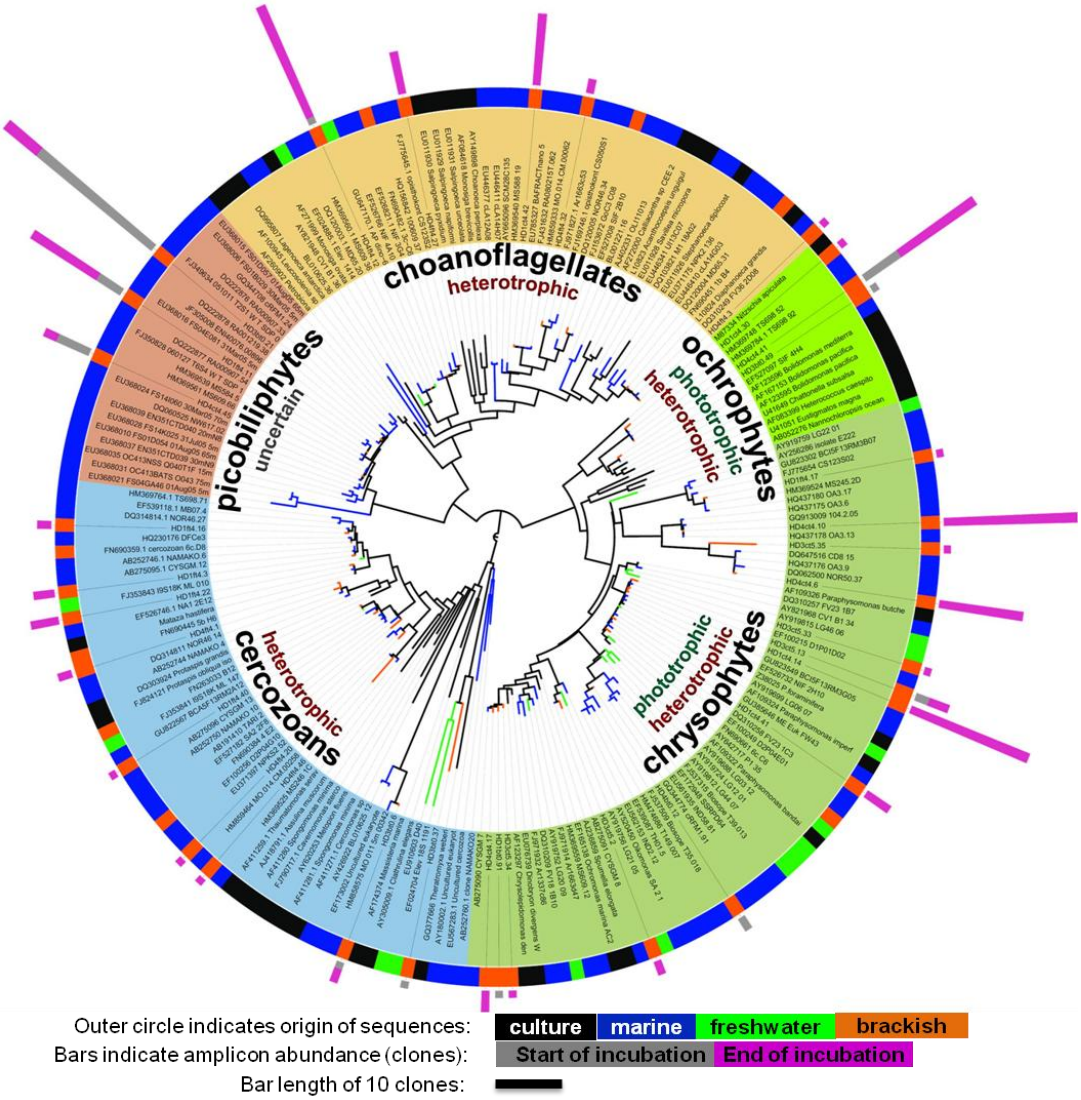

Figure 9. Mid-point rooted maximum likelihood phylogenetic tree showing the affiliation of the most important heterotrophic and presumably heterotrophic protist taxa and their clonal abundance before and at the end of incubation (grey and purple bars, respectively). Additionally, the origin of related phylotypes is given by the color code in the outer circle.

This result is especially striking for chrysophytes and other ochrophytes, which belong to the nutritionally most diverse groups of protists (Cavalier-Smith & Chao 2006), and for picobiliphytes which appeared to be puzzling with regard to their trophic mode (Seenivasan et al. 2013). Early studies on the basis of FISH analysis suggested picobiliphytes to be a new plastid-bearing algal group (Not et al. 2007, Cuvelier et al. 2008). However, flow cytometric cell sorting and single cell genomics later indicated these organisms to be heterotrophs rather than phototrophs (Heywood et al. 2011, Yoon et al. 2011). Recent investigations on *Picomonas judraskeda,* the first cultured member of picobiliphytes (now Picozoa), provided strong evidence that our postulated heterotrophy for these

picoeukaryotes is correct (Seenivasan et al. 2013). The ochropyte related clones were represented in two novel ochrophyte clades, distantly related to bolidophytes but closely related to phylotypes that were suggested to be heterotrophic on account of positive lysosomal marker signals (Heywood et al. 2011). Further, most of our chrysophyte sequences were affiliated with the environmental clade I, closely related to phylotypes that grew in other unamended dark incubation experiments with North Atlantic and Mediterranean Sea water samples (Massana, Guillou, et al. 2006, del Campo, Balagué, et al. 2013). Among the phylotypes that anyway represent heterotrophic protists, we erected four and three novel environmental clades within the choanoflagellates (clades J-M within acanthoecida, craspedida) and cercozoans (clades A-C related to thecofilosea), respectively.

Astonishingly, the bulk of phylotypes after incubation were characterized by an elevated degree of novelty, with an average sequence similarity of less than 94 % to their closest cultured match (CCM) (Fig. 10). This value is much lower compared to the average CCM similarities of our culture collection (Chapter 2) and even lower to the one of the clone library before incubation (Fig. 10). This demonstrates that a culturing bias was largely diminished and repeatedly proofs that unamended dark incubations preferentially promote the growth of uncultured taxa (Massana, Guillou, et al. 2006, del Campo, Balagué, et al. 2013). In addition, unamended dark incubations have been proposed to represent promising preliminary stages for the isolation of uncultured protist taxa (Massana, Guillou, et al. 2006). However, traditional cultivation techniques applied to original water samples and those after unamended dark incubations (Chapter 2) showed no fundamental difference in the yield of novel protist taxa (Figure 8, compare fourth and fifth box plots) and none of the uncultured taxa that dominated the incubation experiments were obtained. Thus, I recommend combining unamended dark incubations with more inventive isolation strategies.

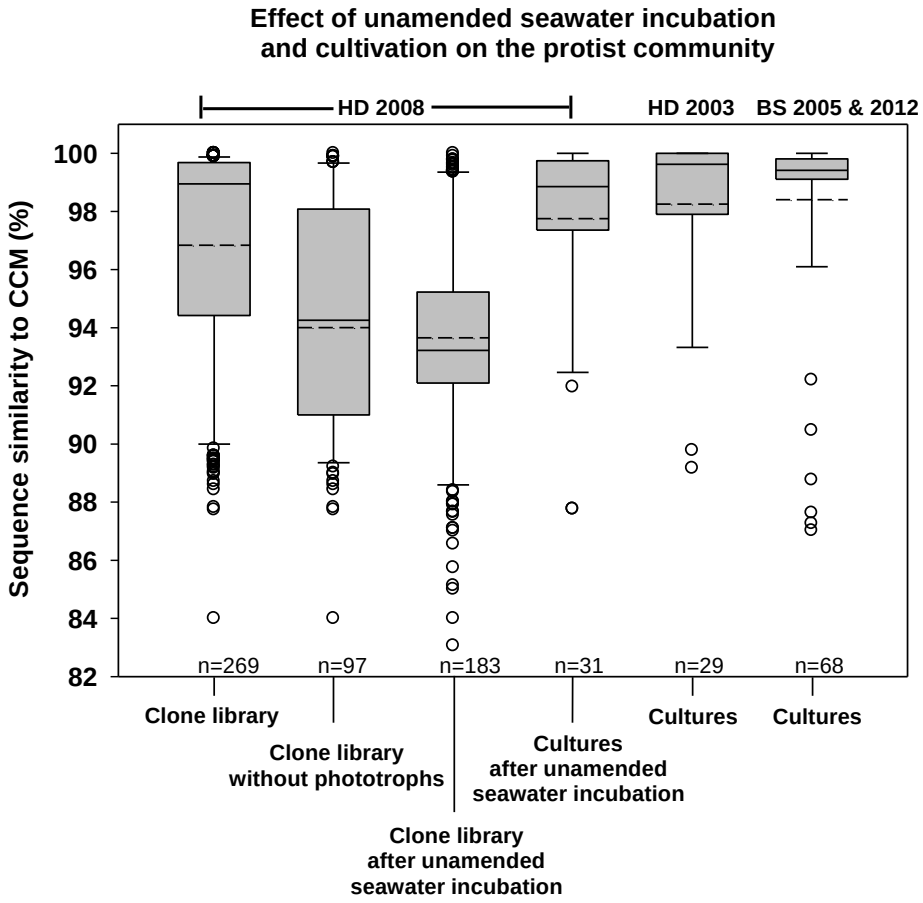

Figure 10. Box plot showing the sequence similarities (in %) to the closest cultured match (CCM) in GenBank for clone libraries and culture collections originating from differently processed water samples from Heiligendamm (HD) in 2003 and 2008 and the central Baltic Sea (BS) in 2005 and 2012. Note that the different sample treatments in HD 2008 were performed on water samples of the same campaign and serve for direct comparison here. The number (n) of sequenced clones or cultures is given. Boxes encompass the 25th–75th percentiles of all data; solid and dashed lines within boxes represent medians and means, respectively; whiskers enclose the 10th–90th percentiles and open circles represent all outliers.

Finally, using unamended dark incubations, trophic functions of uncultured and novel protist lineages, especially in nutritionally diverse and puzzling protist groups, could be revealed. Further, there is evidence that the heterotrophic protistan community in the southern Baltic Sea is largely composed of species with marine origin. Our experimental design leveled out the drawbacks of traditional cultivation and most cultivation-independent approaches, in particular the culturing bias and the loss of functional information, respectively. Thus, unamended dark incubations are confirmed as a powerful tool to bridge the gap between protist diversity, phylogeny and function.

Perspective

Putting a face to uncultured protists by developing "phylogenetic stains"

Cultivation independent techniques on the basis of 18S rRNA genes catalyzed our comprehension of protistan biodiversity in aquatic systems and demonstrated that the majority of taxa have as yet largely refused all attempts of their cultivation (Shi et al. 2009, Massana et al. 2013). Consequently, we are facing a large knowledge gap on account of the ecological properties and functional roles these uncultured protists play in the environment.

Only a few approaches exist that bridge the gap between protistan phylogeny and specific functions. These are unamended seawater incubations in the dark (Chapter 4), stable isotope probing (Frias-Lopez et al. 2009) and sophisticated cell sorting techniques (Heywood et al. 2011), that are all complemented by subsequent 18S rRNA sequencing. Another opportunity is provided by fluorescence *in situ* hybridization (FISH), which, in contrast to the other methods, can be performed in one methodological assay. FISH probes, as a kind of "phylogenetic stain", allow the visualization and quantification of specific protist taxa in natural assemblages and, to some extent, can relate these to basic ecological features (e.g., plastidic, aplastidic, bacterivorous) (Massana, Guillou, et al. 2006). For instance, FISH analysis revealed that uncultured marine stramenopile groups (MASTs) account for the most abundant bacterivorous flagellates in the world's oceans (Massana, Terrado, et al. 2006). However, currently evaluated and published FISH probes are available for only a limited number of taxa and therefore do not reflect the high protistan diversity in the environment explicitly.

Thus, the intention of this study was to close the whole cycle 18S rRNA approach by the development of new fluorescent oligonucleotide probes (phylogenetic stains) for the most abundant phylotypes (at amplicon level) that grew in unamended seawater incubations from the Baltic Sea (Chapter 4). These were uncultured chrysophytes of the environmental clade I (Chapter 4). This clade seems to be highly diverse since it harbours one of the highest numbers of environmental sequences of all chrysophyte clades (del Campo & Massana 2011). Further, members of this clade are apparently widespread and have been detected by other studies in various oceanic regions, e.g., Arctic Ocean, North Atlantic, Norwegian Sea and Mediterranean Sea (Chapter 4). In general Chrysophytes belong to the nutritionally most diverse eukaryotic supergroup, the stramenopiles (heterokonta) (Cavalier-Smith & Chao 2006) and comprise flagellate, amoeboid and coccoid forms (Andersen et al. 1999). These aspects strongly justify putting a face to these uncultured chrysophytes in order to infer some autecological features on taxa of this diverse group.

Due to the high genetic diversity of clade I, individual probes for each of the two subclusters (clade I 01, clade I 02) were designed (Fig. 11) with the PROBE_DESIGN tool of the ARB software package (Ludwig et al. 2004) and named Chryso_I_01 and Chryso_I_02, accordingly.

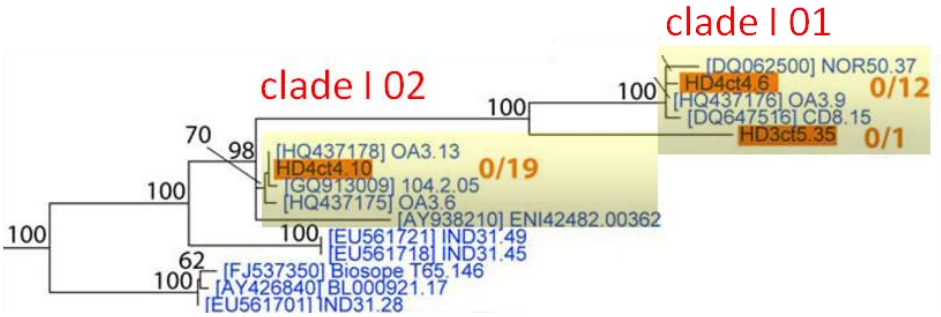

Figure 11. Phylogenetic representation of the environmental chrysophyte clade I and the two subclusters that get targeted by the respective FISH probes.

Submitting the probes to the SILVA Probe Match and Evaluation Tool (TestProbe 3.0) resulted in 56 and 24 environmental sequences of the SILVA database (SSU r119 Parc) that matched the probes Chryso_I_01 and Chryso_I_02 with zero unweighted mismatches, respectively. Among the sequences that matched the probes Chryso_I_01 and Chryso_I_02, a maximum genetic distance of 0,107 and 0,048 was calculated, respectively. Consequently, both probes potentially cover many taxa, probably at species level in the case of Chryso_I_02 and at even higher taxonomic ranks (genera) in the case of Chryso_I_01.

After the evaluation of the probes specificity by the use of thirteen non-target protists (including other chrysophytes) as negative controls, FISH experiments were performed according to the protocol of Pernthaler et al. (2001). Prior to routine application the probes were subjected to a spiking test in order to rule out cross hybridizations of each of the probes with cells belonging to their respective “sisterclade” (Fig 12).

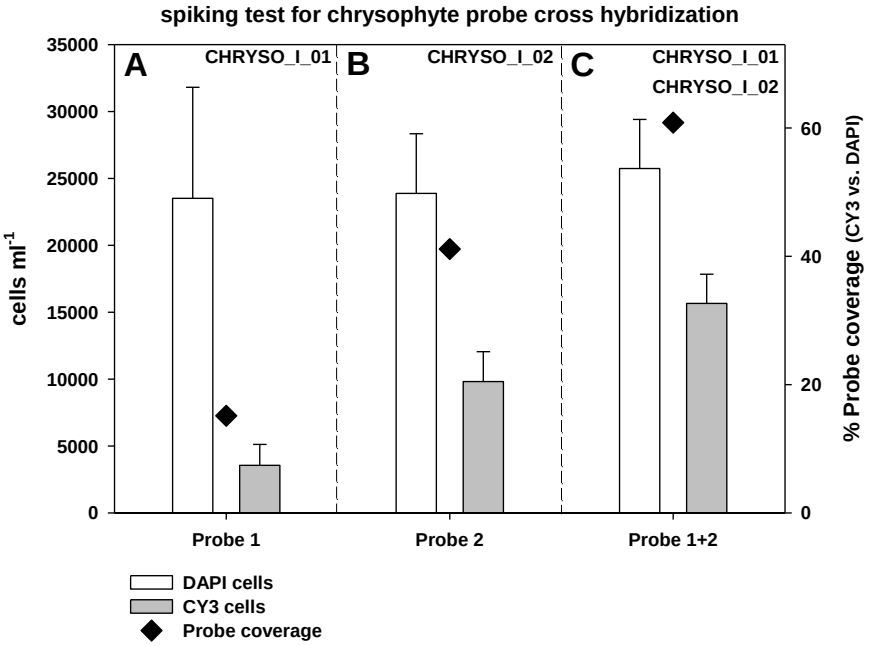

Figure 12: DAPI and CY3 cell counts on the same flagellate enrichment FISH filter. A) and B) show the individual cell numbers hybridized with each of the probes designed for the two chrysophyte clade I subclades. C) shows the cell numbers hybridized simultaneously with a mixture of both probes. Black diamonds in A), B), C) indicate the probe coverage as the quotient of CY3 and DAPI cell counts. Error bars indicate the deviation of cell counts in 10 microscopic fields.

Hybridizations with samples of unamended Baltic Sea water incubations revealed two aplastidic picoeukaryotic, assumingly flagellated and almost spherical cells. Cell size measurements and cell volume calculations revealed Chryso_I_01 to be slightly larger than Chryso_I_02 (Figure 13). Cell size is very basic information but definitely matters in microbes, since it influences food web linkages (Worden et al. 2015) and plays a role in prey selection (Jürgens & Matz 2002).

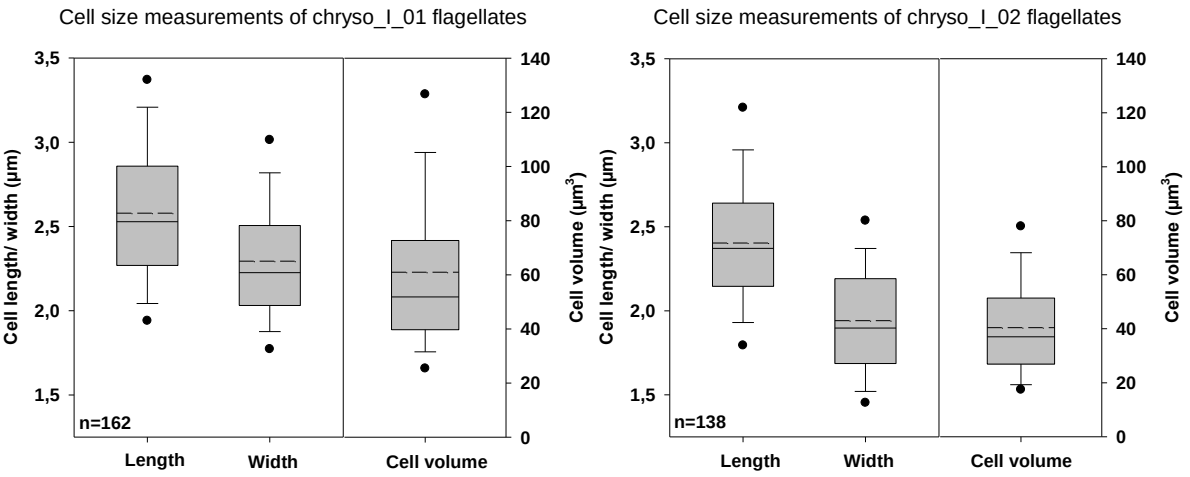

Figure 13. Cell size measurements and cell volume calculations of two uncultured chrysophyte taxa (chryso_I_01, chryso_I_02) in samples from a coastal station of the Baltic Sea (Heiligendamm).

Additionally, we were able to follow the cell number development during unamended dark incubation experiments (Chapter 4). This revealed an adaptation phase of three days for both

chrysophytes and total HNF in general, probably due to low prey availability, followed by a steep increase in cell numbers with Chryso_I_01 and Chryso_I_02 cells, accounting for 17% and 43% of total HNF counts (Figure 14). Assuming bacterivorous growth for both, the smaller Chryso_I_02 cells might be more successful in the hunt for bacteria, since the efficiency in capturing bacteria declines rapidly with increasing size, at least for interception feeding flagellates (Jürgens & Massana 2008).

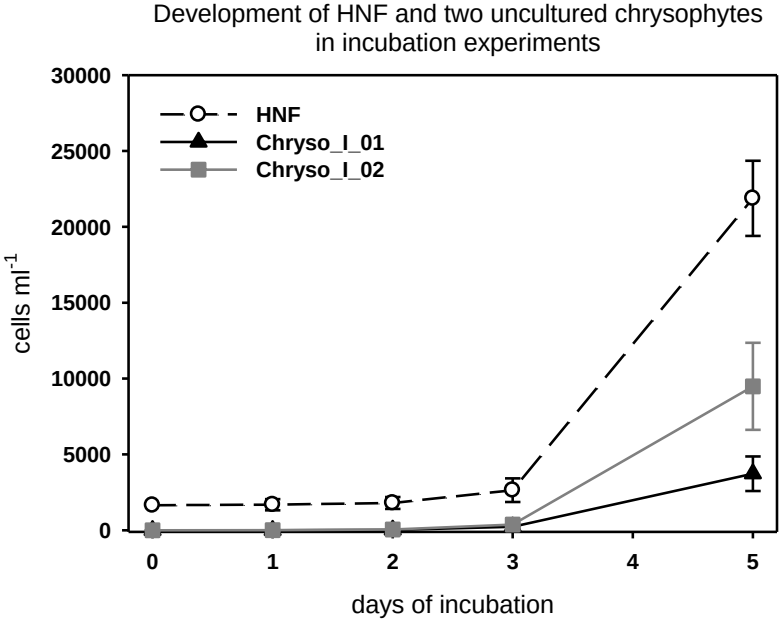

Figure 14. Cell number development of total HNF (assessed by enumeration of DAPI-stained cells) and uncultured chrysophytes of the two subclusters (Chryso_I_01 and 02) within clade I (CY3), enumerated by FISH, in dark unamended seawater incubations.

From the tightly coupled bacterial and HNF succession, and the considerable increase in amplicon abundance of chrysophyte clade I related phylotypes during the incubation, bacterivory can be assumed for this group (chapter 4). The proof of principle was achieved by grazing experiments with fluorescently labelled bacteria (FLB). Exemplarily shown for chryso_I_02, FLBs were detected in the food vacuoles of chrysophyte cells (Figure 15a) and their active ingestion was verified by the proportional increase of hybridized chrysophytes that have ingested FLBs over time (Figure 15b).

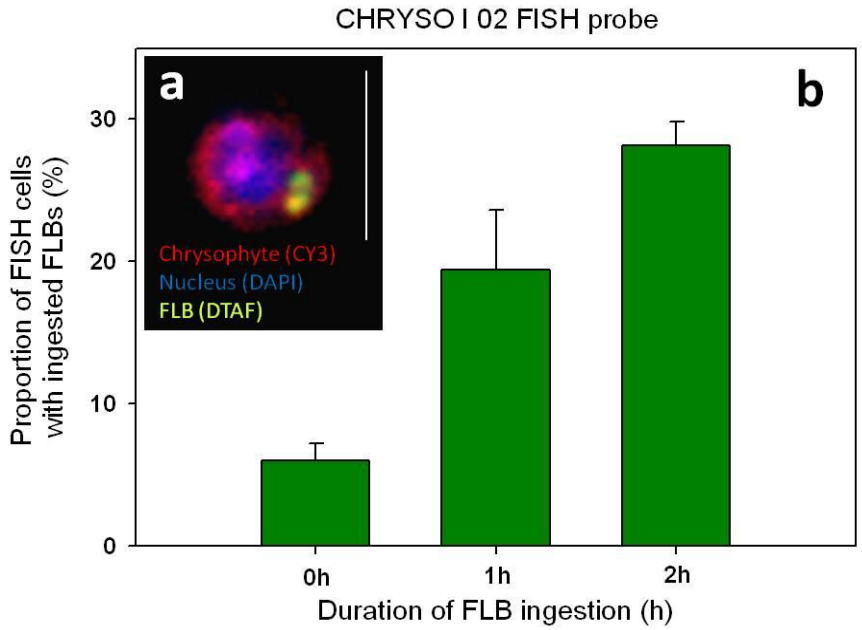

Figure 15. a) Combined epifluorescence micrograph of a hybridized chrysophyte cell (clade I 02) in red, the nucleus in blue and fluorescent labelled bacteria (FLB) in yellow. Scale bar= 4µm. b) the proportion of hybridized chrysophyte cells with ingested FLBs after zero, one and two hours of

Further investigation on the grazing activity included the calculation of specific clearance and ingestion rates based on FLB ingestion for the chryso_I_02 cells in comparison to total, small and large HNF (Figure 16). In general the clearance and ingestion rate of chryso_I_02 was comparable with the one inferred for the whole HNF community, with large HNF (1h) as an exception. Nevertheless, the procedure of determining these rates from FLB ingestion might cause underestimations, due to the fact that the size, quality, and quantity of prey are critical factors that define prey selection (Jürgens & Massana 2008). The average clearance rates for 2 µm-flagellates to sustain growth (one cell doubling per day) is 1nl for when high prey biomass is available (Sherr & Sherr 2002). Indeed MAST-4 cells fed with FLBs also showed rather low clearance and ingestion rates (0,7 nl predator^{-1} hour^{-1} and 1 bacteria predator^{-1} h^{-1}), but when isolated bacteria from the original sampling site were used the rates doubled or tripled (Massana et al. 2009).

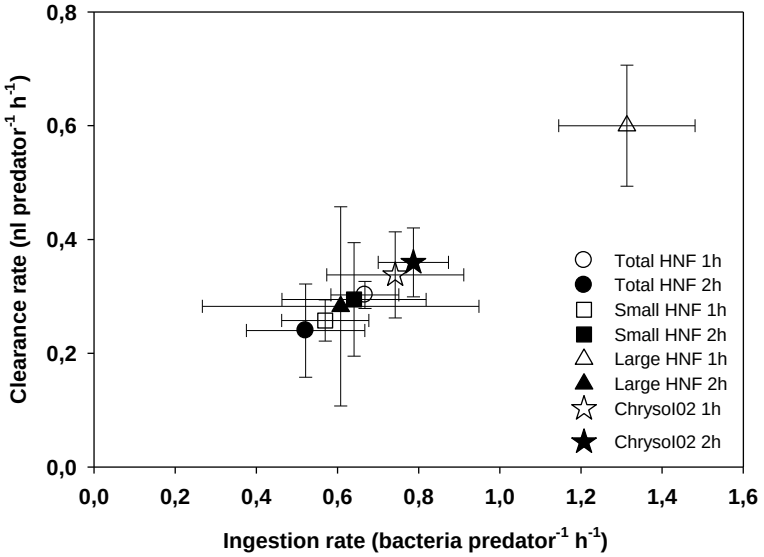

Figure 16. Clearance rates and ingestion rates calculated from FLB ingestion of total, small (< 5µm), large (>5µm) HNF and the uncultured chrysophyte of clade I (02) after 1 and 2 hours.

To date, comprehensive data on the environmental abundance of the two chrysophyte clade I members is missing, and first quantification attempts along the Baltic Sea salinity gradient were handicapped by sudden technical problems with the hybridization procedure (e.g., agglutinated probes or dissociated fluorochromes). However, two cell counts of both chrysophytes from samples of the coastal monitoring station Heiligendamm prior to the incubation experiment suggested low *in situ* cell numbers and a contribution to the total HNF abundance of less than 1%. A reason for that might the high seasonal population dynamics and short-lived blooms of nanoflagellates in coastal surface waters of the southern Baltic Sea (Piwosz & Pernthaler 2010). Interestingly, BLAST searches in GenBank suggest that the closest representatives of our two phylotypes (in the subclusters 01 and 02) were preferably found in coastal or near coastal sites. In addition, the majority of phylotypes that belong to chrysophyte clade I occurred in four different (including the one of this thesis, Chapter 4) dark unamended seawater incubation experiments (Massana, Guillou, et al. 2006, del Campo & Massana 2011, del Campo, Balagué, et al. 2013) and in mesocosm enclosures after an induced phytoplankton bloom (Newbold et al. 2012). This puts strong emphasis on the assumption that chrysophytes of clade I are specialized in exploiting sudden bacterial bursts that are accompanied by phytoplankton post-bloom situation. In these experimental settings Chrysophyte clade I members of both subclusters seem to co-occur. Although both are not closely related to each other they seem to follow a very similar strategy in the environment or, alternatively, there exists some inter-dependency of these two phyla.

With this study I provide first autecological features on two, as yet never seen and uncharacterized chrysophyte taxa of an assumingly very diverse and widespread clade, which consequently remained unrecognized in marine ecological processes. This protist group definitely

requires to be thoroughly studied, especially with respect to its distribution, abundance and its assumed dependency on phytoplankton blooms. Additionally, I encourage other researchers to systematically develop new FISH or CARD-FISH probes for the most abundant phylotypes found in genetic libraries (mainly 18S rRNA genes), in order to follow their distribution and abundance in the environment and to unravel their ecological roles.

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Mylnikov AP, **Weber F**, Jürgens K, Wylezich C (2015). *Massisteria marina* has a sister: *Massisteria voersi* sp. nov., a rare species isolated from coastal waters of the Baltic Sea. *European Journal of Protistology 08/2015; 51:299-310. doi:10.1016/j.ejop.2015.05.002*

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Contributions by authors – concept and experimental design: **FW,** CW, KJ; practical work: **FW** (protist cultivation and sequencing), CW (protist isolation, cultivation and sequencing), AM (protist isolation and cultivation); first edition of the manuscript: FW; revision of the manuscript: CW, KJ.

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Chapter 1) Morphological and molecular approaches reveal highly stratified protist communities along Baltic Sea pelagic redox gradients

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Morphological and molecular approaches reveal highly stratified protist communities along Baltic Sea pelagic redox gradients

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ABSTRACT: The deep basins of the central Baltic Sea are characterized by anoxic and sulfidic bottom water and steep vertical pelagic redox gradients. The highly active prokaryotic assemblages of this and other redox transition zones have been intensely studied, while knowledge on the protistan communities remains fragmentary. Thus, we conducted a multi-annual microscopy-based study, combined in one year with 18S rRNA gene and transcript-based DGGE fingerprints to identify the dominant protist taxa and to assess their vertical distribution. Both approaches, applied in high vertical resolution, demonstrated strong stratifications of the protist community composition along the redox gradient. The suboxic zone was dominated by dinoflagellates and oligotrichous ciliates related to *Strombidium*, whereas the interface and upper sulfidic zone were dominated by ciliates of the genera *Mesodinium* and *Metacystis*. Several flagellate taxa within the jakobids, euglenozoans and choanoflagellates occurred exclusively in sulfidic water. Our morphological approach indicates that the pelagic redoxclines of the central Baltic Sea are inhabited by a stable and characteristic protist community. Incongruously, certain taxa (e.g. *Mesodinium* and *Metacystis* sp.) which remained undetected by the molecular fingerprinting technique could be identified and enumerated by microscopic observations, whereas small and virtually amorphous protists (especially flagellates) were detected only by sequencing DGGE bands. Fine-scaled assessment of dominant protists in distinct redox strata is a crucial step in understanding their impact and interactions with the prokaryotic world and the biogeochemical processes they mediate in these zones.

KEY WORDS: Protists · Ciliates · Flagellates · Metacystis · 18S rRNA · Stratification · Redox gradient · Baltic Sea

INTRODUCTION

Up to 7% of the modern ocean is considered to be devoid of oxygen, including upwelling regions on the western continental shelves and stratified marginal seas (Baltic Sea, Black Sea) as prominent examples (Wright et al. 2012). Oxygen depleted environments, and oxic-anoxic transition zones (redoxclines) in particular, are known as sites of high redox-driven microbial activity, with impacts on the carbon, nitrogen and sulfur cycling, trace gas production and organic matter fluxes (Ulloa et al. 2012, Wright et al. 2012). Recent studies revealed novel insights into the taxonomic and functional diversity of prokaryotes in these systems, with the identification of many novel bacterial and archaeal lineages together with new

aspects of their metabolic activity and their role in the biogeochemical cycles (Ulloa et al. 2012, Wright et al. 2012).

In contrast, our knowledge of protistan communities in oxygen depleted systems is lagging tremendously behind, even though the first record of protists from anoxic sites dates back more than a hundred years (Lauterborn 1901). Protists play a fundamental role in all aquatic environments as grazers that control both the standing stocks and community structure of prokaryal assemblages and the pico- and nanoplankton (Sherr & Sherr 2002, Jürgens & Massana 2008). Protists in anoxic systems also act as hosts for numerous symbiotic archaeal and bacterial cells (Fenchel & Finlay 1995, Bernhard et al. 2000, Orsi et al. 2012a). Therefore, both as predators and as symbiotic hosts, protists can significantly influence ecological processes, such as the fate of prokaryal matter production and the metabolic potential of microbial assemblages.

Morphological characterizations of protists in hypoxic water layers of marine and freshwater systems have shown highly stratified community structures and enhanced protist abundances at oxic-anoxic interfaces (Fenchel et al. 1990, Zubkov et al. 1992, Massana & Pedrós-Alió 1994, Guhl et al. 1996, Finlay & Esteban 1998). However, most of those studies focused on protistan functional guilds or protists with conspicuous morphological features, thus providing only little taxonomical resolution especially for smaller eukaryotes. Overall, this led to the assumption that anoxic waters are rather poor in species richness (Fenchel & Finlay 1990a, 1995).

18S rRNA sequencing approaches changed this view, uncovering an astonishingly high diversity and multiple novel lineages of microbial eukaryotes in the pelagial and sediments of anoxic and oxygen depleted environments (e.g. Dawson & Pace 2002, Edgcomb et al. 2002, Stoeck & Epstein 2003, Stoeck et al. 2003, Zuendorf et al. 2006, Edgcomb et al. 2011a). Furthermore, molecular studies provided the first hints of a fine-scale architecture and seasonal variations in the protistan community structure along marine redox gradients (Behnke et al. 2006, 2010, Orsi et al. 2011, Wylezich & Jürgens 2011). However, with a few exceptions (Coolen & Shtereva 2009, Behnke et al. 2010, Orsi et al. 2011, 2012b), 18S rRNA-based approaches were generally applied as snapshot surveys covering only very few samples within the water column and with low temporal resolution. Additionally, for various reasons, 18S rRNA-based approaches are only marginally suitable for quantitative assessments of a taxon's cellular abundance or biomass contribution to the protistan assemblage (Caron 2009). Consequently, the spatio-temporal changes of protistan communities in such systems remain mostly unresolved by molecular techniques, which have been seldom accompanied by parallel morphology-based approaches (Edgcomb et al. 2011b,c, Orsi et al. 2012a,c, Wylezich et al. 2012).

The brackish Baltic Sea, with its steep pelagic redox gradients, represents an ideal model system to study microbial communities in response to oxygen depletion and sulfide accumulation in deeper waters. In contrast to numerous detailed studies on the prokaryotic assemblages (e.g. Labrenz et al. 2007, 2010, Jost et al. 2008, Grote et al. 2012), protist related studies of Baltic Sea redoxclines are limited to a few microscopy-based approaches (Setälä 1991, Detmer et al. 1993) and one single 18S rRNA-based diversity study, covering 2 water depths of the Gotland Deep (Stock et al. 2009). Two recent studies, which also provide data for different protist functional groups, focused on the role of protist grazing and viral lysis as prokaryotic mortality factors in the redox gradient (Anderson et al. 2012, 2013). Here, we used morphological and molecular techniques to describe the stable and fine-scaled distribution patterns of protists and to identify dominant taxa which are indigenous to distinct redox zones in the Baltic Sea.

MATERIALS AND METHODS

Study sites and sampling

Water samples were collected in the central Baltic Sea at Gotland Deep (Stn 271: 57° 19.2′ N, 20° 03′ E) and Landsort Deep (Stn 284: 58° 35.0′ N, 18° 14.0′ E) onboard the RV 'Alkor' in May 2005, the RV 'Professor Albrecht Penck' in July 2007 and the RV 'Poseidon' in August 2008 (Table 1). Sampling was performed with a CTD rosette (SeaBird Electronics) equipped with 5 l Free-Flow Bottles (Hydrobios). Water masses were sampled at 10 to 50 m and 2 to 5 m depth intervals to obtain whole water column profiles (Gotland Deep 2007) and high resolution profiles of the oxic-anoxic transition zone (Gotland Deep 2005, 2007, 2008 and Landsort Deep 2008). In all cases, inorganic nutrients, oxygen and hydrogen sulfide were measured immediately onboard according to standard methods (Grasshoff et al. 1983). An overview on the different approaches used in the different years is given in Table 1.

Table 1. Sampling effort for different analyses done during 3 cruises in 2005, 2007 and 2008 at Gotland Deep and Landsort Deep. The number of samples for each analysis is given. Redox: high resolution redoxcline profile, Whole: whole water column profile

	Gotland Deep					Landsort Deep
	2005 May		2007 July		2008 August	2008 August
	Redox 180–220 m	Redox 181–221 m	Whole 6–232 m	Redox 119–143 m	Redox 114–136 m	Redox 80–115 m
Physico-chemical parameters	12	11	13	13	12	13
Determination of cell numbers						
Prokaryotes	12	11	13	13	12	13
HNF			13	13	12	13
Dinoflagellates			13	13	12	13
Ciliates (incl. morphotypes)	12	11	13	13	12	13
Taxonomic identification						
Ciliates	2	2				
Molecular analysis						
DGGE fingerprint			13	13		

Determination of microbial cell abundance and biomass

To determine prokaryotic cell abundance, samples (4 ml) were immediately fixed with paraformaldehyde and glutaraldehyde (final conc. 1% and 0.05%, respectively), deep-frozen in liquid nitrogen and stored at −80°C until processing. Upon thawing, prokaryotic cell counts were determined with a FACScalibur (Becton & Dickinson) flow cytometer following a previously described protocol (Gasol & del Giorgio 2000).

To assess the abundance of heterotrophic nanoflagellates (HNF), samples (100 ml) were fixed immediately after retrieval with glutaraldehyde (1.25% final conc. in 2005 and 0.5% final conc. in 2007) or with particle free formaldehyde (1% final conc. in 2008) at 4°C for 2 to 24 h. Aliquots of 40 ml were then vacuum filtered (100 mbar) onto black polycarbonate filters (Nucleopore; 0.8 µm pore-size; Whatman). In 2007, filters were stained onboard with DAPI (0.01 mg ml^{-1} final conc.) prior to storage at −20°C; in 2005 and 2008, filters were first stored at −20°C and stained with DAPI upon thawing (tests were conducted to ensure this did not affect cell counts, F. Weber unpubl. data). Observation and counting of a minimum of 100 HNF cells per sample was performed at a magnification of 630× and an excitation wavelength of 360 nm (filter set 02, Carl Zeiss Microscopy) under a Zeiss Axioskop 2 mot plus epifluorescence microscope (Carl Zeiss Microscopy).

Enumeration of ciliates and dinoflagellates was accomplished following the Utermöhl technique (Utermöhl 1958) with samples (200 ml) previously fixed with Lugol's solution (1% final concentration) (Willen 1962). Samples were processed as described in Anderson et al. (2012) and cells were enumerated by screening between 10 diagonal stripes (representing ~one third of the total area) and the whole counting chamber. Ciliates were classified as morphotypes of the genera *Metacystis*, *Mesodinium*, *Strombidium* (in 2005, 2007 and 2008), *Metopus*, *Tintinnopsis*, *Spathidium* and *Uronema* (in 2005) (Foissner et al. 1999).

Protist biomass estimates were derived from individual cell volume calculations of distinguished morphotypes and size classes using approximate geometric forms. The carbon content per cell was determined by means of specific cell volume to biomass conversion factors from the literature (220 fg C $µm^{-3}$ for HNF, Børsheim & Bratbak 1987; 125 fg C $µm^{-3}$ for dinoflagellates, Pelegri et al. 1999 and 190 fg C $µm^{-3}$ for ciliates, Putt & Stoecker 1989) and was multiplied by the cell abundance to obtain the total biomass for each protist group at the sampled depths. It should be noted that the use of different fixation methods might have affected the identification and enumeration of some protists (Sherr & Sherr 1993).

Taxonomic identification of novel ciliates

A more comprehensive taxonomic identification of dominant ciliates from the oxic- anoxic interface of the Gotland Deep was performed in 2005 (Foissner et al. 1999 and literature cited therein). Live ciliates were concentrated by gravity filtration of 40 to 50 l of seawater through a submerged 10 µm mesh size plankton net, thereby largely reducing air contact. Onboard, the concentrated material was stored at 4°C and then sent on ice to the laboratory in Salzburg. There, samples were checked for the species present, and those which were likely unde-

scribed were studied *in vivo* and by various silver impregnation methods as described in Foissner (1991).

Detection of potential symbiotic bacteria associated with redoxcline ciliates

Water samples from the oxic-anoxic interface (129 m) in 2007 were fixed with 0.2 µm filtered formaldehyde (final conc. 2%) and filtered on 0.8 µm pore diameter polycarbonate filters. Fluorescence *in situ* hybridization (FISH) was accomplished as described in Pernthaler et al. (2001), with 35% of deionized formamide in the hybridization buffer. Either a mixture of eubacterial probes (EUB338 I-III; Daims et al. 1999) or a probe specific for *Gammaproteobacteria* (GAM42a + non labelled competitor probe Bet42a; Manz et al. 1992) was used. DAPI-stained ciliates were inspected for hybridized bacterial cells within the cytoplasm or the cell surface. Among the ciliates that were associated with bacteria, several were unidentified, whereas many belonged to the genus *Metacystis*, according to the prominent morphology.

Nucleic acids extraction and ribosomal complementary DNA (rcDNA) synthesis

Samples for nucleic acids extraction were taken parallel to the samples for microscopical cell counts in the profile from Gotland Deep in 2007. Microbial biomass was collected on 0.2 µm pore size polycarbonate filters (Durapore, Millipore), shock frozen in liquid nitrogen and stored at −80°C until nucleic acid extraction was performed. A combination of mechanical and chemical procedures was used in order to simultaneously extract RNA and DNA as described by Weinbauer et al. (2002).

The separated DNA and RNA extracts were washed twice with 70% ice cold ethanol and dissolved in nuclease free water. Residual environmental DNA was eliminated in RNA extracts by DNase I digestion (DNA-free Kit, Ambion) for 30 min at 37°C. An aliquot of both nucleic acids was quality-checked and quantified using a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop, Thermo Fisher Scientific). Enzymatic degradation of RNA was prevented by treating each RNA extract with 0.9 µl RNase inhibitor (Peqlab) per 20 µl of RNA sample. Complete removal of the DNA in each of the RNA extracts was verified by PCR, as described in the next section, using the RNA extracts as template. To generate cDNA, ~200 ng of template RNA was reverse transcribed at 42°C using the iScript Select cDNA synthesis kit (Bio-Rad) following the manufacturer's recommendations. In addition to the random primer provided with the kit, the eukaryote-specific primer Euk B (Medlin et al. 1988) was used. In each reverse transcription reaction, some of the RNA samples were not supplemented with reverse transcriptase as an additional control for DNA contamination.

Denaturing gradient gel electrophoresis (DGGE)

18S rRNA fragments (~560 bp length) were amplified by PCR using both DNA and cDNA as template with the eukaryote specific primers EukA and Euk516r-GC (Medlin et al. 1988, Amann et al. 1990). The PCR mixture (25 µl) was composed of 200 µM of each deoxynucleoside triphosphate, 0.47 mM of each primer, 1.25 U of *Taq* DNA polymerase (Eppendorf) and the Mg^{2+} self-adjusting PCR buffer supplied with the enzyme (*Taq* Buffer advanced, Eppendorf). PCR was started with an initial denaturation at 94°C for 2 min, followed by 34 cycles of 30 s at 94°C, 45 s at 56°C and 2 min at 72°C. The final extension step was done at 72°C for 6 min.

DGGE was performed as described in Weber et al. (2012) with denaturing conditions ranging from 40 to 55%. For a better comparison of band positions in different gel runs, an environmental DGGE standard with defined bands (loaded to middle and outer lanes) was developed using samples from the same sampling campaign. Gel images were subjected to cluster analysis with the software GelCompare II (Applied Maths). Densiometric curves of each lane were used to calculate dendrograms after Pearson correlation under the UPGMA model. Additionally, cophenetic correlation coefficients were estimated for each node of the dendrogram.

Across lanes, DGGE bands were assigned banding positions, excised from the gel and eluted overnight at 4°C in 50 µl of nuclease free water before they were stored at −20°C. For reamplification of bands, 1 µl of a 1:10 000 dilution of the eluate served as template in a PCR with the previously described conditions. Subsequently, all reamplicons were verified to derive from a single DGGE band by subjecting them to additional DGGE runs. The final reamplification was done with the reverse primer lacking the GC clamp. PCR products were purified with the NucleoSpin® Extract II kit (Macherey-Nagel). The sequencing reactions were carried out with the primer EukA by the sequencing service QIAGEN. Bands were

excised and sequenced from the same band position in several lanes. All bands assigned to a position were quantified by their relative band intensity, translated into diameter values and were plotted as bubbles according to their presence in the depth profiles.

Phylogenetic analysis

Chromatograms supplied with each sequence were reviewed in SeqManII (DNAStar) and ends containing unreliable trace data peaks were trimmed manually. Sequences were submitted to KeyDNA-Tools (www.keydnatools.com) and BLAST (Altschul et al. 1997) for chimera detection and taxonomic affiliation. Representative 18S rRNA sequences of a single operational taxonomic unit (OTU) based on 99% similarity were called by creating a distance matrix and clustering of sequences according to the average-neighbor method in Mothur (Schloss et al. 2009) and were deposited in GenBank under accession numbers KF373741 to KF373752.

Multiple alignments were done using MAFFT version 6 (Katoh et al. 2002) and were inspected and manually refined in BioEdit (Hall 1999). Bayesian and maximum likelihood phylogenetic trees with 57 complete and partial 18S rRNA sequences were calculated using MrBayes version 3.2.1 (Ronquist et al. 2012) and RAxML version 7.0.4 (Stamatakis 2006), launched via the web-based computational resource Bioportal of the University of Oslo (Kumar et al. 2009). Trees generated with both methods were fairly similar in topology (overall topological score = 94.5%) as calculated from Compare2Trees (Nye et al. 2006), and, therefore, only the Bayesian tree is shown. The Bayesian likelihood parameters were set to the substitution model GTR. Rate variation across sites was modeled using a gamma-shaped rate variation with a proportion of invariable sites. Rate variation across the tree was allowed under the covarion-like model. Monte Carlo Markov chain (MCMC) search was run with 4 chains for 1 000 000 generations, with trees being sampled every 100 generations. Bayesian posterior probabilities were calculated under the MCMC method. The first 25% of sampled trees were considered 'burn-in' trees and were discarded prior to tree reconstruction. The maximum likelihood phylogenetic tree and bootstrap analysis in RAxML were done in 1000 replicates on random starting trees under the evolutionary model GTRGAMMA. A consensus tree, displaying the bootstrap values, was computed in MrBayes and respective values were transferred into the Bayesian topology tree.

RESULTS

Physico-chemical characteristics of the central Baltic Sea water column

During our observation period at Gotland Deep in 2007, 2008 and Landsort Deep in 2008, we found a characteristic water column stratification for the central Baltic Sea (Jost et al. 2008, Anderson et al. 2012), as exemplified for the Gotland Deep in 2007 in Fig. 1. The euphotic zone extended down to 16 m, determined as the depth where irradiance represented 1% of subsurface photosynthetically active radiation. The water column was characterized by a thermocline and a deeper halocline. Between these 2, the cold intermediate water layer was situated with higher oxygen concentrations than surface waters. Below the halocline, oxygen declined until its complete depletion at the redoxcline (transition zone between oxygenated and sulfidic waters). The chemocline demarcated the beginning of the sulfidic part of the water column, which was characterized by steadily increasing H_2S content towards the seafloor (Fig. 1B).

Microbial abundance and protistan community structure along the redox gradient based on microscopical analysis

In the water column, the abundance of all microbial cells (prokaryotes, HNF, dinoflagellates and ciliates) was highest in the surface zone; declined drastically with decreasing oxygen tension and showed a second, smaller peak near the chemocline (Fig. 1B,C). Prokaryote abundance (including both *Bacteria* and *Archaea*) at the chemocline was one fourth of the concentration detected at the surface, while HNF, dinoflagellates and ciliates accounted for 6, 13 and 20% of their respective abundances in surface waters. Biomass estimates for different functional groups of protists showed a dominance of ciliates at the oxic-anoxic interface (87 to 97% in all profiles investigated, Fig. 1D). The sulfidic part of the water column was characterized by very low protist cell numbers, while the abundance of prokaryotes remained high and even slightly increased towards the seafloor (Fig. 1B).

High resolution cell counts along the redox zones of Landsort and Gotland Deep from 2007 and 2008, plotted against oxygen concentrations, exhibited a consistent distribution pattern of prokaryotes, HNF, dinoflagellates and ciliates (Fig. 2). Highest prokaryotic cell numbers were detected directly at or around the oxic-anoxic interface (dashed line). At sulfidic depths, cell

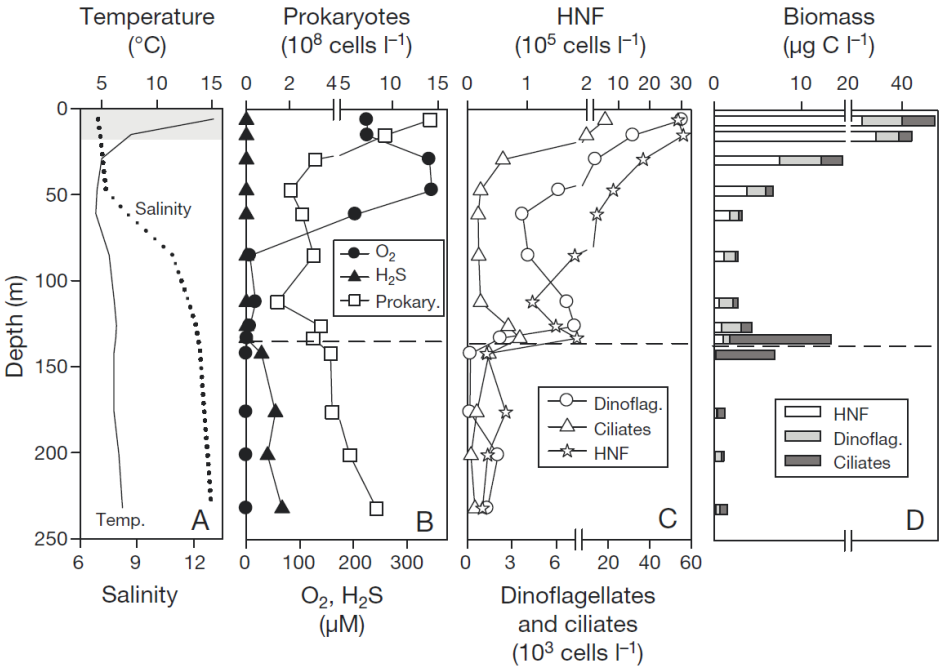

Fig. 1. Whole water column profile at Gotland Deep in July 2007. (A) Temperature and salinity profile. Changes with depth in (B) oxygen, hydrogen sulfide and prokaryote concentrations, and (C) the abundance of the 3 protist functional groups (heterotrophic nanoflagellates (HNF), dinoflagellates and ciliates). (D) Vertical biomass distribution of HNF, dinoflagellates and ciliates. The shaded area in (A) is the extension of the euphotic zone (defined as 1% of subsurface photosynthetically active radiation). The horizontal dotted line indicates the oxic-anoxic interface

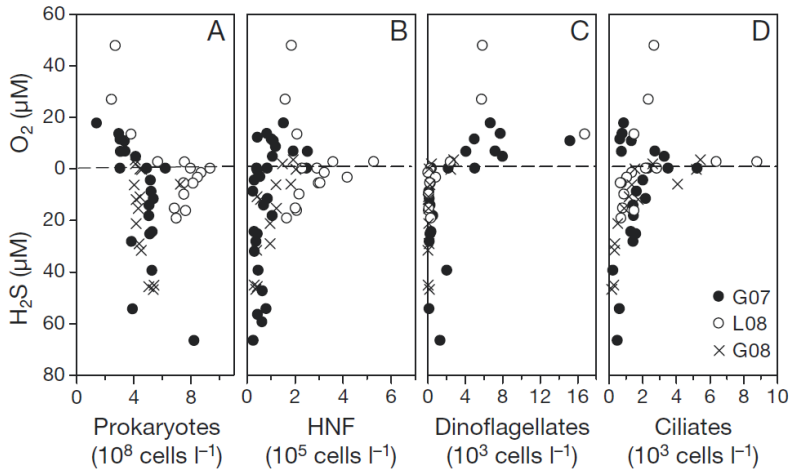

Fig. 2 Changes in the abundance of (A) prokaryotes, (B) heterotrophic nanoflagellates (HNF), (C) dinoflagellates and (D) ciliates along the oxygen gradients of the Gotland Deep in July 2007 (G07), August 2008 (G08) and Landsort Deep in August 2008 (L08). The *y*-axis is split into values for oxygen and hydrogen sulfide concentration, while a value of 0 indicates that both were below detection (detection limit: 2 µM and 0.2 µM, respectively). Dashed line represents the oxic-anoxic interface

numbers declined slightly but still exceeded those found in the suboxic zone. Overall, higher prokaryotic cell counts were obtained in Landsort Deep than in Gotland Deep, with respective maximal abundances of 9.4×10^8 cells l^{-1} and 6.2×10^8 cells l^{-1} (Fig. 2A).

HNF and ciliate abundances followed similar patterns along the oxygen gradient. Both reached the highest numbers around the interface (respective maximum of 5.3×10^5 cells l^{-1} and 8.8×10^3 cells l^{-1}) and higher abundances at Landsort Deep than at Gotland Deep (Fig. 2B,D). In contrast to prokaryotes, HNF and ciliate numbers strongly declined with increasing depth and H_2S content. Dinoflagellate abundance generally showed the highest abundance in the suboxic zone and declined drastically when H_2S appeared in the water column (Fig. 2C). Highest dinoflagellate counts were ~16 $\times 10^3$ cells l^{-1} in the oxygen depleted zones of both Gotland Deep in 2007 and Landsort Deep in 2008.

Ciliate distribution and identification

Beyond the rough classification of protist functional groups, the distinction and enumeration of different ciliate morphotypes allowed us to view the protist zonation at a higher resolution (Fig. 3, Fig. A1 in the Appendix). Although a significant proportion of ciliates remained unidentified at some depths, 3 morphotypes dominated: *Metacystis* spp., *Strombidium* spp. and *Mesodinium* spp., all showing clear shifts in abundance along the oxic-anoxic transition zone (Fig. 3A). *Strombidium* was present solely in the suboxic zone and disappeared with the complete

depletion of oxygen. *Mesodinium* was present at low abundance in the suboxic zone and peaked around the interface. Finally, *Metacystis* peaked at the interface and extended down to depths with high sulfide concentrations (Fig. 3B).

In 2005, a more precise taxonomic identification by means of live observation and silver impregnation allowed the detection of novel ciliate species (Fig. 4A–I), which will be described in a separate paper. The most frequent ciliates belonged to the genus *Metacystis*, which was represented by at least 5 species, as judged from cell sizes and morphological structures. At least one of them was a new species, related to *M. elongata* but differing by possessing a caudal cilium (Fig. 4A,E,F). FISH hybridisations, conducted with samples from the oxic-anoxic interface in 2007, showed *Metacystis* cells to be characteristically associated with large, presumably endosymbiotic *Eubacteria* and covered by numerous small, presumably ectosymbiotic *Gammaproteobacteria* (Fig. 5).

Two other ciliates were classified as potential novel genera. One was re-

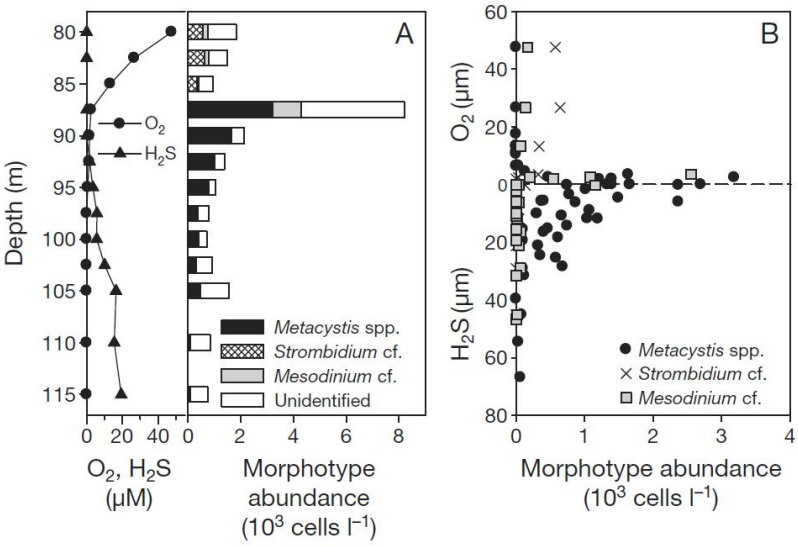

Fig. 3. (A) Changes with depth at the redoxcline in oxygen and hydrogen sulfide concentrations and the relative abundance of the different ciliate morphotypes identified, taken in Landsort Deep in August 2008. (B) Changes in the abundance of the 3 principal ciliate morphotypes distinguished along the redox gradient. Data were pooled for all study sites and sampling years (Gotland Deep in July 2007, August 2008 and Landsort Deep in August 2008). The *y*-axis is split into oxygen and hydrogen sulfide concentration values, with a value of 0 indicating that both were below detection (detection limits: 2 µM and 0.2 µM, respectively)

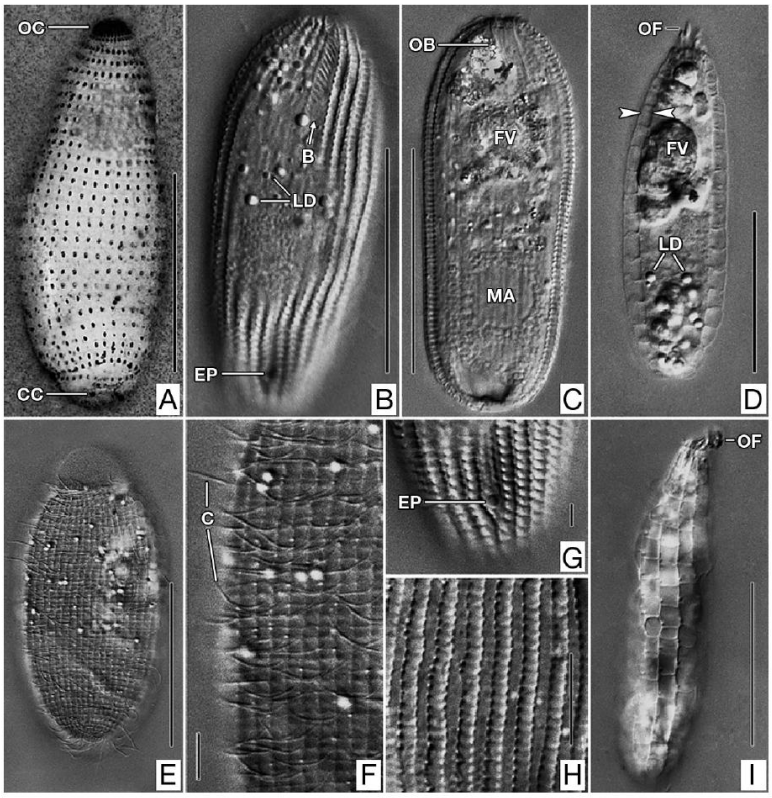

Fig. 4. Novel ciliates from the chemocline at Gotland Deep in May 2005, observed (A) after nitrate silver impregnation and (B–I) *in vivo*. (A,E,F) A potential new species belonging to the genus *Metacystis* (about 100 µm in length). (E,F) The genus is easily recognized by the highly ordered arrangement of the cilia/basal bodies, forming quadrangular meshes. (B,C,G,H) New genus related to *Placus* spp. (about 90 µm in length): (B,G,H) surface views and (C) optical section. (G,H) The cortex structure of the new genus, which resembles that of *Placus* spp., although the brush (as shown in B) is different. (D,I) New plagiocampid genus (about 50 µm in length): (D) optical section and (I) surface view showing a highly conspicuous cortex composed of cubic alveoli (arrows in D). B – brush, C – cilia, CC – basal body of caudal cilium, EP – excretory pore of contractile vacuole, FV – food vacuoles, LD – lipid droplets, MA – macronucleus, OB – oral basket, OC – oral ciliature, OF – oral flaps. Scale bars = (F,G) 5 µm, (H) 10 µm, (D,I) 25 µm, (A–C,E) 50 µm

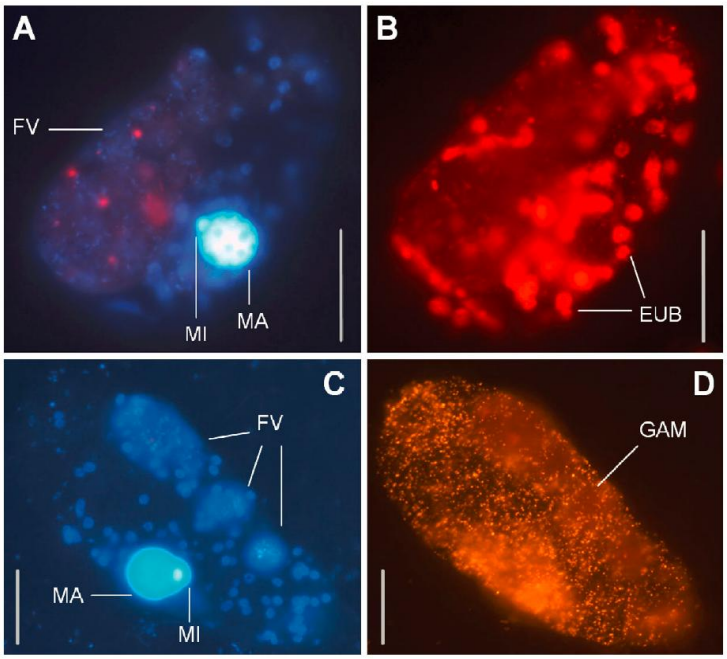

Fig. 5. Epifluorescence micrographs of *Metacystis* spp. with potentially symbiotic bacteria. (A,C) DAPI-stained *Metacystis* cells with bacteria in the cytoplasm (note that bacteria on the cell surface are not in focus in C). (B) Fluorescence *in situ* hybridization (FISH) signal of *Eubacteria* (EUB338I-III) within the cytoplasm. (D) FISH signal of *Gammaproteobacteria* (GAM42a) on the cell surface. FV – food vacuole, MI – micronucleus, MA – macronucleus, EUB – *Eubacteria*, GAM – *Gammaproteobacteria*. Scale bars = 20 µm

lated to *Placus*, having a similar cortex structure but differing in the structure of the brush (Fig. 4B,C,G,H). The other belonged to the family Plagiocampidae, as indicated by the characteristic finger-like oral flaps on the dorsal mouth margin but differs from the known genera of this family by the unusual cortex consisting of large, quadrangular alveoli (Fig. 4D,I).

Vertical protistan community distribution along the redox gradient based on 18S rRNA fingerprints

To determine changes in the protistan community composition along the oxygen gradient, we performed DGGE fingerprints with samples from the whole water column (Fig. 6) and a high resolution redoxcline profile (Fig. 7) of Gotland Deep in 2007. In each case, fingerprints were based on 18S rRNA gene fragments (DNA) as well as the respective gene transcripts (RNA) in order to detect distribution patterns of active protists (Stoeck et al. 2007). Both cluster analysis of RNA- and DNA-based fingerprints illustrated that the protist communities from different depths largely clustered according to their values of oxygen and H_2S concentration (Figs. 6 & 7).

Dendrograms from fingerprints of the whole water column (Fig. 6) separated different water bodies: euphotic surface waters (6 and 15 m) with an oxygen concentration of 230 µM; the cold intermediate water layer (29, 47 and 61 m) where oxygen varied between 200 and 350 µM; suboxic waters (112, 126 and 133 m) with low oxygen content (2 to 18 µM) but devoid of H_2S and sulfidic waters (142, 176 and 201 m), free of oxygen and containing 30 to 70 µM H_2S. Suboxic waters were clearly more similar to the surface samples than to sulfidic or cold intermediate layer samples.

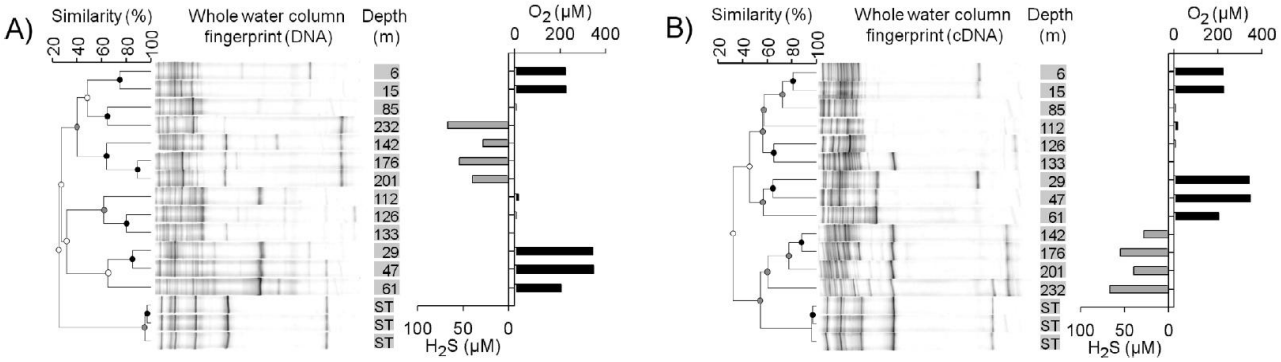

Fig. 6. Dendrograms of (A) DNA-based and (B) cDNA-based DGGE fingerprints of sampled depths in the whole water column of the Gotland Deep in July 2007. Black, grey and white circles at the nodes represent cophenetic correlation coefficients of 100, ≥85 and ≥70, respectively. The O_2 and H_2S values for all depths are given in the bar charts. ST indicates standard lanes

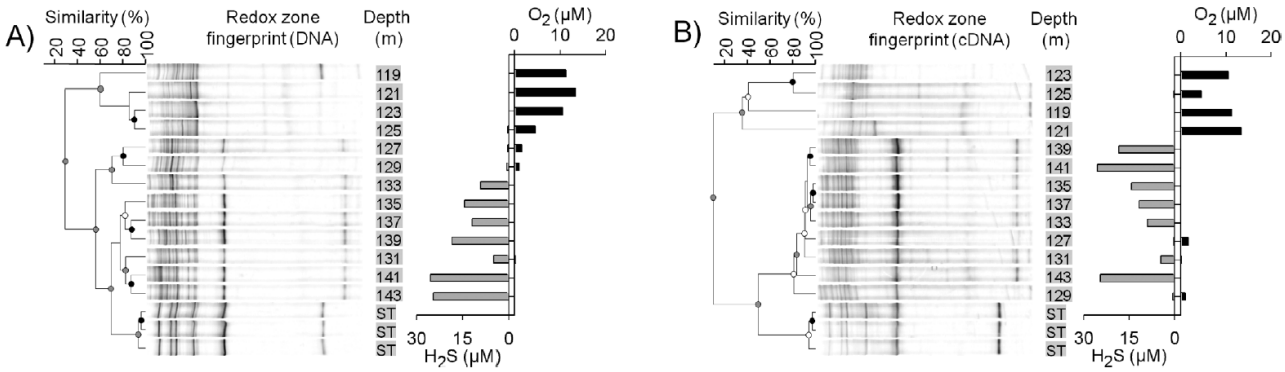

Fig. 7. Dendrograms of (A) DNA-based and (B) cDNA-based DGGE fingerprints of sampled depths in the high resolution redox zone profile of the Gotland Deep in July 2007. Black, grey and white circles at the nodes represent cophenetic correlation coefficients of 100, ≥85 and ≥70, respectively. The O_2 and H_2S values for all depths are given in the bar charts. ST indicates standard lanes

Dendrograms of the high resolution redoxcline profiles showed a clear separation of samples from the suboxic and the sulfidic zone (Fig. 7). Samples from the oxic-anoxic interface (127 and 129 m) were generally more similar to fully sulfidic samples than to suboxic samples, indicating oxygen values around the detection limit of the method applied (1 to 2 µM O_2) to be the demarcation between an oxic and anoxic protistan community (Fig. 7). A clear separation of communities deriving from oxic and anoxic water masses could additionally be confirmed by dendrograms, which were generated solely through the presence and absence of DGGE bands (Jaccard index) for both profiles and through DNA and RNA gels (data not shown).

Phylogenetic identification of protists based on 18S rRNA sequences

Reamplification of excised gel bands from the 2 DNA- and RNA-based DGGE fingerprints resulted in 38 sequences belonging to microbial eukaryotes. These clustered into 12 OTUs (here also defined as DGGE band positions) within the following taxonomic groups: dinoflagellates (4 OTUs), ciliophorans (2 OTUs), euglenozoans (2 OTUs), jakobids (2 OTUs) and choanozoans (2 OTUs). Most OTUs showed a specific distribution pattern with respect to the redox conditions in the whole water column (Fig. 8B) and the redoxcline profile (Fig. 8C).

The euglenozoan phylotype (OTU 10) with 100% sequence identity to *Eutreptiella gymnastica* was found solely in surface water samples (based on DNA and RNA gels). Two dinoflagellate phylotypes (OTU 2 based on DNA, OTU 3 based on DNA and RNA) were detected in the cold intermediate and highly oxygenated water layer as well as in the suboxic zone and reached higher relative amplicon abundances in the latter. OTU 3 was closely affiliated (98% similarity) to the mixotrophic dinoflagellate *Karlodinium micrum* (not shown in tree), while OTU 2 was very distantly related to any cultured species and formed a long branch with clones from oxic and suboxic marine sites.

In the suboxic zone, the most dominant bands belonged to OTU 5 (10 to 20% relative intensity in RNA gels), which was closely affiliated to the ciliate *Strombidium basimorphum* (99% similarity), and to OTU 1 (up to 44% in DNA gels), which had 99% sequence similarity to the pigmented dinoflagellate *Gymnodinium catenatum*. The latter phylotype was additionally present with lower signal intensity (~13% in DNA gel) in sulfidic depths, whereas at the RNA level it was detected only in well oxygenated depths (ca. 3% relative band intensity in RNA gels).

Throughout the whole sulfidic part of the water column, both RNA- and DNA-based approaches revealed 2 phylotypes with high amplicon abundance. One (OTU 7) accounted for up to 40 and 25% of relative band intensity in RNA and DNA gels and was affiliated within the excavate jakobids, very distantly related (82%) to the closest cultured representative *Jakoba incarcerata*. The other phylotype (OTU 9) was represented in RNA and DNA gels with up to 20 and 25% of relative band intensity, branched off within the Symbiontida and was very divergent to cultured species like *Calkinsia aureus* and *Bihospites bacati* (80% and 78% similarity, respectively). Both the jakobid and symbiontid phylotypes were exclusively related to environmental sequences from various anoxic and sulfidic sites (Edgcomb et al. 2002,

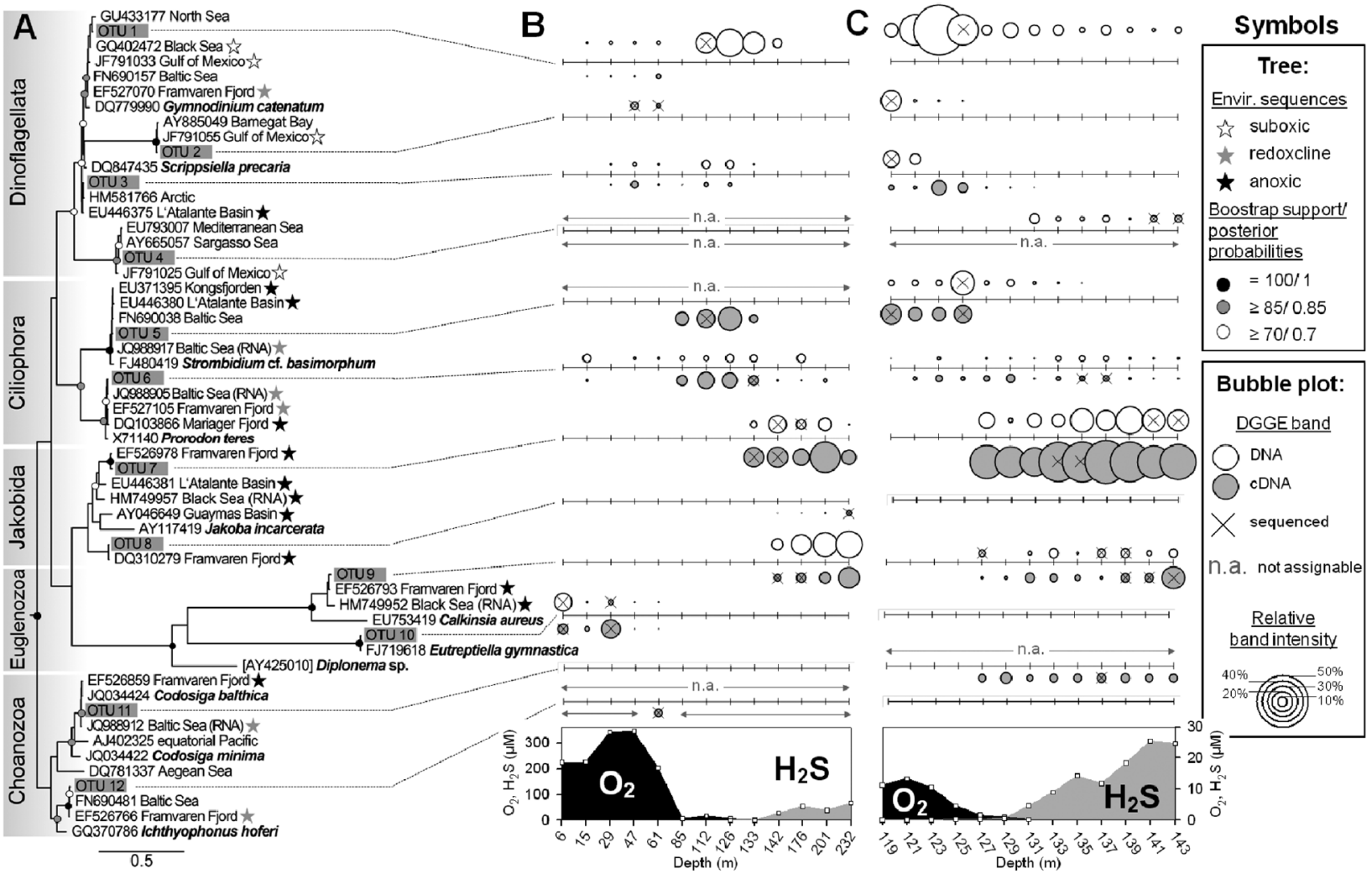

Fig. 8. Phylogenetic affiliation of sequences derived from DNA and cDNA DGGE gel bands of the whole water column and the redoxcline in high resolution of the Gotland Deep in July 2007. (A) Bayesian phylogenetic tree constructed with 57 partial and complete 18S rRNA sequences (1705 alignment patterns). The tree was rooted (not shown) by the cyanobacterium *Prochlorococcus marinus*, AF311220. The scale bar indicates 0.5 substitutions per position. Sequences appearing in shaded boxes originate from this study. Sequences from cultured representatives are shown in ***bold italics.*** Environmental clones are highlighted, according to their origin (see stars in key). (B,C) The occurrence of each OTU within the whole water column and the redoxcline profile is indicated as follows: open and grey circles represent OTUs found in DNA- and cDNA-based DGGE analysis, respectively. The size of the circles corresponds to the relative gel band intensity at the respective depths. Circles marked with an 'X' indicate sequenced DGGE gel bands. n.a. indicates that an unambiguous assignment of bands to the band position of the respective OTU could not be achieved. Note that missing data points represent real absence of bands at the OTUs band position

Alexander et al. 2009, Behnke et al. 2010, Wylezich & Jürgens 2011) (Fig. 8A). Other phylotypes, which were distributed with lower signal intensity (5 to 10%) in the sulfidic zone, belonged to dinoflagellates (OTU 4, only in DNA gel) and choanoflagellates (OTU 11, only in RNA gel). The dinoflagellate phylotype OTU 4 clustered together with environmental syndiniales group 1 sequences from oxic and hypoxic marine systems. The choanoflagellate phylotype (OTU 11) showed 100% sequence similarity to *Codosiga balthica*. Another jakobid (OTU 8), barely affiliated (82%) to described species but closely affiliated (100%) with a clone from the sulfidic boundary in the Framvaren Fjord, was detected solely in the RNA gel from a sample close to the seafloor (232 m) (Fig. 8B). The ciliate phylotype (OTU 6), closely related to *Prorodon teres* (98%), showed a wide distribution from surface waters to the sulfidic zone, with the highest signal intensity of RNA derived bands (up to 15%) in suboxic to slightly sulfidic water layers (Fig. 8B,C). The closest environmental sequences were obtained in DNA- and RNA-based studies from other interface and anoxic samples (Fig. 8A).

DISCUSSION

Protistan community structure along the pelagic redox gradient

Aquatic systems with anoxic deep water generally have a bimodal vertical distribution of prokaryotic and eukaryotic microbial cells. The peak in cell numbers at the oxic-anoxic interface can exceed the one formed at the surface waters, e.g. in the Cariaco Basin (Taylor et al. 2001, Lin et al. 2007) or vice versa like in the Baltic Sea (Setälä 1991, Detmer et al. 1993, Anderson et al. 2012). The latter was also the case for the deep basins of the central Baltic Sea (Fig. 1), which showed a main change in protist composition; whereas heterotrophic flagellates constituted up to 85% of the total protistan biomass at the surface, ciliates accounted for a similar percentage at the oxic-anoxic boundary (Fig. 1). This represents a fundamental change of the microbial food web structure, with flagellates as the dominant bacterial consumers in surface waters and ciliates as the major bacterivores within the redox zone (Anderson et al. 2012). The high abundance of prokaryotes seems to support the growth of larger ciliate taxa, since ciliates at interface depth were represented by only 20% of cell counts but with approximately three-quarters of biomass compared to respective surface layer values. Besides ciliates, dinoflagellates represented a major component in terms of cellular abundance and biomass (up to 56% of total protistan biomass) in suboxic waters. Sulfidic waters, on the other hand, were characterized by low protist numbers and relatively high prokaryote abundance, which is consistent with observations from other anoxic systems (Fenchel et al. 1990, Massana & Pedrós-Alió 1994) and previous estimates of low bacterial grazing losses in sulfidic waters of the central Baltic Sea (Anderson et al. 2012).

The 18S rRNA gene and transcript-based analysis also documented the stratification of the protistan communities along the redox gradient (Figs. 6 to 8). The most pronounced shift was observed at oxygen concentrations of 2 to 5 µM (ca. 1 to 2% atmospheric saturation), providing evidence for a turning point of an aerobic to a facultative or obligate anaerobic metabolism (Fig. 7). Indeed, these oxygen concentrations represent the tolerance level of many obligate anaerobes (Fenchel & Finlay 2008). It is additionally the upper limit where enough reducing power can be supplied for oxygen consumption, effectively acting as an oxygen detoxification mechanism for anaerobic protists (Fenchel & Finlay 1995), and where endosymbiotic methanogens are active and chemosensory phobic response to O_2 is provoked (Fenchel & Finlay 1990b). Aside from redox conditions as the main driving force, other factors such as prey composition and availability might play an additional role in vertical protist zonation. Altogether, 3 different zones (suboxic, oxic-anoxic interface and sulfidic zone) could be distinguished by their protistan assemblages based on DGGE cluster analysis (Fig. 7 A), phylotype (Fig. 8) and morphotype composition (Figs. 2 & 3), confirming previous investigations in this system (Anderson et al. 2012).

Dominant protists in suboxic, interface and sulfidic depths

The suboxic zone appeared to be primarily dominated by dinoflagellates and ciliates of the genus *Strombidium*. Among the 3 dinoflagellate phylotypes, 2 belonged to the order *Gymnodiniales* (OTU 1, 3), whereas one was rather of unknown taxonomic affinity (OTU 2). However, all 3 phylotypes were closely related to many environmental sequences retrieved under similar redox conditions from, e.g., the Black Sea and the Gulf of Mexico (Fig. 7A). However, the parallel 18S rRNA transcript-based approach showed a pronounced discrepancy in phylotype distribution for OTU 1 and 2 raising doubts on their activity in the suboxic zone (Fig. 8). A remark-

ably similar result was obtained with an identical phylotype to our OTU 1 in the Black Sea (Coolen & Shtereva 2009). A mismatch between taxon presence (DNA approach) and activity (RNA approach) seems to be a common phenomenon among certain dinoflagellate representatives in oxygen depleted and anoxic environments (Stoeck et al. 2007, Coolen & Shtereva 2009). It might be that diploid states in their life cycle and the extreme variability of rRNA gene copy numbers (from 65 to 12 000 copies cell^{-1}) in dinoflagellates could cause considerable overestimations of their relative amplicon abundance (Galluzzi et al. 2004, 2010, Zhu et al. 2005). Nevertheless, various dinoflagellate taxa were detected (via RNA) to be preferentially active in the suboxic zone of the Black Sea (Wylezich & Jürgens 2011) and catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) analysis confirmed that they are active grazers in Baltic Sea suboxic depths (Anderson et al. 2012). These facts, and the distinct abundance peak formed by intact dinoflagellate cells in Baltic Sea suboxic zones, as observed via microscopy, point to an important role of dinoflagellates in this zone (Anderson et al. 2012, this study).

Within the ciliate genus *Strombidium*, remarkably similar results were obtained by microscopy and 18S rRNA-DGGE, indicating a high abundance and activity in the suboxic zone. The strong DGGE band of OTU 5 (Fig. 8), related to *Strombidium* cf. *basimorphum*, suggest that the bulk of *Strombidium* spp. cell counts were comprised by this phylotype. *Strombidium* species are common photosynthesizers in surface waters, but they are also known mixotrophs and can be frequently detected in oxygen deficient systems (Fenchel et al. 1990, Stoeck & Epstein 2003, Behnke et al. 2006) where they have been shown to be active grazers (Anderson et al. 2012).

The majority of all ciliate morphotypes detected at the oxic-anoxic interface and the upper sulfidic zone belonged to the genus *Metacystis*, which was represented by at least 5 species, as determined by the microscopic inspections. At least one represents a novel species with features different to those found in the 14 *Metacystis* species described so far from marine, brackish and freshwater environments (summarized in Arregui et al. 2010). *Metacystis* species were often described as sessile or as benthic dwellers (Aladro-Lubel & Martinez-Murillo 1999, Azovsky & Mazei 2003, Prast et al. 2007, Arregui et al. 2010). Aside from the findings in a shallow lake (Guhl et al. 1996) and in nutrient-rich brackish tide pools (Dietz 1964, W. Foissner unpubl.), the Baltic Sea seems to be the only aquatic system where *Metacystis* species are an important component of the pelagic protist community. A high proportion of *Metacystis* spp. were large forms (70 to 100 µm), which dominated the protistan assemblage with up to 80 % of total biomass in the redoxcline. Since protistan grazing in these strata can eliminate up to 100 % of the prokaryal standing stocks per day (Anderson et al. 2012), *Metacystis* species are likely the dominant grazers of prokaryotes. In addition, these ciliates seem to maintain partnerships with large numbers of endo- and ectosymbiotic bacteria (Fig. 5), which argues for multiple functions mediated by this key component of the Baltic Sea redoxclines. However, to date, the phylogenetic identity of the genus *Metacystis* still remains unsolved, since 18S rRNA sequences of all morphologically described *Metacystis* species are missing.

Other ciliates consistently present at interface depths were *Mesodinium* spp. as revealed by the morphological approach. Unfortunately, none of those were detected via DGGE, because the employed primer set does not amplify this genus. *Mesodinium* species have long been recognized as photosynthetically active but are also capable of bacterivory (Myung et al. 2006). *Mesodinium rubrum* has been shown to form abundance maxima at shallow redoxclines (12 to 14 m) of a Baltic Sea inlet in order to exploit high nutrient concentrations before moving back to the euphotic zone (Lindholm & Mörk 1990). However, the fact that the central Baltic Sea's redoxclines are situated between 90 and 130 m of depth, motility costs make such a migration behavior less likely. Several molecular surveys conducted in the Mariager and Framvaren Fjords, hypersaline deep sea basins and the Black Sea revealed *Mesodinium*-related sequences to be frequent at the redoxcline and in anoxic waters, with the deepest RNA signature detected at a depth of 3500 m (Behnke et al. 2006, 2010, Zuendorf et al. 2006, Alexander et al. 2009, Wylezich & Jürgens 2011). Although, the *Mesodinium* species present at deep pelagic redoxclines seem to be pigmented (Anderson et al. 2012), they might represent different taxa to the ones present at mixed layer depths or subpopulations of these, which are able to prosper independent of light energy.

The most prominent flagellate phylotypes which emerged in the sulfidic zone, with strong DNA- and RNA-derived signatures in DGGE gels, were affiliated within jakobids (OTU 7) and euglenozoans (OTU 9). The closest relatives of both have been recorded from various anoxic sites such as the Framvaren Fjord (Behnke et al. 2006) or the Black Sea (Wylezich & Jürgens 2011), and jakobid sequences accounted for almost three-quarters of all clones in a

genetic library from an anoxic sample of the Baltic Sea (Stock et al. 2009). With our high resolution sampling in Gotland Deep, we demonstrate that these 2 phylotypes are important components of the protist community in the entire sulfidic zone (ca. 100 m thickness) in terms of presence and activity. The euglenozoan phylotype belonged to the Symbiontida subgroup, which has been recently shown to harbor numerous sulfur or sulfide-oxidizing *Epsilonproteobacteria* as symbionts (Edgcomb et al. 2011d).

The choanoflagellate phylotype (OTU 11), which was found at the interface and upper sulfidic zone, was very closely related to *Codosiga balthica*, a recently described isolate from the Baltic Sea oxic-anoxic interface. It harbours endosymbiotic bacteria and possess atypical mitochondrial cristae, assumingly as an adaption to oxygen limited and anoxic conditions (Wylezich et al. 2012). Sequences of this taxon were also found in the sulfidic depths of the Gotland Deep in 2005 (Stock et al. 2009), and at the oxic-anoxic interface of Landsort Deep in 2011 (Anderson et al. 2013), indicating that it could be a permanent member and important bacterivore in Baltic Sea hypoxia.

Finally, the dinoflagellate OTU 4 belonged to prominent residents of oxygen deficient and anoxic ecosystems, the syndiniales group 1 (Guillou et al. 2008). Syndiniales comprise a widespread group of obligate parasites with a broad host spectrum ranging from protists to metazoans. Since metazoans generally do not prosper under sulfidic conditions, it is likely that our OTU is a parasite of other anaerobic protists. Further, unlike the assumption that syndiniales are exclusively marine (Guillou et al. 2008), our data suggest this group has a wider salinity tolerance, including at least brackish water conditions.

Currently, for the dominant protists that prosper in distinct redox zones of the Baltic Sea, rather vague ecological functions can be inferred from our findings and the comparative literature. Therefore, comprehensive studies are needed that target these particular taxa and assess their grazing impact, food preferences and symbiotic interactions in order to increase our understanding of the biogeochemical processes of this and other redoxcline systems.

CONCLUSIONS

The application of morphological and molecular techniques in concert has been frequently recommended as a valuable task for better understanding the structuring and composition of protist communities (e.g. Zuendorf et al. 2006, Behnke et al. 2010, Caron et al. 2012) Following this strategy to examine the dominant protist taxa along pelagic redox gradients of the central Baltic Sea turned out to compensate for shortcomings related to the application of just a single method. The semi-quantitative nature of molecular techniques caused by PCR bias and species specific variations in 18S rRNA gene copy number (Caron 2009) was outbalanced by obtaining cell counts and biomass estimates of certain functional and taxonomic protist groups. Furthermore, microscopy was superior in the detection of larger ciliates like *Metacystis* and *Mesodinium* as major components of the redoxcline protist community which remained undetected by the molecular approach. On the other hand, the use of 18S rRNA sequences allowed a more accurate and unambiguous taxonomic identification for some protists. This is especially important for amorphous protists with few diagnostic features (Caron 2009). Like the findings of Savin et al. (2004), the overall congruence of taxa detected by both approaches was unexpectedly low and highlights again the need for such combined approaches to cover a larger portion of protistan assemblages.

The sampling campaigns in different years revealed a remarkably similar morphotype distribution along the redoxclines, which was also highly comparable with the ones observed by Anderson et al. (2012, 2013). Additionally, our study shared 3 phylotypes (affiliated within *Codosiga*, *Strombidium* and *Prorodon*) that were detected at similar redox conditions in the Baltic Sea by previous and later sequencing approaches (Wylezich et al. 2012, Anderson et al. 2013). This argues that the Baltic Sea's pelagic redox zones are inhabited by a characteristic and relatively stable community of dominant protist. Observations in the redox gradients of the Framvaren Fjord (Behnke et al. 2010) and the Cariaco Basin (Orsi et al. 2011) provide some evidence for seasonal fluctuations in the protist communities. Further studies in the Baltic Seas pelagic redox zone, with a higher temporal resolution in the yearly cycle, are needed.

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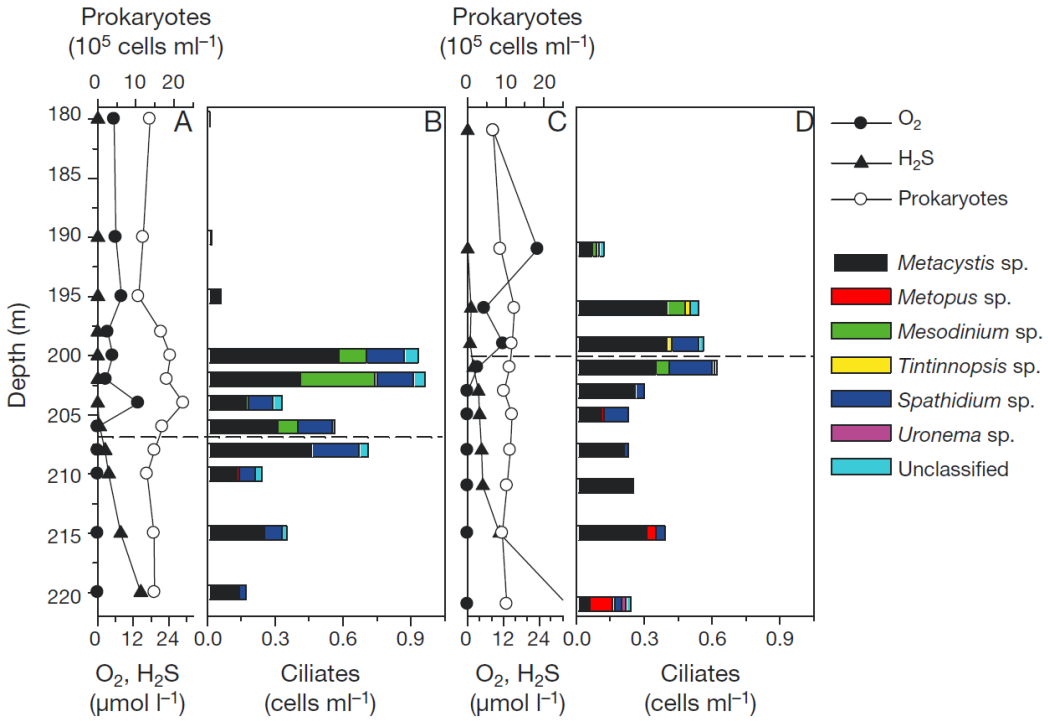

Appendix. Fig. A1. High resolution profile of ciliate morphotype composition during reconstruction process of the redox zone at Gotland Deep on (A,B) 4 May and (C,D) 6 May 2005. Changes with depth in (A,C) oxygen, hydrogen sulphide and prokaryote concentrations and in (B,D) ciliate cell-counts are shown. The horizontal dashed line represents the oxic-anoxic interface

Chapter 2) Culturing of protists from the Baltic Sea: many usual suspects as well as some novelties

Culturing of protists from the Baltic Sea: many usual suspects as well as some novelties

ABSTRACT

The study of cultured strains has a long tradition in protistological research and has largely contributed to establish the morphology, taxonomy and ecology of many protist species. Nevertheless, the application of culturing independent techniques, based on sequencing of 18S rRNA genes, is superior in describing the composition of natural protistan assemblages and thus largely replaced cultivation in environmental diversity studies. From sequencing data we learned that natural protistan assemblages mainly consist of hitherto uncultured lineages and thus cannot be directly brought into accordance with the biological features that were inferred by studies on cultured strains. This knowledge gap requires additional taxa of ecological relevance to be brought into culture in order to assess their ecological functions and to provide reference data for metagenomic and metatranscriptomic studies. In this study, traditional cultivation techniques were applied to samples from coastal and central Baltic Sea waters. In total 128 monoclonal protist cultures were identified based on 18S rRNA gene sequences. The majority of our isolated strains were affiliated to already cultured and described taxa, mainly belonging to Chrysophycea and Bodonida. This was likely a consequence of the so-called "culturing bias" or, in some cases, attributed to the eutrophic nature of the Baltic Sea. However, around 11% of the isolates in our culture collection were characterized by highly divergent 18S rRNA gene sequences compared to known organisms, and therefore represent novel taxa at species or even genus level. There is evidence for some of those isolated taxa to be ecologically relevant, at certain conditions, for the Baltic Sea.

INTRODUCTION

The isolation and cultivation of single cells is one of the most traditional approaches in microbiology. It was Horace-Bénédict de Saussure who established the first monoclonal protist culture in 1769 (Hausmann et al. 2003, N. Hülsmann pers. comm.). This success was a starting point of experimental research in protistology and led to the description of numerous newly discovered species until the present day. Today the general biology of protists as well as their morphology and even our picture of the eukaryotic tree of life are largely based on laboratory studies and gene sequences of cultivated strains. Moreover, many fundamental research questions and biological, ecological and evolutionary concepts were formulated on account of the knowledge that was gained through studies on pure protist cultures.

However, with the introduction of culturing independent techniques, skepticism arose whether or to what extent the protist species kept in culture mirror the species that are abundant and active in natural aquatic assemblages (Moon-van der Staay et al. 2001, Massana et al. 2004). Comparisons of cultivation methods such as the most probable number (MPN) (Sinclair & Ghiorse 1987) with direct microscopical counts generally yielded large underestimations of heterotrophic nanoflagellate (HNF) abundance and raised doubts about the expedience of such methods for environmental studies (Caron et al. 1989). In that way, it has been estimated that on

average only 1% of the natural protistan abundance can be assessed by cultivation approaches (Caron et al. 1989). A closer look on cultivation surveys that have been performed at different environmental sites unveiled that mainly the same species are repeatedly retrieved, which indicated the selective nature of cultivation (Lim et al. 1999, summarized in Jürgens & Massana 2008). Prominent examples of theses opportunistic species which are apparently, unlike to others, able to better cope with laboratory conditions and artificial media can be found for example within the genera *Spumella*, *Paraphysomonas*, *Cafeteria*, *Rhynchomonas* and *Bodo* (Jürgens & Massana 2008, del Campo, Balagué, et al. 2013). Direct proofs for the discrepancy of dominance in heterotrophic enrichment incubations while having low *in situ* relevance was achieved with the application of group or species-specific oligonucleotide probes to enumerate *Bolidomonas* spp., *Paraphysomonas imperforata*, *Cafeteria roenbergensis* and *Caecitellus paraparvulus* in natural samples (Lim et al. 1999, Guillou et al. 1999, Massana et al. 2007). Although the dilemma of the culturing bias in heterotrophic protists circulates in the literature for long, it was just recently systematically investigated with regard to the effects caused by the quality and quantity of organic matter supplements on the protistan community structure (del Campo, Balagué, et al. 2013). This study revealed that the community composition of protists differed more and more from the one in the original sample, the richer the cultivation media were, and developed towards already cultured species.

Studies based on sequencing of 18S rRNA genes uncovered a hitherto unexpected high diversity of natural protistan assemblages and demonstrated that the world's oceans are dominated by as yet uncultured taxa (e.g. Díez et al. 2001, Shi et al. 2009, Massana et al. 2014). Even though molecular approaches, especially next generation sequencing (NGS), allow massive gatherings of environmental sequence data, the organisms behind, including their general biology and ecological traits, still remain unknown. Consequently, the evaluation of ecosystem processes and the establishment of biogeochemical models suffer tremendously from this knowledge gap (del Campo et al. 2014, Worden et al. 2015). Thus, a call in the scientific community currently exists to draw attention on an urgent demand of autecologically characterized cultures of relevant taxa in order to quantify protist activities and their impacts on the global carbon cycle (Heger et al. 2014, del Campo et al. 2014, Worden et al. 2015). Further, cultured strains offer the chance to act as model organisms to establish reference data for the evaluation and interpretation of findings inferred from metagenomic and metatranscriptomic approaches (Keeling et al. 2014, Worden et al. 2015). Due to the high in build fail rate of traditional culturing techniques to retrieve ecologically significant taxa, alternative isolations such as fluorescence-activated cell sorting and mimicking of *in situ* conditions were recently developed to increase the amenability of important taxa to cultivation (Seenivasan et al. 2013, del Campo, Not, et al. 2013). This lead to cultivation success of some widespread planktonic representatives like *Minorisa minuta* (Cercozoa), the assumingly smallest bacterivore in the ocean (del Campo, Not, et al. 2013) and *Picomonas judraskeda* the first described member within the Picozoa (former picobiliphytes) (Seenivasan et al. 2013). Nevertheless, recent achievements show that even traditional cultivation methods are also capable to discover new taxa of ecological relevance. Resulting from such approaches, a member of the abundant marine stramenopile group MAST-3 (Cavalier-Smith & Scoble 2013), ten new strains of vampyrellid-like naked amoebae (Cercozoa) (Berney et al. 2013), 11 new species related to Apusozoa (Glücksman et al. 2013) and several new species of a novel bicosoecid (stramenopiles) cluster (H. Arndt, C. Wylezich, unpublished results) were obtained in culture.

In this study, traditional cultivation attempts were performed to study pelagic heterotrophic protists in the meso- to eutrophic

brackish Baltic Sea. Clonal cultures from coastal and central Baltic Sea water samples were established by picking single cells followed by serial dilutions (MPN) or by the liquid aliquot method (LAM). Cultures were PCR screened applying an armada of primer sets. Resulting 18S rRNA gene sequences were used to search the phylogenetic affiliation of each culture and judge their degree of novelty by comparison with sequences deposited in GenBank.

MATERIALS AND METHODS

Sampling and establishment of cultures

Coastal surface water samples from the south-western Baltic Sea were taken from a phytoplankton monitoring station (sea bridge of Heiligendamm, Germany, 54°08'N, 11°50'E) during several campaigns in 2003 (November 04, 11, 18, 24) and 2008 (October 28; November 11, 18) resulting in 46 and 116 protist cultures respectively. Samples in the central Baltic Sea were taken along depth profiles in the Landsort (IOW station 284, 58° 350'N, 18°140'E) and Gotland Deep (IOW station 271, 57°190'N, 20°100'E) during two cruises in 2005 (May 25, RV Alkor) and 2012 (July 16, 22, RV Meteor) resulting in 24 and 87 cultures respectively.

Before starting with isolation procedures, samples from the coastal site (Heiligendamm) in 2008 were prefiltered, yielding two fractions (≤ 200 µm and ≤ 3µm), and subjected to unamended seawater incubation experiments in the dark as described in detail in Weber et al. (2012). After 5 to 6 days of incubation, protists were isolated from the abundance peak of developing heterotrophic flagellates. At all other sampling sites, culturing was performed from the original untreated water samples. The culturing attempts in 2003, 2005 and 2008 were performed as single cell isolations with a micromanipulator under the microscope and followed by serial dilutions. In 2012 the liquid aliquot method (Butler & Rogerson 1995, Loquay et al. 2009) was used to establish monoclonal cultures.

Protist cultures obtained were routinely kept in sterile 50-ml tissue culture flasks (Sarstedt, Nümbrecht, Germany) containing sterile filtered and autoclaved seawater or F2 medium (Guillard & Ryther 1962, salinity 8–16‰). Autoclaved wheat or quinoa grains and *Vibrio angustum* or the natural assemblage of bacteria from the sampling site were provided as food source. Growth and survival of cultures was regularly checked under the microscope and aliquots of old cultures were transferred into new culture flasks containing fresh media. Several replicates of the cultures were produced in order to keep a backup and to store the cultures in a 10 and 15°C climate chambers simultaneously. Nevertheless, many cultures were lost during several air-condition crashes in both climate chambers or by fungus contamination. Cultures that were further investigated are listed in the appendix with their date and source of isolation (Table S1).

Gene sequence analysis

DNA of harvested cells was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre) or the CTAB method (Clark 1992, modified after Wylezich et al. 2007). The 18S rRNA gene was amplified by polymerase chain reaction (PCR) using various primer combinations depending on the culture (see appendix). The PCR mixture containing 1 µM of each primer, 200 µM dNTPs, 1x PCR buffer and 0.5 µl of Herculase II Fusion DNA Polymerase (Agilent Technologies) or *Tag* DNA polymerase (Fermentas) was heated to 94°C for 2 min, and the 18S rRNA gene was amplified in 35 cycles of 95°C for 20 s, 52-57°C (depending on primers) for 30 s, and 72°C for 70 s. PCR products were run on Agarose gels (1.2%) and in some cases bands were excised and purified with the Nucleospin II Kit (Machery Nagel). Sequencing was carried out by companies (LGC Genomics, QIAGEN) with single or up

to four internal sequencing primers (see appendix).

Obtained 18S rRNA fragments of different length (depending on primers, see Table S1) were carefully corrected and assembled in SeqManII (DNAStar). The resulting whole and partial sequences were submitted to KeyDNA-Tools (www.keydnatools.com) and BLAST (Altschul et al. 1997) for chimera detection and taxonomic affiliation. Novelty analysis of the sequences was performed based on sequence similarities to the closest cultured and environmental match found via BLAST search against GenBank as detailed described by Massana et al. (2011).

Phylogenetic analysis

Some cultures that were characterized by a high novelty degree of their 18S rRNA gene sequences were subjected to further tree reconstruction analysis in order to reveal their exact phylogenetic affiliation. Culture sequences and sequences retrieved from GenBank were aligned using the CLUSTAL_X program (Thompson et al. 1997) or MAFFT version 6 (Katoh et al. 2002). The multiple alignments were inspected and manually refined in BioEdit (Hall 1999). For all sequence data sets, phylogenetic analyses were done using PhyML 3.0 (Guindon et al. 2010, http://www.atgc-montpellier.fr/phyml/) under the GTR model of substitution (Lanave et al. 1984) and gamma-shaped distribution of rates of substitution among sites with eight rate categories and a proportion of invariable sites. To estimate branch support, we performed 1,000 bootstrap replicates for ML analyses. For the cercozoans (Thecofilosea, Granofilosea and *Massisteria*) phylogenetic analyses were additionally performed with MrBAYES (Huelsenbeck & Ronquist 2001). This analysis was performed for 1,000,000 generations and sampled every 100 generations for four simultaneous chains. For the likelihood analysis, all model parameters were estimated from the data set. To estimate branch support, we performed 1,000 bootstrap replicates for ML analyses.

RESULTS AND DISCUSSION

We aimed to study the cultivable fraction of protists and performed an exhaustive culturing effort yielding 273 monoclonal cultures from coastal and central Baltic Sea water samples. Due to climate chamber breakdowns, almost half of the cultures were lost before they were further processed. PCR screening was finally successful for 147 cultures from which about 40% could be amplified by a single primer set (EukA, Euk516r). For the remaining cultures, 25 different combinations of group specific and eukaryote specific primers were needed (see appendix for details). Finally, a total of 128 high quality partial and complete 18S rRNA gene sequences were obtained with 60 sequences originating from the coastal station of Heiligendamm (HD) and 68 from the central Baltic Sea (BS) cultures (Figs. 1 and 2).

Most of the species found in cultures were related to heterotrophic and bacterivorous flagellate groups. Chrysophytes predominated in both culture collections (52% and 69% in HD and BS cultures, respectively) and mainly belonged to the genera *Paraphysomonas*, *Pedospumella* and *Ochromonas* (Figs. 1 and 2). Bodonids were represented in both collections with 10% and 13% in HD and BS cultures, respectively, and comprised the genera *Procryptobia* and *Rhynchomonas.* Bicosoecids in the HD collection (12% of all sequences) were all closely related to *Cafeteria roenbergensis* whereas only one bicosoecid sequence close to *Caecitellus parvulus* occurred in the BS collection. Pedinellales sequences (13%) closely related to *Pteridomonas danica* and cercozoans very distantly related to any cultured and environmental representative were solely found in the HD cultures (Fig. 1).

Otherwise, Choanozoa were exclusively found among the BS cultures (10%), most of which were quite distant to described species (Fig. 2).

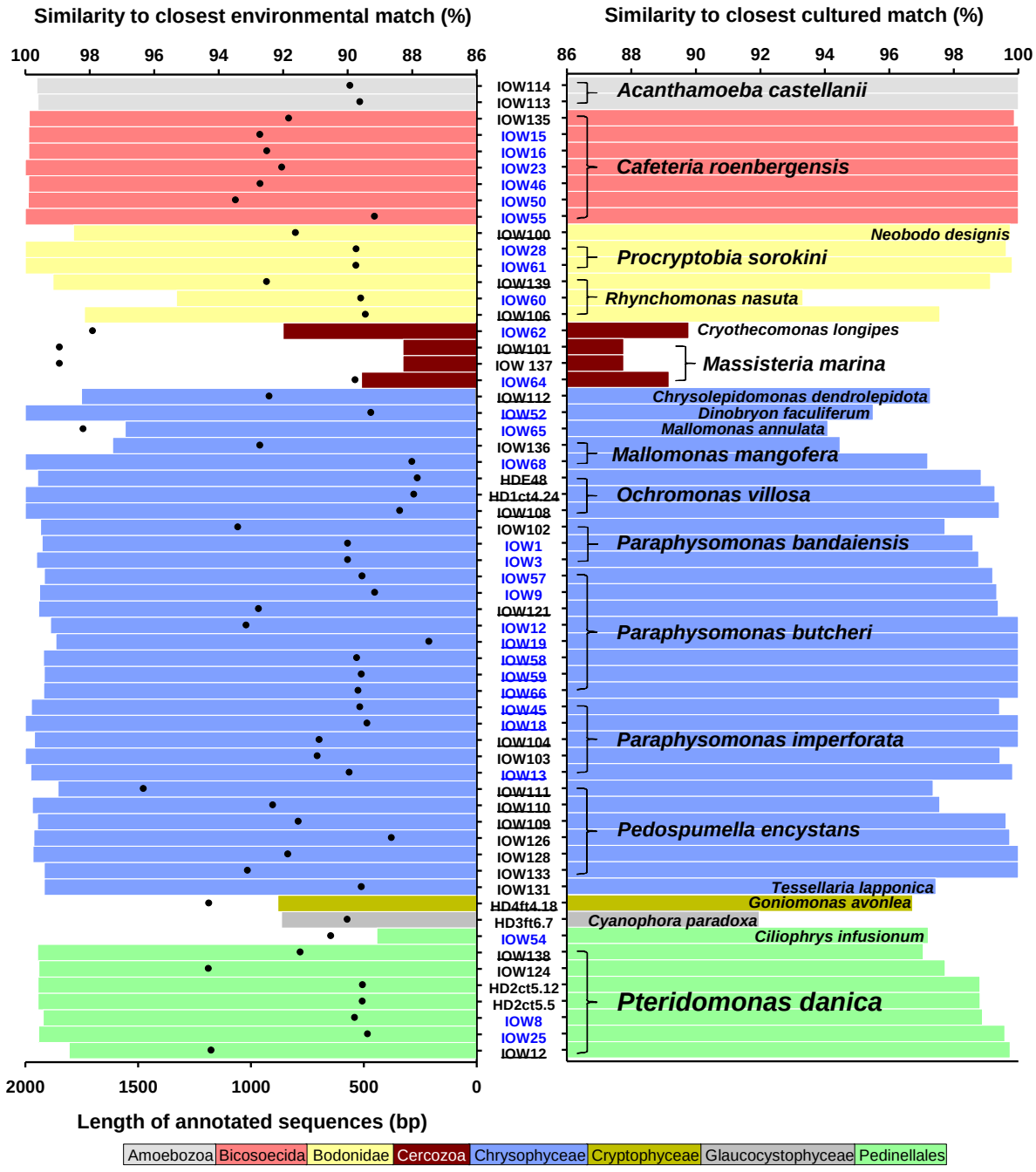

Fig. 1. Bar chart showing the phylogenetic assignment (color code) and sequence similarity to the closest environmental match (CEM, left panel) and the closest cultured match (CCM, right panel) of 18S rRNA gene sequences obtained from cultures isolated at **Heiligendamm**. Black circles in the left panel refer to the length of the annotated sequence. Black and blue culture names (middle panel) indicate the isolation in 2003 and 2008 respectively. Underlined and regular culture names declare the sequence authors F. Weber and C. Wylezich respectively.

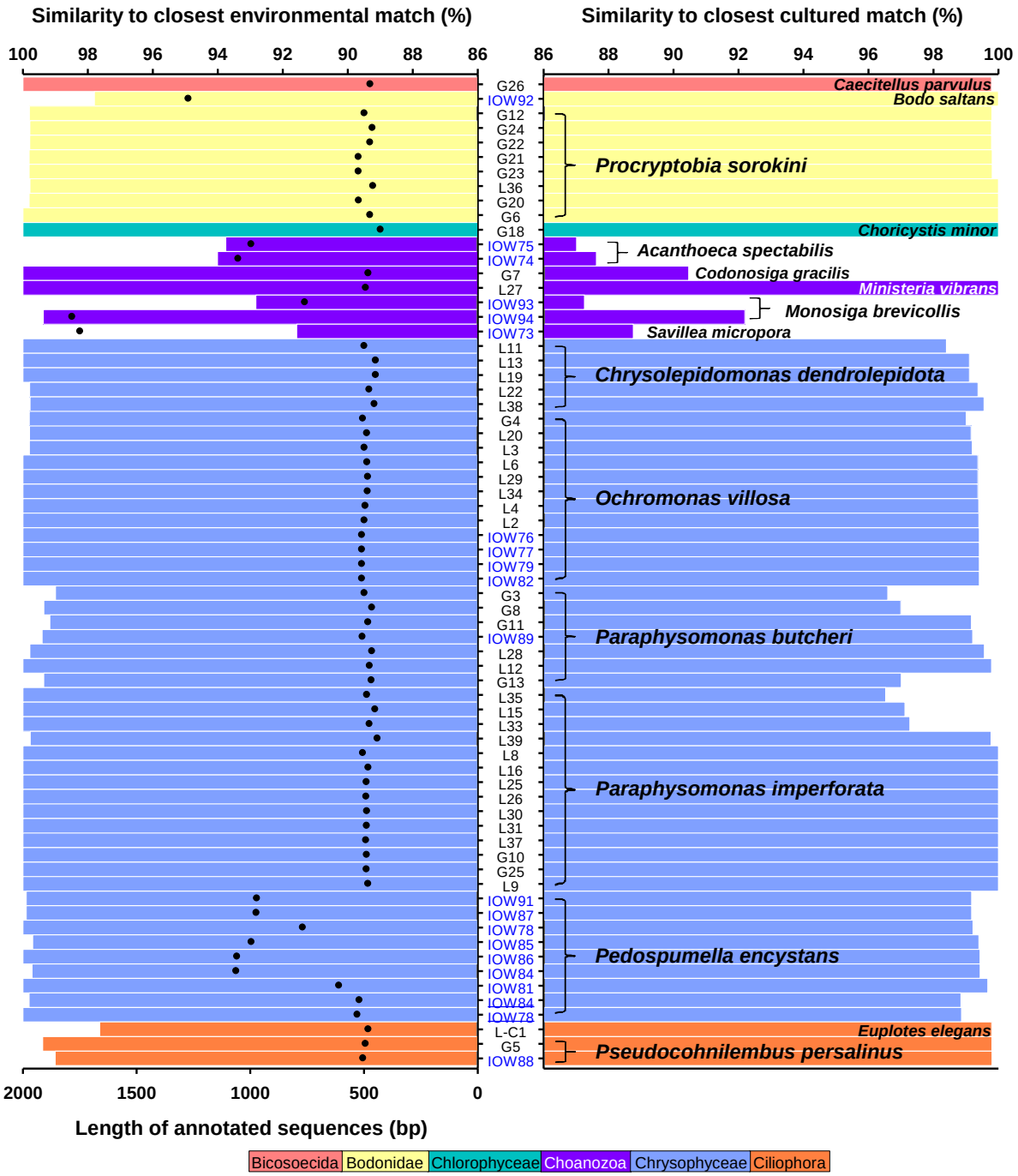

Fig. 2. Bar chart showing the phylogenetic assignment (color code) and sequence similarity to the closest environmental match (CEM, left panel) and the closest cultured match (CCM, right panel) of 18S rRNA gene sequences obtained from cultures isolated in the **central Baltic Sea**. Black circles in the left panel refer to the length of the annotated sequence. Black and blue culture names (middle panel) indicate the isolation in 2005 and 2012 respectively. Underlined and regular culture names declare the sequence authors F. Weber and C. Wylezich respectively.

To what extend are the results affected by culturing bias?

The phenomenon of culturing bias in protists has been described as underestimations of their natural abundance (Caron et al. 1989), their

natural diversity (Groisillier et al. 2006, Massana, Terrado, et al. 2006) and the resulting misinterpretations of the cultured species' ecological relevance (Lim et al. 1999). These biases were revealed through comparisons of culturing with microscopical counts (Caron et al. 1989), environmental sequencing (Massana et al. 2004) or fluorescence in situ hybridization (Lim et al. 1999), although these techniques are not free of their own specific biases (Sherr & Sherr 1993, Caron 2009, Bochdansky & Huang 2010). Cultivation of environmental samples has been shown to cause a shift in the protistan community towards species that are already known from laboratory cultures (del Campo, Balagué, et al. 2013). Thus, the proportion of already cultured species that occur during cultivation can be used as an indication of the culturing bias. Therefore, each culture sequence obtained in this study was aligned against sequences deposited in GenBank in order to display the similarity to the closest culture match (CCM) and closest environmental match (CEM) (Fig. 3). Most of the sequences were closely related to their CCM, resulting in an average similarity of around 98% for the cultures of both sampling sites (Fig. 3).

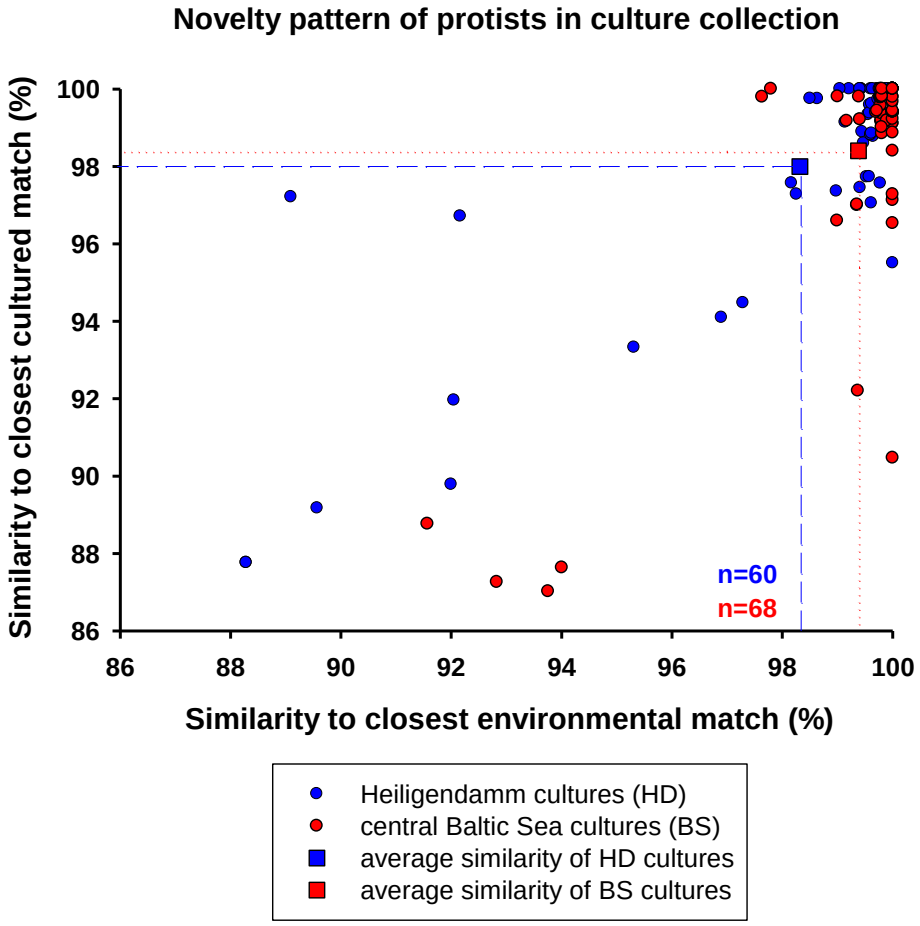

Fig. 3. Novelty pattern determined for protist cultures obtained at the coastal station (Heiligendamm) and in the central Baltic Sea (Landsort and Gotland Deep). Each circle represents one culture and its percent sequence similarity to the closest environmental match (CEM) and the closest cultured match (CCM) in GenBank. The average similarity to both is indicated by the squares.

In comparison, clone library construction of coastal water samples off Heiligendamm (Weber et al. 2012) revealed an almost similar average CCM similarity of 97% (Fig. 4). This

small difference between a culturing independent approach and a traditional cultivation study of the same habitat suggests a low culturing bias at first glance. However, the high CCM value of the clone library is mainly caused by numerous sequences related to algal groups (Weber et al. 2012) which are generally better represented in public culture collections than heterotrophic protists (del Campo et al. 2014). A re-analysis of the same dataset, after removal of all sequences related to phototrophs (trophic assignment after Weber et al. 2012), then resulted in an average CCM similarity of 94% for predominantly heterotrophic species (Fig. 4). Based on these average similarity values, unamended seawater incubations are not affected by culturing bias, whereas subsequent cultivation of single taxa induced a shift towards protists known from laboratory cultures. Additionally, the clone library data (with and without phototrophs) show a much higher degree of dispersion in CCM similarity values indicating the natural protist community to be a heterogeneous assemblage of taxa closely and distantly related to cultured representatives. On the contrary, with the exception of few outliers, cultivation has caused a more homogenous collection of taxa shifted towards already known species. Although data on the original community of the other sampling campaigns are missing, a similar shift can be assumed for the culture collections at Heiligendamm in 2003 and the central Baltic Sea in 2005 and 2012 (Fig. 4).

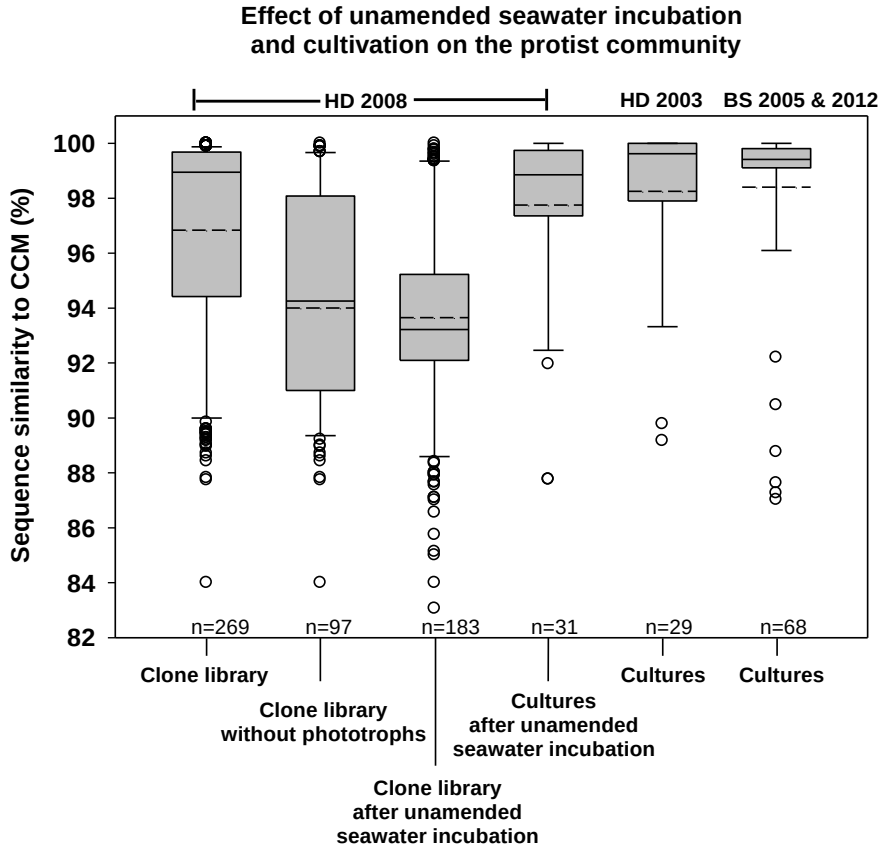

Fig. 4. Box plot showing the sequence similarities (in %) to the closest cultured match in GenBank for clone libraries and culture collections originating from differently processed water samples from Heiligendamm (HD) in 2003 and 2008 and the central Baltic Sea (BS) in 2005 and 2012. Note that the different sample treatments in HD 2008 were performed on water samples of the same campaign and serve for direct comparison here. The number (n) of sequenced clones or cultures is given. Boxes encompass the 25th–75th percentiles of all data, solid lines within boxes and dashed lines represent medians and means, respectively, whiskers enclose the 10th–90th percentiles and open circles represent all outliers.

BLAST searches for the closest cultured representatives in GenBank were also used to separate the strains in our collection into the ones which are closely related and the ones which are distantly related to already cultured species. Therefore, a similarity cutoff was applied and all cultures with a sequence identity below 96% to their CCM were judged as being novel cultured strains. These accounted for 11,5% in our culture collection, with 5 cultures having an astonishingly low similarity (below 88%) to their CCM which makes them potential candidates of novel protistan genera (Figs. 3 and 5).

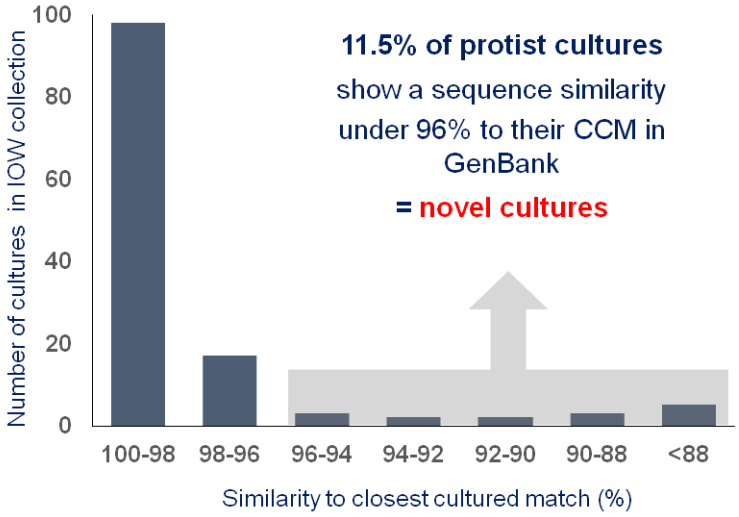

Fig. 5. Distribution of 18S rRNA gene sequence similarities of IOW cultures to their closest cultured match (CCM) in GenBank. Cultures with a similarity less than 96% to their CCM are considered as novel cultures.

Interestingly, most of these novel strains were also characterized by a low similarity (94% on average) to environmental clones in GenBank (Fig. 3) and thus seem to have refused the detection by environmental sequencing, with the possible exception of NGS approaches, for which datasets are typically not deposited in public databases. This could imply that these taxa are only rarely represented in the environment or that they belong to a specialized protist fauna of brackish waters which are vastly understudied compared to freshwater and marine systems. Another explanation might be that environmental sequencing studies typically employ only a single primer set which is known to select for only a fraction of the protistan diversity and bears the risk to exclude certain protist groups from amplification, even at the highest taxonomic ranks (Stoeck et al. 2006, Jeon et al. 2008, Bass et al. 2012). In our study, this primer bias was minimized by the use of 15 eukaryote-specific and group-specific primers applied in 25 different combinations, while the application of a single primer set (e.g., EukA, Euk516r) would have amplified only 40% of our cultures at best. Especially Kinetoplastida related cultures, which represented more than 10% of the strains in our collection, would have been missed to be identified without applying kinetoplastid specific primers. Among eukaryotes, kinetoplastids have undergone a rapid evolution of their small subunit rRNA gene and therefore cannot be accessed by most general eukaryote specific primers or probes at similar success rates compared to other phylogenetic groups (Bochdansky & Huang

2010, Scheckenbach et al. 2010, Mukherjee et al. 2015). Although kinetoplastids play a vital role in various habitats (Arndt et al. 2000) and are often encountered by morphological studies (Tikhonenkov et al. 2006, Risse-Buhl et al. 2013), their diversity might be underestimated and important lineages might have been overlooked by environmental sequencing approaches using a single primer set.

The usual suspects in the culture collection

The majority of our cultures belonged to so called "easy to culture" flagellates for which it remains doubtful whether they represent the most active and dominant species in the ocean (Jürgens & Massana 2008). Such species get selectively enriched by cultivation surveys that use unrealistic high amounts of organic matter supplements which promote the growth of large bacteria in high abundances(Jürgens & Massana 2008). These bacteria support fast growth of only a minor fraction of the natural nanoplankton community, which in turn then outcompete other bacterivores by their rapid response to the changing quality and quantity of prey.

Species in our collection that match a list of marine flagellate taxa for which these high growth rates have been observed (summarized in Jürgens & Massana 2008) were found within the genera: *Paraphysomonas*, *Spumella*, *Cafeteria*, *Caecitellus*, *Bodo*, *Rhynchomonas* and *Pteridomonas.* At first glance, it appears astonishing that there is a considerable share in the species composition of our collection, the list of fast growing cultured flagellates in Jürgens & Massana (2008) and the list of the 20 most commonly reported heterotrophic flagellates in the world (Patterson & Lee 2000). A reason might be that the latter study indeed represents a morphological survey of globally distributed protists, whereas its sample treatment, including enrichment steps, long distance transportations and delayed processing of the obtained material, rather resembles cultivation conditions than live observations of environmental samples. This puts emphasis on the assumption that this pool of protist taxa is an indicator of a culturing bias, which might be as well the major explanation for our results. Besides the ability to exploit high concentrations of bacteria, high tolerances to abiotic factors might also be responsible for some taxa to get repeatedly enriched in several cultivation surveys. For example, high tolerances to factors such as salinity, anoxic conditions or temperature has been reported for several species of *Rhynchomonas, Bodo* and *Paraphysomonas* (Lee & Patterson 1998, Arndt et al. 2000). Indeed our culture collections from the south-western and the central Baltic Sea showed a considerable share with regard to some of these easy to culture flagellates, such as species of *Paraphysomonas, Pedospumella, Ochomonas* and *Procryptobia* (formerly included in the genus *Bodo*). The fact that the depths sampled in the central Baltic Sea derived predominantly from suboxic or sulfidic waters (see appendix) implies a high tolerance towards differing oxygen conditions within the found species. Further, several air condition breakdowns happened during long-term storage, causing the loss of many cultures. We conclude that these events have already selected the most robust taxa to survive in our collection, at least with regard to temperature tolerance.

Besides that, the eutrophic Baltic Sea represents a less stable environment compared to the oligotrophic open ocean, due to considerable seasonal variations (e.g., water temperature and salinity) as well as transient perturbations (e.g., mixing events) and phytoplankton bloom situations that stimulate bacterial food supply for predacious protists. Thus, the life strategies (fast growth on bacterial bursts and high tolerance towards environmental conditions) of some easy to culture flagellates seem to perfectly match some of the Baltic Sea`s environmental characteristics. Consequently, some of these taxa might become sporadically relevant here. Striking examples might be species of the genera *Paraphysomonas* and *Spumella* which

have been frequently detected throughout the Baltic Sea by microscopical observations (Samuelsson et al. 2006). In accordance with that, many strains in our culture collections were closely related to *P. imperforata*, *P. bandaiensis* and *P. butcheri*, provided that these species represent correctly identified species based on morphological and molecular criteria, which is currently hotly debated (Scoble & Cavalier-smith 2013). Further, the occurrence of *P. imperforata* related sequences, detected by cultivation independent approaches (DGGE and clone libraries) in Heiligendamm water samples, (Weber et al. 2012), indicate that this species could be indeed an important member of the Baltic Seas coastal protist community. However, very short-lived blooms of certain flagellate groups have been observed in the southern Baltic Sea, including high abundance peaks of *Pedinella* related flagellates (Piwosz & Pernthaler 2010). Various pedinellales sequences related to *Pteridomonas danica* have also been found among our cultures from the south-western Baltic Sea.

In fact, the efficiency to capture protists in culture largely depends on the trophic status of the habitat under study and seems to be most challenging for oligotrophic systems, while significantly more promising for eutrophic ones (Caron et al. 1989).

The novelties in the culture collection

Around 11% of our cultures were considered to be novel cultured strains (with a CCM similarity ≤ 96%). For these strains and even for some cultures, which were initially not considered to be novel, the taxonomic assignment by BLAST searches and KeyDNATools turned out to be insufficient. Therefore, sequences with a doubtful assignment were subjected to phylogenetic tree reconstruction in order to reveal their exact affiliation.

Within chrysophytes (Fig. 6), which comprised the largest group in our culture collection, we found 12 cultures that were misannotated by BLAST searches. In the phylogenetic tree, 7 cultures branched off together with environmental sequences in clade J, although the BLAST results suggested them to either belong to clade F2 related to the genus *Paraphysomonas* or to clade A related to species of *Mallomonas* and *Tesselaria.* Interestingly, one sequence of clade J was formerly assumed to be a second minor functional 18S rRNA gene variation of an *Oikomonas* strain (Cavalier-Smith & Chao 2006), probably due to sparse data of environmental sequences. However, most strains, including the cryptic *Oikomonas* sequence, most likely represent a novel taxon on species or even genus level that does not belong to the highly divergent *Oikomonas* species within clade B2. Another 4 sequences belonged to clade C forming a cluster distant to cultured representatives and sister to *Dinobryon faculiferum* whereas BLAST searches suggest these sequences to belong to the genera *Pedospumella*, *Dinobryon* and *Chrysolepidomonas*, respectively. Within the clade F1 one sequence (L35) that was supposed to belong to *Paraphysomonas imperforata* showed a long branch somehow related to *Paraphysomonas bandaiensis* (Fig. 6). Astonishingly, most of the sequences that showed a disagreement between BLAST annotations and their placement in the phylogenetic tree had similarity values above 97% to their closest cultured representatives. This indicates misannotation to occur even with higher similarity values, at least within chrysophytes, and proves our novelty cutoff to be rather conservative. Moreover, the newly cultured strains branching in clade J and C are closely related to sequences from clone libraries of the same sampling site (Heiligendamm) (Weber et al. 2012).

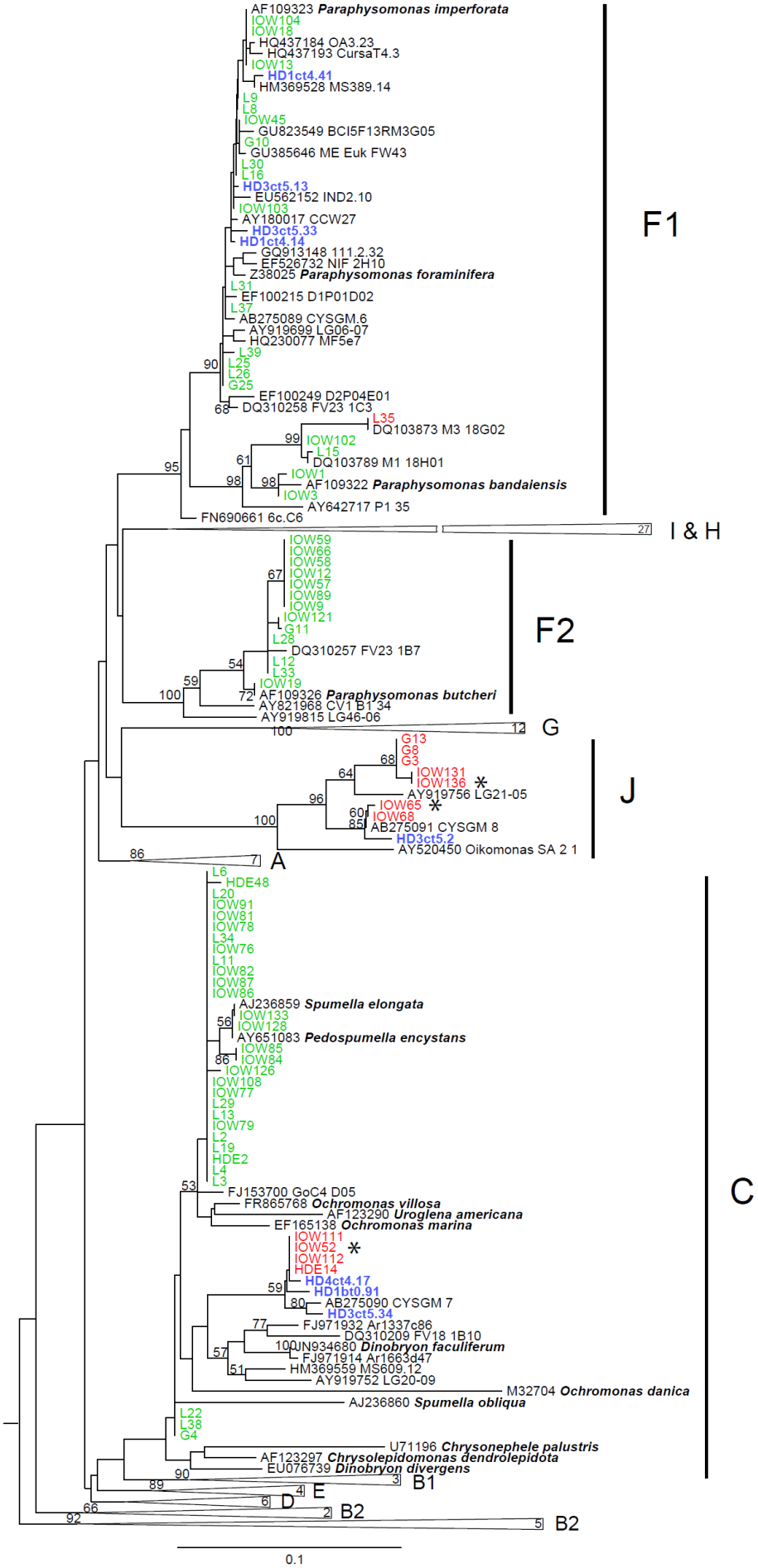
AF109323 Paraphysomonas imperforata
IOW104
IOW18
HQ437184 OA3.23
HQ437193 CursaT4.3
IOW13
HD1ct4.41
HM369528 MS389.14
L9
L8
IOW45
GU823549 BCI5F13RM3G05
G10
GU385646 ME Euk FW43
L30
L16
HD3ct5.13
EU562152 IND2.10
IOW103
AY180017 CCW27
HD3ct5.33
HD1ct4.14
GQ913148 111.2.32
EF526732 NIF 2H10
Z38025 Paraphysomonas foraminifera
L31
EF100215 D1P01D02
L37
AB275089 CYSGM.6
AY919699 LG06-07
HQ230077 MF5e7
L39
L25
L26
G25
EF100249 D2P04E01
DQ310258 FV23 1C3
L35
DQ103873 M3 18G02
IOW102
L15
DQ103789 M1 18H01
IOW1
AF109322 Paraphysomonas bandaiensis
IOW3
AY642717 P1 35
FN690661 6c.C6
F1
I & H
IOW59
IOW66
IOW56
IOW12
IOW57
IOW89
IOW9
IOW121
G11
L28
DQ310257 FV23 1B7
L12
L33
IOW19
AF109326 Paraphysomonas butcheri
AY821968 CV1 B1 34
AY919815 LG46-06
F2
G
G13
G8
G3
IOW131
IOW136
AY919756 LG21-05
IOW65
IOW68
AB275091 CYSGM 8
HD3ct5.2
AY520450 Oikomonas SA 2 1
J
A
L6
HDE48
L20
IOW91
IOW81
IOW78
L34
IOW76
L11
IOW82
IOW87
IOW86
AJ236859 Spumella elongata
IOW133
IOW128
AY651083 Pedospumella encystans
IOW85
IOW84
IOW126
IOW108
IOW77
L29
L13
IOW79
L2
L19
HDE2
L4
L3
FJ153700 GoC4 D05
FR865768 Ochromonas villosa
AF123290 Uroglena americana
EF165138 Ochromonas marina
IOW111
IOW52
IOW112
HDE14
HD4ct4.17
HD1bt0.91
AB275090 CYSGM 7
HD3ct5.34
FJ971932 Ar1337c86
DQ310209 FV18 1B10
JN934680 Dinobryon faculiferum
FJ971914 Ar1663d47
HM369559 MS609.12
AY919752 LG20-09
M32704 Ochromonas danica
AJ236860 Spumella obliqua
C
L22
L38
G4
U71196 Chrysonephele palustris
AF123297 Chrysolepidomonas dendrolepidota
EU076739 Dinobryon divergens
B1
E
D
B2
B2
0.1

Fig. 6. Phylogenetic affiliation of cultures related to chrysophytes. Bayesian phylogenetic tree constructed with 199 partial and complete 18S rRNA sequences. The clades where none of our cultures were represented are collapsed with the number of sequences given in the triangles. Culture sequences from this study appear in green and red, indicating valid and invalid previous BLAST annotations, respectively. Culture sequences marked with a star had a CCM similarity below 96%. Sequences in blue derive from clone libraries of unamended seawater incubation experiments with the same water sample. The tree was rooted by the two diatoms *Skeletonema costatum* and *Chaetoceros rostratus* (not shown). Bootstrap values above 50% are shown. The scale bar represents a distance of 0.1 substitutions per site.

Among the Heiligendamm collection, 4 cercozoan cultures are very distantly related to cultured cercozoan species (88-90% sequence similarity). One was affiliated within an environmental thecofilosean cluster representing a potential novel genus related to the Ventricleftida (sensu lato) (Fig. 7).

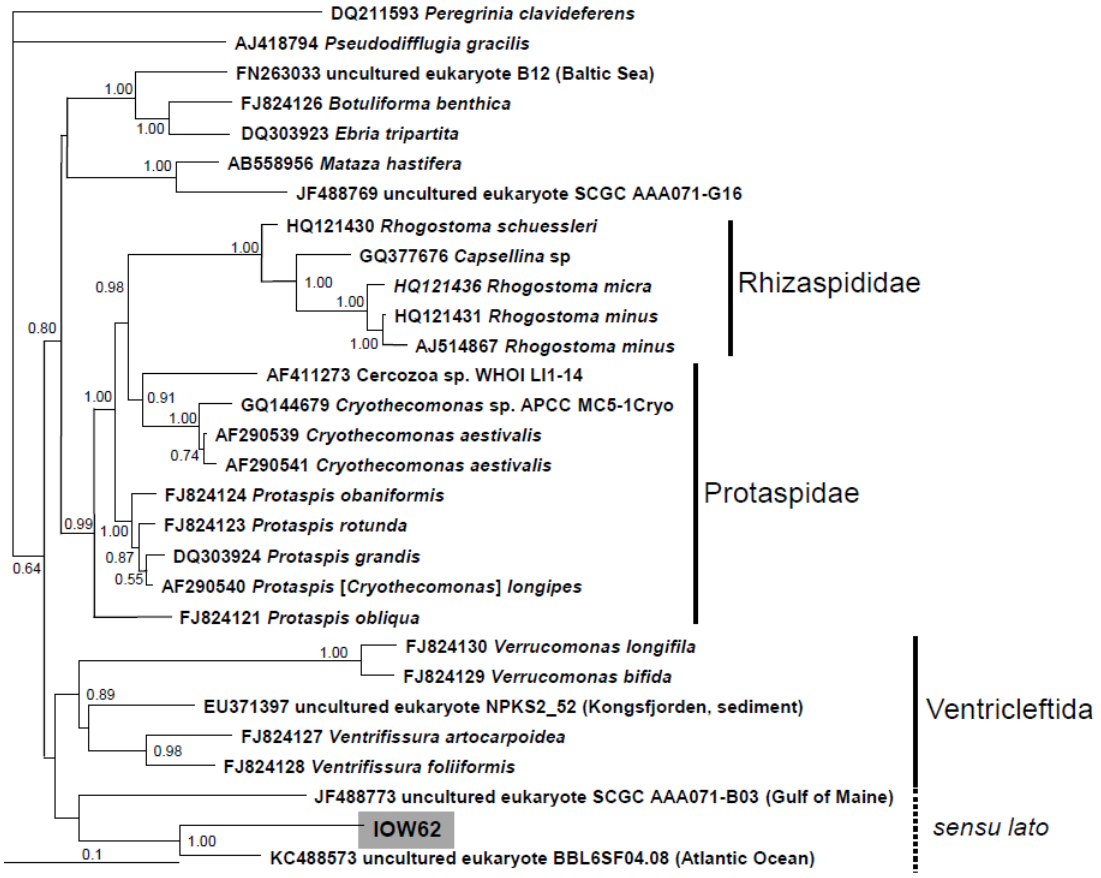

Fig. 7. Phylogenetic affiliation of strain IOW62 within the Thecofilosea (Cercozoa). Bayesian phylogenetic tree constructed with 29 partial and complete 18S rRNA sequences. The tree was rooted by the imbricatean *Peregrinia clavideferns* (Cercozoa). Posterior probability values above 0.5 are shown. The scale bar represents a distance of 0.1 substitutions per site.

The other three identical cultures belonged to the Granofilosea related to *Massisteria marina* (see Fig. 22 in Mylnikov et al. 2015). Based on further electron microscopical investigations of these cultures, we described the new species *Massisteria voersi*, with IOW137 as the type strain (Mylnikov et al. 2015). The fact that *M. voersi* was repeatedly isolated at Heiligendamm in 2003 and 2008 could indicate this species to be a permanent member of this coastal and possibly other sites in the Baltic Sea.

Another culture (HD4-f-t4.18) belonged to the goniomonads, which comprise exclusively phagotrophic flagellates with a sister relationship to cryptophytes. Goniomonads are phylogenetically clearly divided into freshwater and marine taxa that probably differ substantially in their physiology which has prevented either marine or freshwater species to effectively colonize dissimilar habitats since several hundred million years (von der Heyden et al. 2004). Our phylogenetic analysis suggests the culture to be a novel species within the marine *Goniomonas* clade that is obviously able to thrive under brackish water conditions (Fig. 8). Another candidate (HD3-f-t6.7) for a novel genus was found to be affiliated within the glaucophytes (Fig. 8).

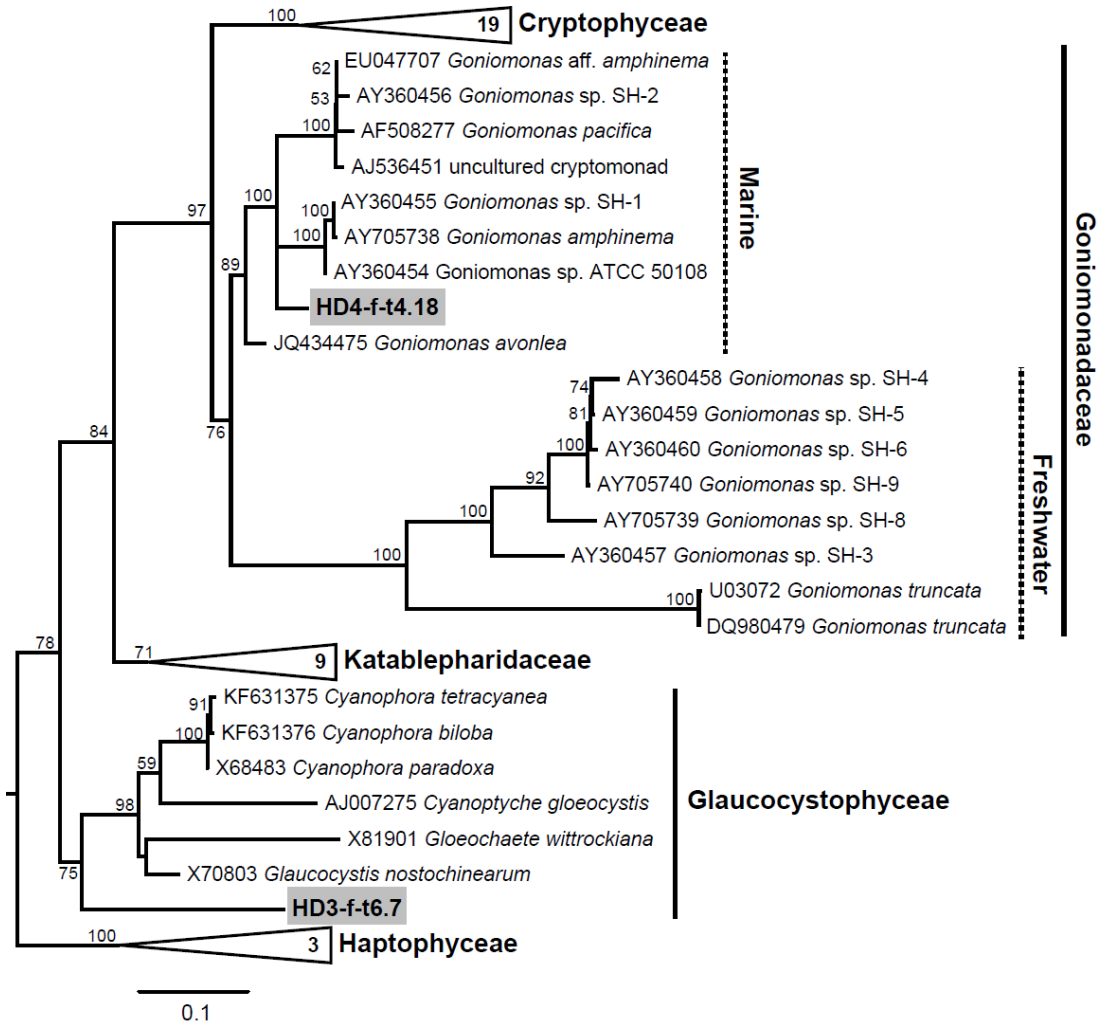

Fig. 8. Phylogenetic affiliation of two novel taxa within the goniomonads and the glaucophytes. Bayesian phylogenetic tree constructed with 56 partial and complete 18S rRNA sequences. The clades of cryptophytes, kathablepharids and haptophytes are collapsed with the number of sequences given in the triangles. The tree was rooted by the bicosoecid *Cafeteria roenbergensis* (not shown). Bootstrap values above 50% are shown. The scale bar represents a distance of 0.1 substitutions per site.

Within strains affiliated with the genus *Rhynchomonas*, one new species was detected, whereas two other strains were closely related to *R. nausea*. The 18S rRNA gene of the new species (strain IOW60) is only 93% similar to the numerous *R. nausea* strains in GenBank (Fig. 9), whereas the morphology resembles a typical *Rhynchomonas* species.

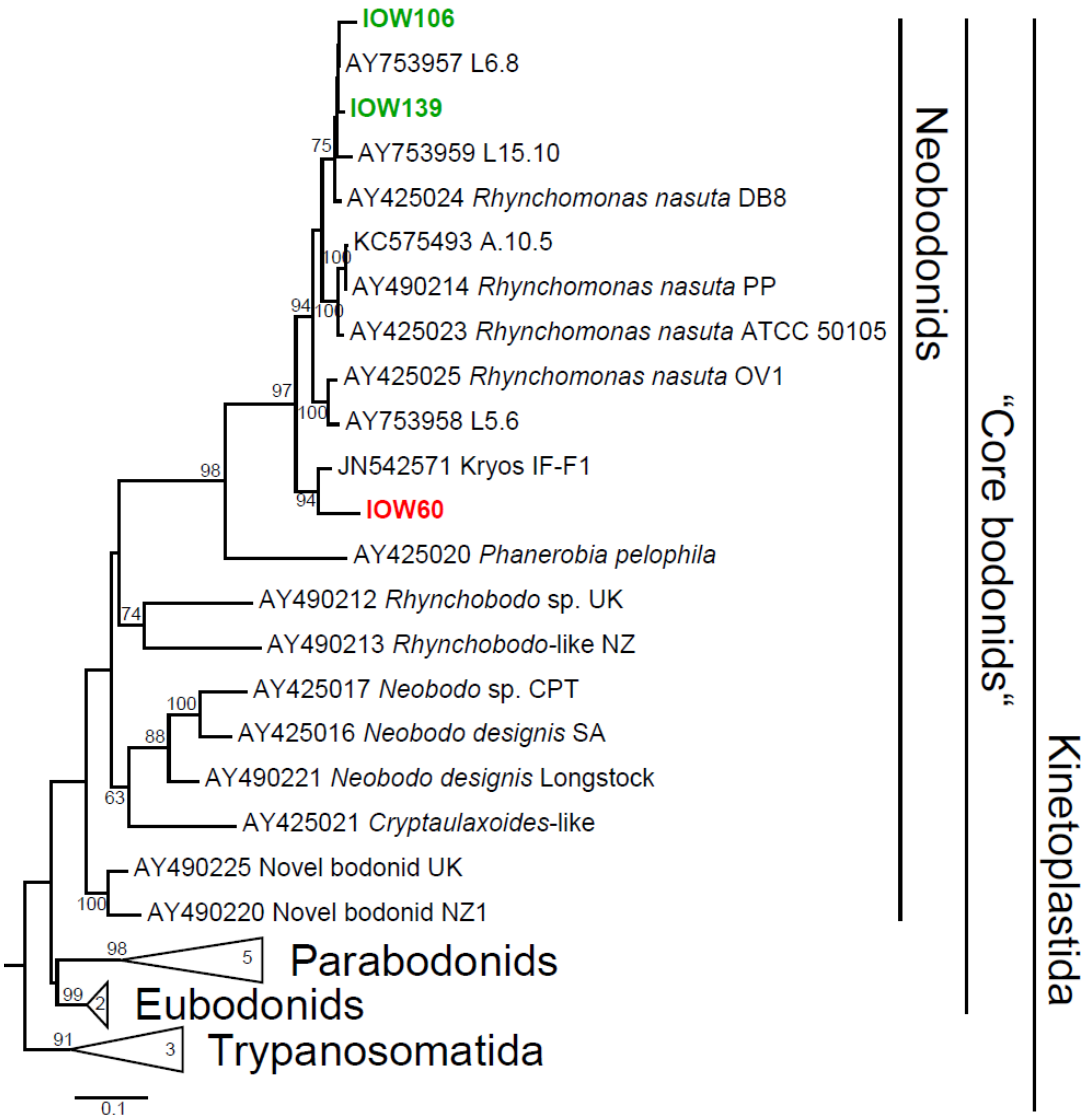

Fig. 9. Phylogenetic affiliation of 3 cultures related to *Rhynchomonas*. Bayesian phylogenetic tree constructed with 33 partial and complete 18S rRNA sequences. The clades of parabodonids, eubodonids and Trypanosomatida are collapsed with the number of sequences given in the triangles. Culture sequences from this study appear colored, green for close relatives of *R. nasuta* and red for the novel taxon. The tree was rooted by the diplonemid *Diplonema ambulator* (not shown). Bootstrap values above 50% are shown. The scale bar represents a distance of 0.1 substitutions per site.

Choanoflagellate related cultures were exclusively obtained from the sampling campaigns in the central Baltic Sea. Among them, 6 strains that were distantly related to cultured representatives were isolated from the redoxclines of the Gotland and Landsort Deep. Based on this cultured material, the two novel Craspedida species *Codosiga balthica* and *Codosiga minima* were erected (Wylezich et al. 2012). In sampling campaigns from several years and using cultivation independent techniques (clone libraries and DGGE), *C. balthica* was detected in redoxclines of the central Baltic Sea (Stock et al. 2009, Anderson et al. 2013, Weber et al. 2014), and it was estimated that this species accounted for about 10% of the whole 18S rRNA amplicon abundance (Weber et al. 2014). Therefore, *C. balthica* seems to be an abundant and permanent member of the protistan community in these zones.

Conclusions

An extensive cultivation effort with coastal and central Baltic Sea water samples yielded a majority of protist taxa which belonged to species of well known and fast growing laboratory cultures. Among them, several species, e.g., of the genera *Paraphysomonas, Pedospumella* and *Procryptobia*, were isolated from fully oxygenated to suboxic and even sulfidic waters, indicating them to be highly tolerant towards different oxygen conditions. However, the remarkable share of phylotypes between these two sampling sites, so different in their environmental conditions (e.g. oxygen conditions), likely implies the effect of culturing bias. Nevertheless, exceeding all expectations, novel protistan taxa at the species and even genus level contributed with 11% to our culture collection and comprised the groups of chrysophytes, cercozoans, goniomonads, glaucocystophytes, bodonids and choanoflagellates. Some 18S rRNA gene sequences of these new cultures lack any closely affiliated counterparts in GenBank and thus probably hold evolutionary key positions in the phylogenetic tree and provide new insights into a hidden protistan diversity. Among both, the "easy to culture" protists as well as the novel taxa, there is some evidence that ecologically relevant representatives have been found (e.g., *P. imperforata*, novel chrysophytes, choanoflagellates). Nevertheless, further investigations using fluorescently labeled oligonucleotide probes are needed to reveal the abundance and distribution of the cultured taxa in the environment. Although the cultivation of protists represents a laborious task with a high failure rate, we encourage other researchers to follow such approaches, or to develop new strategies to increase its eligibility. Pure protist cultures are indeed an important biological resource and represent the gold standard to further study their trophic modes, their interactions with other microbes and their impact on the global biogeochemical cycles.

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Table S1. Overview of cultures obtained from the Baltic Sea with sampling date, location (HD, Heiligendamm, BS-GD, Gotland Deep central Baltic Sea, BS-LD, Landsort Deep central Baltic Sea) and depths. Primers used are indicated (18S-1630Rev (Wylezich & Jürgens 2011), 590F, 600R, 1280F, 1300R (Wylezich et al. 2002), EukA, EukB (Medlin et al. 1988, Euk1A, Euk516R (Diéz et al. 2001), 25F (Bass& Cavalier-Smith 2004), 528F (Elwood et al. 1985), 18SFor-n2, 18SRev-Ch (Wylezich et al. 2012), 18S-Rev2 (Iwashita 2000), 18Sfor-Bodo, 18Srev-Bodo (Scheckenbach et al. 2005), Kineto14f (von der Heyden et al. 2004), EK-82F (Lopéz-García et al. 2001). The closest cultured and environmental matches are given with taxonomic assignment, similarity and accession number.

Culture	Isol. Date	Location	Source	PCR primers	Sequencing primers	Bp length	Closest cultured match (CCM)	Similarity	Accesion Nr.	Group	Closest environmental match (CEM)	Similarity	Accesion Nr.
IOW1	11.11.2003	HD	surface	EukA, 18S-Rev2	25F	570	*Paraphysomonas bandaiensis*	98,60%	AF109322	Chrysophyceae	BIO10_F1	99,47%	FN598224
IOW3	04.11.2003	HD	surface	EukA, 18S-Rev2	25F	570	*Paraphysomonas bandaiensis*	98,77%	AF109322	Chrysophyceae	BIO10_F1	99,65%	FN598224
IOW8	04.11.2003	HD	surface	528F, 18S-1630Rev	18S-1630Rev	539	*Pteridomonas danica*	98,89%	L37204	Pedinellales	MPE1-37	99,44%	AB695480
IOW9	11.11.2003	HD	surface	EukA, Euk516r	Euk516r	450	*Paraphysomonas butcheri*	99,33%	AF109326	Chrysophyceae	ws_143, clone 1809C12	99,56%	FR874662
IOW12	04.11.2003	HD	surface	EukA, 18S-Rev2	25F 528f	1020	*Paraphysomonas butcheri*	100,00%	JQ967291	Chrysophyceae	ws_143, clone 1809C12	99,22%	FR874662
IOW13	04.11.2003	HD	surface	EukA, EukB	528f	563	*Paraphysomonas imperforata* strain VS1	99,82%	AF109323	Chrysophyceae	clone RA5.13	99,82%	KC147387
IOW15	11.11.2003	HD	surface	590F, 18S-1630rev	528F	959	*Cafeteria roenbergensis*	100,00%	FJ032655	Bicosoecida	clone OC4.19	99,90%	EF620525
IOW16	11.11.2003	HD	surface	528F, 18S-1630rev	528F, 18S-1630Rev	928	*Cafeteria roenbergensis*	100,00%	FJ032655	Bicosoecida	OC4.19	99,89%	EF620525
IOW18	04.11.2003	HD	surface	EukA, EukB	528f	484	*Paraphysomonas imperforata* clone C1	100,00%	EF432518	Chrysophyceae	clone RA5.13	100,00%	KC147387
IOW19	04.11.2003	HD	surface	EukA, 18S-Rev2	528f	210	*Paraphysomonas butcheri*	100,00%	AF109326	Chrysophyceae	ws_143, clone 1809C12	99,05%	FR874662
IOW23	04.11.2003	HD	surface	EukA, Euk516r	18S-1630Rev	862	*Cafeteria roenbergensis*	100,00%	FJ032655	Bicosoecida	OC4.19	100,00%	EF620525
IOW25	04.11.2003	HD	surface	EukA, Euk516r and 25F, 18S-Rev2	Euk516r	482	*Pteridomonas danica*	99,59%	AB081640	Pedinellales	MPE1-40	99,59%	AB695483
IOW28	11.11.2003	HD	surface	EukA, Euk516r	EukA	532	*Procryptobia sorokini* strain HFCC16	99,63%	DQ207592	Bodonidae	band B49-5602	100,00%	JF274001
IOW45	11.11.2003	HD	surface	EukA, EukB	528f	516	*Paraphysomonas imperforata* clone C1	99,42%	EF432518	Chrysophyceae	clone SGTB417	99,81%	HQ866893
IOW46	04.11.2003	HD	surface	590F, 18S-1630Rev	528F	958	*Cafeteria roenbergensis*	100,00%	FJ032655	Bicosoecida	clone OC4.19	99,90%	EF620525
IOW50	11.11.2003	HD	surface	18SFor-n2, 18SRev-Ch (nested)	18S-1630Rev	1067	*Cafeteria roenbergensis*	100,00%	FJ032655	Bicosoecida	OC4.19	99,91%	EF620525
IOW52	18.11.2003	HD	surface	EukA, EukB	528f	467	*Dinobryon faculiferum* strain RCC2293	95,50%	JN934680	Chrysophyceae	clone SGSP545	100,00%	HQ866180
IOW54	25.11.2003	HD	surface	EukA, 18S-Rev2	25F	645	*Ciliophrys infusionum*	97,21%	L37205	Pedinellales	clone N4aC37	89,10%	EU333107
IOW55	24.11.2003	HD	surface	528F, 18S-1630Rev	25F	451	*Cafeteria roenbergensis*	100,00%	FJ032655	Bicosoecida	BS16_E11	100,00%	FN598363
IOW57	24.11.2003	HD	surface	EukA, Euk516r	EukA	506	*Paraphysomonas butcheri*	99,21%	AF109326	Chrysophyceae	ws_143, clone 1809C12	99,41%	FR874662
IOW58	24.11.2003	HD	surface	EukA, EukB	528f	530	*Paraphysomonas butcheri* isolate MD03	100,00%	JQ967291	Chrysophyceae	clone SHAH451	99,43%	HQ867832
IOW59	24.11.2003	HD	surface	EukA, EukB	528f	509	*Paraphysomonas butcheri* isolate MD03	100,00%	JQ967291	Chrysophyceae	clone SHAH451	99,41%	HQ867832
IOW60	24.11.2003	HD	surface	EukA, Euk516r and 18Sfor-Bodo, 18Srev-Bodo	Euk516r	512	*Rhynchomonas nasuta* strain HFCC319	93,32%	DQ207598	Bodonidae	Kryos_IF_F1	95,31%	JN542571
IOW61	24.11.2003	HD	surface	EukA, Euk516r	2x EukA	533	*Procryptobia sorokini* strain HFCC16	99,81%	DQ207592	Bodonidae	DGGE gel band B49-5602	100,00%	JF274001
IOW62	24.11.2003	HD	surface	EukA, Euk516r and Euk1A, EukB	1300R, 590F, 1280F, 18S-For-n2, 18S-Rev-Ch, 600R	1700	*Cryothecomonas longipes*	89,78%	AF290540	Cercozoa	BBL6SF04.08	92,00%	KC488573
IOW64	24.11.2003	HD	surface	EukA, Euk516r	Euk516r	537	*Massisteria marina* strain GBB2	89,17%	AF174370	Cercozoa	clone BS19_A3	89,57%	FN598382
IOW65	24.11.2003	HD	surface	528F, 18S-1630Rev and 25F, 18S-Rev2	EukA, 25f, 528f, 18S-1630Rev, Euk516r, 18SRev2, 1280F	1742	*Mallomonas annulata*	94,09%	U73230	Chrysophyceae	TKR07M.39	96,90%	GU290095
IOW66	24.11.2003	HD	surface	EukA, EukB	528f	524	*Paraphysomonas butcheri* isolate MD03	100,00%	JQ967291	Chrysophyceae	clone SHAH451	99,43%	HQ867832
IOW68	24.11.2003	HD	surface	18SFor-n2, 18SRev-Ch (nested)	25F	285	*Mallomonas mangofera var. foveata* strain DMJMMAFo	97,19%	JX946338	Chrysophyceae	clone 268E12	100,00%	KJ925198
IOW100	28.10.2008	HD	surface	18Sfor-Bodo, 18S-1630Rev	1300R	801	*Neobodo designis* isolate Auckland	99,75%	AY753628	Bodonidae	clone L14.7	98,50%	AY753966
IOW101	18.11.2008	HD	surface	Euk1A, EukB	For-n2, Rev-Ch	1847	*Massisteria marina* strain GBB2	87,76%	AF174370	Cercozoa	clone LC103_5EP_6	88,28%	DQ504340
IOW102	28.10.2008	HD	surface	18SFor-n2, 18S-1630Rev (nested)	25F	1057	*Paraphysomonas bandaiensis*	97,73%	AF109322	Chrysophyceae	clone SA2_2D5	99,53%	EF527168
IOW103	28.10.2008	HD	surface	25F, 18S-Rev2	1300R	705	*Paraphysomonas imperforata* clone D1	99,43%	EF432519	Chrysophyceae	ws_101, clone 1807D03	100,00%	FR874603
IOW104	28.10.2008	HD	surface	EukA, EukB	EukA	696	*Paraphysomonas imperforata* clone D1	100,00%	EF432519	Chrysophyceae	ws_101, clone 1807D03	99,71%	FR874603
IOW106	28.10.2008	HD	surface	25F, 18S-Rev2	Euk516r	491	*Rhynchomonas nasuta* strain HFCC322	97,57%	DQ207599	Bodonidae	clone L15.10	98,17%	AY753959
IOW108	28.10.2008	HD	surface	EukA, EukB	EukA	339	*Ochromonas villosa*	99,41%	FR865768	Chrysophyceae	clone BT_Euk_C5	100,00%	KC306554
IOW109	28.10.2008	HD	surface	EukA, 18S-Rev2	1280F EukA	789	*Pedospumella encystans*	99,62%	AY651083	Chrysophyceae	clone: 10182008-Euk18S-Clone9	99,62%	AB520724
IOW110	28.10.2008	HD	surface	EukA, EukB and EK-82f, 18S-1630Rev	EukB	902	*Pedospumella encystans*	97,56%	AY651083	Chrysophyceae	CYSGM-7	99,78%	AB275090
IOW111	28.10.2008	HD	surface	18SFor-n2 18S-Rev-Ch (nested)	25F EK-82f 1630f	1475	*Pedospumella encystans*	97,36%	AY651083	Chrysophyceae	CYSGM-7	98,98%	AB275090
IOW112	28.10.2008	HD	surface	EukA, EukB	EukA	918	*Chrysolepidomonas dendrolepidota*	97,28%	AF123297	Chrysophyceae	clone LA8E2G5	98,26%	JF730832
IOW113	04.11.2008	HD	surface	EukA, Euk516r	Euk516r	516	*Acanthamoeba castellanii* strain ATCC 30011	100,00%	KF318462	Amoebozoa	clone TWII-I-42C-3e	99,61%	JF775336
IOW114	04.11.2008	HD	surface	25F, 18S-Rev2	Euk516r	560	*Acanthamoeba castellanii* ATCC 50374	100,00%	U07413	Amoebozoa	clone TWII-I-30C-3g	99,64%	JF775294
IOW121	11.11.2008	HD	surface	EukA, EukB	EukA	965	*Paraphysomonas butcheri*	99,38%	AF109326	Chrysophyceae	ws_143, clone 1809C12	99,59%	FR874662
IOW122	18.11.2008	HD	surface	EukA, 18S-Rev2	25F 1300R	1175	*Pteridomonas danica*	99,74%	L37204	Pedinellales	MPE1-40	98,64%	AB695483
IOW124	18.11.2008	HD	surface	EukA, 18S-Rev2	25F 1300R	1187	*Pteridomonas danica*	97,73%	L37204	Pedinellales	CYSGM-5	99,58%	AB275088
IOW126	18.11.2008	HD	surface	EukA, 18S-Rev2	25F	376	*Pedospumella encystans*	99,73%	AY651083	Chrysophyceae	clone BT_Euk_C5	99,73%	KC306554
IOW128	18.11.2008	HD	surface	25F, 18SRev-Ch	1300R	835	*Pedospumella encystans*	100,00%	AY651083	Chrysophyceae	Cn-St.1-51	99,76%	AB749100
IOW131	18.11.2008	HD	surface	EukA, 18S-Rev2	1300R	509	*Tessellaria lapponica*	97,45%	HF549063	Chrysophyceae	SIFG675_N9D4_18S_E	99,41%	LN587906
IOW133	18.11.2008	HD	surface	EukA, 18S-Rev2	25F	1014	*Pedospumella encystans*	100,00%	AY651083	Chrysophyceae	905st23-30	99,41%	JQ782092
IOW135	18.11.2008	HD	surface	EukA, 18S-Rev2	25F	831	*Cafeteria roenbergensis*	99,88%	AF174364	Bicosoecida	clone BS16_E11	99,88%	FN598363
IOW136	18.11.2008	HD	surface	EukA, 18S-Rev2	25F	959	*Mallomonas mangofera var. foveata* strain DMJMMAFo	94,47%	JX946338	Chrysophyceae	MIF_CilE6	97,29%	EF526986
IOW 137	11.11.2008	HD	surface	Euk1A, EukB	For-n2, Rev-Ch	1847	*Massisteria marina* strain GBB2	87,76%	AF174370	Cercozoa	clone LC103_5EP_6	88,28%	DQ504340
IOW138	11.11.2008	HD	surface	EukA, EukB and Ek-82f, 18SR1300	EK-82F	780	*Pteridomonas danica*	97,05%	L37204	Pedinellales	CYSGM-5	99,62%	AB275088
IOW139	28.10.2008	HD	surface	Kineto14f, 18S-1630Rev	1630R	929	*Rhynchomonas nasuta* strain HFCC316	99,14%	DQ207597	Bodonidae	clone L6.8	99,14%	AY753957
HD1ct4.24	28.10.2008	HD	surface	EukA, EukB	EukA	277	*Ochromonas villosa*	99,28%	FR865768	Chrysophyceae	clone BT_Euk_C5	100,00%	KC306554
HD2ct5.12	04.11.2008	HD	surface	EukA, Euk516r	Euk516r	504	*Pteridomonas danica*	98,81%	L37204	Pedinellales	clone XMAB11	99,60%	DQ667614
HD2ct5.5	04.11.2008	HD	surface	EukA, Euk516r	Euk516r	505	*Pteridomonas danica*	98,81%	L37204	Pedinellales	clone XMAB11	99,60%	DQ667614
HD3ft6.7	11.11.2008	HD	surface	EukA, EukB	EukA	572	*Cyanophora paradoxa*	91,96%	X68483	Glaucocystophyceae	clone MLBA11.21	92,05%	FJ410565
HD4ft4.18	18.11.2008	HD	surface	EK-82f, 18S-1630 and Ek-82f, 18SR1300	EK-82f 590F 600R 1300R	1185	*Goniomonas avonlea*	96,71%	JQ434475	Cryptophyta	clone 5-A1	92,17%	FN689953
HDE48	28.10.2008	HD	surface	EukA, EukB	EukA	261	*Ochromonas villosa*	98,85%	FR865768	Chrysophyceae	clone KF_Euk_E4	99,62%	KC306595

Table S1. Continued.

Culture	Isol. Date	Location	Source	PCR primers	Sequencing primers	Bp length	Closest cultured match (CCM)	Similarity	Accesion Nr.	Group	Closest environmental match (CEM)	Similarity	Accesion Nr.
IOW73	25.05.2005	BS-GD	150 m	528F, 18S-1630Rev, and 18SFor-n2, 18SRev-Ch	528F, 18S-1630Rev, 18SFor-n2, 1280F, 18SRev-Ch, 600R	1748	*Savillea micropora*	88,76%	EU011928	Choanozoa	OLI11041	91,57%	AJ402325
IOW74	25.05.2005	BS-GD	208 m	EukA, 18S-Rev2	25F	1053	*Acanthoeca spectabilis*	87,63%	AF084233	Choanozoa	clone BLACKSEA_cl_56	94,01%	HM749958
IOW75	25.05.2005	BS-LD	261 m	528F, 18S-1630Rev and 18SFor-n2, 18SRev-Ch	25F	996	*Acanthoeca spectabilis*	87,02%	AF084233	Choanozoa	clone BLACKSEA_cl_56	93,76%	HM749958
IOW76	25.05.2005	BS-LD	101 m	EukA, Euk516r	EukA	509	*Ochromonas villosa*	99,41%	FR865768	Chrysophyceae	BT_Euk_C5	100,00%	KC306554
IOW77	25.05.2005	BS-LD	111 m	EukA, Euk516r	EukA	509	*Ochromonas villosa*	99,41%	FR865768	Chrysophyceae	BT_Euk_C5	100,00%	KC306554
IOW78	25.05.2005	BS-LD	116 m	25F, 18SRev-Ch	528f	529	*Pedospumella encystans* isolate JBM/S11	98,87%	AY651083	Chrysophyceae	clone 905st23-30	100,00%	JQ782092
IOW78	25.05.2005	BS-LD	116 m	EukA, EukB	1300R	769	*Pedospumella encystans*	99,22%	AY651083	Chrysophyceae	clone 905st23-30	100,00%	JQ782092
IOW79	25.05.2005	BS-LD	5 m	EukA, Euk516r	EukA	509	*Ochromonas villosa*	99,41%	FR865768	Chrysophyceae	BT_Euk_C5	100,00%	KC306554
IOW81	25.05.2005	BS-GD	195 m	528F, 18S-1630Rev	18S-1630Rev, 18S-Rev2	610	*Pedospumella encystans*	99,67%	AY651083	Chrysophyceae	clone: 10182008-Euk18S-Clone18	100,00%	AB520733
IOW82	25.05.2005	BS-LD	11 m	EukA, Euk516r	EukA	509	*Ochromonas villosa*	99,41%	FR865768	Chrysophyceae	BT_Euk_C5	100,00%	KC306554
IOW84	25.05.2005	BS-LD	11 m	EukA, 18S-Rev2	528f	520	*Pedospumella encystans* isolate JBM/S11	98,85%	AY651083	Chrysophyceae	clone KF_Euk_F4	99,81%	KC306600
IOW84	25.05.2005	BS-LD	11 m	EukA, EukB	25F	1062	*Pedospumella encystans*	99,44%	AY651083	Chrysophyceae	KF_Euk_F4	99,72%	KC306600
IOW85	25.05.2005	BS-LD	71 m	EukA, 18S-Rev2	25F	995	*Pedospumella encystans*	99,40%	AY651083	Chrysophyceae	clone KF_Euk_F4	99,70%	KC306600
IOW86	25.05.2005	BS-GD	204 m	EukA, 18S-Rev2	25F	1058	*Pedospumella encystans*	99,43%	AY651083	Chrysophyceae	clone 905st23-30	100,00%	JQ782092
IOW87	25.05.2005	BS-GD	195 m	528F, 18S-1630Rev	528F, 18S-1630Rev	973	*Pedospumella encystans*	99,18%	AY651083	Chrysophyceae	clone 905st23-30	99,90%	JQ782092
IOW88	25.05.2005	BS-GD	207 m	EukA, Euk516r	EukA	504	*Pseudocohnilembus persalinus* strain QD 5	99,80%	JQ956554	Ciliophora	clone CA13	99,01%	KC922162
IOW89	25.05.2005	BS-LD	126 m	EukA, Euk516r	EukA	507	*Paraphysomonas butcheri*	99,21%	AF109326	Chrysophyceae	ws_143, clone 1809C12	99,41%	FR874662
IOW91	25.05.2005	BS-GD	212 m	528F, 18S-1630Rev	528F, 18S-1630Rev	971	*Pedospumella encystans*	99,18%	AY651083	Chrysophyceae	clone 905st23-30	99,90%	JQ782092
IOW92	25.05.2005	BS-GD	208 m	528F, 18S-1630Rev	528F- 18S-1630Rev	1272	*Bodo saltans* strain HFCC14	100,00%	DQ207571	Bodonidae	Bodo sp. TGS2	97,80%	AB585965
IOW93	25.05.2005	BS-GD	208 m	EukA, 18S-Rev2	25F	760	*Monosiga brevicollis*	87,26%	KM387288	Choanozoa	clone BLACKSEA_cl_56	92,83%	HM749958
IOW94	25.05.2005	BS-GD	207 m	EukA, Euk516r and 18S-For-n2, 18S-Rev-Ch	EukA, 18SFor-n2, 590F, 1280F, 18SRev-Ch, 600R, 1300R	1783	*Monosiga brevicollis*	92,20%	KM387288	Choanozoa	NA1_3C12	99,37%	EF526833
G10	16.06.2012	BS-GD	80 m	EukA, Euk516r	Euk516r	488	*Paraphysomonas imperforata* clone C1	100,00%	EF432518	Chrysophyceae	clone SGYT1294	100,00%	KJ761892
G11	16.06.2012	BS-GD	80 m	EukA, Euk516r and 25F, Euk516r	Euk516r x2	482	*Paraphysomonas butcheri*	99,17%	AF109326	Chrysophyceae	ws_143, clone 1809C12	99,17%	FR874662
G12	16.06.2012	BS-GD	80 m	EukA, Euk516r	Euk516r	498	*Bodo sorokini* strain ATCC 50641	99,80%	AY425018	Bodonidae	DGGE gel band B49-5602	99,80%	JF274001
G13	16.06.2012	BS-GD	80 m	EukA, Euk516r	Euk516r	467	*Paraphysomonas butcheri*	97,01%	AF109326	Chrysophyceae	clone LG21-05	99,36%	AY919756
G18	16.06.2012	BS-GD	120 m	EukA, Euk516r	Euk516r	427	*Choricystis minor*	100,00%	X89012	Chlorophyta	clone A0Esp_2_11	100,00%	KC911759
G20	16.06.2012	BS-GD	80 m	EukA, Euk516r	Euk516r	523	*Procryptobia sorokini* strain HFCC98	100,00%	DQ207593	Bodonidae	DGGE gel band B49-5602	99,81%	JF274001
G21	16.06.2012	BS-GD	80 m	EukA, Euk516r	Euk516r	524	*Bodo sorokini* strain ATCC 50641	99,81%	AY425018	Bodonidae	DGGE gel band B49-5602	99,81%	JF274001
G22	16.06.2012	BS-GD	80 m	EukA, Euk516r	Euk516r	473	*Bodo sorokini* strain ATCC 50641	99,79%	AY425018	Bodonidae	DGGE gel band B49-5602	99,79%	JF274001
G23	16.06.2012	BS-GD	80 m	EukA, Euk516r	Euk516r	524	*Bodo sorokini* strain ATCC 50641	99,81%	AY425018	Bodonidae	DGGE gel band B49-5602	99,81%	JF274001
G24	16.06.2012	BS-GD	120 m	EukA, Euk516r	Euk516r	463	*Bodo sorokini* strain ATCC 50641	99,78%	AY425018	Bodonidae	DGGE gel band B49-5602	99,78%	JF274001
G25	16.06.2012	BS-GD	120 m	EukA, Euk516r	Euk516r	489	*Paraphysomonas imperforata* clone C1	100,00%	EF432518	Chrysophyceae	clone SGYT1294	100,00%	KJ761892
G26	16.06.2012	BS-GD	120 m	EukA, Euk516r	Euk516r	472	*Caecitellus parvulus*	99,79%	AY520446	Bicosoecida	clone BK322	100,00%	GU433132
G3	16.06.2012	BS-GD	80 m	Euk1A, Euk516r	EukA	498	*Paraphysomonas butcheri*	96,59%	AF109326	Chrysophyceae	clone LG21-05	99,00%	AY919756
G4	16.06.2012	BS-GD	80 m	Euk1A, Euk516r	EukA	505	*Ochromonas villosa*	99,01%	FR865768	Chrysophyceae	clone Es8	99,80%	JX645154
G5	16.06.2012	BS-GD	80 m	Euk1A, Euk516r	EukA	493	*Pseudocohnilembus persalinus*	99,80%	AY835669	Ciliophora	clone CA13	99,39%	KC922162
G6	16.06.2012	BS-GD	80 m	Euk1A, Euk516r	EukA	473	*Procryptobia sorokini* strain HFCC98	100,00%	DQ207593	Bodonidae	clone AT1-3	100,00%	AF530519
G7	16.06.2012	BS-GD	120 m	Euk1A, Euk516r	EukA	481	*Codonosiga gracilis*	90,47%	AY149897	Choanozoa	DGGE gel band OTU 11	100,00%	KF373752
G8	16.06.2012	BS-GD	80 m	EukA, Euk516r	Euk516r	465	*Paraphysomonas butcheri*	97,00%	AF109326	Chrysophyceae	clone LG21-05	99,35%	AY919756
L11	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	499	*Chrysolepidomonas dendrolepidota*	98,40%	AF123297	Chrysophyceae	clone T08S1C3EukS3	100,00%	JF829209
L12	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	475	*Paraphysomonas butcheri*	99,79%	AF109326	Chrysophyceae	ws_143, clone 1809C12	100,00%	FR874662
L13	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	448	*Chrysolepidomonas dendrolepidota*	99,11%	AF123297	Chrysophyceae	clone T08S1C3EukS3	100,00%	JF829209
L15	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	451	*Paraphysomonas imperforata* clone C1	97,12%	EF432518	Chrysophyceae	clone BS17_D2	100,00%	FN598370
L16	22.06.2012	BS-LD	105 m	25F, Euk516r	Euk516r	481	*Paraphysomonas imperforata* clone C1	100,00%	EF432518	Chrysophyceae	clone SGYT1294	100,00%	KJ761892
L19	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	448	*Chrysolepidomonas dendrolepidota*	99,11%	AF123297	Chrysophyceae	clone T08S1C3EukS3	100,00%	JF829209
L2	22.06.2012	BS-LD	105 m	Euk1A, Euk516r	EukA	498	*Ochromonas villosa*	99,40%	FR865768	Chrysophyceae	clone BT_Euk_C5	100,00%	KC306554
L20	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	487	*Ochromonas villosa*	99,16%	FR865768	Chrysophyceae	clone T08S1C3EukS3	99,79%	JF829209
L22	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	477	*Chrysolepidomonas dendrolepidota*	99,37%	AF123297	Chrysophyceae	clone B.12.1	99,79%	KC575364
L25	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	489	*Paraphysomonas imperforata* clone C1	100,00%	EF432518	Chrysophyceae	clone SGYT1294	100,00%	KJ761892
L26	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	490	*Paraphysomonas imperforata* clone C1	100,00%	EF432518	Chrysophyceae	clone SGYT1294	100,00%	KJ761892
L27	22.06.2012	BS-LD	105 m	EukA, EukB	Euk516r	492	*Ministeria vibrans strain* ATCC 50519	100,00%	AF271998	Choanozoa	clone G1205-0219	100,00%	KF534594
L28	22.06.2012	BS-LD	105 m	25F, Euk516r	Euk516r	465	*Paraphysomonas butcheri*	99,57%	AF109326	Chrysophyceae	ws_143, clone 1809C12	99,78%	FR874662
L29	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	483	*Ochromonas villosa*	99,37%	FR865768	Chrysophyceae	clone T08S1C3EukS3	100,00%	JF829209
L3	22.06.2012	BS-LD	105 m	Euk1A, Euk516r	EukA	498	*Ochromonas villosa*	99,20%	FR865768	Chrysophyceae	clone BT_Euk_C5	99,80%	KC306554
L30	22.06.2012	BS-LD	78 m	EukA, Euk516r	Euk516r	487	*Paraphysomonas imperforata* clone C1	100,00%	EF432518	Chrysophyceae	clone SGYT1294	100,00%	KJ761892
L31	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	488	*Paraphysomonas imperforata* clone C1	100,00%	EF432518	Chrysophyceae	clone SGYT1294	100,00%	KJ761892
L33	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	476	*Paraphysomonas imperforata clone C1*	97,27%	EF432518	Chrysophyceae	ws_143, clone 1809C12	100,00%	FR874662
L34	22.06.2012	BS-LD	78 m	EukA, Euk516r	Euk516r	484	*Ochromonas villosa*	99,37%	FR865768	Chrysophyceae	clone T08S1C3EukS3	100,00%	JF829209
L35	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	487	*Paraphysomonas imperforata* clone C1	96,53%	EF432518	Chrysophyceae	clone M3_18A12	100,00%	DQ103874
L36	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	460	*Procryptobia sorokini* strain HFCC98	100,00%	DQ207593	Bodonidae	band B49-5602	99,78%	JF274001
L37	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	491	*Paraphysomonas imperforata* clone C1	100,00%	EF432518	Chrysophyceae	clone SGYT1294	100,00%	KJ761892
L38	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	454	*Chrysolepidomonas dendrolepidota*	99,56%	AF123297	Chrysophyceae	clone B.12.1	99,78%	KC575364
L39	22.06.2012	BS-LD	78 m	EukA, Euk516r	Euk516r	441	*Paraphysomonas imperforata* clone C1	99,77%	EF432518	Chrysophyceae	clone SGYT1294	99,77%	KJ761892
L4	22.06.2012	BS-LD	105 m	Euk1A, Euk516r	EukA	494	*Ochromonas villosa*	99,39%	FR865768	Chrysophyceae	clone BT_Euk_C5	100,00%	KC306554
L6	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	486	*Ochromonas villosa*	99,37%	FR865768	Chrysophyceae	clone T08S1C3EukS3	100,00%	JF829209
L8	22.06.2012	BS-LD	105 m	EukA, Euk516r	Euk516r	505	*Paraphysomonas imperforata* clone C1	100,00%	EF432518	Chrysophyceae	clone SGYT1294	100,00%	KJ761892
L9	22.06.2012	BS-LD	78 m	25F, Euk516r	Euk516r	482	*Paraphysomonas imperforata* clone D1	100,00%	EF432519	Chrysophyceae	clone SGYT1294	100,00%	KJ761892
L-C1	22.06.2012	BS-LD		EukA, Euk516r	Euk516r	481	*Euplotes elegans*	99,79%	DQ309868	Ciliophora	clone ME_Euk_FW35	97,63%	GU385637

Chapter 3) *Massisteria marina* has a sister: *Massisteria voersi* sp. nov., a rare species isolated from coastal waters of the Baltic Sea

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Massisteria marina has a sister: *Massisteria voersi* sp. nov., a rare species isolated from coastal waters of the Baltic Sea

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Abstract

For many years, the genus *Massisteria* (Cercozoa, Leucodictyida) comprised only one species, *M. marina*. This small species has a biphasic life cycle and feeds through filose, radiating pseudopodia. It has a distinct swimming form and is regularly detected in association with detritus aggregates. However, environmental sequences closely related to this species indicate a larger species richness than hitherto described for the genus *Massisteria*. Here we provide the first report of *Massisteria voersi* sp. nov., investigated with microscopic and molecular methods. Several strains of this new species were isolated from brackish water at a Baltic Sea coastal monitoring station. Their characteristics are typical of the genus. *Massisteria voersi* differs from *M. marina* by smaller cell size (2.3–3 μm vs. 2.5–9 μm) and absent fused motile cells. Additionally, in contrast to *M. marina*, the new species lacks a paranuclear body and its kinetosomes are arranged in parallel. Both species are quite distantly related regarding their 18S rRNA gene sequences. The sparse availability of environmental sequences closely related to *M. voersi* as well as our preliminary results from fluorescence in situ hybridization studies suggest that this new species is a representative of low-abundance populations comprising the so-called "rare biosphere."

Keywords: Benthic–pelagic coupling; Brackish; Detritus; *Massisteria voersi*; Rare species

Introduction

Heterotrophic nanoflagellates (HNF) are important and abundant components of marine and freshwater ecosystems. They are the main consumers of prokaryotes as well as the link between prokaryotic biomass and higher trophic levels, such as the larger suspension feeders in aquatic food webs (Azam et al. 1983; Fenchel 1982). Detritus particles provide a further potential food source for some protists, either by the microbial prey items associated with the particles or, in the case of detritus compounds with nutrient value, by their direct digestion (Fenchel 1987; Posch and Arndt 1996; Scherwass et al. 2005). Larger detritus aggregates may additionally serve as microhabitats for prokaryotic and eukaryotic microorganisms. An example of a protist that is invariably associated with detritus particles is the marine genus *Massisteria*. This genus, based on its hitherto single known species, *M. marina*, was established by Larsen and Patterson (1990) and electron microscopically investigated by Patterson and Fenchel (1990). *Massisteria marina* alternates between immotile amoeboid and swimming flagellate stages. During the amoeboid stage, the two

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naked flagella are short and relatively inactive whereas in actively swimming cells they are considerably longer and the extrusome-bearing pseudopodia are retracted (Larsen and Patterson 1990). Swimming stages are not often seen in nature but they do appear in culture (Ekebom et al. 1995/96; Larsen and Patterson 1990). The two life stages are thought to be associated with different life strategies: Amoeboid cells attached to detritus particles feed on associated bacteria whereas actively swimming cells can explore other particles and aggregates or migrate to the benthos (Patterson and Fenchel 1990).

The bacterivorous genus *Massisteria* was suggested to be related to the cercomonads because both possess a paranuclear body (Karpov 1997; Patterson and Fenchel 1990; Patterson and Zölffel 1991). However, the radiating, filose pseudopodia of *Massisteria* are often branched and contain granules (extrusomes) dissimilar to those in the pseudopodia of other cercomonads (Mylnikov and Karpov 2004). In 1993, Cavalier-Smith erected the separate family Massisteriidae for this genus belonging to the Cercomonadida within the new phylum Opalozoa (Cavalier-Smith 1993). The first analysis of the 18S rRNA gene of *Massisteria marina* was based on six cultures established from different deep-sea sites. The results corroborated the assumed relationship to cercomonads in the broadest sense (Atkins et al. 2000). Following additional taxon sampling using 18S rRNA gene sequences, a group of sequences clustering with the so-called *Nuclearia*-like filose amoeba strain N-Por (Bhattacharya and Oliveira 2000; Vickerman et al. 2002), now re-named *Nanofila marina* (Bass et al. 2009), and with other cercozoan sequences was identified (Cavalier-Smith and Chao 2003). In 2009, the new class Granofilosea Cavalier-Smith et Bass, 2009 was erected, thus uniting taxa with a paranuclear microbody and with concentric extrusome-bearing filose pseudopodia (Bass et al. 2009). The genus *Massisteria* was included in this class (order Leucodictyida) based on these characters and on a molecular phylogenetic analysis. Howe et al. (2011) discussed the phylogenetic relationship of *Massisteria* within the scope of the affiliation of the newly described genus *Minimassisteria*.

Within 10 years after its first description based on a strain isolated from a tropical marine sediment (Larsen and Patterson 1990), *Massisteria marina* was detected worldwide, in benthic and in pelagic marine habitats. In fact, it was one of the 20 most frequently detected flagellate species in the world (Patterson and Lee 2000). Most of the *Massisteria marina* detections were made microscopically, in samplings from marine waters (Arndt et al. 2003; Atkins et al. 2000; Ekebom et al. 1995/96; Hausmann et al. 2002; Ikävalko and Gradinger 1997; Larsen and Patterson 1990; Patterson and Lee 2000; Patterson and Simpson 1996; Patterson et al. 1993; Tong 1997; Tong et al. 1998), whereas there are fewer reports in which *M. marina* was isolated from lower-salinity to brackish-water sites (Aydin and Lee 2012; Garstecki and Arndt 2000; Ikävalko and Gradinger 1997; Vørs 1992a,b).

In this study, we established several cultures of *Massisteria* sp. from coastal waters of the brackish Baltic Sea and investigated the strains genetically. The type strain was additionally studied by light and electron microscopy. Here we present the description of a second species of the genus *Massisteria, M. voersi* sp. nov. In contrast to its sister species, the newly discovered species seems to be rare in the environment.

Material and Methods

Isolation and cultivation

Surface-water samples were collected and prefiltered (200-µm plankton net) at a Baltic Sea coastal monitoring station (sea bridge of Heiligendamm, Germany, 54°08′ N, 11°50′ E) in November 2008. During sampling, temperature and salinity were measured with a portable conductivity meter (Cond 1970i, WTW GmbH, Weilheim Germany). Prefiltered (<200 µm) and 3-µm-fractionated water samples were subjected to dark and unamended seawater incubation experiments as described in Weber et al. (2012). During these experiments, culturing was attempted by isolating single cells with a micromanipulator fitted with a glass micropipette, allowing the establishment of clonal cultures. Pre-cultures were established in 24-well microtiter plates filled with 0.2 µm-filtered and autoclaved seawater or F2 medium (Guillard and Ryther 1962) and a quinoa grain and were observed daily. Two cultures of *Massisteria voersi* sp. nov. were thus obtained: IOW101, isolated from the prefiltered fraction of experiment 3, and IOW137, from the 3-µm-filtered fraction of experiment 2, on incubation days 6 and day 4, respectively (see Weber et al. 2012). The cultures were deposited in the IOW culture collection and were routinely kept in sterile 50-ml tissue culture flasks (Sarstedt, Nümbrecht, Germany) containing F2 medium (salinity 8–16‰), a mixture of bacteria from the sampling site as food source, and wheat or quinoa grains as a carbon source for the bacteria.

Another strain (IOW64) of the newly described species was isolated earlier (November 2003) from the same sampling site but did not grow well in culture. Before its loss, we obtained a partial 18S rRNA gene sequence, which allowed its molecular identification.

Microscopy investigations

Living cells were observed by light microscopy with an AxioScope A1 microscope (Carl Zeiss, Jena, Germany). For transmission electron microscopy of the sectioned cells, the flagellates were concentrated by centrifugation and fixed by incubation in a solution containing 2% osmium tetroxide and 0.6% glutaraldehyde in Schmaltz-Pratt medium (28.15 g/l NaCl, 0.67 g/l KCl, 5.51 g/l $MgCl_2 \times 6\ H_2O$, 6.92 g/l $MgSO_4 \times 7\ H_2O$, 1.45 g/l $CaCl_2 \times H_2O$, 0.1 g/l KNO_3 and 0.01 g/l $K_2HPO_4 \times 3H_2O$ in deionized water) for 15–30 min at 1 °C. After dehydration in a series of alcohols

and anhydrous acetone, the specimens were embedded in an araldite–epon mixture and examined using a JEM-1011 microscope (JEOL, Tokyo, Japan). The sections were cut using a diamond knife (LKB Ultramicrotome, Sweden).

For whole-mount preparations, drops of suspended cells were placed on copper grids coated with Formvar film and prepared according to the method described by Moestrup and Thomsen (1980).

Gene sequence analysis

DNA was extracted from the harvested cells using the MasterPure Complete DNA and RNA purification kit (Epicentre, WI, USA). The 18S rRNA gene was amplified by polymerase chain reaction (PCR) using the primer pair 18SFor-n2 and 18SRev-Ch (Wylezich et al. 2012). The reaction mixture, containing 0.1 mM of each primer, 200 mM dNTPs, 1× PCR buffer, and 0.5 µl of Herculase II Fusion DNA polymerase (Agilent Technologies), was heated to 94 °C for 2 min after which the 18S rRNA gene was amplified in 35 cycles of 95 °C for 20 s, 57 °C for 30 s, and 72 °C for 70 s. The PCR products were separated on an agarose gel (1.2%) and the excised bands were purified with the Nucleospin II kit (Machery Nagel). Sequencing was carried out externally (LGC Genomics, Qiagen, Germany) using the same primers as in the PCR and four different internal sequencing primers (590F, 600R, 1280F, 1300R; Wylezich et al. 2002).

The obtained sequence fragments were carefully corrected and assembled in Bioedit (Hall 1999). The resulting complete 18S rRNA gene sequences of *Massisteria* and other 18S rRNA sequences retrieved from GenBank were aligned using the CLUSTAL_X program (Thompson et al. 1997) and manually edited with Bioedit. Two data sets of sequence alignments were created on different taxonomic levels: a "Granofilosea" data set (1641 positions) to investigate the relationship of the genus *Massisteria* within the granofiloseans and a "*Massisteria*" data set (1915 positions) to demonstrate the composition of the genus *Massisteria*, including environmental sequences. Genetic distances (*p*-distances) were calculated using the "*Massisteria*" data set and MEGA (Kumar et al. 2004). Phylogenetic analyses were performed for both data sets using MrBAYES 3 (Ronquist and Huelsenbeck 2003) and PhyML 3.0 (Guindon et al. 2010, http://www.atgc-montpellier.fr/phyml/). The GTR model of substitution (Lanave et al. 1984) and a gamma-shaped distribution of substitution rates among sites with eight rate categories and a proportion of invariable sites were used. The Bayesian analysis was performed for 1,000,000 generations and sampled every 100 generations for four simultaneous chains. For the likelihood analysis, all model parameters were estimated from the data set. To estimate branch support, 1,000 bootstrap replicates were performed for ML analyses. The 18S rRNA gene sequences obtained in this study were deposited in GenBank under the accession numbers KM065451 (IOW101) and KM065452 (IOW137).

Fluorescence in situ hybridization (FISH) analysis

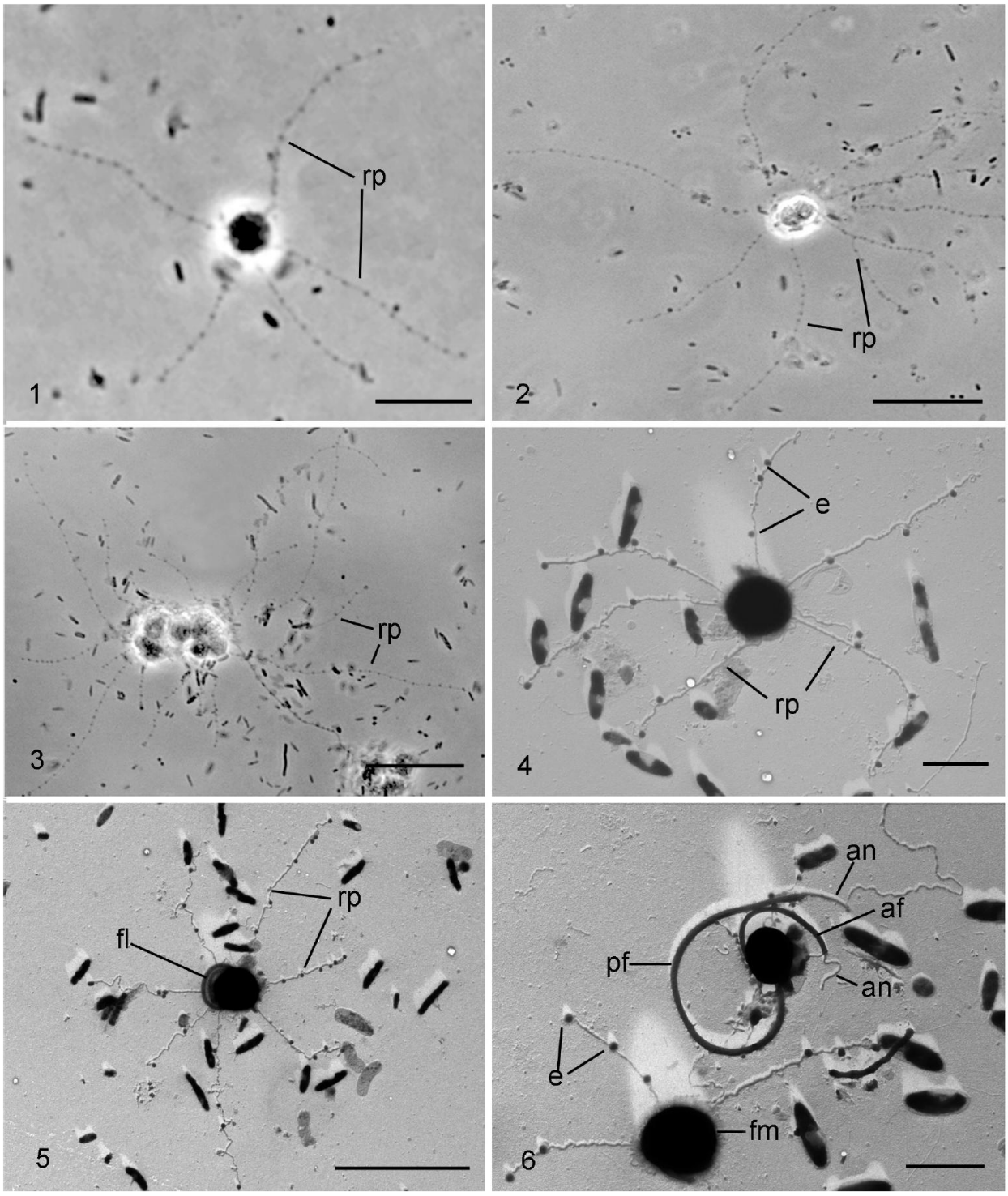
Complementary to the 18S rRNA gene sequence of *M. voersi* at region 1431–1450, a specific oligonucleotide probe was manually designed in Bioedit. As indicated by BLAST searches and the probe-match tool of the ARB software package (Ludwig et al. 2004), the resulting probe, Mv_1431 (5′ GTT GGG TGA GCC TTG CAC GG 3′), was very robust *in silico*, with ten unweighted mismatches compared to the closest cultured representative *M. marina*. The probe was purchased with a 5′ end Cy3 fluorophore (Eurofins MWG Synthesis, Ebersberg, Germany) and FISH experiments were performed according to the protocol of Pernthaler et al. (2001). In brief: subsamples of both *Massisteria* cultures were fixed for 2 h in the dark with particle-free formaldehyde (2% final concentration) and filtered (with gentle pressure) through 0.6-µm pore size polycarbonate filters (Whatman, via VWR International, Weiterstadt, Germany). Hybridization was carried out at 46 °C for 3 h using 30% formamide in the hybridization buffer. The filters were counterstained and mounted on microscopic slides in a mixture of 4′,6-diamidino-2-phenylindole (DAPI), Citiflour, and Vectashield and observed at 1000× magnification with a Zeiss Axioskop 2 mot plus epifluorescence microscope (Carl Zeiss Microscopy) at an excitation wavelength of 360 nm (DAPI) and 546 nm (Cy3). The probe gave a negative signal (in the presence of 20% and 30% formamide) with the non-target rhizarian strain IOW62 (Thecofilosea sp.) as the closest relative available in our culture collection.

Results

Three strains of the new species were isolated during two sampling campaigns (2003 and 2008) at the coastal monitoring station (Heiligendamm, see Table 1). During the entire sampling campaign in autumn 2008, the salinity ranged between 13 and 18, whereas the type strain IOW137 was isolated from a water sample with a salinity of 14.5. Although the first strain, isolated in 2003, was lost, both surviving strains (IOW101 and IOW137) were included in more detailed molecular investigations. Strain IOW137 was used in microscopy investigations and was therefore designated as the type strain.

Light microscopy and shadowing preparations

The biphasic life cycle of *M. voersi* sp. nov. consists of two stages: an amoeboid attached, immotile (or very slightly motile) stage and an actively swimming flagellate stage. Cysts have not been found. The swimming cells seen in freshly inoculated cultures are short-lived and generally rare in culture. The roundish and flattened amoeboid cells are 2.2–2.5 µm or, rarely, 3 µm in diameter ($n = 109$, Figs 1–5).



Figs 1–6. Amoeboid cells (solitary and grouped) with thin branched rhizopodia (light microscopy, **1–3**). (**4–6**) Whole-mount preparations of amoeboid and flagellar cells as seen on transmission electron microscopy. Abbreviations: an, acroneme; af, anterior flagellum; e, extusomes; fl, flagellum; fm, fibrous material; pf, posterior flagellum; rp, rhizopodia. Scale bars: Figs 1 and 5: 5 μm, Figs 2 and 3: 10 μm, Figs 4 and 6: 2 μm.

Table 1. Overview of *Massisteria* strains from marine (*M. marina*) and brackish (*M. voersi*) sites, including isolation source and references.

Strain	Accession number	Reference	Isolation
Massisteria marina Larsen et Patterson, 1990			
GBB2	AF174370 (1762 bp)	Atkins et al. 2000	Guaymas Basin Beggiatoa Mat, Pacific Ocean
DFS1	AF174371 (1726 bp)	Atkins et al. 2000	Dante Flange substratum, Pacific Ocean
LFS1	AF174372 (1731 bp)	Atkins et al. 2000	Lobo Flange substratum, Pacific Ocean
CAS1	AF174373 (1744 bp)	Atkins et al. 2000	ClamAcres Spire, Pacific Ocean
TPC1	AF174374 (1718 bp)	Atkins et al. 2000	Twin Peaks Chimney, Pacific Ocean
ATCC50266	AF174369 (1699 bp)	Atkins et al. 2000	Hudson Canyon, Atlantic Ocean
	AF411286 (1760 bp)	Cavalier-Smith and Chao 2003	
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IOW64	(not submitted)	This study	Baltic Sea, coastal surface water, 2003
IOW101	KM065451 (1878 bp)	This study	Baltic Sea, coastal surface water, 2008
IOW137	KM065452 (1878 bp)	This study	Baltic Sea, coastal surface water, 2008

A thin layer of fibrous substance (Fig. 6) surrounds the cell surface. In most of these cells, flagella are not visible by light microscopy. Obvious movement of the amoeboid cells has not been detected. Freshly inoculated amoeboid specimens associate in groups of two to four and, rarely, six cells (Figs 2, 3). The five to eight pseudopodia are very thin, sometimes branched, and radiate from the cell surface such that they are seen only on the bottom of the Petri dishes. They contracted after fixation. Extrusomes are clearly visible in the pseudopodia, appearing as darkly colored (dense) granules (Figs 1–4). A contractile vacuole has not been found. The two flagella of the flagellate stage differ in length and bear thin terminal threads (acroneme) (Fig. 6). The anterior flagellum is 9–12 μm and the posterior flagellum 3–4 μm in length but, as noted above, the flagellate stage is apparently rare. The swimming course of elongated cells was straight, sometimes arched, very rapid and with sudden stops. After fixation and desiccation, swimming cells were rounded (Fig. 6).

Electron microscopy

Because of the infrequently seen flagellate stage, only amoeboid specimens could be investigated on thin sections. The two flagella of amoeboid stages are naked and without mastigonemes (Fig. 6), lie next to each other close to the cell body (Fig. 7), and usually have the typical 9 + 2 arrangement of microtubular axonemes (Fig. 7). A small amount of amorphous material is present inside the proximal part of at least one of the flagella (Figs 7, 11). The transverse plate of each flagellum (located on the cell surface) has a small axosome (Fig. 9). The two flagella emerge in parallel to each other (Fig. 10). The kinetosomes are very short (0.22 μm), without the characteristic cartwheel structure at their basal ends, and are connected by at least one fibril (Fig. 8). The kinetosomes are closely packed, separated by a distance of 0.2 μm (Figs 8, 10). Small satellites are seen near the kinetosomes (Fig. 9). The cytoplasmic microtubules, as groups of parallel units, were not observed to be connected with the kinetosomes (Figs 11–13). Thus, microtubular roots have yet to be detected. In some cases, an axoneme of the flagellum is present in the cell (Fig. 12). The mitochondria have tubular cristae (Figs 12, 13, 15–17). The vesicular nucleus is 1.0–1.2 μm in diameter, has a central nucleolus, and is typical in its appearance (Figs 13, 18). Extrusomes 0.12–0.13 μm in diameter are located inside the pseudopodia and just beneath the cell surface (Figs 14–16, 20). A plasmalemma of usual structure and an external layer of fibrous material (Figs 17, 18) cover the cell. Vesicles with rudimentary fibrous material are present in the cytoplasm (Figs 16, 19). Small flattened vesicles lie beneath the plasmalemma (Fig. 17). The Golgi apparatus is close to the nucleus (Fig. 18). At least two microtubules pass through the rhizopodes (Figs 15, 20). In some cells, a spherical (concentric) structure is slightly visible in these extrusomes (Fig. 21). Food vacuoles contain bacteria (Fig. 21).

Gene sequence data and phylogenetic analysis

The two identical 18S rRNA gene sequences of both *Massisteria voersi* strains (IOW101, IOW137) are 1878 nucleotides long and have a GC content of 50.3%. The short 5′ sequence fragment of the strain isolated in 2003 is identical to both *M. voersi* sequences. The new species shows a similarity of 93.5–93.8% to the *M. marina* strains (which have a slightly lower GC content of 48.2–48.5%). This low similarity for the two species of a single genus is due to the highly divergent V4 region of *M. voersi*. The V4 regions of the two species have only 88.5–89.1% similarity with the V4 primer-binding sites used for 454 pyrosequencing. However, phylogenetic analysis clearly supports a relationship between these species, as shown in Fig. 22. In addition, *M. voersi* occurs along a relatively long branch within the *Massisteria* clade, mainly because of its V4 variable region. Several environmental clonal sequences (GU821524, AB275101) of different habitats, including those that are oxygen depleted, are closely related to the two *Massisteria* species (Fig. 22B).

Environmental sequences from Pacific Ocean samples form a branch with *M. marina* strain GBB2 (AF174370), which is slightly distantly related to the other *M. marina* strains. The 18S rRNA genes of the latter show a similarity of ~99.6% to each other and similarities of 98.4–98.8% to strain GBB2. Interestingly, there are no environmental clonal sequences in the NCBI database that are closely related to the new species *M. voersi*. A second, careful survey using short fragments of the *M. voersi* sequence in the NCBI (BLAST analysis) yielded two 145-nucleotide sequences (FJ929414 and FJ988252) showing 99.2–100% sequence identity to *M. voersi*. Both sequences derived from a 454 pyrosequencing approach using sediment samples of Sydney Harbour (Chariton et al. 2010). Our own 454 pyrosequencing results from oxygen-depleted waters of the Black Sea did not contain sequences similar to those of *M. voersi*. Furthermore, we found one sequence (OTU86, Wylezich et al. unpublished results) with 95% similarity to the clonal sequence GU821524 (included in Fig. 22) from anoxic water samples of the Cariaco Basin (Edgcomb et al. 2011).

Quantification attempt of *M. voersi* at the sampling site

The newly designed probe Mv_1431 was tested (in the presence of 20% and 30% formamide) in cultures of *M. voersi* and yielded strong Cy3 signals for both strains (IOW101 and IOW137) (Figs 23, 24). However, when the probe was applied to environmental samples taken during the sampling campaigns in autumn 2008 and spring 2010 (200-µm prefiltered), only very few and weak signals were obtained and did not allow reliable quantification of cells. This implies a very low abundance in Baltic Sea plankton.

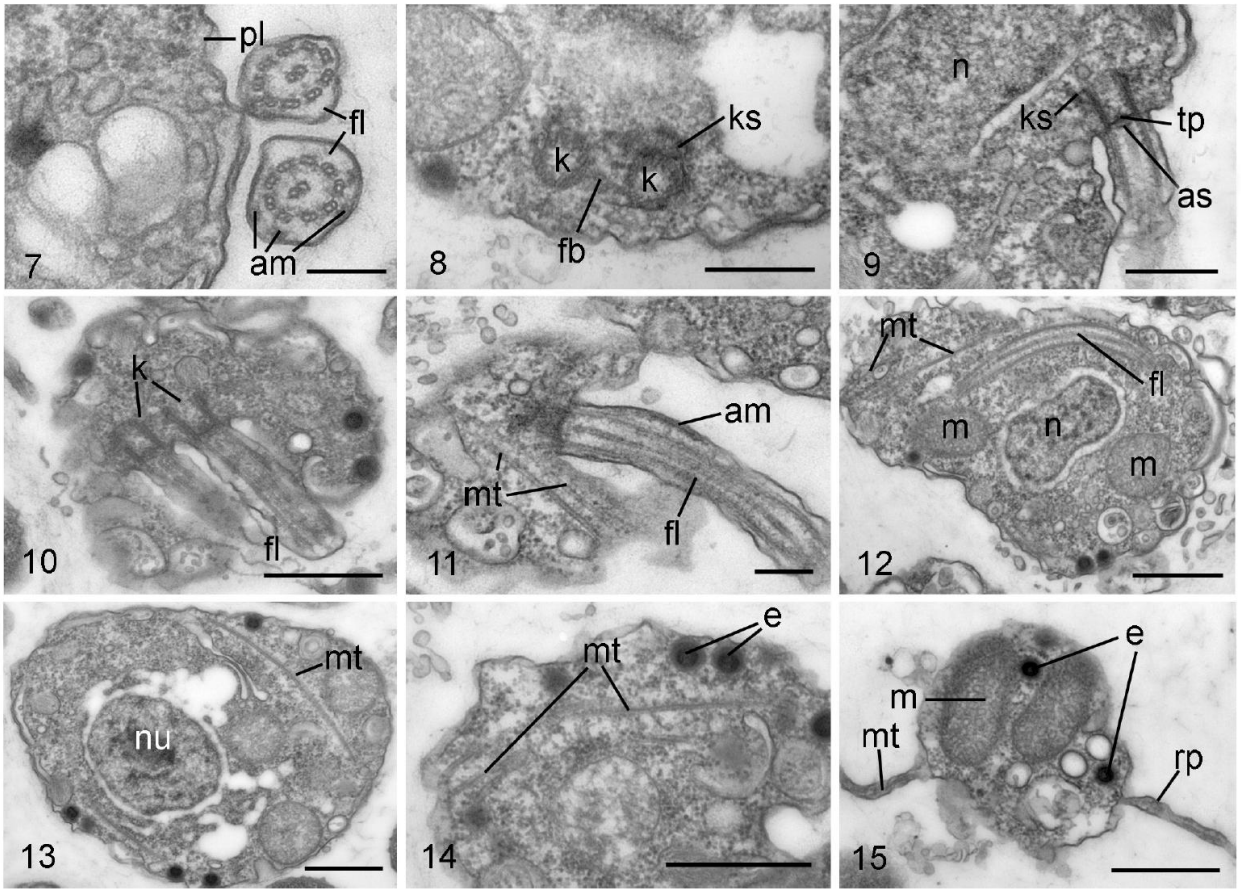
Diagnosis

Phylum Cercozoa Cavalier-Smith, 1998, emend. Adl et al., 2005

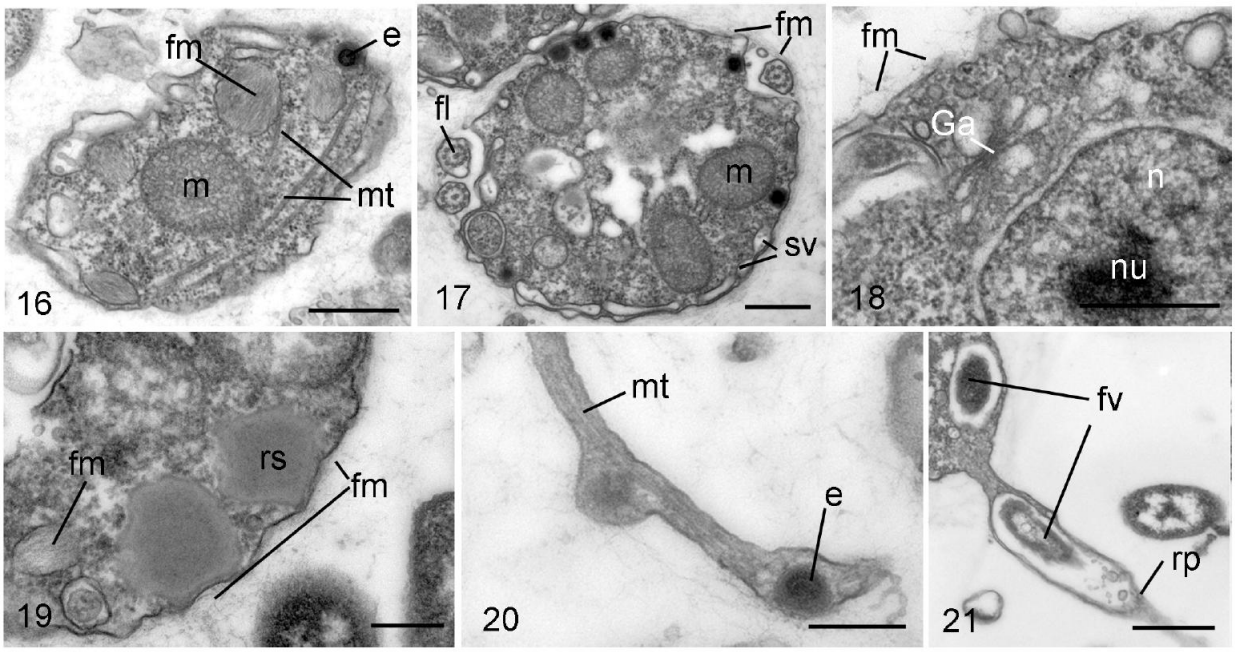
Class Granofilosea Cavalier-Smith et Bass, 2009

Order Leucodictyida Cavalier-Smith, 1993, emend. 2003

Family Massisteriidae Cavalier-Smith, 1993



Figs 7–15. The structure of the flagellar apparatus (**7–11**). (**12, 13**) Cross-sections of the cells. (**14**) The extrusomes lie beneath the plasmalemma. (**15**) Mitochondria and rhizopodia. Abbreviations: am, amorphous material inside a flagellum; as, axosome; e, extusomes; fb, fibril connecting kinetosomes; fl, flagellum; k, kinetosome; ks, kinetosome satellite; m, mitochondria; mt, microtubule; n, nucleus; nu, nucleolus; pl, plasmalemm; rp, rhizopodia; tp, transverse plate; Scale bars: Figs 7 and 11: 0.2 µm, Figs 8–10, 12, 13–15: 0.5 µm.



Figs 16–21. Mitochondria and cytoplasmic microtubules (**16**). (**17**) The flagella are twisted (coiled) around the cell. (**18**) The positions of the Golgi apparatus and nucleus. (**19**) Granules of the reserve material. (**20**) Microtubules and extrusomes located inside rhizopodia. (**21**) Food vacuoles containing a single bacterium. Abbreviations: e, extusomes; fl, flagellum; fm, fibrous material surrounding the cells; fv, food vacuole; Ga, Golgi apparatus; m, mitochondria; mt, microtubule; n, nucleus; nu, nucleolus; rp, rhizopodia; rs, reserve substance; sv, small vesicles beneath the plasmalemma. Scale bars: Figs 16–18, 21: 0.5 μm, Figs 19 and 20: 0.2 μm.

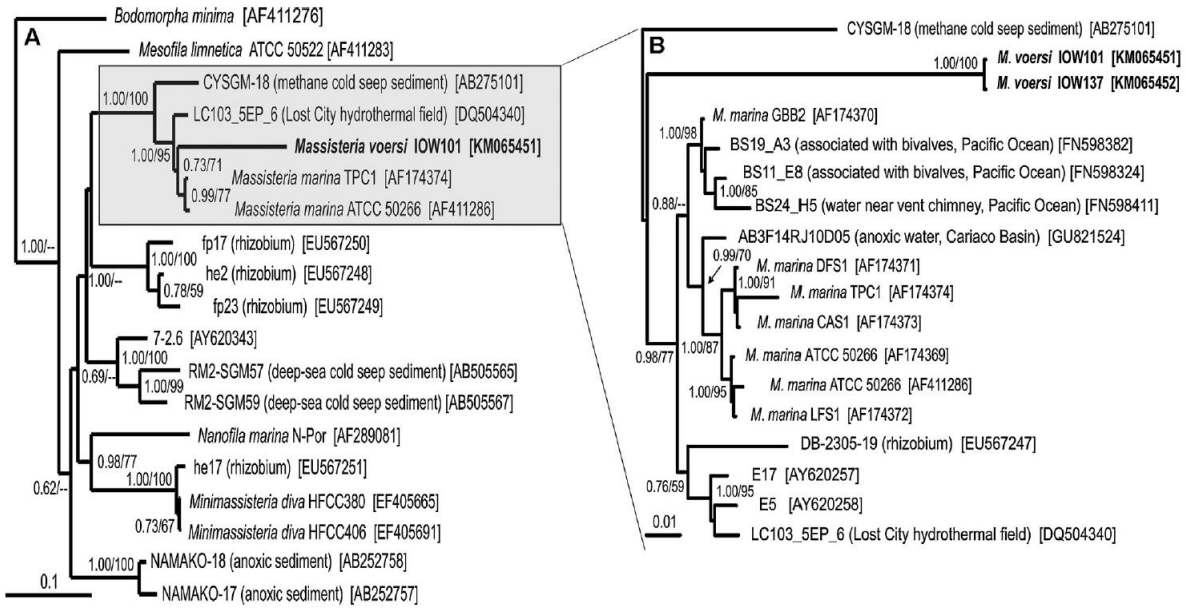
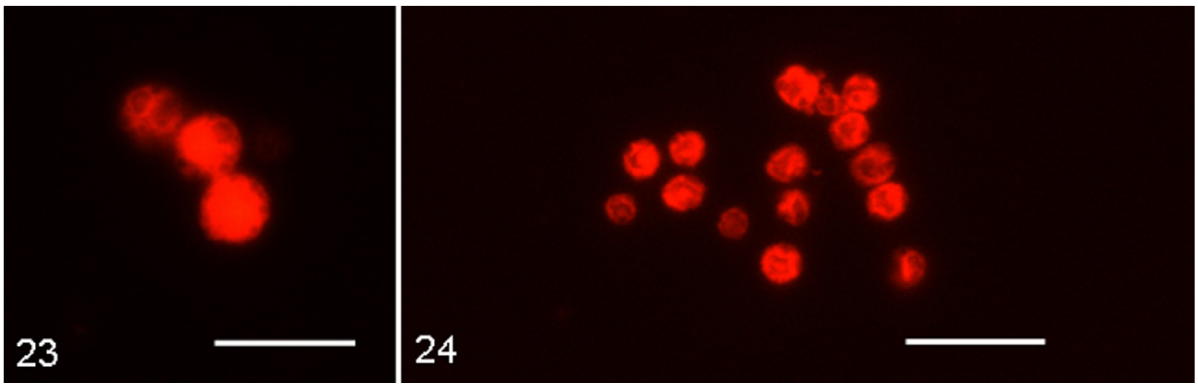


Fig. 22. Phylogenetic affiliation of *Massisteria voersi* sp. nov. and the composition of the genus *Massisteria*. **(A)** Bayesian phylogenetic tree of Granofilosea, including *Massisteria* species. The tree was rooted using *B. minima*. **(B)** Bayesian phylogenetic tree of the genus *Massisteria*, including environmental sequences belonging to this genus. Clonal sequence CYSGM-18 was used as the outgroup, as suggested in tree A. The posterior probability values for 1,000,000 generations and corresponding maximum-likelihood bootstrap values for 1000 replicates are shown. Support values less than 0.5 and 50 are not shown (–). The scale bar represents a distance of 0.1 substitutions per site. Genbank accession numbers of all species used are indicated in square brackets.



Figs 23 and 24. Epifluorescence micrographs. Fluorescence in situ hybridization (FISH) signal of *Massisteria voersi* sp. nov. IOW137 (**23**) and IOW101 (**24**) using probe Mv_1431. The cells are associated with detritus flocks. Scale bar = 10 μm.

Genus *Massisteria* Larsen et Patterson, 1990

Massisteria voersi sp. nov. Mylnikov et Wylezich

Amoeboid cells are roundish, flattened, almost immotile, and 2.2–2.5 μm, rarely 3 μm, in diameter. The slightly visible flagella are curved laterally around the flagellate cell. Groups of two to six daughter cells form. The five to eight pseudopodia are thin, branched threads bearing dark granules (extrusomes). In flagellates, the anterior and posterior flagella are, respectively, 9–12 μm and 3–4 μm in length. Cyst formation is unknown. Mitochondria have tubular cristae. A paranuclear body is absent.

Observed habitat

Coastal surface water of the southwestern Baltic Sea (Heiligendamm, Germany; 54°08′ N, 11°50′ E), salinity range 13–35 (this study and Chariton et al. 2010).

Type material

Type strain: IOW137; holotype: Fig. 5 (fixed); hapantotype: embedded specimens are kept in the Laboratory of Microbiology of the Institute for the Biology of Inland Waters, Russian Academy of Sciences, Borok, Yaroslavskaya Obl., Russia (slide 560, 561 and 562).

Etymology

The new species has been named *Massisteria voersi*, after Naja Vørs, who intensively studied the Baltic flagellate fauna (Vørs 1992a) where the type strains were isolated.

Differential diagnosis

Massisteria voersi sp. nov. strongly resembles *M. marina* on light microscopy with respect to cell shape and the presence of flagellate and amoeboid stages. *M. voersi* differs from *M. marina* by the absence of a paranuclear body in the cytoplasm, the absence of fused motile cells, cells that are smaller than those of *M. marina* (2.2–3 μm vs. 2.5–9.5 μm), and the parallel arrangement of the kinetosomes. Based on their 18S rRNA gene sequences (similarity 93.5–93.8%), the two species are very distantly related.

Remarks

Live strains are held as clonal cultures (strains IOW101, IOW137) in the laboratory of the IOW-Leibniz Institute for Baltic Sea Research in Rostock-Warnemünde, Germany. The species description is deposited in ZooBank under urn:lsid:zoobank.org:act:6F96ED7C-1309-4CBA-963A-020088E608DD.

Discussion

Here we describe a new species, *Massisteria voersi*, isolated from the Baltic Sea. It is the second species belonging to the genus *Massisteria*, after *Massisteria marina*. The morphological, genetic, and especially ultrastructural dissimilarities between *M. marina* and our isolated strains justify the establishment of this new species. Most reports of *Massisteria* specimens come from morphological identifications, which identified all specimens as *M. marina*. With the identification of a second species, the identity of some of those specimens may need correction, since the characters used in their classification are only genus-specific. Even cell-size differences between the two species are only partially suitable as a distinguishing criterion. Although *M. marina* cells can be as large as 9 μm in diameter, the majority of environmental records are smaller (2.5–5 μm) and thus overlap with the size of *M. voersi* cells (2.3–3 μm). Since most of the reports of *M. marina* included just size ranges (e.g., Larsen and Patterson 1990; Tong 1997; Tong et al. 1998; Vørs 1992a,b), it may well be that *M. voersi* was mistakenly identified as *M. marina* in some reports.

Another potentially distinguishing criterion is the occurrence of fused flagellate cells in *M. marina* (Patterson and Fenchel 1990). However, since the flagellate stage seems to be rare for *M. voersi*, the existence of similar fusions cannot be completely excluded. By contrast, ultrastructural

characteristics strongly justify the erection of a new species. *M. voersi* differs from *M. marina* by the absence of a paranuclear body, an ancestral character of Filosa that is absent in many of its genera or species (Cavalier-Smith and Chao 2003). Furthermore, the parallel arrangement of the kinetosomes of *M. voersi* differs from the acutely angled kinetosomes of *M. marina* (Patterson and Fenchel 1990).

Members of the recently described flagellate genus *Minimassisteria* Arndt et Cavalier-Smith, 2011 (Howe et al. 2011) resemble those of *Massisteria* with respect to cell shape whereas a thicker, non-granular, anterior tractional pseudopodium characterizes the crawling forms of *Minimassisteria* but has not been found in *Massisteria*. Additionally, *Massisteria* species differ from all cercomonads by the absence of a visible gliding motion of the cells. Among the cercomonads, three species (*Cercomonas cometa* Holland, 1942; *Eucercomonas ramosa* Karpov et al., 2006 and *Paracercomonas virgaria* Cavalier-Smith and Karpov, 2012) consist of slightly motile amoeboid cells that form thin branched rhizopodia (Cavalier-Smith and Karpov 2012; Karpov et al. 2006; Mylnikov 1986, 1990). These rhizopodia in cercomonads contain extrusomes that are similar in structure to those of *Massisteria*. In the case of *M. voersi*, it specifically differs from cercomonads by its simplified (reduced) flagellar root system, which in cercomonads consists of two kinetosomes arranged at a right angle, four, rarely five, nucleating flagellar microtubular roots, a composite root, fibrous satellites near kinetosomes, fibrils connecting kinetosomes, and one microtubular cone (rarely, two cones) directed towards the nucleus (Cavalier-Smith and Karpov 2012; Karpov et al. 2006). These features may be related to the lack of motility of the amoeboid life cycle stages of *M. voersi*. Unfortunately, we do not have any data on the flagellar apparatus in the very rare, motile flagellate cells of this newly identified species.

The molecular phylogenetic analysis based on 18S rRNA gene sequences showed that the two *Massisteria* species (*marina* and *voersi*) branch off together (Fig. 22A). Additionally, several environmental sequences were found to be closely related to *M. marina* strains or to branch off as its sister group (clonal sequences DB-2305-19, E5, E17 and LC103_5EP_6; Fig. 22B, pyrosequencing reads from the Black Sea; Wylezich, unpublished results). Hence, the genus *Massisteria* is probably species-rich, containing more than two species. Surprisingly, no sequences closely related to *M. voersi* were found using a BLAST search of its complete gene sequence in Genbank. Given the large number of 18S rRNA genes sequences of culture and culture-independent origin deposited in NCBI, the discovery of a species with <94% similarity was unexpected. Moreover, despite both belong to the same genus, *M. voersi* has diverged remarkably from *M. marina* with respect to 18S rRNA gene sequences, especially within the hypervariable V4 region. Indeed, the detection of an unexpectedly divergent V4 sequence using a next-generation sequencing approach without reliable culture-derived sequences would have raised suspicion that the finding was artifactual. Accordingly, we sequenced the complete 18S rRNA gene of another strain (IOW101) and were thus able to confirm the sequence of the type strain IOW137. A subsequent refined BLAST search using short fragments of the *M. voersi* 18S rRNA gene sequence yielded two closely related sequences (FJ988252 and FJ929414; 99–100% identical to *M. voersi* at nucleotide position 1360–1500) from a Sydney Harbour 454 pyrosequencing approach (Chariton et al. 2010). In an earlier study, Tong et al. (1998) reported *M. marina* from Sydney Harbour. Since their description was based only on microscopy observations, it cannot be ruled out that this record of *M. marina* (Tong et al. 1998) included specimens of *M. voersi* and that the two species occupy the same niche, as indicated by the 454 data of Chariton et al. (2010). Similarly, the Baltic records of *M. marina* could also contain *M. voersi* specimens and possibly further yet unidentified species. Vørs (1992a) described cells larger than those of *M. voersi* whereas Garstecki and Arndt (2000) did not provide any information on cell size. In general, without reliable genetic or ultrastructural data, a *Massisteria* species cannot be reliably identified.

The hitherto known records of *M. voersi* based on cultivation (this study) and environmental sequencing (Chariton et al. 2010) originated from sites of different salinities: 13–18 and 28–35 (A. Chariton, personal communication), respectively. Thus, the new species apparently tolerates at least brackish to marine conditions. Based on the observations from the Baltic Sea coast and Sydney Harbour, *M. voersi* seems to be a coastal inhabitant. Coastal habitats are well-suited for protists that exhibit a bentho-pelagic life style, given the high rate of resuspension of the sediment surface layer. The life style of *M. marina* consists of an amoeboid stage hidden in sediment or in detritus particles (Patterson and Fenchel 1990), as evidenced by the frequent isolation of this species from sediment samples (Arndt et al. 2003; Garstecki and Arndt 2000; Hausmann et al. 2002; Larsen and Patterson 1990; Patterson and Simpson 1996) and coastal sites (Ekebom et al. 1995/96; Tong et al. 1998; Vørs 1992a,b). Similar to thaumatomonads, rapid shifts to the flagellate stage enable *Massisteria* to actively migrate, which suggests its involvement in both benthic and pelagic food webs (benthic–pelagic coupling). Specimens of *M. voersi* have not been isolated from original water samples but during incubations of 200-μm pre-filtered water samples. Therefore, there is no information on the association of *M. voersi* with detritus particles in the natural environment, as known for *M. marina*. However, in long-term cultures *M. voersi* showed a tendency to colonize detritus particles (derived from wheat or quinoa grains) whereas in freshly inoculated cultures in which detritus particles were absent, swimming (flagellate) stages were seen, albeit only rarely. The absence of *Massisteria*-related 18S rRNA gene sequences in clone libraries prepared from the corresponding incubation experiments at the sampling station (Weber et al. 2012), where water samples were pre-filtered, strongly suggests that the proliferation of *Massisteria* depends on the presence of detritus particles.

Massisteria marina is also found in extreme environments, such as anoxic marine waters (Edgcomb et al. 2011) and hydrothermal fields (López-García et al. 2007), and in some cases in association with other organisms (Bass et al. 2009; Sauvadet et al. 2010). The accumulated observations indicate that members of the genus *Massisteria* are able to colonize a wide range of niches, indicating a broad ecological capacity. Nevertheless, according to the microscopy records, they also settle in less extreme environments and do not seem to be dependent on other organisms to permanently host or protect them during their life cycle.

In our FISH analysis, strong signals were obtained from both *M. voersi* cultures using the newly designed probe Mv_1431 (Figs 23, 24). However, hardly any signal could be detected at the sampling site using environmental plankton samples (200 µm-prefiltered) (data not shown), which suggests that the abundance of swimming stages of *M. voersi*, at least in the pelagial, is extremely low. Because this species lives, albeit perhaps only transiently, in the sediment or is associated with detritus particles (as discussed above), abundances in these settings might be much higher. Alternatively, the detection of only two closely related sequence fragments in the GenBank, resulting from high throughput sequencing, could indicate that *M. voersi* is a representative of the so-called "rare biosphere." This term was introduced by Sogin et al. (2006) to describe the large number of highly diverse taxa with low abundances. Recently, Weisse (2014) distinguished two types of rare species for ciliates. The present description would place *M. voersi* in the background fauna of a habitat, i.e., as a species in universal environments but exhibiting only low abundances. A more detailed investigation of the sediment at the sampling site may show that *M. voersi* is not a representative of a specialist fauna living in dense populations in extreme habitats. A search in the V4 BioMarKs data including sediment samples (details are given in Logares et al. 2012) resulted in closely related sequences to *M. marina* (99% similarity) whereas *M. voersi* was not found in this dataset (highest similarity to *M. voersi* was 93%; D. Bass, unpublished data). In fact, Garstecki and Arndt (2000) already considered *M. marina* to be a rare species at their sampling stations. It should be noted that, thus far, there is no evidence for a cyst stage in either *M. marina* (Patterson and Fenchel 1990) or *M. voersi* (this study). However, cyst formation would be essential for a rare species to withstand competition (Weisse 2014) as well as adverse environmental conditions. Interestingly, cyst formation has been reported for the related genus *Minimassisteria* (Howe et al. 2011).

Taken together, our description of *M. voersi* and the comparison with *M. marina*, the first described species of this genus, provide important insights into the morphological and genetic diversity within *Massisteria.* The hitherto available data indicate a wide distribution of the new species but also its infrequent occurrence. However, *Massisteria* species are very delicate and are often difficult to detect (Larsen and Patterson 1990; Vørs 1992a). Therefore, proof of the abundance and distribution of *M. voersi* in the environment requires further studies that include FISH or CARD-FISH analyses with the newly designed probe Mv_1431. In addition, investigations of the life cycle of members of the genus *Massisteria* will reveal the ecological consequences of their ability to exploit very different habitats (pelagic, benthic, detritus, and metazoan hosts) depending on the life stage (flagellate vs. amoeboid) or on the suitability of the environment.

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Chapter 4) Unveiling trophic functions of uncultured protist taxa by incubation experiments in the brackish Baltic Sea

Unveiling Trophic Functions of Uncultured Protist Taxa by Incubation Experiments in the Brackish Baltic Sea

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Abstract

Background: Our knowledge of the phylogeny and diversity of aquatic protists is rapidly increasing due to molecular surveys and next-generation sequencing approaches. This has led to a considerable discrepancy between the taxa known from cultures and those known from environmental 18S rRNA gene sequences. Hence, it is generally difficult to assign ecological functions to new taxa detected by culture-independent molecular approaches.

Methodology/Principal Findings: A combination of unamended dark incubations and 18S rRNA sequencing was chosen to link molecular diversity data of uncultured protists with heterotrophic, presumably bacterivorous, growth. The incubations, conducted with Baltic Sea brackish water, resulted in a consistent shift from a protistan community dominated by phototrophs to one in which heterotrophs predominated. This was determined on the basis of cell abundance and 18S rRNA sequences derived from fingerprint analysis and clone libraries. The bulk of enriched phylotypes after incubation were related to hitherto uncultured marine taxa within chrysophytes, ochrophytes, choanoflagellates, cercozoans, and picobiliphytes, mostly represented in recently established or here defined environmental clades. Their growth in the dark, together with coinciding results from studies with a similar objective, provides evidence that these uncultured taxa represent heterotrophic or mixotrophic species.

Conclusions/Significance: These findings shed some light into the trophic role of diverse uncultured protists especially within functionally heterogeneous groups (e.g., chrysophytes, ochrophytes) and groups that appear to be puzzling with regard to their nutrition (picobiliphytes). Additionally, our results indicate that the heterotrophic flagellate community in the southwestern Baltic Sea is dominated by species of marine origin. The combination of unamended incubations with molecular diversity analysis is thus confirmed as a promising approach to explore the trophic mode of environmentally relevant protist taxa for which only sequence data are currently available.

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Introduction

In terms of abundance and biomass, heterotrophic protists are an essential component of planktonic communities in aquatic systems [1]. Among them, heterotrophic nanoflagellates (HNF) are the main grazers of prokaryotic cells and other picoeukaryotes [2,3], and are able to shape bacterial community structure [4,5]. Additionally, heterotrophic protists transfer significant amounts of bacterial production to higher trophic levels [6], and serve as important agents for nutrient remineralisation in aquatic food webs [7].

Traditional approaches such as microscopy and cultivation techniques have provided valuable data on the distribution and abundance of aquatic protists [8,9,10], and on the autecological properties of several cultured representatives [11,12]. However, they have proven to be insufficient to describe the diversity and taxonomic composition of natural protistan assemblages [13]. In particular, small protists often lack distinctive morphological characteristics, making them hard to distinguish by light or electron microscopy, even at the class level [14]. Their identification is further complicated by the fact that cultivation and isolation attempts are known to be very selective for opportunistic species, which can cope better with altered *in vitro* conditions [15,16]. Such culture conditions often involve media that differ substantially from the organisms' aquatic surroundings, while surplus in nutrients can favor the growth of species that play only a minor role in the environment [17,18]. Therefore, a growing consensus exists that the majority of microbial eukaryotes are yet to be cultured, and, consequently, groups without any cultured representatives may dominate in various oceanic regions [19,20].

Just over a decade ago, molecular techniques based on the analysis of small-subunit rRNA genes began to be applied to diversity analyses of microbial eukaryotes [21,22,23,24]. Nowadays, fingerprinting techniques and the construction of genetic libraries are routinely used to investigate protist communities, and they have considerably advanced our knowledge of the diversity and distribution of marine and freshwater protists [25,26]. For

example, several novel lineages within the marine stramenopiles (MAST), novel alveolates (MALV), and the recently discovered picobiliphytes, have been detected through environmental surveys [27,28,29,30]. The application of extensive genetic libraries, high-throughput sequencing, and metagenomics has continued to expand the datasets of partial 18S rRNA gene sequences [26,31,32,33]. Together, these comprehensive methodologies have revealed an unexpected magnitude of protist biodiversity in aquatic environments and resolved important aspects of their biogeography [34,35,36,37].

Nonetheless, linking ecological functions to this diversity data is still a major challenge. This is due to the considerable discrepancy between the taxa known from cultures and those inferred from environmental 18S rRNA sequences [38]. Sometimes, environmental sequences affiliate with well-known groups and can therefore be assigned a tentative functional mode (e.g., prototroph or heterotroph). However, environmental sequencing frequently recovers phylotypes distantly related to cultured representatives, e.g., within the stramenopiles, and consequently their ecological function remains unknown [39]. Only a few studies have combined cultivation-independent techniques with approaches aimed at revealing the functional characteristics of different protists groups, such as the application of specific oligonucleotide probes for fluorescent in situ hybridization (FISH) [30,40], sometimes in combination with bacterial uptake experiments [27,41,42]. Other methodologies, such as those designed to identify phagotrophic protists, include stable isotope probing [43] and sophisticated cell sorting analyses that are complemented by subsequent 18S rRNA sequencing [44].

As an alternative approach, unamended seawater incubations in the dark have been used. These stimulate the growth of heterotrophic flagellates (HF) that are abundant *in situ*, and are generally mostly uncultured, but does not trigger the mass growth of typical cultivable flagellates [45,46]. Subsequent 18S rRNA analysis in these incubation experiments have allowed the identification of otherwise undetected protistan taxa [47,48].

In the present study we followed the approach of unamended dark incubations for the first time in a more nutrient rich and brackish water environment in order to detect those uncultured protists that exhibit a heterotrophic life style. Thereby, we succeeded in preferentially enriching uncultured heterotrophic flagellates, while minimizing the culturing bias [46]. Subsequent 18S rRNA analysis revealed that most of the taxa at the end of the incubations were only distantly related to cultured representatives. Unexpectedly, most of the enriched phylotypes of this brackish water site showed highest similarities to environmental clones of marine origin. Overall, our study provides valuable information on the heterotrophic protist community of the Baltic Sea.

Results

Microbial Successions

The development of bacteria, *Synechococcus*, phototrophic eukaryotes, and HF showed a consistent pattern in the three incubation experiments, with a remarkably low variance within the triplicates (Fig. 1). Total bacterial abundance increased to form a peak after about 3 days, with cell numbers decreasing after 5–6 days to values similar to or below the initial ones. Changes in bacterial abundance were due to the rapid response and subsequent decline of high nucleic acid containing bacteria (HNA) whereas low nucleic acid containing bacteria (LNA) remained relatively constant throughout the experiments (Fig. 1A). Additional experiments revealed that leucine incorporation, as a measure of bacterial production, also increased considerably during the incubations, reaching a maximum one day before the peak in bacterial abundance (Weber *et al.*, unpublished data). Coincidently with the decrease of bacteria, HF increased significantly and were enriched 5- to 13-fold at the end of the incubation. The cell numbers of HF detected in unfiltered treatments were generally lower (1,300–15,000 cells ml^{-1}) than those enumerated in the 3-µm filtered samples (1,200–21,000 cells ml^{-1}). Additively, larger forms of HF (>3 µm) as well as acanthoecid choanoflagellates (with silicious loricae) were observed primarily in the unfiltered treatments. Phototrophic eukaryotes were initially 5–15 times more abundant than HF but began to decline after 2–3 days of incubation such that by the end of the experiments their cell numbers were lower than those of HF (Fig. 1B).

Compositional Shift Towards Heterotrophic Taxa Revealed by DGGE

Both molecular techniques, i.e., DGGE and clone libraries, were RNA-based in order to principally detect actively growing protists. The DGGE fingerprints of 18S rRNA obtained at the start (t_0) and the end (t_{end}) of incubation revealed compositional shifts in the protist community, again with very low variance in the triplicates (Fig. 2, S1).

The presence or absence of bands at a certain position was used to calculate the similarity of all samples, which was then displayed in a multidimensional scaling plot (Fig. S1). The 3-µm filtration step caused only minor changes in community structure whereas the incubation process was critical, since samples of the same time point were more similar than those obtained from different time points (Fig. S1). This finding was consistent across all three experiments and in triplicate samples, with two exceptions: the unfiltered t_{end} sample of Exp1 (black open triangle) was very similar to the respective t_0 sample (black open square), and the unfiltered t_{end} sample of Exp2 (dark gray open triangle) was an outlier to all other samples due to the presence of the ctenophore *Mnemiopsis leidyi*, represented by one very dominant DGGE band (band 6, Fig. 2).

By quantifying the relative intensities of the sequenced bands in each DGGE lane we obtained a first impression of the taxa contributing to the community shift during the incubation (Fig. 2). Among the dominant bands in the t_0 samples were three belonging to the phototrophic prasinophytes *Micromonas pusilla* (band 1) and *Ostrecoccus tauri* (bands 2 and 3), and one belonging to the mixotrophic oligotrich ciliate *Laboea strobila* (band 4). Together, these four bands accounted for 10–30% of the relative band intensity in unfiltered treatments and 30–45% in the 3-µm filtered treatments of the t_0 samples. The same bands were absent or contributed <3% in most of the t_{end} samples (except for the unfiltered t_{end} sample of Exp1, in which *Laboea strobila* still accounted for 10% of the relative band intensity).

In contrast, bands representing known heterotrophic taxa such as *Paraphysomonas imperforata* (band 7) or an uncultured choanoflagellate (band 5) as well as various bands belonging to uncultured chrysophytes (bands 8–12) of unknown trophic function seemed to benefit from the incubation, as they were of low signal intensity or undetected in the t_0 samples but became dominant by the end of the incubation. For instance, all bands affiliated with uncultured chrysophytes (related to environmental clade I) contributed a maximum of 2% to the relative band intensity in the t_0 samples and 20–50% to that of the 3-µm filtered samples processed at the end of the incubation (Fig. 2).

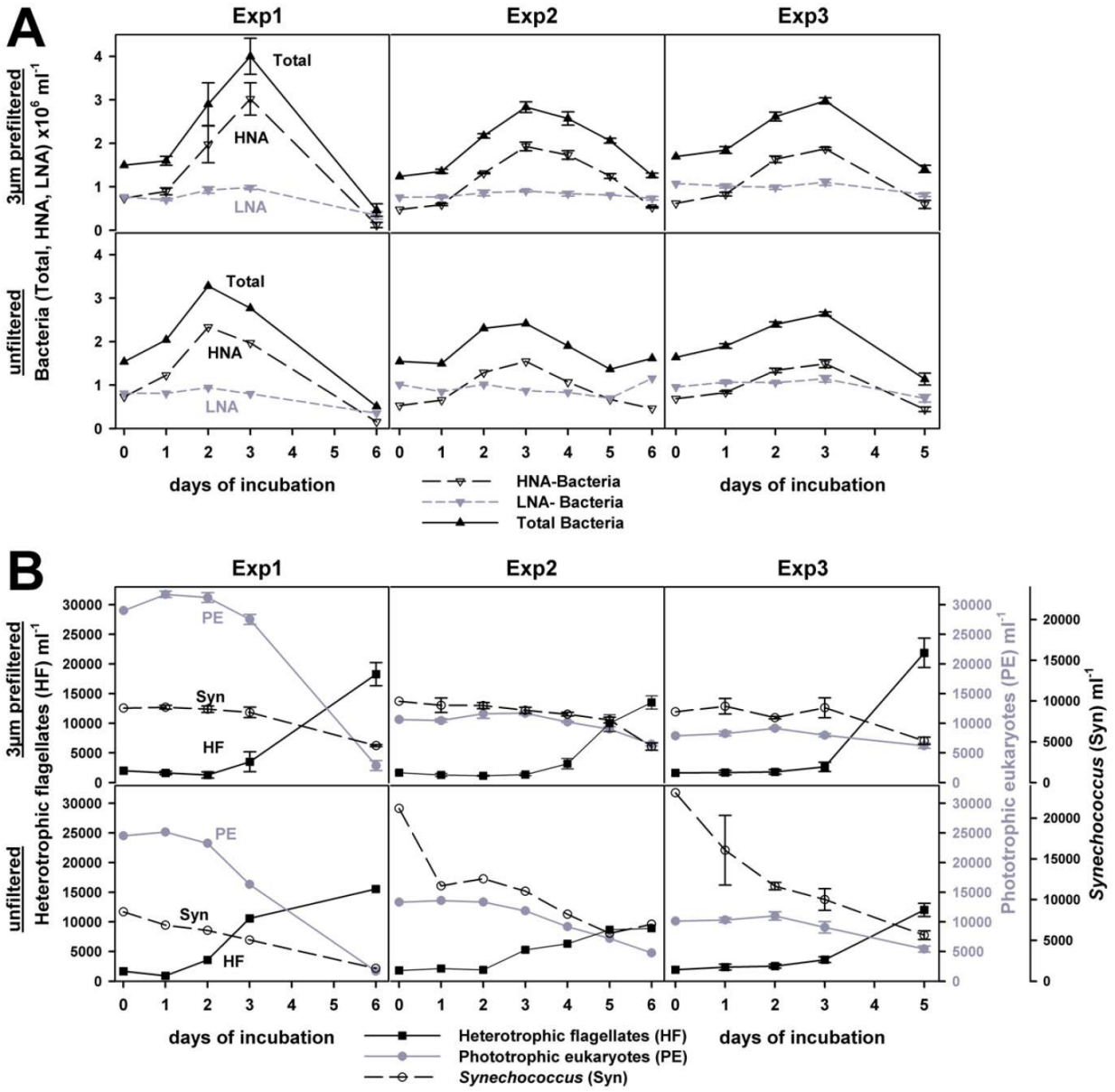
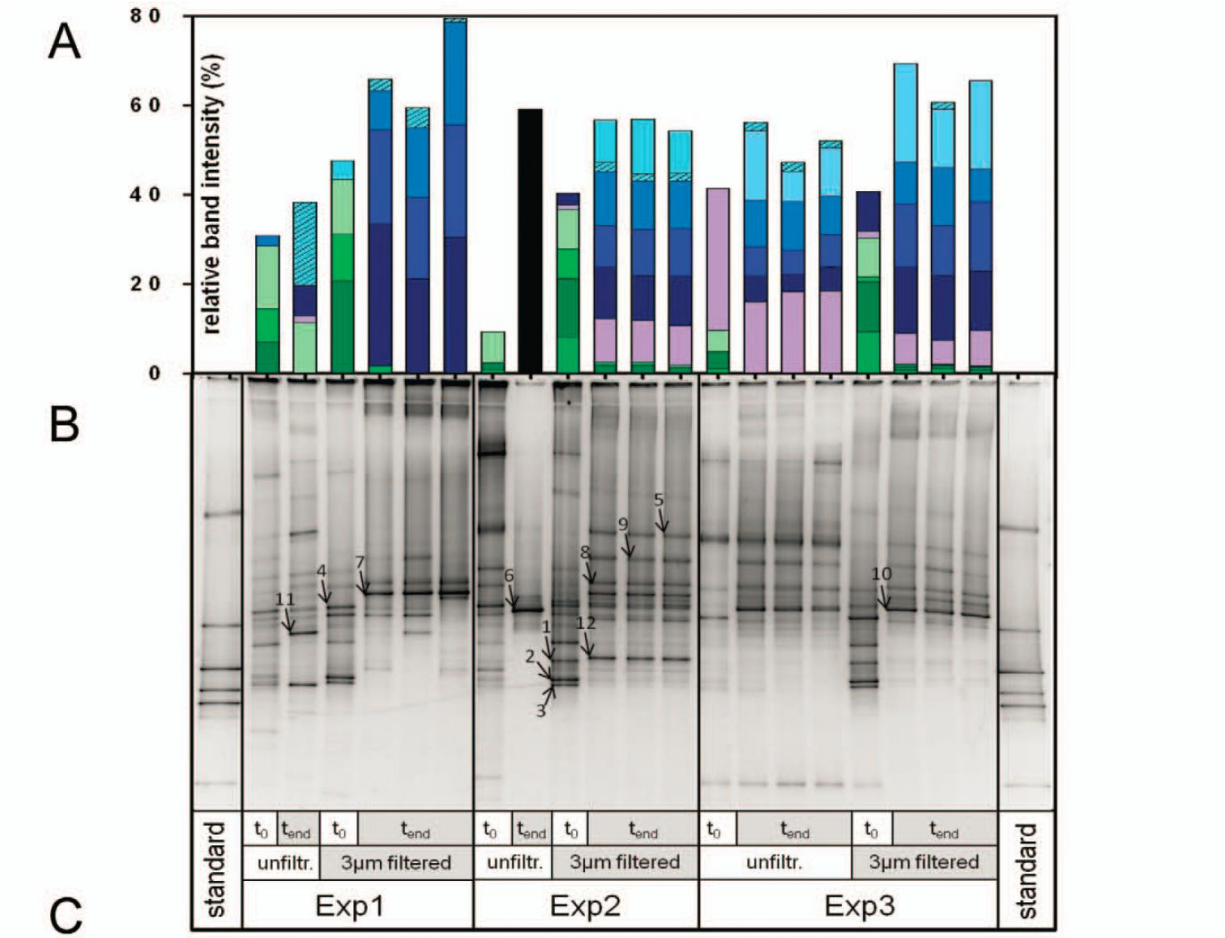


Figure 1. Cell number development in the course of three incubation experiments. (A) the abundance of total, high, and low nucleic acid containing bacteria (total, HNA, and LNA) for the 3-µm filtered and unfiltered treatments, respectively. (B) Cell numbers of *Synechococcus* (Syn), phototrophic eukaryotes (PE), and heterotrophic flagellates (HF) in the 3-µm filtered and unfiltered treatments. Error bars indicate the standard deviation in triplicate incubation bottles.
doi:10.1371/journal.pone.0041970.g001

Compositional Shifts Towards Heterotrophic Taxa Revealed by Clone Libraries

To achieve a finer resolution and gain more valuable phylogenetic information on the protistan community composition at the start and end of the incubation, 18S rRNA clone libraries were prepared. In total, eight clone libraries from the three experiments were constructed; resulting in 269 and 182 analyzed clones for t_0 and t_{end} samples, respectively (Fig. 3). In the three initial (t_0) clone libraries from the 3-µm filtered samples, Viridiplantae sequences were dominant (35%, 52% and 65%, respectively), with those of the prasinophytes *Ostreococcus tauri*, *Ostreococcus lucimarinus*, and *Micromonas pusilla* representing the largest fraction. Among the CCTH group (including cryptomonads, centrohelids, telonemids, haptophytes and picobiliphytes, as defined by Burki *et al.* [49]), which accounted for 16%, 20%, and 30% in the t_0 libraries, half of the sequences belonged to cultured microalgal species within the cryptophytes and haptophytes while the other half of the sequences originated from yet uncultured picobiliphytes. Alveolate sequences mostly belonged to spirotrich ciliates related to *Laboea strobila* and *Strombidium biarmatum* and other spirotrichs that could not be further classified. Whereas only a single



color code	band	length (bp)	closest match	similarity in %	accession number	culture match	similarity in %	accession number	phylogentic placement	trophic function
	1	499	clone BK477	98.4	GU433157	*Micromonas pusilla*	98.2	AB183589	prasinophyte	phototroph
	2	487	clone 1b-C4	99.6	FN690726	*Ostreococcus tauri*	99.6	AY329635	prasinophyte	phototroph
	3	501	clone CN207H3_5m	99	HQ847732	*Ostreococcus tauri* strain CB6	99	GQ426346	prasinophyte	phototroph
	4	505	clone BK262	99.8	GU433119	*Laboea strobila*	99.8	AF399153	oligotrich Ciliate	mixotroph
	5	364	clone Elev_18S_1414	97	EF024885	*Volkanus costatus*	94	EU011923	choanoflagellate (clade L)	heterotroph
	6	528	*Mnemiopsis leidyi*	99.8	AF293700	*Mnemiopsis leidyi*	99.8	AF293700	metazoa- ctenophora	heterotroph
	7	514	*Paraphysomonas imperforata*	100	EF432519	*Paraphysomonas imperforata*	100	EF432519	chrysophyte (clade F1)	heterotroph
	8	472	clone OA3.9	98.1	HQ437176	*Chrysonebula flava* strain CCMP2765	91.7	EF165104	chrysophyte (clade I)	unknown
	9	501	clone Biosope_T65.104	93	FJ537343	*Paraphysomonas imperforata*	91.4	EF432519	chrysophyte (clade I)	unknown
	10	498	clone OA3.6	100	HQ437175	*Ochromonas tuberculata*	93.3	AF123293	chrysophyte (clade I)	unknown
	11	480	clone AD6S.18	97.3	DQ647542	*Chrysolepidomonas dendrolepidota*	93.4	AF123297	chrysophyte (clade H)	unknown
	12	497	clone MIF_CilE6	99.4	EF526986	*Mallomonas akrokomos*	97.2	MAU73229	chrysophyte (clade J)	unknown

Figure 2. Comparison of 18S rRNA fingerprints at the start and end of three incubation experiments. (A) Stacked bar chart showing the relative intensity of the sequenced gel bands in each lane from filtered and unfiltered samples taken at the start and end of the incubation within the three experiments. Each color of the stacked bars represents one band and the length corresponds to the relative band intensity. (B) Numbered arrows indicate sequenced bands in the inverted DGGE image. (C) Information on the phylogenetic affiliation and trophic functions of sequenced bands with the band numbers referring to the bands in the DGGE image and the color code referring to the stacked bars chart.
doi:10.1371/journal.pone.0041970.g002

choanoflagellate-related sequence was found in the three t_0 samples, choanoflagellate-related sequences accounted for 14–35% in the t_{end} samples, except for the unfiltered sample of Exp1, in which no choanoflagellates were found. The contribution of cercozoans to the protistan assemblage was low in the t_0 samples but increased especially in the unfiltered samples to as much as 10–33% by the end of incubation. Stramenopiles comprised up to 12% of the clones in the t_0 libraries and 30–73% of those in the final samples. This change was mainly driven by a vast increase of sequences related to chrysophytes and novel ochrophytes.

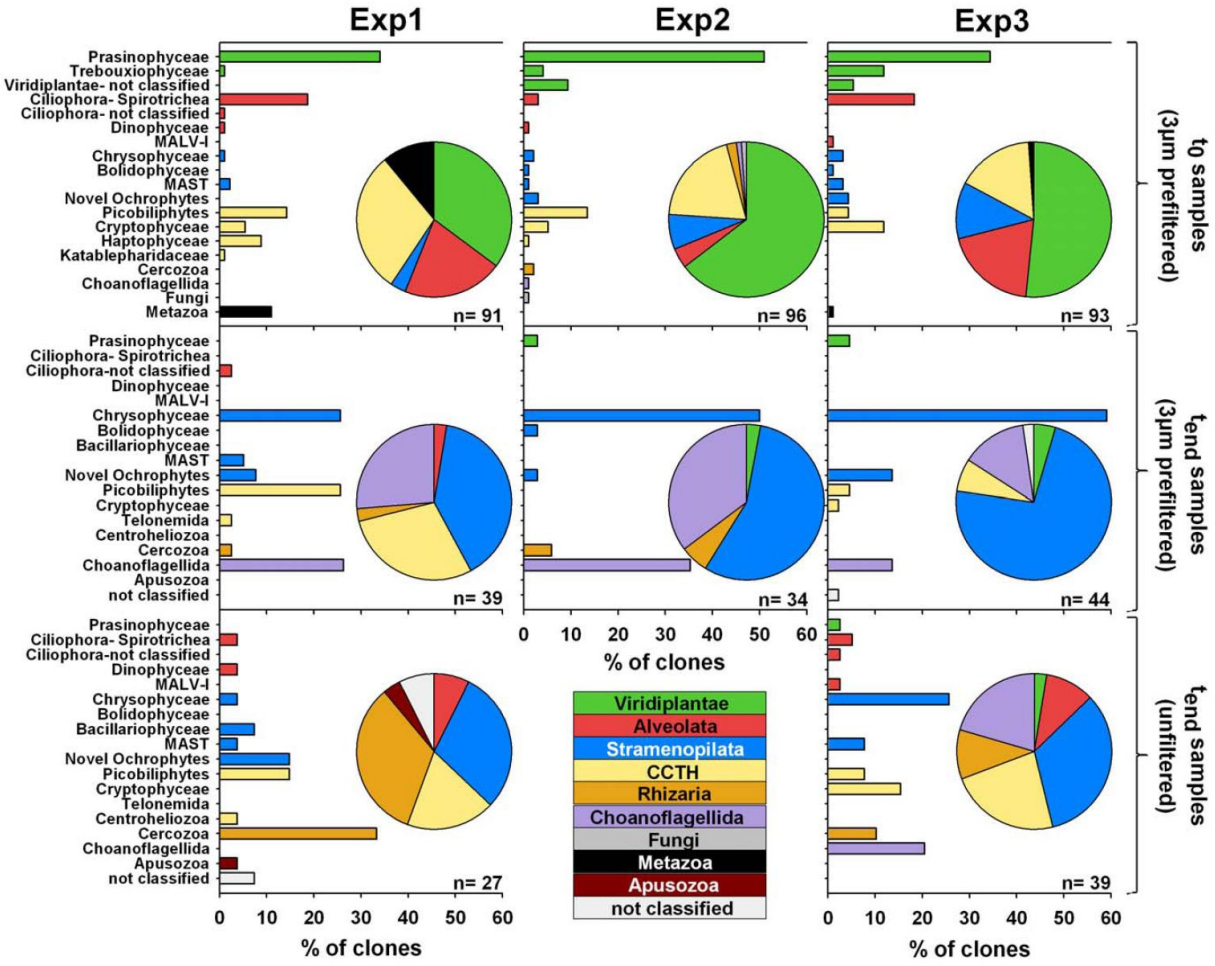


Figure 3. Phylogenetic composition in clone libraries constructed from the three incubation experiments. Upper and middle panels show the composition of clones in the 3-µm filtered initial and final incubation samples, respectively. The unfiltered final incubation samples are represented by the lower panels. For each library, the proportion of clones within the major taxonomic groups is displayed as pie charts. A finer resolution of the taxonomic groups and their contribution to each library is shown in the bar charts.
doi:10.1371/journal.pone.0041970.g003

Assignment of Trophic Functions

To assign a potential trophic function to the detected sequences, and particularly to distinguish photo- and heterotrophs, we searched for the closest cultured representatives in GenBank via BLAST. A similarity value ≥98% was used as the basis for attributing the reported trophic function (phototroph or heterotroph) of cultured species to the sequence. Irrespective of this similarity value to the closest cultured representative, all sequences closely affiliated with choanoflagellates, cercozoans (excluding sequences affiliated with chlorarachneans), and to members of the MAST cluster were assumed to be heterotrophic. All other sequences belonging to different phylogenetic groups and not complying with the similarity criterion were classified as unknown in their trophic function. This classification revealed a clear shift from phototrophic to heterotrophic species during the incubation, as evidenced by sequences derived from the clone libraries (Fig. 4A) and from excised DGGE bands (Fig. 4B). In clone libraries, sequences related to phototrophs decreased from 64% to 3% of all clones whereas sequences related to heterotrophs increased from 11% to 44%. The fraction of protists with an unassigned trophic function increased from 25% to 53%. The bulk of these unassigned sequences belonged to uncultured chrysophytes, novel ochrophytes, and picobiliphytes. A similar pattern was observed in the DGGE band analysis.

Phylogenetic Affiliation of Enriched Protist Taxa

BLAST searches for the closest cultured representative and the closest environmental sequence in GenBank resulted in two similarity values for each clone. These were represented in a scatter plot displaying distinct novelty patterns for clones obtained before and after the incubation (Fig. 5). Clones of the t_{end} samples were less similar to cultured representatives than t_0 clones (average similarities: $t_0 = 96.5\%$; $t_{end} = 93.6\%$). Additionally, there was a slight trend among clones at the end of the incubation to be less similar to environmental sequences than clones prior to incubation (average similarities: $t_0 = 98{,}9\%$; $t_{end} = 97{,}8\%$).

To identify the phylogenetic affiliation of sequences representing taxa seemingly able to grow in the dark, we constructed phylogenetic trees for ochrophytes, choanoflagellates, cercozoans, and picobiliphytes. Within these groups most of our sequences

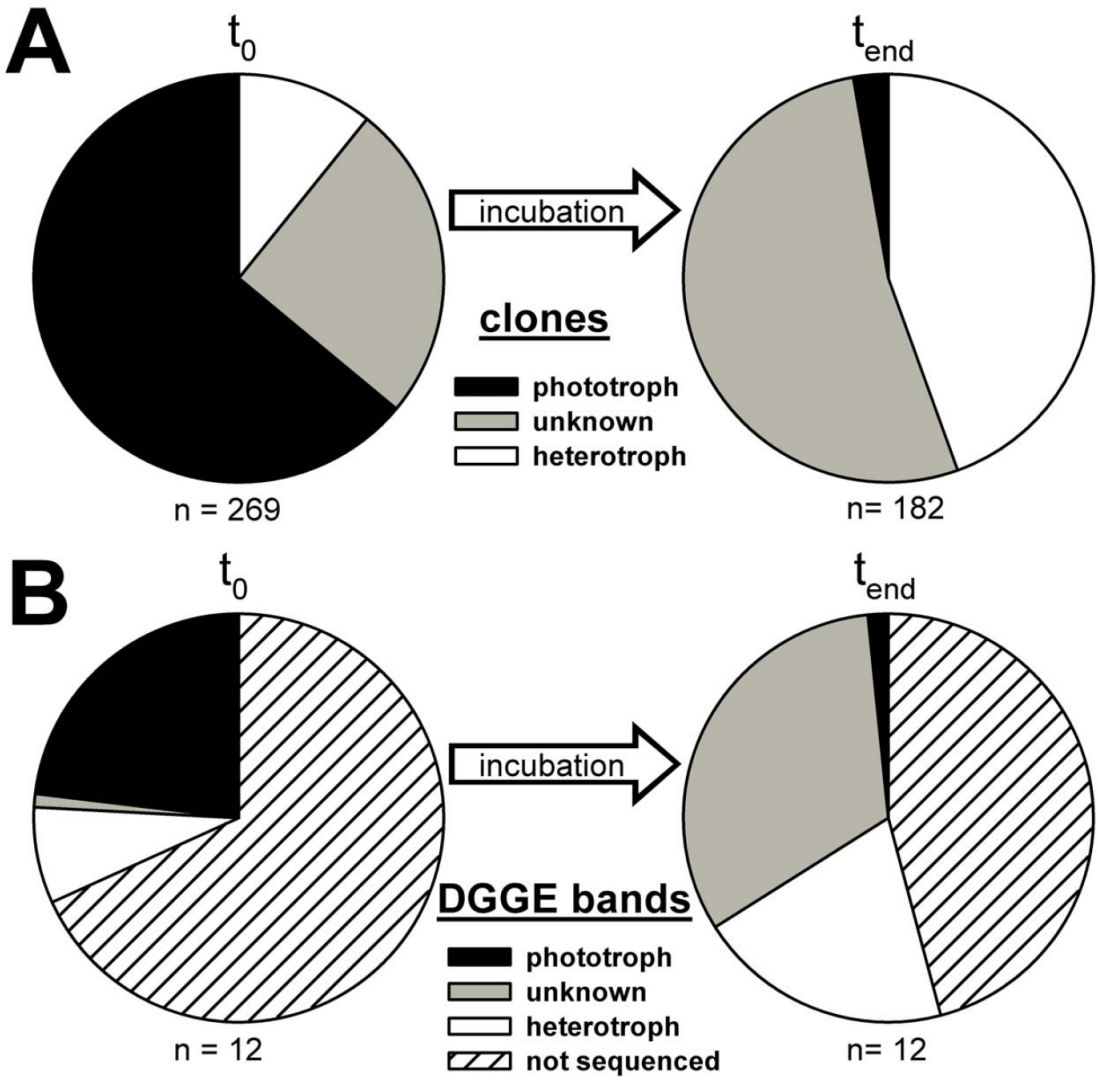


Figure 4. Community shift towards heterotrophic taxa revealed by assignment of trophic functions to 18S rRNA sequences. The proportion of sequences assigned a trophic function (phototroph, heterotroph, unknown) at the start and end of incubation, as determined in clone libraries (A) and DGGE analysis (B).
doi:10.1371/journal.pone.0041970.g004

were rather closely related to clones of marine origin than to clones of freshwater or brackish water systems (Fig. 6, 7, 8, S2). The bulk of the ochrophyte sequences affiliated with chrysophytes. The topology of the latter in the phylogenetic tree (Fig. 6) was in accordance with the branching order reported by del Campo and Massana [38]. Less than half of the chrysophyte sequences belonged to clades (F1, C, J) containing both, cultured representatives of the genera *Paraphysomonas*, *Dinobryon*, *Spumella* and *Oikomonas*, and environmental freshwater and marine sequences. Rather, most of the chrysophyte sequences affiliated with clades that exclusively consisted of environmental sequences, as determined based on good bootstrap support (>84%). Some sequences fell into clades G and H, related to sequences originating from various marine and freshwater habitats, while the majority appeared in clade I, presumably an exclusively marine cluster [38]. Within clade I, 19 and 12 sequences clustered at two distinct positions, respectively, building a long branch with sequences from the North Atlantic (clone 104.2.05) and the Arctic Ocean (clone NOR50.37). Furthermore, clade I included sequences derived from a HF peak determined in two other unamended dark incubation experiments that had been carried out with water samples collected in the Mediterranean Sea [38] and the Norwegian Sea [50]. Another large fraction of sequences (20 clones) grouped in novel ochrophyte clades distantly related (94% on average) to *Bolidomonas* species and closely related to heterotrophic protists sorted by flow cytometry (clones TS698-92 and TS698-52 [44]).

The choanoflagellate tree (Fig. 7) recovers nine clades (A–I) as defined by del Campo and Massana [38] and four additional clades (J–M) as defined by the present study. All clades comprise solely environmental sequences, including those from our experiments. Only one choanoflagellate clone (HD4ft4.3) was closely

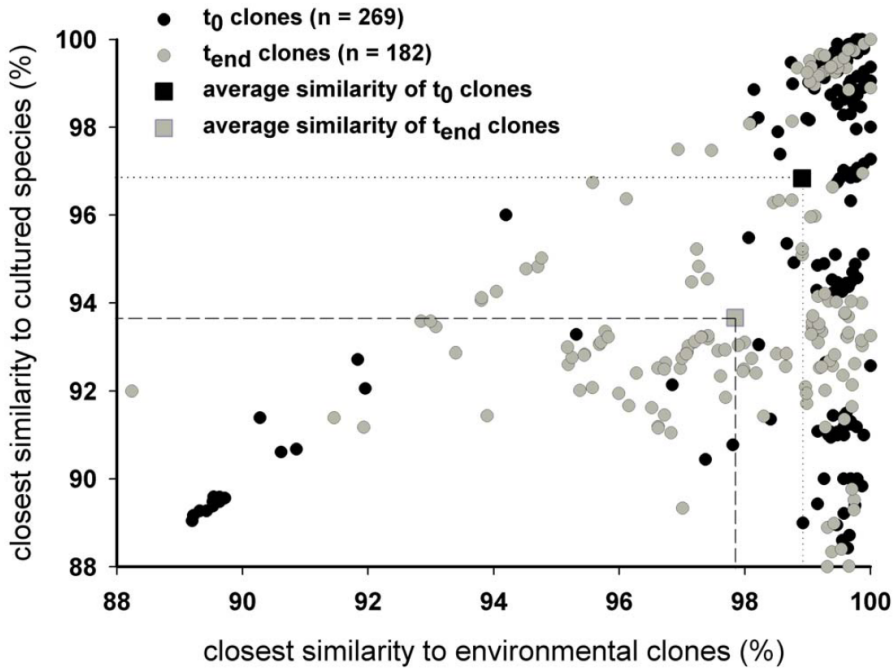


Figure 5. Novelty pattern determined for all clones obtained at the start and end of incubation experiments. Each circle represents one clone and its percent similarity to the closest environmental clone and the closest cultured species in GenBank. The mean similarity to both is indicated by the squares.
doi:10.1371/journal.pone.0041970.g005

affiliated with a cultured organism (*Diaphanoeca grandis*), in clade H. The remaining 36 clones clustered within the four novel clades, mostly within clades K and L. Whereas clades J and K contain sequences of marine origin only, clade L also comprises sequences from freshwater and soil habitats. In addition to marine sequences, clades H and M contain sequences from the Baltic Sea ice (clone 3c-C6) and wintertime water (clone 1b-B4) of the Bothnian Sea and Gulf of Finland.

The bulk of cercozoan-related sequences branches off within Thecofilosea *sensu lato*, made up of Thecofilosea *sensu stricto* and the environmental clades A (defined by Yabuki and Ishida [51]), B, and C (defined herein) (Fig. 8). All sequences related to Thecofilosea *sensu lato* were detected in t_{end} samples, and preferentially in the unfiltered treatments. Most of our sequences belonged to environmental clade A, represented by three OTUs related (96–99% similarity) to the recently described organism *Mataza hastifera* [51]. The environmental sequences within clades A and B derive from a variety of systems, including marine, freshwater, and brackish water sites (Baltic Sea). However, our sequences showed higher similarity to marine representatives. Clade C solely contains sequences of marine origin. The Thecofilosea *sensu stricto* contains one OTU that is closely affiliated (98%) to Baltic Sea and marine clones and to *Cryothecomonas aestivalis* strain 1. Among the marine sequences within Thecofilosea *sensu lato*, several were detected in sediment samples. Sequences within the Chlorarachnea and Endomyxa were related to either solely marine or solely freshwater clones, respectively.

Among marine picobiliphytes (Fig. S2), 49 sequences (30 originating from the t_0 samples and 19 from the t_{end} samples) affiliated exclusively with clones of clade BP1, as defined by Cuvelier *et al.* [52]. The effect of growth during incubation probably only applied to the OTU HD1ft4.11, represented by four clones in the t_0 sample and by 11 clones in the t_{end} sample. Furthermore, clade BP1 also contains sequences representing putatively heterotrophic taxa, as identified by Heywood *et al.* [44].

Discussion

In this study, the composition of heterotrophic protists in Baltic Sea surface water was analyzed through an approach based on unamended dark incubations. These were used to create a "functional filter" able to promote a slight enrichment of heterotrophic (bacterivorous) flagellates due to a stimulation of the naturally occurring bacterial assemblage. The growth of heterotrophic bacteria was driven by HNA-containing bacteria, thought to represent the active bacterial community and responsible for increases in bacterial production [53]. The maximum bacterial abundance in the experiments was in the range of $2.5-4\times10^6$ cells ml^{-1}, consistent with that commonly reported for the Baltic Sea during summer/autumn phytoplankton blooms [54,55,56]. We assume that the dark bottle incubations induced a moderate nutrient pulse, with the bulk of organic matter likely deriving from algal degradation–comparable to productive periods in the Baltic Sea.

The development of HF in the course of the incubations was obviously linked to an increase in bacterial abundance. Although the 3-µm filtered and unfiltered treatments showed a similar succession, the growth of HF was more pronounced in the former. In the unfiltered treatments, interactions of HF with larger protists probably became important, evident from the increased contribution of ciliate sequences to the clone libraries. Ciliates can be considered to affect HF abundance by top-down control [6], or simply by prey exhaustion when they act as competitors for bacterial prey. Another group of protists that was predominantly represented in clone libraries of the un-

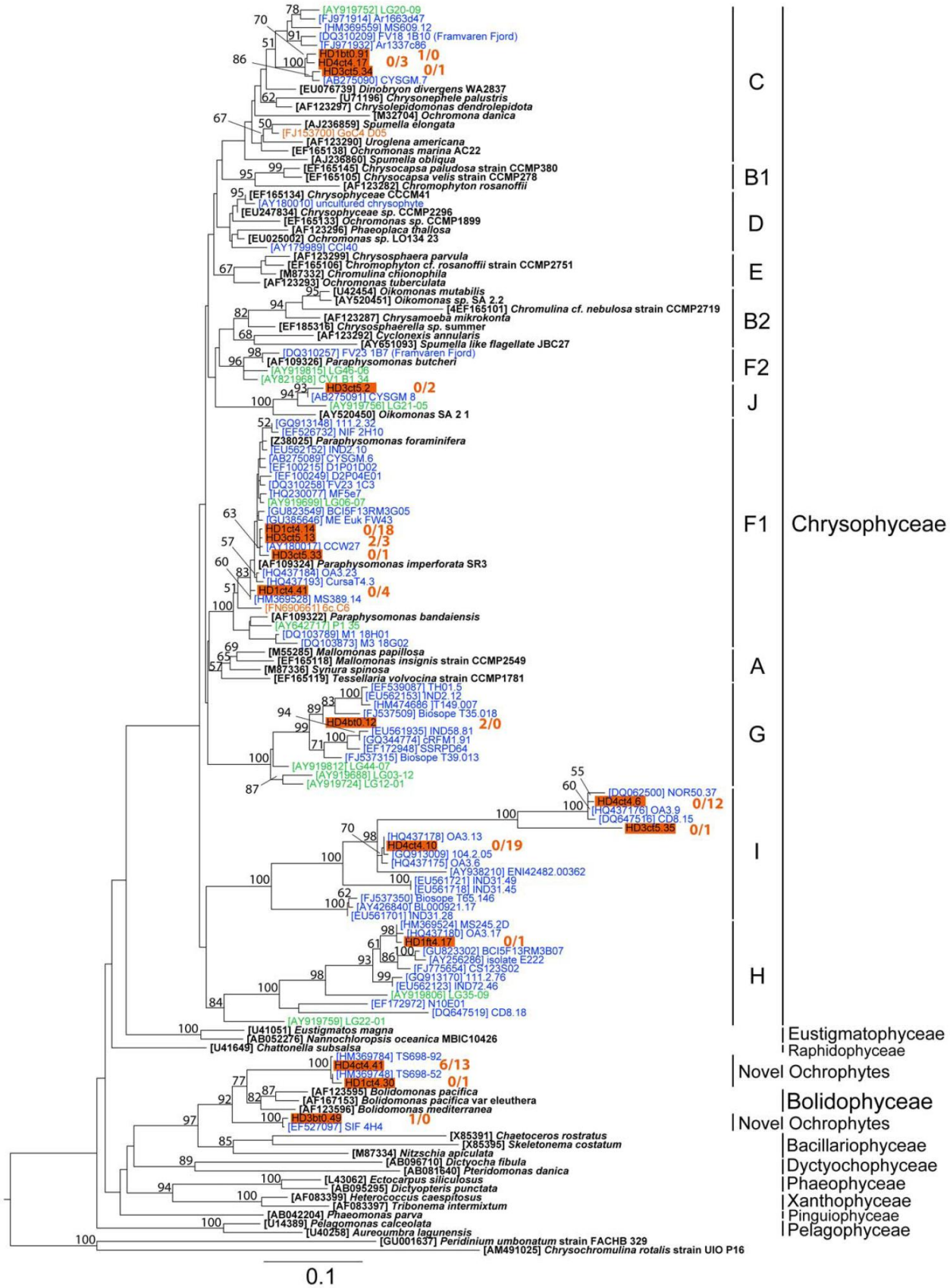

C
B1
D
E
B2
F2
J
F1
Chrysophyceae
A
G
I
H
Eustigmatophyceae
Raphidophyceae
Novel Ochrophytes
Bolidophyceae
Novel Ochrophytes
Bacillariophyceae
Dyctyochophyceae
Phaeophyceae
Xanthophyceae
Pinguiophyceae
Pelagophyceae
0.1

Figure 6. Phylogenetic affiliation of clones within ochrophytes. Maximum-likelihood phylogenetic tree constructed with 142 partial and complete ochrophyte 18S rRNA sequences (962 informative positions). Bootstrap values >50% are displayed. Clades within the chrysophytes follow the notation of del Campo and Massana [38]. Sequences indicated by red bars originate from this study; the numbers refer to the found clones in t_0 and t_{end} samples, respectively. Sequences from cultured representatives are shown in italics. Environmental clones are highlighted, according to their origin, in blue for marine clones and green for freshwater clones. The scale bar indicates 0.1 substitutions per position.
doi:10.1371/journal.pone.0041970.g006

filtered samples consisted of the Thecofilosea-related cercozoans, which might have been omnivores and were thus preying also on HF.

Even though primer systems targeting different regions of the 18S rRNA were used in the DGGE and clone libraries, the two techniques identified similar phylotypes contributing to the protistan community shift. This was particularly true for chrysophytes clade I and choanoflagellates clade L, which constituted a considerable proportion of the protist community at the end of the incubations. This latter point indicates that both groups profited from the bacterial stimulation and are thus very likely bacterivorous. In general, many of the phylotypes in the incubated samples were characterized by an elevated degree of novelty with respect to cultured representatives and even to environmental records (Fig. 5). In the initial samples, many of these novel sequence types were absent, while others occurred at low clonal abundance. In these samples, they were largely masked by the presence of numerous well-known phototrophic protists in the clone libraries. In contrast, the higher number of novel sequences (mostly chrysophytes, ochrophytes, choanoflagellates, cercozoans and picobiliphytes) at the end of the incubation allowed their detection within the clone libraries. As reported from other studies using bottle incubations, our experiment also enabled the identification of protistan taxa that otherwise might have been undetected [47,48].

It has long been pointed out by microbial ecologists that experiments which include containment of water samples may influence cell concentrations and the activity of microbial cells [57,58]. Moreover, changes in the bacterial and protistan community structure after one to three days of enclosure have been reported [48,59]. These multiple impacts on microbial assemblages under confined conditions have been compiled in the literature as the so called "bottle effect". Currently there is no consensus whether the bottle size, the volume of enclosed water and the resulting surface to volume ratio has an influence on the outcome of bottle effects [60]. In our study we observed shifts in the protistan community structure (from phototrophs to heterotrophs) as a consequence of the experimental manipulation

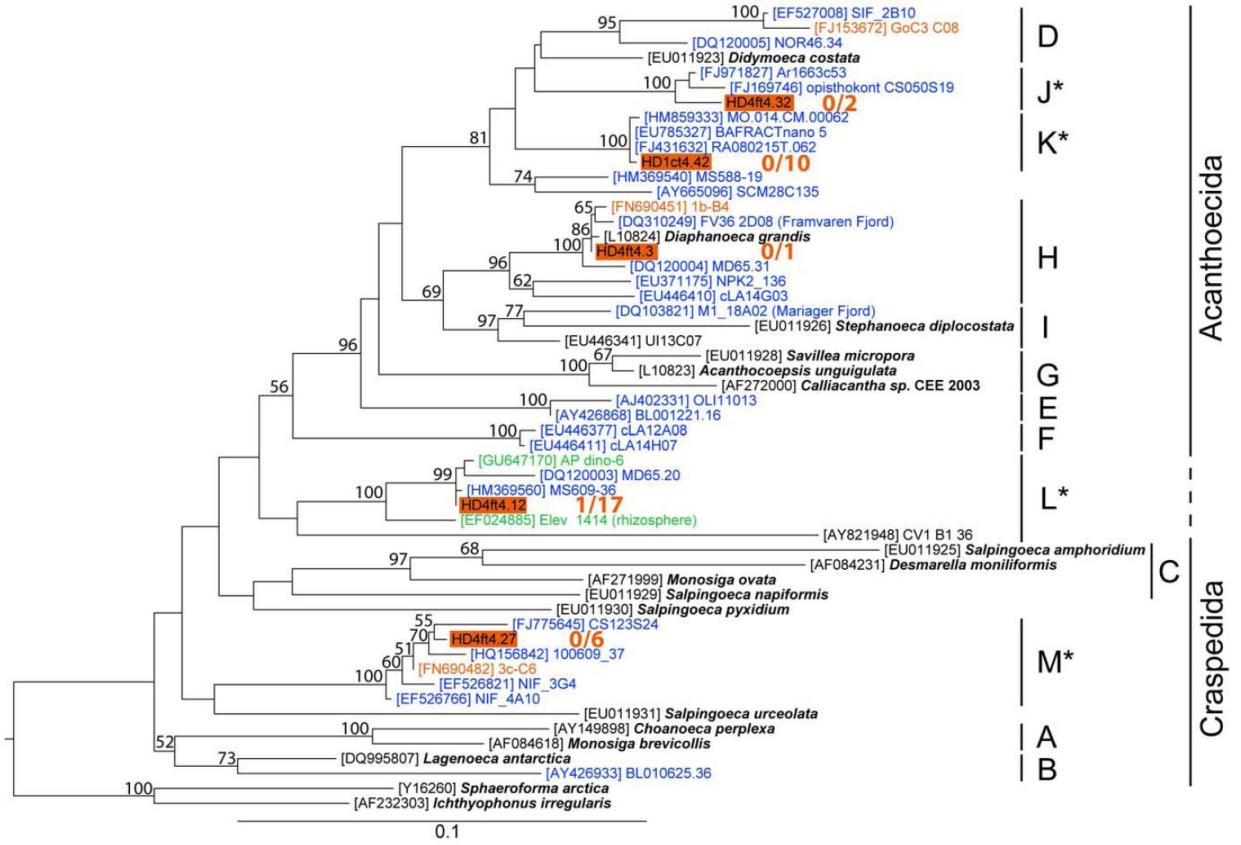


Figure 7. Phylogenetic affiliation of clones within choanoflagellates. Maximum-likelihood phylogenetic tree constructed with 54 partial and complete choanoflagellate sequences (733 informative positions). Clades A–I follow the notation of del Campo and Massana [38]. Four additional clades (J–M) are introduced. For further description see legend of Figure 6.
doi:10.1371/journal.pone.0041970.g007

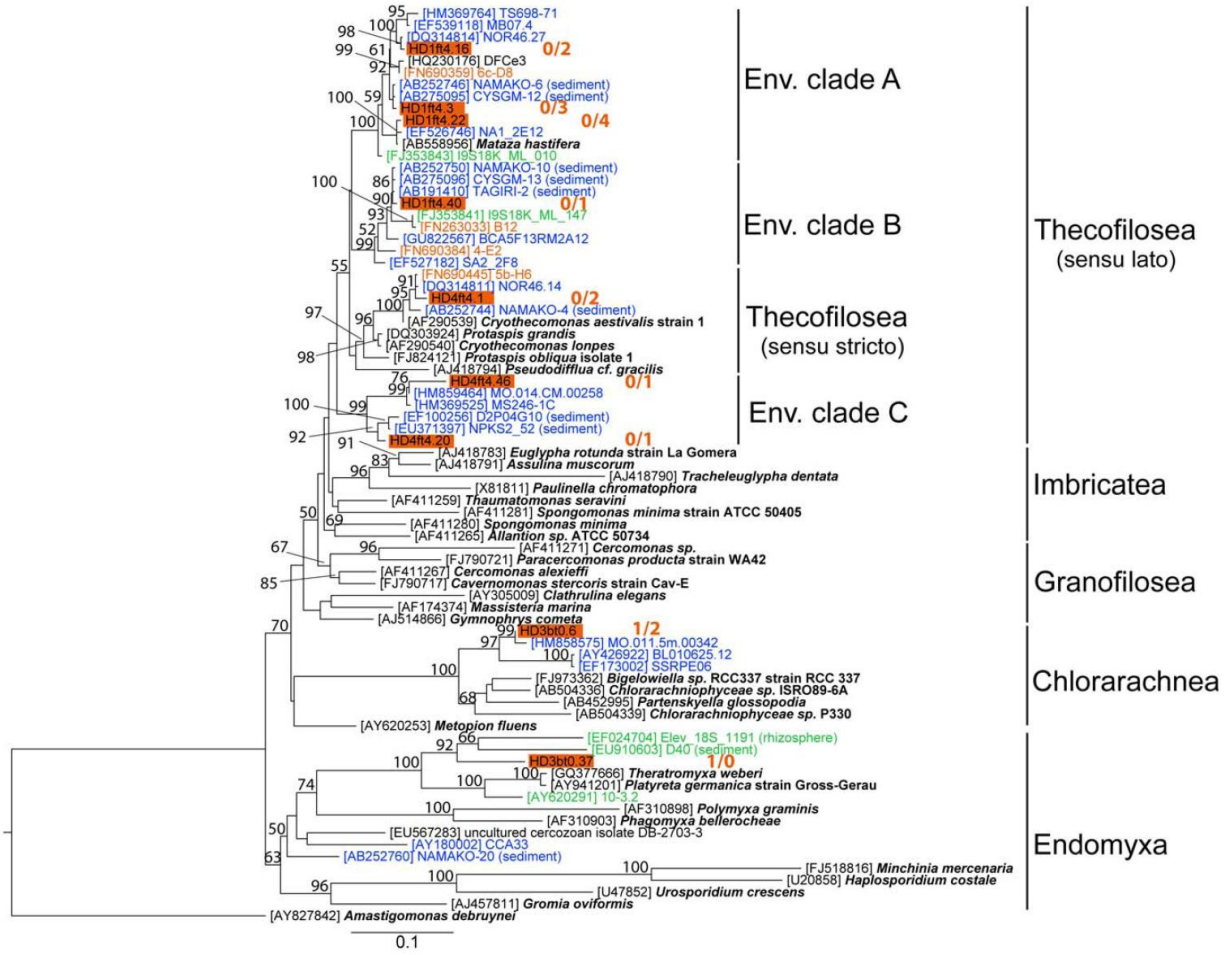


Figure 8. Phylogenetic affiliation of clones within cercozoans. Maximum-likelihood phylogenetic tree constructed with 77 partial and complete cercozoan sequences (1059 informative positions). For further description see legend of Figure 6.
doi:10.1371/journal.pone.0041970.g008

(incubation in the dark). Although we cannot preclude container effects during our incubation and do not exactly know to what extend our results reflect the *in situ* diversity of the heterotrophic protist community, we think that these incubations enable to study an important portion of this community which is generally hidden by most other approaches.

Several methodological limitations, specifically during nucleic acid extraction and PCR amplification, may block the possibility of an absolute and detailed quantification of certain taxa based on their clonal abundance [61]. Nevertheless, by comparing the communities at the start and end of the incubation we obtained relative estimates of the taxa that respectively grew or declined during the course of the experiment. Moreover, RNA-based approaches, as were employed here, are known to provide a more accurate picture of community structure than DNA-based approaches since the latter can suffer from the taxon-specific rDNA copy number and the amplification of DNA derived from inactive and dead cells or even extracellular genetic matter [26,62].

Does a Culturing Bias Apply to Unamended Dark Incubations?

Seawater enclosures, sometimes in combination with size fractionation, have a long tradition of use in ecological studies. They have contributed greatly to our understanding of carbon fluxes in the oceans, trophic interactions in general, and the regulatory mechanisms of bacterial biomass distribution by either nutrient supply or protist consumption [58,63,64,65]. They have also served as precursors for isolation attempts aimed at studying and describing a single microbial species obtained in pure culture [9,66]. On the other hand, the selectivity of this approach towards opportunistic species has led to misinterpretations of the quantitative contribution and widespread dispersal of various protist groups [17,18]. This issue was addressed in two recent studies that investigated the effect of nutrient supply in incubation experiments. Both revealed increasingly rapid and pronounced community shifts as the incubation medium became richer and more complex [48,46]. Specifically, whereas unamended incubations promoted the growth of uncultured HF, these organisms were displaced towards cultured representatives in incubations altered by allochthonous nutrient addition [38,45].

In our experiments a considerable proportion of clones (12% of all clones) at the end of the incubation were closely affiliated with *Paraphysomonas imperforata*. This could indicate a culturing artifact, such as shown by Lim *et al.* [18] for Atlantic seawater incubations with substrate addition. However, based on the occurrence of sequences related to *P. imperforata* in both the DGGE and the clone libraries prior to the incubation, we conclude that this organism was a naturally dominant member of the protist community at the sampling site and remained so throughout the incubation. In addition, during our sampling campaign in Heiligendamm, well known and easily cultivable species like *Cafeteria roenbergensis* and various *Bodo* species were frequently isolated and subsequently maintained in wheat grain cultures (F. Weber and A.P. Mylnikov, unpublished data) but none of these flagellate sequences were detected in our unamended incubation experiments.

Another line of evidence that speaks against a culturing bias is the fact that most of the sequences at the end of the incubation belonged to taxa that were only distantly related to cultured representatives (Fig. 5), some of which were already present in the initial samples. A culturing artifact would have yielded sequences closely affiliated with well known cultured representatives, which are only seldom found in natural samples [46]). With regard to that our findings are more consistent with those obtained by culturing-independent environmental approaches. This allows the conclusion that culturing bias can be disregarded as the main explanation for our results.

Novel Heterotrophic and Putatively Heterotrophic Taxa

Similar to other dark incubations [45,46,67], the experimental design of our study promoted the growth of HF while suppressing that of phototrophic species. Furthermore, based on the tightly coupled bacteria and HF successions, a strong potential for bacterivory can be assumed. This enabled us to link the phylogenetic data obtained at the end of the incubation with the presence of a heterotrophic, phagotrophic life style.

The proportion of sequences unambiguously assigned an obligate heterotrophic function increased during the incubation from 11% to 44% of all found clones. One reason for that was the large contribution of sequences closely related to the bacteria-consuming species *P. imperforata*. Another reason why a heterotrophic function could be inferred for various other clones indeed distantly related to cultured organisms was their phylogenetic placement within obligate heterotrophic protist groups such as the choanoflagellates [68] and cercozoans (excluding chlorarachneans).

Interestingly, the relative abundance of sequences assigned an unknown trophic function doubled during the dark incubation, indicating that at least some should be regarded as facultative heterotrophic flagellates. Prime examples are two OTUs within environmental clade I of chrysophytes, both of which had increased in clonal abundance by the end of the incubation (HD4ct4.10 and HD4ct4.6, represented by 19 and 12 clones, respectively). This clade has been shown to comprise pigmented representatives (clone Biosope_T65.146 [19]) as well as putative heterotrophic flagellates (clone CD8.15 [45]; clones OA3.9 and OA3.13, [46] that grew in samples collected from two distinct oceanic regions and then subjected to unamended dark incubation. Our clones are distantly related to the ones inferred from pigmented cells but very closely affiliated with the sequences deriving from putative heterotrophic flagellates. Therefore, the fact that virtually the same taxa have been detected in three independent studies using the same technique to target heterotrophic and bacterivorous protists provides strong evidence that these chrysophytes of environmental clade I are heterotrophic or mixotrophic flagellates.

Another set of clones was designated as novel ochrophytes according to their phylogenetic placement next to well known photosynthetic ochrophyte groups [69] such as bacillariophytes and raphidophytes. These sequences were represented by three OTUs having sister group relationships to bolidophytes, a group of photosynthetic flagellates that putatively contain mixotrophic members [43]. Similar to the other groups discussed herein, certain OTUs (HD4ct4.41 and HD1ct4.30) contributed more to clone libraries prepared at the end of the incubation than to those derived from the initial samples. Moreover, the closest relatives (clones TS698-92 and TS698-52) of these OTUs were reported from a study in which heterotrophic eukaryotes were sorted by the presence of lysotracker fluorescence and the absence of chlorophyll fluorescence before they were characterized with respect to their 18S rRNA genes [44]. Additionally, a closely (99% similarity) related sequence (clone BLACKSEA_cl_48, HM749950, not shown in the tree) was recently detected by an rRNA-based study of the non-illuminated depth of the Black Sea redoxcline [70]. It therefore seems likely that these novel ochrophyte species have a phagotrophic potential.

Another group of uncultured protists that apparently grew in our experiment consisted of picobiliphytes, which represent a possible first-rank taxon phylogenetically related to cryptophytes and katablepharids [30,52]. Based on microscopy observations in combination with specific 18S rRNA FISH probes, picobiliphytes were suggested to be a new plastid-bearing algal group [30,52]. Evidence to the contrary is that picobiliphytes represented a significant fraction of single amplified genomes (SAG)–determined subsequent to flow cytometric cell sorting of heterotrophic eukaryotes. [44]. Furthermore, the results of whole-genome shotgun sequencing of one representative from each of the three picobiliphyte sub-clusters suggested that they are heterotrophic because no indication for plastid DNA or nuclear-encoded plastid-targeted proteins could be found [71]. The growth of picobiliphytes (especially OTU HD1ft4.11) in our unamended dark incubations supports the postulated heterotrophy of these organisms from another perspective.

Which Taxa are Likely to Constitute the Assemblage of Heterotrophic Protists at the Sampling Site?

Our study represents only a spatial and temporal snapshot of the protistan assemblage in the southwestern Baltic Sea. Nonetheless, it offers an impression of the taxa that presumably play an important role as heterotrophic and bacterivorous protists at this coastal site. Indeed, taxa capable of growth in dark incubations likely belong to flagellate groups able to control and rapidly react to sporadic bacterial bursts, thus providing insight into the heterotrophic protist community during naturally occurring pulses of bacterial food supply. In the Baltic Sea, these pulses are typically observed after phytoplankton blooms. However, frequent fluctuations in the protistan community composition can be expected, particularly for coastal areas in the southern Baltic Sea, due to seasonal variations and to transient perturbation events (mixing, upwelling, riverine influx, salinity changes) [42]. Hence, this may explain why the phylotypes found in this study are not in general agreement with those in the study of Piwosz and Pernthaler [42], carried out in the Gulf of Gdansk, Southern Baltic Sea. Only a more frequent sampling can resolve whether this discrepancy can be attributed to the lower salinities of their study site (6–7 PSU in the Gulf of Gdansk vs. 13–16 PSU in Heiligendamm), to seasonal variations, to sporadic events, or to a combination of all three factors.

As revealed by phylogenetic analysis and BLAST searches, most of the closest relatives to our OTUs originate from marine systems and only very few from freshwater or brackish water habitats (see Fig. 6, 7, 8, S2). A similar pattern was seen in the clone libraries obtained from the Gulf of Gdansk [42]. However, the typical representatives within MAST and MALV, frequently reported in various marine studies, rarely occurred in our samples. Therefore, most phylotypes shown in our experiment to be actively growing, including those within chrysophytes, novel ochrophytes, choanoflagellates, cercozoans, and picobiliphytes, apparently represent euryhaline taxa that tolerate salinities ranging from fully marine to brackish water conditions. Further analysis employing newly designed oligonucleotide probes for fluorescent in situ hybridization will resolve the *in situ* abundance, dispersal within the salinity gradient of the Baltic Sea and seasonal fluctuations of the respective taxa.

Conclusions

Unamended dark incubation caused two effects in our experiments: (1) a shift towards a community made up of heterotrophic protists and (2) the enrichment of primarily uncultured taxa, whose presence may have otherwise been obscured by the high abundances of phototrophs. Bringing both observations together, unamended dark incubations appear to be a powerful tool to bridge the gap between our understandings of natural protistan assemblages provided by culturing and environmental sequencing surveys. Indeed, these incubations level out the major drawbacks of both approaches, i.e., culturing bias and the loss of functional information. Additionally, our results suggest that the heterotrophic protist community in the southwestern Baltic Sea is constituted by a large proportion of as yet uncultured marine flagellates, including some that thus far have been only rarely detected, alongside with well known cultured representatives (e.g., *Paraphysomonas imperforata*). Our study therefore provides important insights into both the trophic function of various uncultured protists, and their dispersal capacity with respect to salinity.

Materials and Methods

Sampling

Sampling was conducted at three different dates in autumn 2008 (October 28; November 11; November 18) at the coastal monitoring station of Heiligendamm, located in the Mecklenburg-bight of the southwestern Baltic Sea (54°08,55′ N; 11°50,60′ E). Surface water samples were lifted in a bucket from a sea bridge 200 m off the sandy littoral, filtered through a 200-µm nylon mesh, and collected in sterile plastic containers that were transferred within 1 h to climate chambers. Temperature and salinity were measured during sampling by a portable conductivity meter (Cond 1970i, WTW GmbH, Weilheim Germany).

Seawater Incubation and Experimental Setup

All plastic containers and glass bottles used during sampling and incubation were washed with 10% HCl and with water purified using the Milli-Q lab water system (Millipore), then rinsed with the seawater fraction used for the incubation. Prior to incubation, one fraction of seawater was filtered through a 3-µm polycarbonate filter (Whatman GmbH) under pressure so gentle that it did not register on the barometer attached to the vacuum pump. Both fractions of seawater (<200 µm and <3 µm) were partitioned into triplicate 1 L glass bottles. Those bottles used in the immediate analysis of the starting parameters (t_0) were filled in the same manner but not as triplicates. All filtration steps and transfers to other receptacles were done conservatively through a tubing connection to prevent damage to fragile organisms in the samples. Incubation was carried out in the dark under near in situ temperature conditions in a 10°C climate chamber.

Cell Numbers in the Course of Incubation

The development of heterotrophic flagellates (HF), bacteria, *Synechococcus*, and phototrophic eukaryotes (nano- and picoeukaryotes) in the incubation bottles was monitored by an almost daily subsampling. Subsamples (30–50 ml) for epifluorescence microscopy were fixed with glutaraldehyde (1% final concentration), stained with DAPI, and filtered on 0.2- and 0.8-µm pore size black polycarbonate filters (Whatman GmbH). HF enumeration was carried out with an epifluorescence microscope (Axio Imager.M1, Zeiss). For bacterial determinations, *Synechococcus* and phototrophic eukaryotes subsamples (4 ml) were fixed with paraformaldehyde and glutaraldehyde (final concentration 1% and 0.05%, respectively), deep-frozen in liquid nitrogen, and analyzed by flow cytometry [72].

RNA Extraction and cDNA Generation

Cell material from water samples (200–350 ml) of the start (t_0) and the end (t_{end}) of the incubation was collected on 0.2-µm pore size polycarbonate filters (Durapore, Millipore). The filters were shock frozen in liquid nitrogen and stored at −80°C for subsequent extraction of nucleic acids.

RNA was extracted according to Weinbauer et al. [73] using an acidic extraction buffer. The washed and dissolved RNA was quality-checked on an agarose gel and quantified using a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop, Thermo Fisher Scientific). Residual environmental DNA was eliminated in RNA extracts by DNase I digestion (DNA-free-kit, Ambion) for 75 min at 37°C. Thereby, enzymatic degradation of RNA was prevented by treating each sample with 0.9 µl of an RNase inhibitor (Peqlab) per 20 µl of RNA sample. Complete removal of the DNA in each of the RNA extracts was verified by PCR, as described in the next section, using the RNA extracts as template. In all cases, amplification was negative (results not shown). To generate cDNA, 200 ng of template RNA was reverse transcribed at 42°C using the iScript Select cDNA synthesis kit (Bio-Rad) following the manufacturer's recommendations. In addition to the random primer provided with the kit, the eukaryote-specific primer Euk B [74] was used. In each reverse transcription reaction, some of the RNA samples used as controls in the PCR were not supplemented with reverse transcriptase, in order to rule out DNA contamination.

Denaturing Gradient Gel Electrophoresis (DGGE)

The well established eukaryote specific primers EukA and Euk516r-GC [21,74,75] were used to amplify the 18S rRNA fragments by PCR. The PCR mixture (50 µl) was composed of 200 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.3 mM of each primer, and 1.25 U of *Taq* DNA polymerase (Fermentas). PCR was started with an initial denaturation at 94°C for 2 min, followed by 28 cycles of 30 s at 94°C, 45 s at 56°C, and 2 min at 72°C. Final extension was done at 72°C for 6 min. DGGE was performed with a vertical electrophoresis system (PhorU, Ingeny). Two previously prepared stock solutions were used to cast a 6% (wt/vol) polyacrylamide gel (ratio of acrylamide to bisacrylamide 37.5:1) with a linear gradient of denaturing conditions ranging from 25 to 55%. After loading equivalent amounts of PCR product in each lane, the gel was run at 100 V for 16 h at 60°C submerged in 0.5× TAE buffer, followed by staining with SybrGold for 1 h in the dark and visualization under

UV radiation in a gel documentation system (Geldoc, BioRad). The resulting image was analyzed with the software GelCompare II (Applied Maths) in order to assign all detected DGGE bands to a certain position in the gel and to quantify their relative intensity in each lane. A 0–1 matrix was constructed based on the presence and absence of bands at a certain position and then used to create a non-metric, multidimensional scaling (MDS) ordination plot with respect to the calculated Bray-Curtis similarity coefficients using PRIMER (v6). Representative DGGE bands excised from the gel and eluted overnight at 4°C in 50 µl of nuclease-free water were stored at −20°C. To reamplify the bands, 1 µl of the eluate served as template in a PCR run under the above-described conditions, except that the reverse primer lacked the GC clamp. In most cases, bands assigned to a certain position in the gel were excised and sequenced from separate lanes to verify whether they belonged to the same taxon.

Clone Library Construction

In total, eight clone libraries from three incubation experiments (Exp1, Exp2, and Exp3) were constructed prior to incubation (t_0) and at the time of enriched flagellate abundance (t_{end}) with samples from the 3-µm and 200-µm filtered treatments. Due to the contamination of *Mnemiopsis leidyi* in Exp2, we were able to obtain only two libraries at the end of the incubation from the unfiltered treatments.

Reverse transcribed 18S rRNA was used as template in PCRs with the primer pair Euk528F [76] and 18S-1630Rev [70], amplifying fragments of almost 1,000 base pairs. The PCR mixture was heated to 95°C for 10 min, and the target then amplified within 30 cycles of 95°C for 30 s, three different annealing temperatures (52°C, 55°C, and 58°C) applied in single reactions for 1 min followed by 70°C for 2 min, and a final extension of 10 min at 72°C. PCR products treated with the three different annealing temperatures were pooled and purified with the NucleoSpin® Extract II kit (Macherey-Nagel). In each of the genetic libraries, similar amounts of purified PCR product were cloned using the StrataClone PCR cloning kit (Stratagene) according to the manufacturer's recommendations. Putatively positive transformants were screened by colony PCR using the vector-specific primers T3 and T7. Cells containing inserts of proper lengths were transferred to LB medium containing 96-well plates and sent to the sequencing service (LGC Genomics, Berlin) for plasmid preparation and Sanger sequencing with the primer Euk528F.

Phylogenetic Analysis

Each chromatogram received by the sequencing service was inspected. Unreliable sequence ends were trimmed and low-quality sequences were excluded from further analysis. Chimera detection and taxonomic affiliation were done using KeyDNA-Tools (http://www.keydnatools.com/) and BLAST [77] searches. Representative 18S rRNA sequences of a single operational taxonomic unit (OTU) based on 99% similarity were searched by creating a distance matrix and clustering the sequences according to the average-neighbor method in Mothur [78]. Multiple alignments were done using MAFFT version 6 [79] and poorly aligned positions were eliminated by Gblocks [80]. The alignments were inspected and manually corrected in BioEdit [81]. Maximum-likelihood phylogenetic trees with complete and partial 18S rRNA sequences were calculated using RAxML version 7.0.4 [82], which was run at the freely available Bioportal of the University of Oslo [83]. Both, tree construction and bootstrap analysis were done in 1,000 replicates on random starting trees under the evolutionary model GTRGAMMA. A consensus tree, displaying the bootstrap values, was computed in MrBayes [84] and the values were transferred into the topology tree having the best likelihood of 1,000 replicates. Sequences representing OTUs based on 99% similarity were deposited in GenBank under accession numbers JQ782278–JQ782384.

Novelty Analysis

The novelty of the clonal sequences was analyzed as described by Massana et al. 2010 [85]. Briefly, each sequence was subjected to BLAST searches to extract the closest similarity to cultured organisms and to the closest environmental representative in GenBank. Both similarity values of each clone and the calculated average similarities of all t_0 and t_{end} clones were displayed in a scatter plot indicating the similarity to the closest environmental representative on the x-axis and the similarity to the closest cultured representative on the y-axis.

Supporting Information

Figure S1 Similarity of samples before and after incubation based on presence and absence of DGGE bands. Two-dimensional representation of a nonmetric multidimensional scaling plot based on the binary DGGE matrix for the three experiments (Exp1 in black, Exp2 in dark gray, Exp3 in light gray). Squares and triangles refer to t_0 and t_{end} samples, respectively. Open and filled symbols represent unfiltered and 3-µm filtered samples, respectively. Note that the dark gray filled triangle represents the identical triplicate samples of Exp2.
(TIF)

Figure S2 Phylogenetic affiliation of clones within picobiliphytes. Maximum likelihood phylogenetic tree constructed with 32 partial and complete picobiliphyte sequences (241 informative positions). Clades follow the notation of Cuvelier et al. [52]. For further description see legend of Figure 6.
(TIF)

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Author Contributions

Conceived and designed the experiments: FW RM KJ. Performed the experiments: FW. Analyzed the data: FW JdC CW RM. Contributed reagents/materials/analysis tools: RM KJ. Wrote the paper: FW JdC CW RM KJ.

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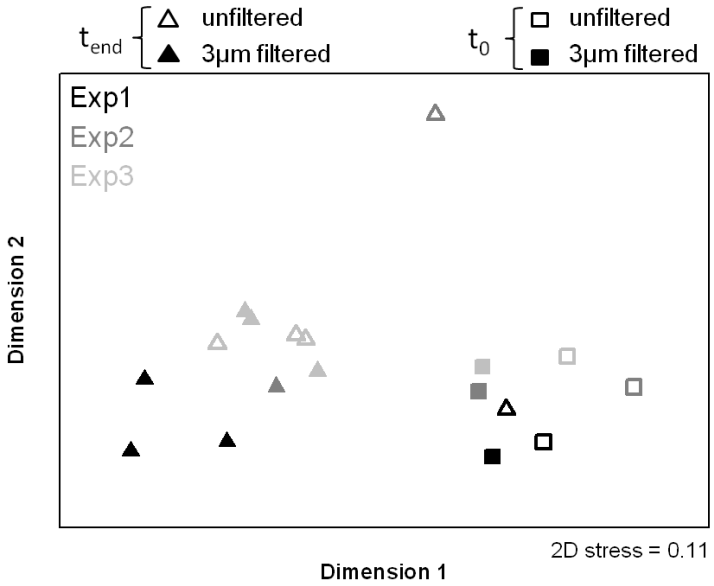
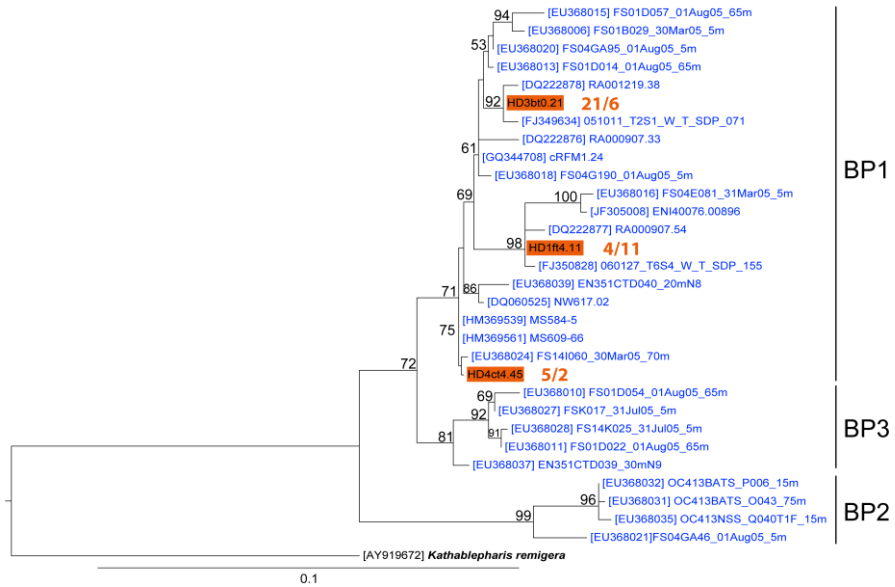
Supporting Information

Figure S1



Similarity of samples before and after incubation based on presence and absence of DGGE bands. Two-dimensional representation of a nonmetric multidimensional scaling plot based on the binary DGGE matrix for the three experiments (Exp1 in black, Exp2 in dark gray, Exp3 in light gray). Squares and triangles refer to t_0 and t_{end} samples, respectively. Open and filled symbols represent unfiltered and 3-µm filtered samples, respectively. Note that the dark gray filled triangle represents the identical triplicate samples of Exp2.

Figure S2



Phylogenetic affiliation of clones within picobiliphytes. Maximum likelihood phylogenetic tree constructed with 32 partial and complete picobiliphyte sequences (241 informative positions). Clades follow the notation of Cuvelier et al. [52]. For further description see legend of Figure 6.

Publications and Conference contributions

Publications

Journal Articles (peer-reviewed)

- **Weber F**, del Campo J, Wylezich C, Massana R, Jürgens K (2012) *Unveiling Trophic Functions of Uncultured Protist Taxa by Incubation Experiments in the Brackish Baltic Sea.* PLoS ONE 7(7): e41970. doi:10.1371/journal.pone.0041970

- **Weber F**, Anderson R, Foissner W, Mylnikov AP, Jürgens K (2014) *Morphological and Molecular Approaches Reveal Highly Stratified Protist Communities in Baltic Sea Pelagic Redox Gradients.* Aquat Microb Ecol 73:1–16

- Mylnikov AP, **Weber F**, Jürgens K, Wylezich C (2015) *Massisteria marina has a sister: Massisteria voersi sp. nov., a rare species isolated from coastal waters of the Baltic Sea*. Eur J Protistol 51(4), 299–310.

Manuscripts in preparation

- **Weber F**, Wylezich C, Mylnikov AP, Jürgens K (to be submitted) Culturing of protists from the Baltic Sea: many usual suspects as well as some novelties

- **Weber F**, Jürgens K. (in prep.) High abundance and grazing activity of uncultured chrysophytes in seawater incubations as revealed by newly designed phylogenetic probes.

Press Releases

- *Die kleinsten "Raubtiere" der Ostsee.* IOW-Pressemitteilung vom 02. August 2012.

- *The smallest predators in the Baltic Sea.* IOW Press Release of 9 August 2012.

Conference Contributions

Oral Presentations:

- Wylezich C, **Weber F**, Anderson R, Herlemann D and Jürgens K. *Assessing the structure and function of protist communities in oxygen depleted waters of the Black and the Baltic Sea.* 14th International Congress of Protistology (ICOP XIV), Vancouver, BC, Canada, 2013.

- **Weber F**, del Campo J, Wylezich C, Massana R and Jürgens K. *Taxonomic novelty of heterotrophic protists revealed by unamended brackish water incubations from the Baltic Sea.* 12th Symposium on Aquatic Microbial Ecology (SAME), Rostock-Warnemünde, Germany, 2011.

- **Weber F**, del Campo J, Wylezich C, Massana R and Jürgens K. *Taxonomic novelty of heterotrophic protists revealed by unamended brackish water incubations from the Baltic Sea.* 6th European Congress of Protistology (ECOP VI) Berlin, Germany, 2011.

- Anderson R, **Weber F**, Wylezich C, Mylnikov A, Foissner W and Jürgens K. *Protist diversity, distribution and bacterivory in Baltic Sea Pelagic Redoxclines.* Aquatic Sciences Meeting (ASLO), San Juan, Puerto Rico, 2011.

Poster Presentations:

- Wylezich C, Marcuse M, Karpov SA, Mylnikov AP, Anderson R, **Weber F** and Jürgens K. *Ecologically relevant choanoflagellates collected from oxygen depleted water masses of the Baltic Sea have untypical mitochondrial cristae.* 14th International Congress of Protistology (ICOP XIV), Vancouver, BC, Canada, 2013.

- **Weber F**, del Campo J, Wylezich C, Massana R and Jürgens K. *Detecting novel taxa of heterotrophic protists by dark incubation experiments in the Baltic Sea.* Wissenschaftliche Beiratssitzung am Leibniz Institut für Ostseeforschung Warnemünde, Germany, 2012.

- **Weber F**, del Campo J, Wylezich C, Mylnikov A, Massana R and Jürgens K. *Identification of the dominant bacterivorous protists in the Baltic Sea.* 29th Annual Conference of the National German Protozoology association (DGP), Düsseldorf, Germany, 2010.

- Anderson R, **Weber F** and Jürgens K. *Grazing patterns in Baltic Sea Redoxclines.* 29th Annual Conference of the National German Protozoology association (DGP), Düsseldorf, Germany, 2010.

- **Weber F**, Anderson R, Wylezich C and Jürgens K. *Protist Diversity, Distribution and Bacterivory in Baltic Sea Pelagic Redoxclines.* 28th Annual Conference of the National German Protozoology association (DGP), Naumburg, Germany, 2009.

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- **Weber F**, Brockmöller H, Mylnikov A, Foissner W, and Jürgens K. *Distribution and diversity of protists in pelagic redoxclines of the central Baltic Sea.* 27th Annual Conference of the National German Protozoology association (DGP), Warnemünde, Germany, 2008.

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Declaration of Authorship

Ich versichere hiermit an Eides statt, dass ich die vorliegende Promotionsschrift selbstständig angefertigt und ohne fremde Hilfe verfasst, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, den 27.11.2015