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Analysis of the unique protozoan communities of the abyssal sea floors

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"Wer sich Steine zurechtlegen kann, über die er stolpert, hat Erfolg in den Naturwissenschaften."

Erwin Chargaff

UNIVERSITY OF COLOGNE

Abstract

Faculty of Mathematics and Science

Analysis of the unique protozoan communities of the abyssal sea floors

Although the abyssal seafloor (3-6 km depths) is the largest benthic habitat on this planet covering around 54% of the Earth's surface, knowledge about deep-sea protists, their ecological function, species-level distribution and diversity in these depths is scarce. This is in striking contrast to their potential importance regarding the material flux and bacteria consumption in the deep sea.

The aim of this study was to extend the knowledge of biodiversity and biogeography of benthic deep-sea protist communities, with a focus on abyssal regions. We established a protocol combining several available qualitative and quantitative methods to receive a detailed information on biodiversity and biogeography of benthic deep-sea protist communities. For a global comparative study on benthic protist diversity we sampled sediment from 20 deep-sea basins located in the Pacific and Atlantic Ocean and extracted the DNA for next-generation sequencing (NGS). Comparing our deep-sea OTUs to reference sequences indicated the existence of a specific deep-sea protist fauna (sequence similarity of 90.6% on average). The majority (91%) of our total operational taxonomic units (OTUs) could be assigned to protists. Dominant protist OTUs belonged to Discoba (Diplonemea and Kinetoplastea), Alveolata (Dinophyceae, MALV-I, MALV-II, Ciliophora) and Rhizaria (Foraminifera and Endomyxa). Comparative analysis of the different deep sea basins revealed distinct protist communities on a large and small spatial scale. Only 0.8% of protist OTUs occured in all stations. Molecular studies of cultured deep-sea protists helped us to assess the quality of deep-sea sampling. Several protist OTUs could solely be assigned to our isolated strains underlining the importance of cultivation and taxonomic assignment of protists. In addition, the enhanced cultivation of deep-sea and surface water protists led to a deeper evaluation of the phylogenetic relationship within the Cafeteriaceae. Based on multigene analysis, we regrouped the Cafeteriaceae into eight species of which six were newly described. We could cultivate the first phagotrophic euglenid, *Keelungia nitschei* sp. nov., from the deep sea. Phagotrophic euglenids are known to be typical components in marine sediments and shallow waters, but have seldom been reported, and not yet cultivated from deep-sea samples. Cultured isolates were further used to verify the potential deep-sea origin of the protists recovered by the NGS technique with the aid of pressure and temperature experiments. The deep-sea strain *Keelungia nitschei*, for instance, was better adapted to high hydrostatic pressures at low temperatures (4°C) than the two surface water euglenids *Petalomonas acorensis* gen. et sp. nov. and *Ploeotia costaversata* sp. nov..

While our metagenome data and literature review on ciliates indicated a deep-sea specific nanofauna, we could cultivate and isolate the same genotypes for the bicosoecid *Cafeteria burkhardae* sp. nov. and the ciliate *Pseudocohnilembus persalinus* from marine surface

waters as well as the deep sea. These genotypes, despite their isolation of depths, survived high hydrostatic pressure indicating their potential to live and reproduce in the abyss. There might be a possible exchange of several protist groups between surface waters and the deep sea. Aggregations such as sinking detritus, besides being a food source, may serve as transportation vehicles to the deep sea for different protist species. For the southern North Atlantic Ocean we could show that there is a large biomass of sedimented *Sargassum* algae on the seafloor which can be in the same range as that at the surface. Analysis of fatty acids and stable isotopes indicated that the benthic macrofauna might not directly consume *Sargassum* in the abyss, but probably via the components of the abyssal microbial food web including bacteria and protists.

To summarize, we could establish methodological approaches to cultivate and analyze deep-sea protist communities (morphological and molecular techniques), describe new species and analyze abiotic (pressure) and biotic conditions (food sources) in deep-sea environments. We showed that protist diversities differed on a local and global scale and are distinct from surface water communities, while several genotypes occured in cultures from surface waters and the deep sea.

UNIVERSITÄT ZU KÖLN

Kurzzusammenfassung

Mathematisch-Naturwissenschaftliche Fakultät

Analysis of the unique protozoan communities of the abyssal sea floors

Obwohl der abyssale Meeresboden (3-6 km Tiefe) 54% der Erdoberfläche bedeckt und somit den größten benthischen Lebensraum auf diesem Planeten darstellt, ist der Kenntnisstand bezüglich der Tiefseeprotisten, ihrer ökologischen Funktion, Artenverteilung und Diversität gering. Dies steht in krassem Widerspruch zur potentiellen Bedeutung der Protisten für den Stoffffluss und den Bakterienkonsum in der Tiefsee.

Ziel dieser Arbeit war es, diesen Kenntnisstand der Biodiversität und Biogeographie von Tiefseeprotistengemeinschaften, besonders in den abyssalen Regionen, zu erweitern. Dafür wurde ein Protokoll etabliert, das verschiedene aktuell verwendete qualitative und quantitative Methoden miteinander kombiniert. Für einen globalen Vergleich der benthischen Protistengemeinschaft wurden Sedimentproben aus 20 verschiedenen Tiefseebecken des Atlantiks und Pazifiks gesammelt und die DNA für Next-Generation-Sequenzierung extrahiert. Im Durchschnitt wiesen die gewonnenen taxonomischen Tiefsee-Gruppen ("operational taxonomic units", OTUs) nur eine Übereinstimmung von 90,1% mit Referenzsequenzen aus der Datenbank auf. Dies deutet auf die Existenz einer spezifischen Protistenfauna in der Tiefsee hin. Der Großteil (91%) unserer gesamten Tiefsee-OTUs konnte den Protisten zugeordnet werden. Dominante Protisten OTUs gehörten zu den Discoba (Diplonemea und Kinetoplastea), Alveolata (Dinophyceae, MALV-I, MALV-II, Ciliophora) und Rhizaria (Foraminifera und Endomyxa). Der Vergleich der Protistengemeinschaften der beprobten Tiefseebecken zeigte signifikante Unterschiede auf einer globalen und lokalen räumlichen Skala. Nur 0,8% unserer Protisten OTUs konnten in allen Stationen gefunden werden. Die molekulare Untersuchungen der kultivierten Tiefsee-Protisten halfen dabei, die Qualität der Tiefseeproben einzuschätzen. Einige Protisten OTUs konnten ausschließlich unseren isolierten Stämmen zugeordnet werden, was die Bedeutung der Kultivierung und taxonomischen Zuordnung von Protisten unterstreicht. Zusätzlich konnte durch die Kultivierung von Protisten aus Tiefsee- und Oberflächenwasser eine tiefere Evaluierung der phylogenetischen Verwandtschaftsbeziehungen innerhalb der Cafeteriaceae erfolgen. Multigenanalysen führten zu einer Revision der Cafeteriaceae in acht Arten, von denen sechs neu beschrieben wurden. Weiterhin konnte die erste phagotrophe Euglenide *Keelungia nitschei* sp. nov. aus der Tiefsee kultiviert werden. Phagotrophe Eugleniden sind als typische Komponenten in marinen Sedimenten und flachen Gewässern bekannt, wurden jedoch selten aus Tiefseeproben dokumentiert, geschweige denn kultiviert. Weiterhin wurden die Isolate dafür verwendet, die potentiell in der Tiefsee aktiven Protisten aus unseren Metagenomdaten durch Druckexperimente zu verifizieren.

Der Tiefsee-Stamm *Keelungia nitschei* war besser an hohe hydrostatische Drücke bei niedrigen Temperaturen (4°C) angepasst als die beiden Eugleniden-Stämme *Petalomonas acorensis* gen. et sp. nov. und *Ploeotia costaversata* sp. nov., die aus Oberflächenwasser isoliert wurden.

Während die Metagenomdaten und die Literaturrecherche über Tiefseeciliaten auf eine tiefseespezifische Nanofauna hindeuteten, konnten identische Genotypen für die Bicosoecide *Cafeteria burkhardae* und den Ciliaten *Pseudocohnilembus persalinus* aus Oberflächenwasser als auch aus der Tiefsee isoliert werden. Darüber hinaus waren diese Genotypen in der Lage, hohe hydrostatische Drücke zu überstehen, was auf ihre potentielle Aktivität in der Tiefsee hindeutet. Ferner deutet dies auf einen möglichen Austausch zwischen Oberflächenwasser und der Tiefsee hin. Aggregate wie z.B. sinkender Detritus dienen nicht nur als Nahrungs- quelle, sondern können auch als Transportmittel in die Tiefsee für verschiedene Protisten dienen. Für den südlichen Nordatlantik konnte gezeigt werden, dass auf dem Meeresboden eine ähnlich große Biomasse von sedimentierten *Sargassum* - Algen wie an der Oberfläche vorhanden ist und diese daher vermutlich eine wichtige Rolle für die benthische Produktion spielt. Die Analyse von Fettsäuren und stabilen Isotopen zeigte, dass die benthische abyssale Makrofauna *Sargassum* nicht direkt, sondern wahrscheinlich über Bakterien und Protisten des mikrobiellen Nahrungsnetzes konsumiert.

Zusammenfassend konnten innerhalb dieser Arbeit Methoden zur Kultivierung und Analyse von Tiefseeprotistengemeinschaften (morphologisch und genetisch) etabliert, neue Arten beschrieben sowie abiotische (Druck) und biotische (Nahrung) Bedingungen in der Tiefsee analysiert werden. Die Protistendiversität unterschied sich auf einer räumlichen Skala lokal und global und wies Unterschiede zu Oberflächenwassergemeinschaften auf, während einige Genotypen sowohl in Kulturen aus Oberflächenwasser und aus der Tiefsee auftraten.

Contents

General Introduction

Biodiversity in the Deep Sea

The deep sea is divided into different benthic and pelagic depth profiles, expanding beneath continental shelf depths (200 m depths), with an average depth of approximately 4,200 m. Benthic bathyal regions (1,000 - 3,000 m) have higher temperatures compared to deeper parts of the ocean. In benthic abyssal (3,000-6,000 m) regions as well as hadal trenches (>6,000 m) fairly constant environmental conditions including low temperatures are prevailing. The abyssal sea floor, consisting of muddy soft sediment, covers around 54% of the Earth surface. Even though its extent is orders of magnitude greater representing the largest and most remote biome of Earth (Gage and Tyler, 1991), less than 1% of the deep-sea has been investigated up to now. The abyssal seafloor was formerly assumed to be a featureless and continuous habitat with stable environmental conditions and the lack of physical barriers. Nowadays it is known that deep-sea systems are extremely heterogeneous at all spatial scales and frequently characterized by sudden changes (Levin et al., 2001). In addition to small geological features (e.g. seamounts, deep-water coral reefs, pockmarks), deep trenches, Mid-Ocean Ridges and Fracture zones form a highly complex environment at the landscape level (Watling et al., 2013; Devey et al., 2018). Early scientific studies have presumed the deep sea to be devoid of any biological activity, a paradigm that has been refuted (Sanders, 1968; Grassle, 1989).

Large-scale studies demonstrated a close link between benthic eukaryotic biodiversity and ecosystem functioning and the negative impact of biodiversity loss in deep-sea ecosystems (Danovaro et al., 2008). Effects of climate change (warming, acidification, deoxygenation) are already impacting this vast and deep ecosystem (Figure 1). In addition, the growing global demand for mineral extraction and the significant improvement of mining methodologies is enhancing the plans to mine the deep sea for manganese nodules, cobalt-rich ferromanganese crusts, gas hydrates and polymetallic massive sulphides (Van Dover, 2011; Collins et al., 2013), although deep-sea mining is accompanied with a great risk (Figure 1). Deep gas hydrate extraction might result in a potential destabilization of the deep-sea floor (Lee et al., 2010; Song et al., 2014). Vent dwelling communities together with diverse associated organisms are thought to be impacted by mineral extraction at hydrothermal vent sites (Van Dover, 2011; Collins et al., 2013). A consequence of bottom trawling has already been associated with declines in faunal biodiversity, cover and abundance by removing most of the benthic fauna (Thrush and Dayton, 2002; Pusceddu et al., 2014; Clark et al., 2015). The seabed disturbances at the DISCOL area in the Peru Basin in 1989 and at the Clarion Clipperton Fracture Zone in 1978 to test mining of nodules are

still clearly visible today. In those areas where the top layer of sediment was removed, the microbial communities still showed reduced metabolic activity and biomass (Miljutin et al., 2011).

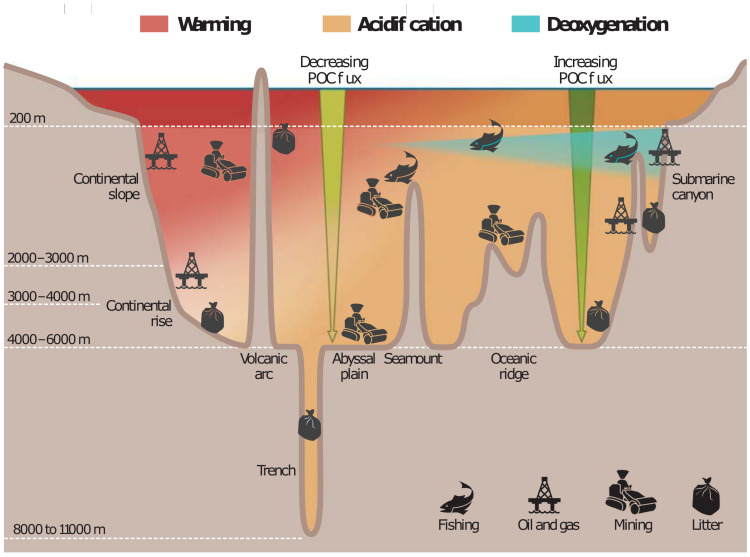

Figure 1: Impact of human activities and climate change on the deep ocean. Human exploitation activities (mining, waste disposal, fisheries) and associated increase of CO^2 levels will negatively impact the temperature, pH, and oxygenation of the deep ocean. (from Levin and Le Bris, 2015).

Emerging evidences point at the dark ocean as a site with a major role in ocean biogeochemistry and an "untapped reservoir" (Arístegui et al., 2009) of high genetic and metabolic microbial diversity (Bernard et al., 2018). Because microbes have distinct dispersal limitations in comparison to large organisms (e.g. megafuna), the geographical distribution of eukaryotic microbial life is substantial for understanding overall microeukaryotic diversity (Logares et al., 2014; Zhang et al., 2017). Most microbial communities consist of highly diverse and abundant species of which several are highly dominant and many that are represented by only a few individuals, the so called "rare biosphere" (Logares et al., 2014). Rare species are likely to be more influenced by different factors in comparison to the more dominant species and can have important ecological roles, serving as nearly limitless reservoirs of genetic and functional diversity (Pedrós-Alió, 2012; Lynch and Neufeld, 2015; Zhang et al., 2017). Besides bathymetric features, environmental factors such as water masses and time may be decisive factors in shaping the abyssal microbial community structure. There is a huge effort of researchers to understand deep-sea ecosystems (Levin et al., 2001; Brandt et al., 2007; Brandt and Ebbe, 2009; Caron and Countway, 2009; Ebbe et al., 2010; Ramirez-Llodra et al., 2010), but we are still lacking a firm understanding of deep-sea ecosystem processes, diversity and spatial distribution on regional and global scales.

Protists in the Deep Sea

Protits are unicellular eukaryotic organisms with a size range from 2-200 μm, while some can be up to cm's. Due to their ability to form resting stages (cysts) they can overcome harsh environmental conditions (Goineau and Gooday, 1970; Rivera et al., 1994). They are ubiquitiously distributed and abundant in terrestrial and aquatic systems fulfilling critical ecosystem functions (del Campo and Massana, 2011; Bates et al., 2013; Geisen et al., 2015). Marine protists consist of extremely diverse taxa which have evolved complex behavioural strategies and multiple trophic modes including autotrophy, heterotrophy, saprotrophy, parasitism and symbioism (Worden et al., 2015). In marine ecosystems protists play an important role within the microbial food web as link for higher trophic levels and are important nutrient remineralizers in biogeochemical cycles (Azam and Malfatti, 2007; Landry and Calbet, 2004).

Establishment and improvements of molecular methods have finally led to a widely accepted consensus in protist taxonomy, but the deeper phylogeny of several groups still remains unresolved (Adl et al., 2012; Archibald et al., 2017). Protists are divided in several major "supergroups" including Archaeplastida, SAR (including Stramenopiles / Heterokonta, Alveolata, and Rhizaria), Discicristata, Metamonada, Amoebozoa, and Obazoa (inlcuding animals and fungi) (Figure 2). Several groups of uncertain placement (Incertae Sedis) exist within the tree of life. While the Cryptista (cryptophyte algae and their relatives), Haptophyta, and Centrohelida, are most closely related to Archaeplastida and/or SAR, Ancyromonadida seem to be more closely related to Amoebozoa and Obazoa (Archibald et al., 2017). Recent estimates indicate a global existence of 70.000 - 150.000 protist species of which only a small fraction is yet known (de Vargas et al., 2015; Grossmann et al., 2016; Mahé et al., 2017).

Expensive ship time and extreme environmental conditions such as depths and resulting pressure make sampling the deep sea a challenging task. There is no standardized protocol for sampling and analyzing benthic deep-sea protists. Cultivation-based methods miss a majority of taxa since most species require specific cultivation conditions, but sometimes even novel taxa appear (del Campo et al., 2013; Weber et al., 2017). The analysis of bulk DNA from deep-sea sediments allows for both a qualitative analysis and a rough assignment of trophic functions of deep-sea nanofauna. Scientists can now easily reveal microbiomes with high coverage, including rare species. However, chimeric sequences derived from PCR amplification artifacts (Kunin et al., 2010; Haas et al., 2011; Mahé et al., 2015b), random sequencing errors, restriction of 'general' primers to detect all protist groups (Pawlowski et al., 2012) and the patchy coverage of reference databases currently present substantial challenges for robust taxonomic interpretations of eukaryotic datasets. Several methods (e.g. mock communities) and pipelines have been developed to remove, mitigate and estimate those biases and problems for downstream analyses (Yeh et al., 2018). Thus, a combination of quantitative and qualitative methods including culture-dependent and culture-independent studies is required to gain a high resolution of deep-sea protist communities.

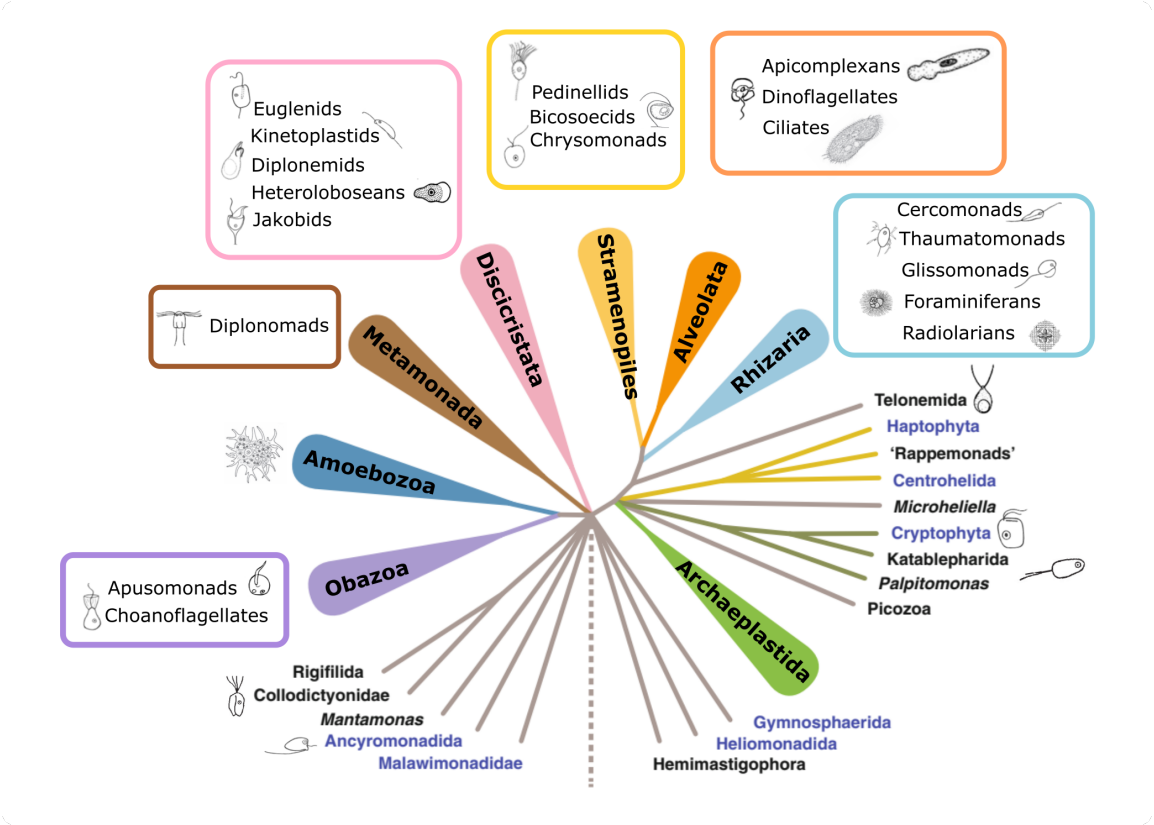

Figure 2: Phylogenetic tree of eukaryotes, highlighting protist diversity occuring in all major supergroups. Tree adapted from Archibald et al. (2017), protist schemes from Jeuck and Arndt (2013).

Genetic approaches like next-generation sequencing have turned out to be reliable tools in identifying novel and uncultured protistan lineages in surface waters and the deep sea (López-García et al., 2001; del Campo and Massana, 2011; Edgcomb et al., 2011; Triadó-Margarit and Casamayor, 2013). The ecological roles of several of these novel lineages might be responsible for critical environmental processes (del Campo et al., 2016). Many new lineages, such as marine alveolates MALV or marine stramenopiles MAST (Massana et al., 2014) have been described as new groups in the phylogeny. MAST contain a diverse range of polyphyletic groups branching at the base of stramenopiles, with each lineage likely occupying slightly different ecological niches (Massana et al., 2014). MAST groups are comprised of bacteria feeding flagellates, with a few groups preferring algae (MAST-6, (Piwosz and Pernthaler, 2010)) and parasitic species (Gómez et al., 2011). While several MAST groups could be detected mainly in the plankton (Massana et al., 2004), MAST-1, -9 and -12 could also be found in deep-sea sediments (Takishita et al., 2007). The MALVs include five independent lineages branching at the base of dinoflagellates in phylogenetic lineages. Known representatives of this cluster belong to Syndiniales (Dyndinids, Dinophyta) and are all parasitic. MALVs have already been identified in sediment at hydrothermal vents (Edgcomb et al., 2002; López-García et al., 2003). The potential ecological role of unicellular opisthokonts both in the water column and in the sediment is limited. A new lineage, the marine fonticulids (MAFO), appear to be abundant in coastal sediments (del Campo et al., 2015). Rhizaria play an important role worldwide in marine plankton

communities, some of which live in symbiosis with eukaryotic microalgae. One group within the Rhizaria, the foraminiferans, are typical benthic species, which dominate in biomass and abundance in the deep sea. Some morphotypes of deep-sea foraminifera have broad geographic boundaries, and some of them were even genetically identical across the global ocean (Pawlowski et al., 2007; Lecroq et al., 2009). Monothalamids, foraminiferans with a single chamber, have often been described as the most abundant form within the foraminifera in the deep sea not only by morphological determinations but also by molecular studies of environmental samples (Lecroq et al., 2011; Goineau and Gooday, 2017). In deeper waters diplonemids, belonging to the Discicristata, have been observed as major taxonomic group (Flegontova et al., 2016).

Comparative analyses of microbial communities of benthic shelf and deep-sea habitats showed differences in protistan assemblages indicating the existence of specific deep-sea heterotrophic protist communities (López-García et al., 2001; Countway et al., 2007; Scheckenbach et al., 2010). In addition, previous studies of deep-sea nanofauna have documented the possible existence of a specific abyssal nanofauna (Arndt et al., 2003; Scheckenbach et al., 2005, 2006). However, in some cases even similar genotypes of heterotrophic flagellates had been isolated from the deep sea and surface waters (Scheckenbach et al., 2005). We already have some information regarding the large-scale patterns of protist (Countway et al., 2007; Scheckenbach et al., 2010) and prokaryotic communities (Kouridaki et al., 2010; Schauer et al., 2010), as well as temporal patterns of prokaryotic communities (Fuhrman et al., 2006; Treusch et al., 2009), but knowledge on both spatial and temporal patterns and their link to environmental processes is scant. While one study on abyssal seafloor bacteria from bathyal and abyssal depths on a global scale already exist (Bienhold et al., 2016), a global deep-sea approach from abyssal depths for protist does not exist. Studies on benthic deep-sea protist assemblages mainly concentrated on assumed hot spots like hydrothermal vents, cold seeps and anoxic regions mostly from bathyal zones at a local scale (Takishita et al., 2005, 2007; Edgcomb et al., 2009; Stoeck et al., 2009; Bernhard et al., 2014) or one a single group, the foraminiferans (Pawlowski et al., 2011; Gooday and Jorissen, 2012; Goineau and Gooday, 2017). A global survey of bathypelagic microbial eukaryote communities identified a few groups as the dominant part of deep-sea communities, whereas the proportional composition of the dominant groups varied on a global scale (Pernice et al., 2016). Only a few studies focused on protist life at abyssal depths (Edgcomb et al., 2002; Scheckenbach et al., 2005, 2010). One study from Salani et al. (2012) focused on kinetoplastids patterns in abyssal regions, a group which is making a major fraction of protist deep-sea communities with only minor contributions from epipelagic depths. They found relatively similar communities of kinetoplastids in the range of hundreds and thousands of kilometers for the South-West Atlantic (Argentine and Brazil Basin) and the South-East Atlantic (Angola and Guinea Basin) (Salani et al., 2012). They recorded statistically significant differences among several communities (i.a., the Mediterranean communities) indicating that deep-sea communities of kinetoplastids are shaped at larger spatial scales by multiple differences in environmental conditions.

Another study focused on the overall diversity of protists using environmental sequencing and showed that the environmental differences between the Cape/Namibian Abyssal Plain and both the Angola- and Guinea Abyssal Plain are paralleled by significant differences in the community structure of protists (Scheckenbach et al., 2010). This supports the idea of restricted distribution areas of, at least less opportunistic species, in the abyss. One study in the abyssal regions of the Pacific Ocean showed, that ciliate communities shared up to 78% of their OTUs from the shallowest site (813 m) to sediments deeper than 3800 m. No significant effects of measured environmental factors on ciliate community composition and structure were observed, unlike observed for benthic ciliates in shallow seafloors (Zhang et al., 2017). Pelagic ciliate communities from abyssopelagic zones did not show a distance-decay relationship along the horizontal scale, but a distinct vertical distribution within the ciliate communities was revealed (Zhao et al., 2017). Only a few studies exist on bacterial communities, none has been reported for protist communities at hadal trenches deeper than 8 km.

Overall, the existence of spatial patterns in biodiversity, shaped by the heterogeneity of deep-sea habitats at large and small spatial scales is far from being clarified. Underexplored areas of ocean sediment include abyssal deep-sea floors and hadal oceanic trenches. The main challenge in deep-sea microbial ecology is to elucidate patterns of biodiversity at a global as well as temporal scale (Logares et al., 2014; Nunes-Alves, 2015). It is a necessity to understand ecological factors/processes and mechanisms that govern and maintain ecosystem functions before deep-sea ecosystems are irreversible damaged (Hanson et al., 2012). Benthic protist communities and the environmental factors shaping their distribution are far from being completely understood and surveys of protists in the dark ocean are still at the beginning.

Aims

The main objective of this thesis was to investigate and extend the scarce knowledge about deep-sea protists and to conduct a global comparison of biodiversity and biogeography of the undersampled deep-sea nanofauna. Therefore, we sampled sediment from 20 deep-sea basins located in the Atlantic and Pacific Ocean at bathyal, abyssal and hadal depths. While this study mainly focused on abyssal protist communities, two-thirds of the sediment samples were obtained from abyssal regions. Sediment samples were taken with a Multi-Corer to obtain samples from the upper 2 mm sediment and the overlaying water (Figure 3). While no standardized protocol for sampling benthic deep-sea protists exist, we combined several culture-dependent and culture-independent methods for a qualitative and quantitative estimation of deep-sea protists. Sediment samples were used to isolate cultivable protists (also from the overlaying water of the cores), and fix samples for DNA analysis for next generation sequencing (Figure 3). Furthermore, isolated and cultivated deep-sea nanofauna from different sites were used to study their morphological and molecular identity, first, as a reference for next generation sequencing studies and, second, to study aspects of the global distribution of genotypes.

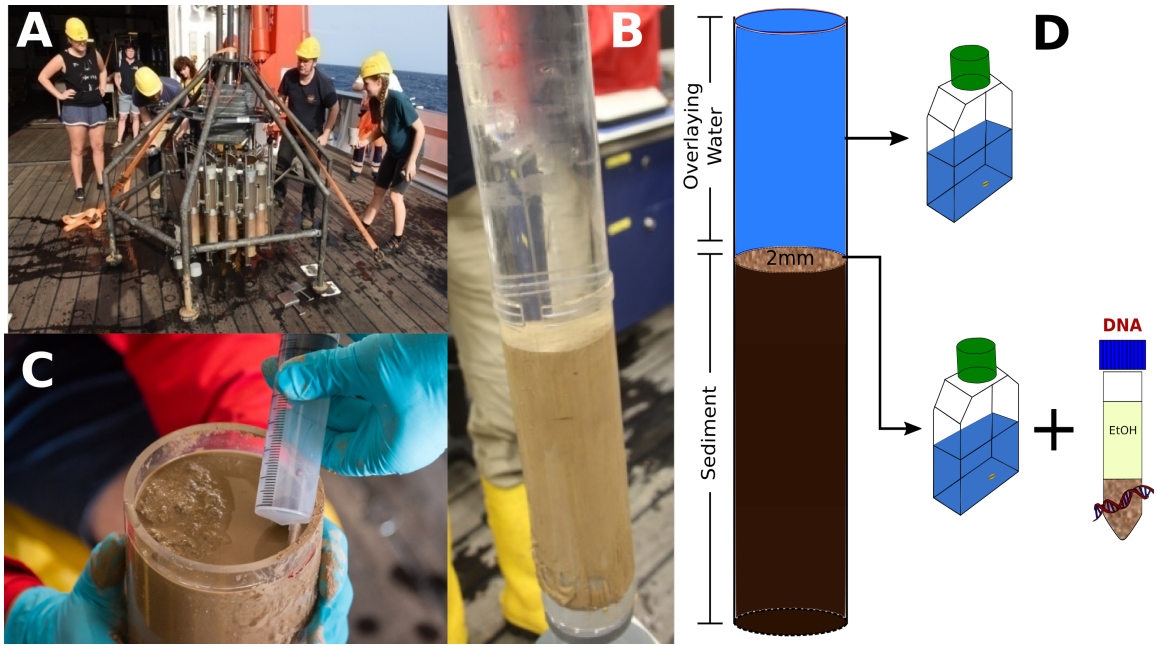

Figure 3: Sampling of benthic deep-sea protist communitues. (A) Sediment samples were taken with a Multi-Corer system equipped with several plexiglas cores. (B) Only cores filled with several centimeters of undisturbed sediment and overlaying water were used. (C) The upper 2 mm of surface sediment were taken with a sterile syringe. (D) Overlaying water and sediment samples were used for cultivation of protists. Sediment samples were fixated for next-generation sequencing (DNA analysis).

We aimed to analyze whether the isolated deep-sea fauna showed significant divergences between the different deep-sea basins, being adjacent and far apart from each other, and between the different depths. Therefore, we addressed the following hypotheses:

1. The spatial distance is reflecting the diversity of protist communities of the abyssal sea floors.
2. The different deep-sea basins harbour specific protist communities.
3. Certain protists show a biogeography in the different deep-sea basins.
4. There are specific groups of protists which occur only in the abyssal plains.
5. There is a specific and unique community of protists which differs from those of other marine habitats.

To answer these hypotheses, to underline the importance of a combination of several methods for analyzing deep-sea protist communities and the importance and potential influence of sinking aggregations (in this case the brown algae *Sargassum*) on protist communities, the thesis is divided into four chapters. These chapters cover the aspects of methodological approaches on analyzing deep-sea protists (Chapter 1), the results of establishing clonal cultures of deep-sea and surface water protists combined with ecological experiments (Chapter 2) as well as deep-sea protist diversity on a global scale by next-generation-sequencing (Chapter 3). In Chapter 4 the potential importance of *Sargassum*-algae in the deep-sea and the role of protists is discussed.

Chapter 1

Methodological Studies on Estimates of Abundance and Diversity of Heterotrophic Flagellates from the Deep-Sea Floor

This study aimed at providing a summary and recommendation of currently used culture-dependend and culture-independent methods in the study of benthic deep-sea protist communities in order to obtain the highest possible qualitative and quantitative resolution.

Chapter 2: Cultivation of Protists

2.1 Deep-Sea Ciliates: Recorded Diversity and Experimental Studies on Pressure Tolerance

To get an overview of the ciliate fauna in the deep sea we summarized the available information on ciliate occurrences in the deep sea. Species that occur both in the deep sea and in surface waters are rarely found to our knowledge to date. We could isolate two scuticociliate species (*Pseudocohnilembus persalinus* and *Uronema* sp.) from both the surface and the deep ocean (2687 m, 5276 m, 5719 m) of the Pacific. We aimed at comparing these strains by means of molecular studies and their adaptation to deep-sea conditions by conducting high pressure experiments. We aimed to show the possibility of these species to survive deep-sea environmental conditions to underline the potential of an exchange between the surface and deep-sea habitats.

2.2 New Phagotrophic Euglenids from Deep Sea and Surface Waters of the Atlantic Ocean (*Keelungia nitschei*, *Petalomonas* gen. nov., *Ploeotia costaversata*)

This study aimed to enlarge the taxon sampling of an underrepresented group of euglenids by describing three new phagotrophic euglenids from marine surface waters and the deep sea. We aimed to gain a deeper evaluation of phagotrophic euglenids, which are important for understanding the eukaryotic early evolution. Although molecular barcoding studies have identified euglenozoans (comprising kinetoplastids, euglenids and diplonemids) as a specifically diverse group among deep-sea protists, only a very few euglenids have been described up to now. Whether euglenids and other protists are actually active under deep-sea conditions or might only be resting as cysts in deeper waters is still unknown. Thus, we investigated the species' reactions towards high hydrostatic pressure.

2.3 Global Comparison of Bicosoecid *Cafeteria*-like Flagellates from the Deep Ocean and Surface Waters, with Reorganization of the Family Cafeteriaceae

Cafeteria is known to be one of the most common and ecologically significant species of heterotrophic nanoflagellates in marine plankton. Within this study, we conducted a global comparison of 29 cultivated *Cafeteria*-like strains by means of molecular and morphological characterization. While the type species of *Cafeteria roenbergensis* has never been deposited at any GenBank, sequencing of this type species resulted in a revision within the

Cafeteriaceae. Our 29 strains were isolated from deep sea and surface water regions in the Mediterranean, Pacific, Atlantic, South East Atlantic and Indian Ocean. We aimed to gain more insight in the global distribution pattern of this important heterotrophic flagellate.

Chapter 3

High and Specific Diversity of Protists in the Deep-Sea Basins

Knowledge on protist communities in abyssal depths are scarce and global comparisons of deep-sea protists as has been conducted for marine surface water protist communities has yet not been conducted. Thus, this paper aimed to extend the scarce knowledge on abyssal protists on a global scale and to be the first report of protist communities from hadal regions. In addition, we investigated small scale patterns of protist communities in the deep sea. We hypothesized that the benthic deep-sea protist communities in our 20 examined deep-sea stations at bathyal, abyssal and hadal regions located in the Atlantic and Pacific Ocean are reflected by their spatial distance and that the different deep-sea basins harbour specific protist communities. We assumed that there are specific groups of protists which are endemic to abyssal and hadal plains and that the overall deep-sea protist community is different from those of other marine habitats.

Chapter 4

Potential Contribution of Surface-Dwelling *Sargassum* Algae to Deep-Sea Ecosytems in the Southern North Atlantic

Deep-sea ecosystems, limited by their inability to use primary production as a source of carbon, rely on other sources to maintain life. Sedimentation of organic carbon into the deep sea has been previously studied. However, we encountered a high biomass of sedimented *Sargassum* on the deep-sea floor during a deep-sea expedition in the North Atlantic Ocean. Its potential as a regular carbon input has been an underestimated phenomenon. Thus, this study aimed to provide insights into the potential of sedimented *Sargassum* algae as a carbon source in the deep sea. We hypothesized that deep-sea organisms can use *Sargassum* as carbon source and investigated the trophic interactions between the algae and macrozoobenthos organisms by fatty acids and stable isotope analyzes. In addition, we aimed to investigate the epifauna on surface *Sargassum*, which might be used as transportation vehicle for protists into the deep sea.

Chapter 1

Methodological Studies on Estimates of Abundance and Diversity of Heterotrophic Flagellates from the Deep-Sea Floor

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Review

Methodological Studies on Estimates of Abundance and Diversity of Heterotrophic Flagellates from the Deep-Sea Floor

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Abstract: Extreme environmental conditions in the deep sea hamper access to protist communities. In combination with the potentially highly diverse species composition, it demands a wide range of methods to be applied at the same time to guarantee a high resolution of quantitative and qualitative studies of deep-sea heterotrophic flagellates (HF). Within this study, we present a possible combination of several culture-independent and culture-dependent methods available for investigating benthic deep-sea HF communities. Besides live-counting and fixation of HF, we refer to cultivation methods and molecular surveys using next generation sequencing. Laboratory ecological experiments under deep-sea conditions (high pressure, low temperature) could allow the approval of the potential deep-sea origin of sampled HF. The combination of different methods offers a unique possibility to receive detailed information on nanofaunal life in the deep sea. Specific fixation techniques to preserve samples directly at the sampling depth must be applied in further studies to reflect the real biodiversity of the largest habitat on earth.

Keywords: live-counting; liquid-aliquot; fixation; next generation sequencing; pressure; deep-sea nanofauna

1. Introduction

Although deep-sea ecosystems represent the largest and most remote biome of the Earth [1], only about 5% have been explored so far, even less have been sampled [2]. We lack a firm understanding of species-level distribution (cosmopolitan *vs.* local) for deep-sea communities [3] as well as the functioning of and the interactions between biodiversity and ecological processes in this vast environment [2]. Marine heterotrophic flagellates contribute not only a major part within the microbial food web and are important nutrient remineralizers in biogeochemical cycles in surface waters [4,5] with densities ranging between 10^2 and 10^4 cells mL^{-1}, but are also potentially important regarding material flux and bacterial consumption in the deep sea. Recent studies of microbes have shown that even the deepest parts of our Earth are populated with a large variety of life [6]. Nevertheless, qualitative studies from eukaryotic deep-sea communities concerning diversity, spatial distribution and ecological function are still scarce. Concerning investigations of the bathypelagic deep sea, analysis revealed the occurrence of heterotrophic protists (11 ± 1 cells mL^{-1}) at depths down to 4000 m [7]. A global survey of bathypelagic microbial eukaryote communities identified a few groups as the dominant part of deep-sea communities, whereas the proportional composition of the dominant groups varied on a global scale [8]. Until the end of the last century, besides morphology based studies of foraminiferans [9], only anecdotal reports for other protists existed [10,11] mainly due to

methodological issues. While some authors reported a lack of flagellate occurrence in samples from the deep-sea floor [11], other studies revealed densities of HF [10,12,13] up to 10^5 cells cm^{-3} [14,15].

There is no standardized protocol for the sampling and analysis of benthic deep-sea protists available at present. Cultivation-based methods miss a majority of taxa since most species require specific cultivation conditions [16]. Molecular barcoding approaches employing PCR introduce significant biases in reported community compositions of marine protists due to the restriction of 'general' primers to detect all protist groups [17]. Since molecular surveys cannot yet provide any information on the morphology and abundance of the organisms, culture-dependent and culture-independent investigations are required to gain quantitative and qualitative results concerning deep-sea protist biodiversity.

The aim of this review is the study of benthic flagellated protists, those living in deep-sea sediments/seafloor. We will illustrate the above mentioned methodological problems presenting own recent results and will recommend a combination of methods to get a more reliable estimate of deep-sea benthic nanofauna.

2. Quantification and Qualification of Deep-Sea Protists

Several methods have been applied during the last decades to characterize deep-sea communities. Main procedures for flagellate detection in the past included live-counting of samples immediately after sampling, occurrences in laboratory cultures and molecular surveys using Sanger or next generation sequencing (NGS) (Figure 1). However, the taxonomic identification of protists, especially nanoprotists, in routine samples is difficult due to the general lack of conspicuous morphological features and the selectivity of sampling and counting methods [18–20]. The taxonomic identity of heterotrophic flagellates is generally based on cultivated strains, on which ultrastructural, physiological and molecular studies have been performed [16,21]. However, most deep-sea organisms are extremely difficult to cultivate due to their slow *in-situ* growth rates and their likely strict adaptation to extreme environmental conditions (oligotrophy, low temperatures, high pressure, anoxia) [2]. The role of these cultured strains as representatives within deep-sea protist communities is unclear. Molecular surveys frequently recover novel eukaryotic lineages that have not been recorded from cultures so far [16,22]. Environmental molecular surveys in microbial ecology have revolutionized our knowledge, indicating how far we are from understanding this "untapped reservoir" [23] of microbial diversity in the depth [19,22,24]. A major problem up to now is the assignment of these obtained sequences to species level with existing databases. A better annotation will improve the knowledge that comes from such analysis and sequence libraries. Therefore, such data will become more valuable as better gene annotations become available [25]. Molecular environmental diversity studies of the deep-sea floor have mainly been focusing on assumed "hot spots" of activity (e.g., hydrothermal vents, methane seeps) mostly from the bathyal zone carried out on a local scale [26–28]. Our previous studies of deep-sea nanofauna [29–33] indicated the existence of a specific abyssal nanofauna which contains a large number of endemic taxa [31,33].Recent comprehensive studies [34] indicated protists as the most diverse eukaryotic organisms. The diversity of phyla (Figure 1) with their specific differences (e.g., ultrastructure) makes it necessary to consider specifically designed fixatives or molecular techniques.

2.1. How to Sample Deep-Sea Protists

The main tool used up to now to collect benthic deep-sea protist communities is the Multi-Corer system. Due to a closing mechanism at the top and bottom of the cores, the risk of contamination with organisms and cysts from upper water layers is reduced. However, the problem is that samples have to be treated immediately after sampling which means within minutes. We microscopically observed living nanoflagellates within the first 30 min after sampling. Protists are stressed by tremendous physical changes, e.g., varying pressures and temperatures, during sampling. Therefore, it is likely that several flagellate species adapted to deep-sea conditions die, while being raised through the

water column. Morgan-Smith *et al.* [35] sampled deep-sea protists with 200 mL titanium chambers retaining *in situ* pressure from depths of 2750 and 4000 m to investigate the effect of pressure on protist abundances prior to fixation. Although depressurization pre-versus post fixation did not significantly affect the number of eukaryotes counted, cell physiology might be greatly impacted by changes in pressure. Future methodological studies must be applied to solve these problems. Potential solutions could be the usage of specific fixations of samples already in the depth of sampling. Furthermore, samples might generally be obtained under pressure in special containers to ensure observation of living flagellates under prevailing environmental conditions.

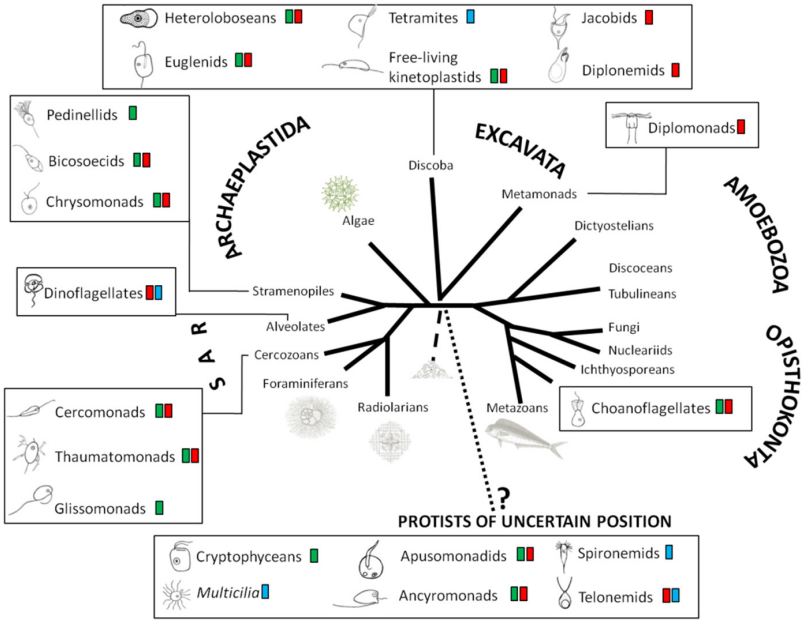

Figure 1. Taxonomic composition of heterotrophic flagellate groups (according to [25]) known from the deep sea. Colors indicate different kinds of quantification and/or qualification methods used for detection: Live-counting (blue), cultures (green), molecular surveys (red) (compilation of literature and own results; scheme derived from [33]).

2.2. Live-Counting

Live-counting techniques offer the opportunity to detect living cells up to the morphospecies level in addition to quantitative estimates. Although this method is difficult regarding a limited available time frame for observation and the need of a high amount of expertise, it is useful for obtaining high taxonomic morphotype resolutions [36].

Generally, untreated sediment samples are stored on ice and used to detect living flagellates immediately after sampling. The direct counts can serve as an estimate of deep-sea protistan abundance and as a cultivation-independent record of species. Inspections and counting of 5–10 µL subsamples of sediment suspensions can be conducted using light microscopes (40–63× phase-contrast objectives) combined with video recording [20]. However, it has to be considered that several flagellates die under the microscope during counting, probably caused either by rising temperatures due to microscopic light exposure or exposure to low atmospheric pressure (1 bar). These observations also underline the limitation of culture-dependent studies discussed in more detail in section 2.4. Due to the fact that only a few individuals can be detected within this short time frame after sampling, the low abundances lead to possibly severe underestimations of actual protist abundances as can be seen by comparing the numbers obtained from live-counts with those obtained from the analysis of fixed and stained samples (Figure 2). On the other hand, counts of fixed samples could overestimate real abundances when not fluorescence in-situ hybridization techniques are applied [35], but unspecifically binding

fluorochromes are used which may also stain free-floating nuclei and other DNA containing particles. An advantage of live-counting and observation is that the presence of living specimens of genotypes only known from clone libraries and metagenomic studies can be verified. In addition, new taxa can be detected.

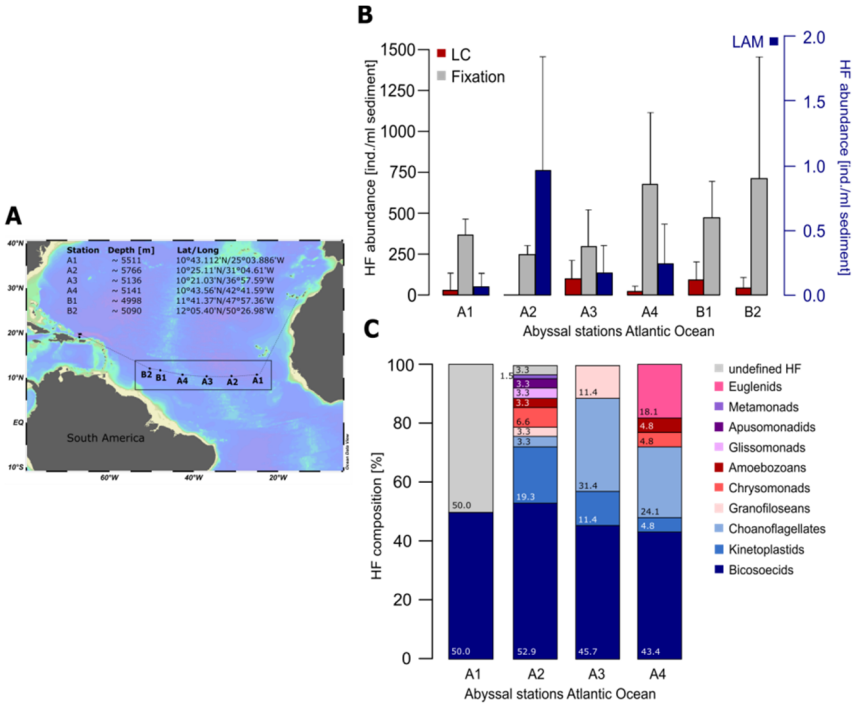

Figure 2. Comparison of methods applied for investigations of deep-sea heterotrophic flagellates in the VEMA fracture zone, southern North Atlantic. (**A**) Station map (created with Ocean Data View [37]) of the research cruise with *R/V Sonne II* (SO 237, 14.12.2014–26.01.2015). Sampling stations are indicated by black dots and station labelling (A1–A4, B1, B2). (**B**) Mean heterotrophic flagellates (HF) abundance (n = 3) of live, fixed and cultivation (liquid aliquot method, LAM) counts (ind./mL sediment). LAM counts were plotted with a separate y-axis. (**C**) Percentage of taxonomic HF group composition for stations A1–A4 revealed with LAM within the first 2–4 weeks. Live-counting: Inspections and counting of 5–10 µL subsamples of sediment suspensions was conducted using light microscopes (40–63× phase-contrast objectives) combined with video recording. Fixation: Sediment subsamples were fixed with formaldehyde (2%), stained with DAPI (4′,6-Diamidin-2-phenylindol, Sigma-Aldrich, Munich, Germany) and filtered on 0.2 µm membrane filters. Following criteria were defined for the detection of flagellates: roundish shape, larger than 1.5 µm and clear blue coloration. Cultivation (LAM): Subsamples of 2 mL of the sediment suspension were cultivated in 50 mL tissue-culture flasks (Sarstedt, Nümbrecht, Germany) filled with 30 mL autoclaved sea water. Additionally, 650 mL culture flasks were filled with 400–500 mL overlaying water.

2.3. Fixation and Staining

Fixation and staining methods are advantageous due to the possible long-term storage and observation of samples. Generally, glutaraldehyde (1%) or formaldehyde (1%–2%) are used as fixatives combined with staining fluorochromes (e.g., DAPI, FITC, Proflavine) which bind to cell components such as DNA or proteins [38–40] to detect potentially eukaryotic cells under epifluorescent microscopes [41]. Morgan-Smith et al [35] suggested the fixation of deep-sea water column samples overnight at room temperature followed by filtration on polycarbonate filters at a vacuum of −200 mbar to ensure the escape of supersaturated gases and, thus, avoid bubble formation on filters. Hondefeld *et al.* [42] proposed a suitable method to detect protists in marine sediment samples resuspending fixed samples and taking subsamples of the supernatant after a few minutes when

the majority of inorganic particles had been settled, alternative methods could be density gradient centrifugation to separate protists from inorganic particles [43,44]. Although fixed counts are up to three orders of magnitude larger than live counts (see Figure 2), no methodological tests of the reliability of this method exist for deep-sea conditions [30]. Quantifying eukaryotic cells in fluorescently stained fixed samples is accompanied by several disadvantages. Critical comparisons of fixed samples of heterotrophic flagellates have found significant discrepancies between parallel counts of each other (*cf.* Figure 2). One has to keep in mind that obtained abundances might be underestimated due to the fact that cells may not survive the pressure changes during sampling. Several groups of HF are very sensitive to the fixation procedure. A significant part of HF might be disrupted by fixation and difficult to detect [45]. Although several authors emphasize the more accurate identification of protists with DAPI-staining due to the discrimination between the nucleus and cytoplasm and sometimes the display of flagella, a clear identification of all protists is still limited and in several cases doubtful, because large bacteria [7] and free-floating nuclei might also be stained. Thus, staining the nucleus with DAPI in combination with FITC [39,40] or Proflavine [38], which stain entire cell body, seem to be a more accurate way to explicitly identify flagellates.

One improvement in detecting protists is the usage of fluorescence-*in-situ*-hybridization (FISH). Although FISH is a huge development in identifying microbial eukaryotes as well as prokaryotes, there are still several disadvantages of FISH such as insufficient sensitivity due to the low number of target molecules in cells, low probe permeability of cells and poor probe hybridization efficiency [46]. The catalyzed reporter deposition fluorescence-*in-situ*-hybridization (CARD FISH) allows characterization of communities in terms of abundance and taxonomy and specifically targets protists, while large bacteria are not confounded [47]. This technique has already been used for analyzing eukaryotic deep-sea microbes together with universal oligonucleotide probes (e.g., EUK516) [35]. The universal probes EUK516 (5′-ACCAGACTTGCCCTCC-3′, [48,49]) and EUK1209 (5′-GGGCATCACAGACCTG-3′, [50,51]) are missing the detection of kinetoplastids within the eukaryotic phylogenetic tree. Thus, the exclusive usage of these two probes would lead to a lack of detection of some free-living protists in at least some marine systems. The overall specificity and reliability of the detection of protists can be increased with a combination of oligonucleotide probes KIN516 (5′-ACCAGACTTGTCCTCC-3′, [52]) and EUK516.

2.4. Cultivation

Cultivation methods offer the possibility of detailed morphological characterizations and the establishment of clonal cultures for molecular studies. Water originating from the sampling depth is autoclaved and bacterial growth is supported by adding organic substances (e.g., yeast extract, glucose) to allow cultivation of bacterivorous species. Generally, not all species appear in cultures due to selective conditions like enrichment of bacteria or the lack of suitable other food sources (e.g., other protists). This results in a support of r-strategists among HF favoring similar genera/species such as *Cafeteria, Caecitellus, Rhynchomonas, Neobodo* during cultivation [53]. However, sometimes even seldom recorded species may appear, showing that a massive cultivation effort is needed to enhance successful cultivation. To partially overcome this problem, molecular investigations such as next generation sequencing are applied to detect uncultivable organisms.

One suitable method of cultivating protists is the liquid aliquot method (LAM, [54]) inoculating defined aliquots small enough to place one cultivable organism into each culture vessel. Aliquots of deep-sea sediment or overlaying water can be cultivated to estimate the abundance and diversity of cultivable deep-sea nanoprotists (Figure 3B,C).

2.5. Next Generation Sequencing (NGS)

Molecular surveys have revolutionized our understanding of deep-sea protist communities. The methodological spectrum of next-generation sequencing (NGS) and DNA-barcoding for HF has increased significantly in the last years [17,34]. Conserved samples for bulk analysis of RNA (active

organisms, metatranscriptomics) and DNA (whole metagenome studies) can be used to analyze the presence of protist genotypes in the deep sea [31,33]. However, there are still some unsolved problems like specific instead of general primers, different rRNA copy numbers for protists, PCR biases, the difficulty of differentiating active from inactive forms (e.g., cysts), and incomplete databases containing incorrect labeled species [45,55,56].

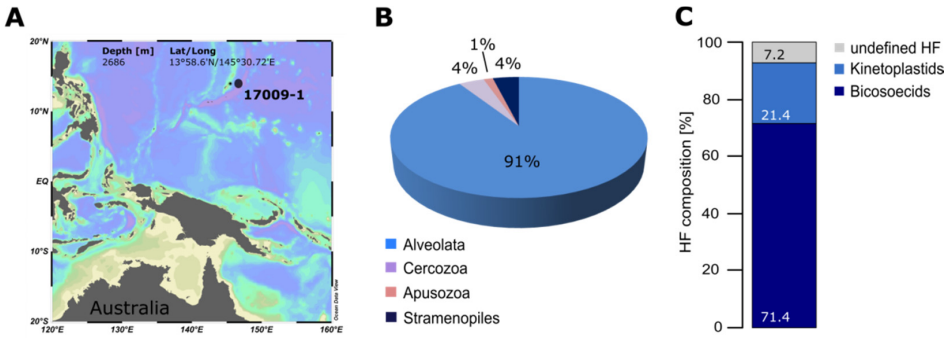

Figure 3. Comparison of methods applied for investigations of deep-sea heterotrophic flagellates in the Mariana Basin, Central Northern Pacific. (**A**) Position of the sampling station (2686 m depth), *R/V Sonne I* (SO223T, 09.09.2012–10.10.2012). Created with Ocean Data View [37]. (**B**) Percentage contribution of sequence reads of HF groups obtained by 454 sequencing. (**C**) Percentage contribution of HF groups revealed with the liquid aliquot method. NGS: Whole genomic DNA extracted from sediment samples using the PowerSoil® DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) and quantified using a spectrophotometer. The highly variable V4 region of the 18S rRNA gene was directly amplified from the samples using the eukaryotic specific primers 590F (5′-CGGTAATTCCAGCTCCAATAGC-3′) and 1300R (5′-CACCAACTAAGAACGGCCATGC-3′). Each sample was PCR'ed in triplicate and pooled to a final concentration of 20 ng/µL to reduce possible PCR biases. NGS using the GS-FLX Titanium sequencer (Roche, Mannheim, Germany) was performed by GATC Biotech AG, (Cologne, Germany). Sequencing was done as from adaptor A (forward primer or 5′-end). Obtained sequences (100% query coverage) were clustered in OTUs using a pairwise identity of 80% on the "class" level, since most reads did not yet have hits in public databases. Cultivation: see Figure 2.

While the usage of quantitative PCR of 18S rRNA genes in conjunction with FISH for marine picoeukaryotes, seemed to be a very promising way to quickly obtain data on the ecological distribution of important phytoplankton groups, primer specificity and varying rRNA gene copy numbers among eukaryotes need to be considered [57]. The potentially selective amplification needs to be incorporated in the interpretation of obtained results concerning species composition and abundances. Thus, the use of multiple sets of primers is required to recover the major part of environmental microbial diversity. Comparison of artificial and environmental 18S rRNA gene libraries revealed, that environmental PCR-based techniques might be sufficient to compare samples, but the total diversity will probably always be underestimated [58]. High amounts of ribosomal sequence data can be obtained by next generation sequencing (e.g., 454-pyrosequencing, Illumina), which has the potential to uncover more organisms including rare species. Both methods, 18S clone libraries and 18S amplicon sequencing, showed significant similarities in protist community composition [59].

An alternative which does not require PCR steps is the metagenomic approach. The analysis of bulk DNA from deep sea-sediments [3] allows for both a qualitative analysis and a rough assignment to trophic functions of deep-sea nanofauna. However, it has to be considered that a large proportion (estimations up to 90%) of the DNA in deep-sea sediments is extracellular [60,61]. Thus, it is uncertain, if detected benthic protist communities are actually thriving under these conditions or are rather an artifact by deposited cells from the upper water column, encysted cells or extracellular DNA [62]. Thus, metagenomics might introduce biases in actual protist biodiversities, because they are accompanied by two major issues, rDNA copy number and extracellular DNA [63]. One major bias of

rDNA diversity surveys, the extracellular rDNA, is reduced significantly by rRNA libraries, but such libraries are exclusively recovering the active part of the communities. A solution might be the RNA and DNA extraction from the same sample to assess the composition of the microeukaryotic assemblage by distinguishing between active cells and signals from inactive or even dead organisms [62]. A comparison with transcriptome data from similar sampling sites in the deep sea could help to detect a "passive seed bank" which might contain species which are able to grow in the sampled habitat, but might be inactive due to actually unfavorable conditions. Investigations of sympagic as well as surface protist communities revealed activity patterns of specific groups by comparing rDNA and rRNA libraries [64,65].

The results from our study of deep-sea sediments from the central Pacific indicate the advantages and disadvantages of molecular and cultivation methods. Cultivation recovered only a very minor part in comparison with the diversity obtained by NGS. Cultivation (and Sanger sequencing) allowed the assignment of sequences to species level (Figure 3). Within our studies 91% of all NGS sequences belonged to alveolates (undetermined dinoflagellates) which amplify preferentially with universal primers, whereas they did not occur in cultures. The same was true for cercozoans. Contrary, kinetoplastids were not recovered by NGS due to primer mismatch, but could be detected in cultures (new species). Bicosoecids (new species) occurred in cultures but were obviously too seldom for registration by NGS.

3. Protocol for Detecting Nanofaunal Abundance and Diversity

Estimates of abundance and diversity should be accomplished by culture independent methods such as live-counting of untreated samples as well as counting of fixed and stained samples. Furthermore, cultivation of defined aliquots of the diluted sample (LAM) offer the possibility of morphological characterization and later molecular surveys (PCR, single-cell genomics/transcriptomics) for identifying corresponding genotypes. This addition of known sequences to molecular database is a very important step to increase the knowledge of diversity of protists in the deep ocean. To get an idea regarding the active genotypes in deep-sea samples, NGS applied to RNA is necessary. Clone libraries or next generation sequencing are helpful tools to detect diversity but the results must be verified regarding the origin of the organisms. From an ecological point of view, pressure (>200 bar) and temperature (<4 °C) experiments may confirm the deep-sea origin of sampled HF [66]. At least for some organisms isolated from the deep sea it should be tested in the laboratory whether they are able to survive at deep-sea conditions. Thus, a combination of several methods is recommended when analyzing deep-sea nanofauna (Figure 4).

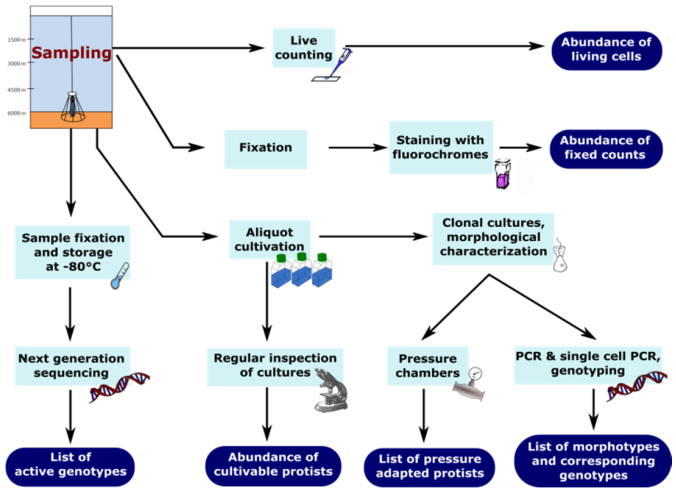

Figure 4. Proposed protocol for diversity and abundance estimates of deep-sea nanoprotists including molecular surveys (such as environmental RNA), fixation, live-counting and aliquot cultivation.

4. Conclusions

Molecular methods are an appropriate way to investigate deep-sea protistan diversity. With metagenome analysis and 18S rDNA amplicon sequencing, the active as well as inactive fraction of protist communities in the deep sea can be recorded. However, one has to keep in mind, that extracellular DNA is also detected. Thus, it is recommended to add analysis of the RNA (rRNA amplicon sequencing and metatranscriptomics) of the recorded genepool to filter for the active organisms. As DNA is well preserved in this environment and protists may form cysts in the deep sea because of unfavorable conditions, one should consider data from metagenomic analysis as a seed bank analysis. This way, a comparison between spatial and temporal separated samplings in the deep sea can be used to detect theoretically viable protists which were not active during sampling due to environmental factors such as lack of resources. To apply NGS for the analysis of species and hence biodiversity, the need of reliable reference databases is a major hindrance, which has to be overcome. A close combination of NGS together with culture dependent methods, morphological observations, single-cell investigations, as well as ecological studies is a prerequisite for a profound understanding of the diversity and the role of protists in deep-sea food webs. We tried to provide a recommendation of methods for investigating abundance and diversity of deep-sea nanoprotists by combining six different techniques available at present (Figure 4). Each method has its own advantages and disadvantages concerning investigations of HF. The combination of different methods offers a unique possibility to receive detailed information on nanofaunal life in this extreme and hardly accessible environment. However, the fact that protists are usually exposed to high variations in pressure and temperature during sampling procedures compared to their constant original environment may potentially lead to a disruption of flagellates. Future studies must solve these methodological problems. Therefore, the usage of specific fixations of samples already at the depth of sampling should be considered. For investigation concerning diversity and the ecological role of HF, samples should be obtained under pressure from the deep sea to ensure observation of living flagellates.

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Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 2

Cultivation of Deep-Sea Protists

2.1 Deep-Sea Ciliates: Recorded Diversity and Experimental Studies on Pressure Tolerance

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Deep-sea ciliates: Recorded diversity and experimental studies on pressure tolerance

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ABSTRACT

Microbial eukaryotes play an important role in biogeochemical cycles not only in productive surface waters but also in the deep sea. Recent studies based on metagenomics report deep-sea protistan assemblages totally different from continental slopes and shelf waters. To give an overview about the ciliate fauna recorded from the deep sea we summarized the available information on ciliate occurrence in the deep sea. Our literature review revealed that representatives of the major phylogenetic groups of ciliates were recorded from the deep sea (> 1000 m depth): Karyorelictea, Heterotrichea, Spirotrichea (Protohypotrichia, Euplotia, Oligotrichia, Choreotrichia, Hypotrichia), Armophorea (Armophorida), Litostomatea (Haptoria), Conthreep (Phyllopharyngea incl. Cyrtophoria, Chonotrichia, Suctoria; Nassophorea incl. Microthoracida, Synhymeniida, Nassulida; Colpodea incl. Bursariomorphida, Cyrtolophosidida; Prostomatea; Plagiopylea incl. Plagiopylida, Odontostomatida; Oligohymenophorea incl. Peniculia, Scuticociliatia, Hymenostomatia, Apostomatia, Peritrichia, Astomatia). Species occurring in both habitats, deep sea and shallow water, are rarely found to our knowledge to date. This indicates a high deep-sea specific ciliate fauna. Our own studies of similar genotypes (SSU rDNA and *cox1* gene) revealed that two small scuticociliate species (*Pseudocohnilembus persalinus* and *Uronema* sp.) could be isolated from surface as well as deep waters (2687 m, 5276 m, 5719 m) of the Pacific. The adaptation to deep-sea conditions was investigated by exposing the ciliate isolates directly or stepwise to different hydrostatic pressures ranging from 1 to 550 atm at temperatures of 2 °C and 13 °C. Although the results indicated no general barophilic behavior, all four isolated strains survived the highest established pressure. A better survival at 550 atm could be observed for the lower temperature. Among microbial eukaryotes, ciliates should be considered as a diverse and potentially important component of deep-sea microeukaryote communities.

1. Introduction

The deep sea represents the world's largest biome on earth. However, there is still a huge lack of understanding concerning biodiversity and ecology of the deep-sea floor. The abyssal sea floor (3000–6000 m) covers around 54% of the earth surface and is the most common benthic environment with remarkably constant conditions (Gage and Tyler, 1991). Deep-sea organisms have to cope with extreme environmental conditions including low food availability, low temperatures, permanent darkness and high hydrostatic pressure.

Microbial eukaryotes or protists play an important role in the ocean matter flux in productive surface waters and are major components of biogeochemical cycles. Controlling half of the primary production, heterotrophic flagellates and ciliates are extremely important for remineralization of nutrients in the ocean (Azam and Malfatti, 2007; Landry and Calbet, 2004). Although there is a high variability of recorded abundances, surface waters are typically inhabited by 10^5 bacteria and 10^3 protists per milliliter (Azam and Malfatti, 2007). Considering the order of geographic magnitude of the deep sea, little is known about protists in these depths (e.g. Edgcomb et al., 2002; Scheckenbach et al., 2005, 2010). Studies mainly concentrated on deep-sea areas with extreme environmental conditions like hydrothermal vents and cold seeps (Alexander et al., 2009; Edgcomb et al., 2009). Comparison of the microbial community structure of shelf and deep-sea habitats showed differences in protistan assemblages (Countway et al., 2007). Genetic approaches like next-generation sequencing and clone libraries have turned out to be reliable tools in identifying previously unknown protistan lineages in the deep sea (Edgcomb et al., 2002; López-García et al., 2001). Deep-sea organisms are dependent on sinking detritus. Besides being a food source such aggregations may serve as transportation vehicle to the deep sea for different protozoan species (Alldredge and Silver, 1988; Arndt et al., 2003; Bochdansky

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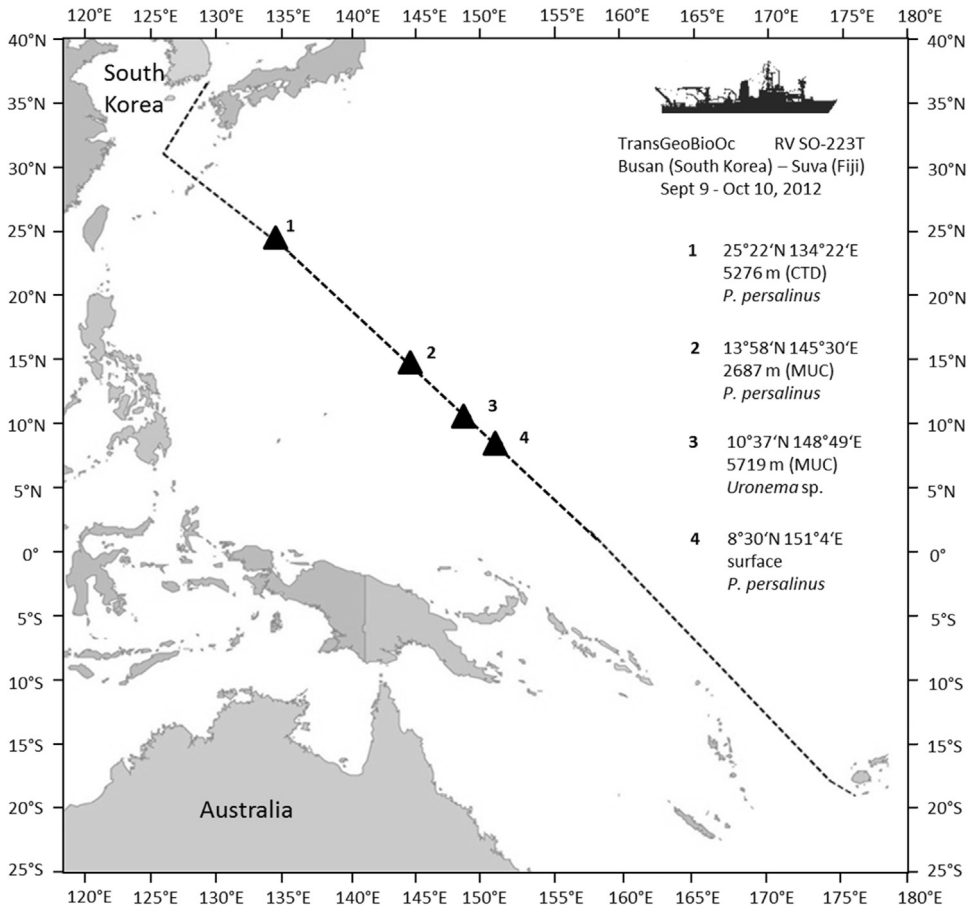

Fig. 1. Station map - Locations of four sampling stations during the research cruise *RV Sonne I* SO 223-T from Busan, South Korea, to Suva, Fiji (Sept 9 – Oct 10, 2012). Geographical positions, depth, sampling procedure and isolated ciliate species are given for each station.

et al., 2017; Caron et al., 1982; Lochte and Turley, 1988) in addition to sinking carcasses (Dayton and Hessler, 1972; Smith, 1985). Protists are known to encyst to overcome periods of unfavorable environmental conditions. This process potentially plays an important role for survival in the deep sea (Atkins et al., 1998).

Protist records from the deep sea are mainly available for foraminiferans (Danovaro et al., 2010; Gooday and Jorissen, 2012; Pawlowski et al., 2011b), while there is surprisingly little information available regarding the diversity of ciliates in the deep sea. Several studies indicate the presence of ciliates in deep-sea sediments (e.g. Pawlowski et al., 2011a). Furthermore, ciliates have been found as epibionts on benthic deep-sea arthropods (Bartsch and Dovgal, 2010; Sedlacek et al., 2013). Large-scale studies demonstrated a close link between benthic biodiversity and ecosystem functioning in the deep sea (e.g. Danovaro et al., 2008a). They estimated that a biodiversity loss of 20–30% can result in a 50–80% reduction of key processes of deep-sea ecosystems. Therefore, it is important to understand the role of ciliated protists as a potentially very important group of abyssal microbial food webs. Earlier studies had shown that deep-sea protists like heterotrophic flagellates are able to survive pressures up to 300 atm (Atkins et al., 1998). Some flagellates and ciliates even preferred moderate pressure (Kitching, 1957; Turley et al., 1988). The ciliate *Tetrahymena* is able to survive deep-sea pressures of up to 250 atm (Hemmingsen, 1982; Hemmingsen and Hemmingsen, 1983).

To give an overview about the ciliate fauna recorded from the deep sea, we summarized the available information on ciliate occurrence in the deep sea. Species occurring in both habitats, deep sea and shallow waters, are rarely found to our knowledge to date. The isolation of two scuticociliates commonly found in surface waters isolated from the deep sea raised the question whether or not the same ciliate species or even genotypes might populate surface as well as deep-sea waters. Thus, there might be a possible exchange or permanent sedimentation of surface individuals to the deep. Therefore, we monitored possible pressure tolerances and discussed distribution mechanisms.

2. Material and methods

2.1. Sampling

Samples were taken with a Multi-Corer system and a Seabird SBE *9plus* CTD from the North Pacific Ocean as well as the South Pacific Ocean during the *RV Sonne I* cruise SO-223T (9th September – 8th October 2012) from Busan, South Korea, to Suva, Fiji (see Fig. 1). Samples of the Multi-Corer system were taken from the overlaying water as well as from the upper 2 mm sediment layer by means of a sterile syringe. A closing mechanism at the top and bottom of the cores reduces the risk of contamination with organisms and cysts from upper water layers and, thus, a contamination should be negligible. Subsamples of a few milliliters of the sediment suspension were cultivated into 50 ml tissue-culture flasks (Sarstedt, Nümbrecht, Germany) filled with 30 ml autoclaved sea water. In addition, 500 ml aliquots of the overlaying water were incubated in 650 ml tissue-culture flasks. All cultures were supplied with sterilized quinoa grains as an organic food source for autochthonous bacteria. Furthermore, surface water was collected to compare surface with deep-sea isolates. These samples were also transferred to 50 ml tissue culture flasks filled with 30 ml autoclaved sea water and two quinoa grains.

Four ciliate strains isolated from different depths were investigated: *Pseudocohnilembus persalinus* HFCC (Heterotrophic Flagellate Cultures Cologne) 816 (0 m), *Pseudocohnilembus persalinus* HFCC 814 (2687 m), *Pseudocohnilembus persalinus* HFCC 822 (5276 m) and *Uronema* sp. HFCC 823 (5719 m). The isolates were cultivated in 50 ml tissue culture flasks (Sarstedt, Nümbrecht, Germany) filled with 30 ml Schmaltz-Pratt

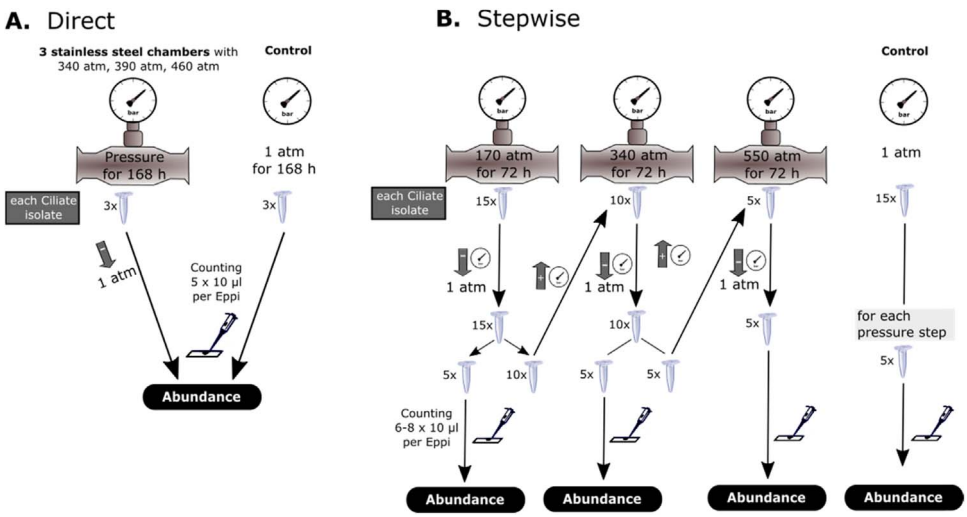

Fig. 2. Set-up of pressure experiments and respective controls with (A) direct exposure to three different hydrostatic pressures and (B) stepwise increase pressure experiments.

medium (35 PSU; per liter: 28.15 g NaCl, 0.67 g KCl, 5.51 g $MgCl_2 \times 6H_2O$, 6.92 g $MgSO_4 \times 7H_2O$, 1.45 g $CaCl_2 \times 2H_2O$, 0.10 g KNO_3, 0.01 g $K_2HPO_4 \times 3H_2O$) containing two quinoa grains. The cultures were stored at 13 °C without light.

2.2. DNA Extraction, purification and sequencing

Cultured ciliates were concentrated by centrifugation (2000 × *g* for 20 min at 4 °C, Megafuge 2.0 R, Heraeus Instruments). Genomic DNA from each isolated ciliate was extracted using the QuickgDNA™MiniPrep (Zymo Research, USA). For amplification of the SSU rDNA of *Pseudocohnilembus persalinus* species and *Uronema* sp. following primers were used: forward 18S-For (5′-AACCTGGTTGATCCTGCCAGT-3′) and reverse 18S-Rev (5′-ACCTACGGAAACCTTGTTACG-3′). PCR reactions were performed in 50 µl PCR reaction mixtures containing 13 µl ddH_2O, 1.5 units TAQ (Mastermix, VWR Germany), 2 µl DNA and 5 µl of each primer (forward and reverse) at a final concentration of 1.6 nM. The PCR conditions were as follows: pre-denaturation at 98 °C for 2 min, 35 cycles of 98 °C for 30 s, 55 °C for 45 s and 72 °C for 2 min and 30 s; final extension at 72 °C for 10 min. The amplified products were analyzed by electrophoresis on 1% agarose gel and fragment sizes were determined by comparison with 100 bp DNA ladder. The PCR products were purified by PCR Purification Kit (Jena Bioscience, Jena, Germany).

The *cox1* region was amplified from *Pseudocohnilembus persalinus* using the primers OXO9-146 (5′-TAAATCTAATCATCGTAAT AATAGAGAATTGTTAG-′3) and MOU08-122 (5′-TARTATAGGATC MCCWCCATAAG-3′) (Whang et al., 2012). Reactions were setup as described for SSU rDNA (see above). PCR was performed with pre-denaturation at 98 °C for 2 min, 30 cycles of 98 °C for 30 s, 48 °C for 1 min and 72 °C for 2 min followed by a final extension at 72 °C for 5 min.

Furthermore, we conducted nested PCRs for the SSU rDNA and *cox1* gene with single cells of the *Pseudocohnilembus persalinus* isolates to get a better understanding of the differences among and within isolates. Single cells (triplicates) were isolated with a micromanipulator and transferred in 500 µl Eppendorf tubes filled with 10 µl ddH_2O. For the first SSU rDNA PCR the primers 18S-For and 18S-Rev were used. Reactions were setup as described above, while the volume of DNA (single cell in 10 µl ddH_2O) and ddH_2O (5 µl) were changed accordingly. Reamplification was conducted using the primers forward 590For (5′-CGGTAATTCCAGCTCCAATAGC-3′) and reverse 1300 R (5′-CACCAACTAAGAACGGCCATGC-3′). PCR conditions were as follows: pre-denaturation at 98 °C for 2 min, 35 cycles of 98 °C for 30 s, 55 °C for 45 s and 72 °C for 2 min and 30 s; final extension at 72 °C for 10 min. For the *cox*1 gene single cell PCR following primer pairs were used: CoxTetrahy2F (5′-GCACATCCTGGAAGTCCATT-3′) and CoxTetrahy1R (5′-TCATCCCATAAAAACAACTGGA-3′) for the first PCR (Jones et al., 2010), OXO9-146 (5′-TAAATCTAATCATCGTAATAATAGAGAATTGTT-AG-′3) and MOU08-122 (5′-TARTATAGGATCMCCWCCATAAG-3′) (Whang et al., 2012) for reamplification. PCR setups for CoxTetrahy2F and CoxTetrahy1R were as follows: 98 °C for 2 min, 35 cycles of 98 °C for 30 s, 46 °C for 45 s and 72 °C for 2 min and 30 s; final extension at 72 °C for 10 min.

2.3. Phylogenetic analysis

For the phylogenetic analysis of our strains of ciliates based on the SSU rDNA gene additional available sequences of ciliates were downloaded from GenBank database. Alignments were carried out using ClustalX (Thompson et al., 1997); corrections to optimize the alignment were done with BioEdit v7.0.5.3 (Hall, 1999). Ambiguously aligned regions were manually corrected in the alignment previously to the phylogenetic analysis.

The Maximum likelihood (ml) phylogenetic tree of the SSU rDNA was inferred in PhyML v3.0.1 (Guindon and Gascuel, 2003) applying 1000 bootstrap replicates under the General Time Reversible + I (G-TRI) nucleotide substitution model as suggested by MrAIC (Nylander, 2004). Bayesian inference (BI) analysis was run with Mr. Bayes v3.2.1 (Ronquist and Huelsenbeck, 2003) using a GTR+I+Γ model and a four-category gamma distribution to correct for among site rate variation. The search consisted of two parallel chain sets run at default temperatures with a sample frequency of 10 and run so that the average standard deviation of split frequencies dropped below 0.01. The analysis consisted of 1,000,000 generations, with a burnin of 87,500, before calculating posterior probabilities.

The hyper-variable region of the *cox1* gene was used for differential identification of various ciliate species. A neighbor-joining (NJ) phylogenetic tree was calculated for *cox1* sequences of *P. persalinus* species using the Kimura two parameter (K2P) distance model. The corresponding bootstrap values of the neighbor joining analyses (1000 bootstrap replicates) were calculated with MEGA6 (Tamura et al., 2011).

2.4. Pressure experiments

A pressure generating system was established based on a pneumo-hydraulic pressure intensifier with a transfer ratio of 1:400. Stainless steel chambers were used to pressurize the obtained isolates. Two different pressure experiments (direct, stepwise) were conducted (s. Fig. 2). Due to the fact that deep-sea conditions vary on a global scale influencing metabolic processes of organisms, we decided to conduct the first set of experiments (direct pressure exposure) under

Mediterranean deep-sea conditions at 13 °C to ensure higher growth rates. However, ambient temperatures in the Pacific deep sea were 2 °C. Thus, the second set of experiments with the stepwise pressure increase was run at both temperatures, 13 °C and at 2 °C. Isolates were pre-cultivated in 50 ml tissue culture flasks filled with 30 ml Schmaltz-Pratt medium (35 PSU) and two quinoa grains at 13 °C. For the experiments at lower temperature, ciliates were acclimatized for 24 h at 2 °C. Ciliates were studied within their logarithmic growth phase.

The first set of experiments included the direct exposure of the ciliates from 1 atm to pressures of 340 atm, 390 atm, 460 atm for 168 h (Fig. 2 A). Aliquots of the isolates were filled into 200 µl Eppendorf tubes (Eppendorf, Wesseling, Germany) and stored in the pressure chambers. Each of the three different pressurized (340 atm, 390 atm and 460 atm) strainless steel chambers contained triplicates from each isolated ciliate strain. Control samples (same replicate number as in experiment) were exposed under the same conditions at atmospheric pressure (1 atm). Five 10 µl subsamples of each tube were counted under a light microscope (ZEISS; Axioskop 2) at the beginning and at the end of the experiment.

In the second experiment (see Fig. 2 B), the pressure was raised by steps about approximately 170 atm every 72 h via 340 atm up to 550 atm with an intervening return to 1 atm before increasing the pressure again (see also Atkins et al., 1998). Each of the four ciliate isolates had to be pressurized separately in one stainless steel chamber, because of the holding capacity of the chamber (max. 15 × 200 µl tubes). For each ciliate isolate 15 aliquots in 200 µl Eppendorf tubes were stored in the stainless pressure chamber. Control samples (same aliquot number as in experiments) were exposed under the same conditions at atmospheric pressure (1 atm). After the first 72 h under 170 atm the pressure was released. Five aliquots were stored on ice for counting, while the other 10 aliquots were further exposed to 340 atm. In addition, 5 aliquots of the control samples were removed from the experiment for counting. The same procedure was conducted for the other pressure steps (Fig. 2 B). Thus, we had 5 replicates for each ciliate at each pressure step and control. Six to eight 10 µl subsamples of each tube were counted under a light microscope (ZEISS; Axioskop 2) at the beginning and after each pressure step.

For further details (including number of living individuals, cysts and respective changes in individual numbers) see Table S3.

2.5. Data analysis

All abundance data were plotted as changes relative to values obtained from the corresponding controls at 1 atm. One-way ANOVAs and posthoc Tukey-tests were conducted to determine the significance of the effect of different hydrostatic pressures on the relative abundance [%] of protists with the open-source Software R (Version 2.15.3, http://www.r-project.org/). Furthermore, two-way ANOVAs were used to define the effect on the relative abundance of ciliates by both experimental factors, hydrostatic pressure and temperature.

3. Results

3.1. Literature survey on records of deep-sea ciliates

Deep-sea ciliate records (> 1000 m, see Table 1, Table S1 and Fig. 3) obtained from literature and NCBI Genbank comprise ciliates from nearly all taxonomic groups (see Table 1). Reference sequences for recorded deep-sea ciliate sequences were used to calculate the *p*-distance within the alignments. Our literature review revealed representatives of Karyorelictea, Heterotrichea, Spirotrichea (Protohypotrichia, Euplotia, Oligotrichia, Choreotrichia, Hypotrichia), Armophorea (Armophorida), Litostomatea (Haptoria), Conthreep (Phyllopharyngea incl. Cyrtophoria, Chonotrichia, Suctoria; Nassophorea incl. Microthoracida, Synhymeniida, Nassulida; Colpodea incl. Bursariomorphida, Cyrtolophosidida; Prostomatea; Plagiopylea incl. Plagiopylida, Odontostomatida; Oligohymenophorea incl. Peniculia, Scuticociliatia, Hymenostomatia, Apostomatia, Peritrichia, Astomatia). We decided to cluster available deep-sea sequences with following taxonomic rank thresholds: ≥ 99.7% pairwise identity of OTUs to NCBI database as a proxy for species level, 97–99.7% as a proxy for genus level, and ≤ 97% as a proxy for subclass/order level. To our best knowledge, only one ciliate could be identified (and newly described) down to species level from the abyss up to now, the suctorian *Corynophrya abyssalis* (Bartsch and Dovgal, 2010). Our literature research and new blast of available deep-sea sequences revealed, that 4.2% of the total 429 sequences within this literature review had a similarity of 99.7% or higher to already known species including *Pseudocyrtolophosis alpestris, Strombidium* sp., *Sinistrostrombidium cupiformum, Parauronema virginianum, Coleps* sp., *Cyclidium varibonneti, Amphisiella annulata, Tunicothrix brachysticha, Ancistrum crassum, Madsenia indomita, Pseudocohnilembus persalinus, Uronema marinum* and *Trichodina sinonovaculae* (see Table 1). One out of these seven sequences (DQ918623; Countway et al., 2007) showed a 100% similarity to two species, *Sinistrostrombidium cupiformum* and *Strombidium* sp., probably due to a short available sequence length (379 bp). Furthermore, 7.9% of the deep-sea sequences had a similarity within 99–99.7%, while a larger proportion (12.0%) had a similarity within 98–99%. A similarity of 97–99% could be assigned to 25.63% of the sequences. Furthermore, Orsi et al. (2012b) even proposed a novel ciliate class, Cariacotrichea, isolated from the anoxic Cariaco Basin (Caribbean Sea, Venezuela) at depths of 900 m (not mentioned in the table due to depths range), presumably restricted to anoxic marine environments.

3.2. Genetic analysis

We isolated ciliates from the abyssal sea floor after sampling and cultivating them at 1 atm. Two species could be cultivated and determined using morphological and genetic characteristics. The two common oligohymenophorean ciliates *Pseudocohnilembus persalinus* and *Uronema marinum* could be identified from several samples from the abyss of the Pacific (see Fig. 1). To get a hint if the obtained ciliates originated either from living deep-sea communities at the respective depth or might originate from cysts sedimented from surface waters we investigated the pressure tolerance of our isolates with a pressure generating system.

The ciliates isolated from various depths were analyzed regarding their SSU rDNA gene. Three respective isolates from depths of 0 m, 2687 m and 5276 m (see Fig. 1) showed a p-distance of maximum 0.2% by comparison with a deposited sequence for *P. persalinus* (GenBank accession number AY835669, Fig. 4). A comparison within our three *P. persalinus* isolate 18 S sequences revealed no p-distance. One isolate could be assigned to the genus *Uronema* with a p-distance of 0.7% to a deposited sequence of *Uronema marinum* (GenBank accession number GQ259747). For comparison of the single *Pseudocohnilembus persalinus* isolates, the cytochrome oxidase 1 (*cox1*) gene was sequenced. The hyper-variable region within the *cox1* gene was sequenced to distinguish between related species (Fig. 5). We did not find significant differences within the *cox1* gene of our different *P. persalinus* isolates. A comparison with one available *cox1* gene sequence from the NCBI gene bank (accession number GQ500579) revealed a *p*-distance of 0.7%.

3.3. Experiments on pressure tolerance

Two kinds of experiments were carried out with the four different ciliate strains: direct exposure from 1 atm to high hydrostatic pressures (340 atm, 390 atm, 460 atm) and stepwise increase of pressure from 1 atm to 550 atm via steps of 170 atm and 340 atm with an intervening return to 1 atm between each pressure step. Living individuals and cysts were counted separately, but the total abundance of survival was comprised of living ciliates and cysts. For detailed information on numbers of living ciliates, cysts, total abundance of ciliates (living +

Table 1
List of ciliate records detected by morphology and molecular studies from the deep sea (> 1000 m), ordered by classification of eukaryotes of Adl et al. 2012 and Gao et al. 2016 (for a more detailed description see Table S1). Besides depths and map points (see Fig. 2, Table S2), the number of sequences are given. The *p*-distances from the available deep-sea sequences to the closest named blast match were calculated. Following similarities and taxonomic ranks were used for clustering available sequences within this table: ≥ 99.7% (species), 97–99.7% (genus), ≤ 97% (subclass/order). Morph.: detected by morphology, —: unknown.

Depth [m]	Taxonomy	No. of sequences	Map point	Depth [m]	Taxonomy	No. of sequences	Map point
3148	Ciliophora	——	18	3000	Ciliophora	morph.	13
3700	Ciliophora	——	19	3300	Ciliophora	morph.	40
2292	Ciliophora	——	20	3500	Ciliophora	morph.	16
4060	Ciliophora	——	21	3624	Ciliophora	morph.	4
6326	Ciliophora	——	22	5498	Ciliophora	morph.	8
2155	Ciliophora	morph.	41	5498	Ciliophora	morph.	8
2426	Ciliophora	morph.	3				
SUBPHYLUM - POSTCILIODESMATOPHORA							
CLASS - KARYORELICTEA							
1178	*Kovalevaia*	1	43	1008	Karyorelictea	1	50
1178	*Prototrachelocerca*	1	43	1178	Karyorelictea	5	43
1998–2002	*Prototrachelocerca*	1	12	1527, 1598	Karyorelictea	1	51
1070	*Tracheloraphis*	1	23	1998–2002	Karyorelictea	8	12
1998–2002	*Tracheloraphis*	12	12	3607	Karyorelictea	morph.	34
1178	*Trachelocerca*	2	43				
CLASS - HETEROTRICHEA							
1178	*Anigsteinia*	1	43	1998–2002	*Peritromus*	1	12
1998–2002	*Condylostoma*	5	12	1178	Heterotrichea	1	43
1550	*Folliculinopsis* sp.	morph.	44	1998–2002	Heterotrichea	5	12
SUBPHYLUM - INTRAMACRONUCLEATA							
Superclade - SAL							
CLASS - SPIROTRICHEA							
	Protohypotrichia						
1070	*Caryotricha*	1	23				
	Euplotia						
1008	*Aspidisca*	1	49	1998–2002	*Uronychia*	1	12
1178	*Aspidisca*	2	43	1178	Euplotia	6	43
1998–2002	*Aspidisca*	2	12	1527, 1589	Euplotia	3	51
1998–2002	*Euplotes*	4	12	1998–2002	Euplotia	5	12
2000–2600	*Euplotes*	1	42	2585–2593	Euplotia	2	24,27
2593	*Euplotes*	1	24	2623	Euplotia	1	25
2757	*Euplotes*	1	26	2757	Euplotia	2	26
2933, 3744	*Euplotes vannus*	morph.		3499–3501	Euplotia	3	1
1998–2002	*Paradiscocephalus*	2	12	5033–5038	Euplotia	2	33
2500	*Paradiscocephalus*	1	10	5136–5142	Euplotia	2	30
	Oligotrichia						
1527, 1589	*Cyrtostrombidium*	1	51	2500	*Spirostrombidium*	1	10
2500	*Cyrtostrombidium*	4	10	2500	*Strombidium* sp.	2	9,11
3415	*Cyrtostrombidium*	2	35	4260	*Strombidium* sp.	morph.	
5033–5038	*Cyrtostrombidium*	1	33	2000–2600	*Strombidium*	1	42
5060–5066	*Cyrtostrombidium*	1	31	2500	*Strombidium*	9	10
5136–5142	*Cyrtostrombidium*	1	30	3258	*Strombidium*	1	35
3501	*Mytilophilus*	1	1	3499–3501	*Strombidium*	4	1
2500	*Navistrombidium*	3	10,11	1527, 1589	*Varistrombidium kielum*	1	51
1998–2002	*Pseudotontonia*	1	12	1008	Oligotrichia	1	49
3415	*Pseudotontonia*	1	35	1998–2002	Oligotrichia	4	12
2500	*Sinistrostrombidium*	7	9,10	3415	Oligotrichia	2	35
2500	*Sinistrostrombidium cupiformum/ Strombidium* sp.	1	10	5646–5655	Oligotrichia	1	32
	Choreotrichia						
~2000–2600	*Rimostrombidium*	1	42	4276	Choreotrichia	1	52
2500	Choreotrichia	1	10	5136–5142	Choreotrichia	1	30
	Hypotrichia						
1527, 1589	*Amphisiella annulata*	1	51	1178	*Spirotrachelostyla*	1	43
1527, 1589	*Tunicothrix brachysticha*	1	51	1008	*Trachelostyla*	1	49
1527, 1589	*Anteholostica*	1	51	1527, 1589	*Trachelostyla*	1	51
1998–2002	*Amphisiella*	2	12	1527, 1589	Hypotrichia	4	51
1008, 1527, 1589	*Kahliella*	2	49,51	1178	Hypotrichia	1	43
5646–5655	*Gonostomum/Kahliella*	1	32	1695	Hypotrichia	1	15
5033–5038	*Gonostomum/Kahliella*	1	33	1998–2002	Hypotrichia	2	12
3499	*Halteria*	1	1	3499	Hypotrichia	1	1
1178	*Holosticha*	1	43	5033–5038	Hypotrichia	3	33
1998–2002	*Protogastrostyla*	1	12	5060–5066	Hypotrichia	1	31
	Hypotrichia						
1998–2002	*Trachelostyla*	1	12	5136–5142	Hypotrichia	5	30
2593	*Trachelostyla*	1	24	5646–5655	Hypotrichia	3	32
CLASS - ARMOPHOREA							
1178	Armophorea	1	43	2000–2600	Armophorea	1	42
1998–2002	Armophorea	2	12				
CLASS - LITOSTOMATEA							

(continued on next page)

Table 1 *(continued)*

Depth [m]	Taxonomy	No. of sequences	Map point	Depth [m]	Taxonomy	No. of sequences	Map point
	Haptoria						
1998–2002	*Acineria*	1	12	5646–5655	*Trachelotractus*	1	32
1998–2002	*Enchelyodon*	1	12	1000	Haptoria	1	52
1178	*Epiphyllum*	2	43	1008	Haptoria	1	49
1998–2002	*Epiphyllum*	1	12	1178	Haptoria	5	43
1998–2002	*Litonotus*	4	12	1998–2002	Haptoria	10	12
1178	*Loxophyllum*	2	43	2264	Haptoria	1	14
1998–2002	*Loxophyllum*	2	12	5136–5142	Haptoria	3	30
5136–5142	*Trachelotractus*	1	30	5646–5655	Haptoria	2	32
SUPERCLADE - CONthreeP							
CLASS - PHYLLOPHARYNGEA							
	Cyrtophoria						
1070	Cyrtophoria	1	23	2623	Cyrtophoria	2	25
1998–2002	Cyrtophoria	1	12	2757	Cyrtophoria	1	26
2585	Cyrtophoria	7	27	3499	Cyrtophoria	2	1
2589	Cyrtophoria	2	28				
	Chonotrichia						
1178	Chonotrichia	1	43	1695?	Chonotrichia	1	15
1692	Chonotrichia	1	29	2585	Chonotrichia	1	27
	Suctoria						
4087	*Corynophrya abyssalis*	morph.	6	1695?	Suctoria	2	15
3520	*Thecacineta*	morph.	5	2589	Suctoria	1	28
1070	Suctoria	2	23	3607	Suctoria	morph.	34
1130	Suctoria	morph.	7	5033–5038	Suctoria	1	33
1527, 1589	Suctoria	1	51	5136–5142	Suctoria	2	30
	Synhymenia						
1178	*Orthodonella*	1	43	5136–5142	Synhymenia	1	30
1178	Synhymenia	2	43	5646–5655	Synhymenia	1	32
CLASS - NASSOPHOREA							
1008	Nassophorea	1	49	1998–2002	Nassophorea	16	12
1178	Nassophorea	5	43	2500	Nassophorea	1	10
CLASS - COLPODEA							
3499–3501	*Pseudocyrtolophosis alpestris*	2	1	5136–5142	Colpodea	1	30
5060–5066	Colpodea	1	31				
CLASS - PROSTOMATEA							
3499	*Coleps* sp.	2	1	1998–2002	Prostomatea	13	12
1178	*Cryptocaryon*	1	43	2000–2600	Prostomatea	1	42
1178	Prostomatea	2	43	2500	Prostomatea	2	9,10
1695?	Prostomatea	1	15				
CLASS - PLAGIOPYLEA							
2000–2600	*Plagiopyla*	1	42	1998–2002	Plagiopylea	20	12
3501	*Trimyema*	1	1	2000–2600	Plagiopylea	1	42
1008	Plagiopylea	6	50	3415	Plagiopylea	1	35
1178	Plagiopylea	4	43	3501	Plagiopylea	1	1
CLASS - OLIGOHYMENOPHOREA							
	Peniculia						
5033–5038	*Urocentrum*	1	33	5646–5655	*Urocentrum*	1	32
5136–5142	*Urocentrum*	1	30				
	Scuticociliatia						
1008	*Ancistrum crassum*	1	49	3415	*Pleuronema*	2	35
1527, 1598	*Ancistrum crassum*	1	51	1527, 1598	*Pseudocohnilembus persalinus*	1	51
3258	*Cyclidium*	1	35	2687	*Pseudocohnilembus persalinus*	1 (+ morph.)	37
3501	*Cyclidium varibonneti*	1	1	5719	*Pseudocohnilembus persalinus*	1 (+ morph.)	39
1998–2002	*Entorhipidium*	1	12	1008	*Uronema marinum*	1	50
1008	*Madsenia indomita*	1	49	2623	*Uronema*	1	25
1527, 1598	*Madsenia indomita*	1	51	5276	*Uronema*	1 (+ morph.)	38
3499–3501	*Mytilophilus*	3	1	1008	Scuticociliata	5	49, 50
2757	*Paranophrys*	1	26	1178	Scuticociliatia	9	43
2585–2589	*Parauronema*	5	27,28	1527, 1598	Scuticociliata	1	51
2623	*Parauronema virginianum*	1	25	1998–2002	Scuticociliatia	16	12
1008	*Philasterides*	1	50	3500	Scuticociliatia	morph.	17
1527, 1598	*Philasterides*	1	51	3499–3501	Scuticociliatia	4	1
1008	*Pleuronema*	2	49,50	5060–5066	Scuticociliatia	2	31
1998–2002	*Pleuronema*	3	12	5136–5142	Scuticociliatia	3	30
3258	*Pleuronema*	2	35				
	Hymenostomatia						
1998–2002	*Frontonia*	2	12				
	Apostomatia						
2500	*Pseudocollinia*	1	10	4053	Apostomatia	1	53
1692	Apostomatia	1	29	5033–5038	Apostomatia	1	33
2500	Apostomatia	2	9	5136–5142	Apostomatia	2	30
3499	Apostomatia	1	1	5646–5655	Apostomatia	1	32
	Peritrichia						
1008	*Trichodina sinonovaculae*	1	49	1070	*Vaginicola*	1	23

(continued on next page)

Table 1 *(continued)*

Depth [m]	Taxonomy	No. of sequences	Map point	Depth [m]	Taxonomy	No. of sequences	Map point
1527, 1598	*Trichodina sinonovaculae*	1	51	3607	Peritrichia	morph.	34
	Astomatia						
1998–2002	Astomatia	4	12	5033–5038	Astomatia	1	33
2500	Astomatia	1	11	5136–5142	Astomatia	1	30
SUPERCLADE – not assigned							
CLASS - MESODINIEA							
	Mesodiniida						
1000	Mesodiniida	1	52				
4260	*Mesodinium* spp.	morph.					

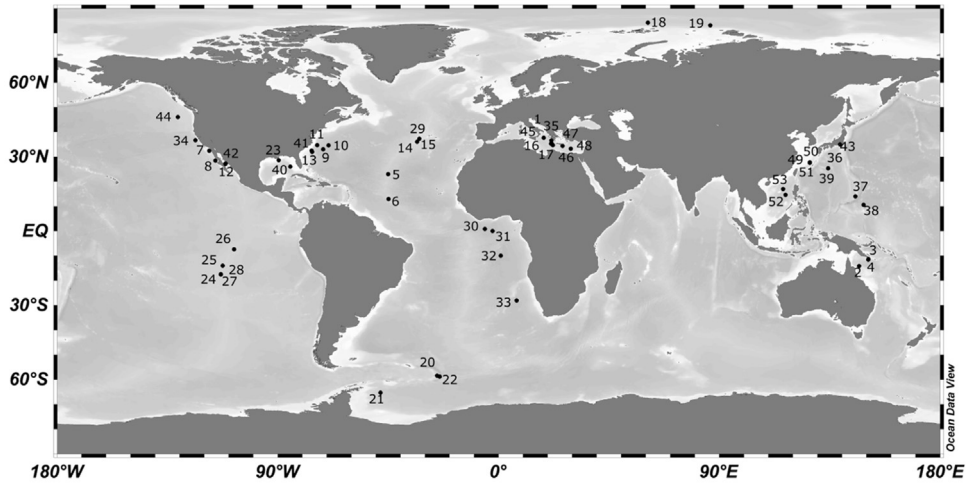

Fig. 3. Station map of ciliate records (morphology and molecular analysis) from the deep sea (> 1000 m) revealed by literature review. Map was created with Ocean Data View (Schlitzer, 2012). Legend for map point numbers (stations): 1 Alexander et al. (2009), 2–4 Alongi (1990), 5 Bartsch (1994), 6 Bartsch and Dovgal (2010), 7 Burnett (1973), 8 Burnett (1977), 9–11 Countway et al. (2007), 12 Coyne et al. (2013), 13 Cunningham and Ustach (1992), 14–15 López-García et al. (2003), 16–17 Orsi et al. (2012a), 18–22 Pawlowski et al. (2011a), 23–29 Sauvadet et al. (2010), 30–33 Scheckenbach et al. (2010), 34 Sedlacek et al. (2013), 35 Stock et al. (2012), 36 Takishita et al. (2010), 37–39 this study, 40–41 Ward et al. (2004), 42 Edgcomb et al. (2002), 43 Takishita et al. (2007), 44 Kouris et al. (2007), 45–48 Hausmann et al. (2002), 49–51 Zhao and Xu (2016), 52–53 Xu et al. (2017). For further station details see Table S1.

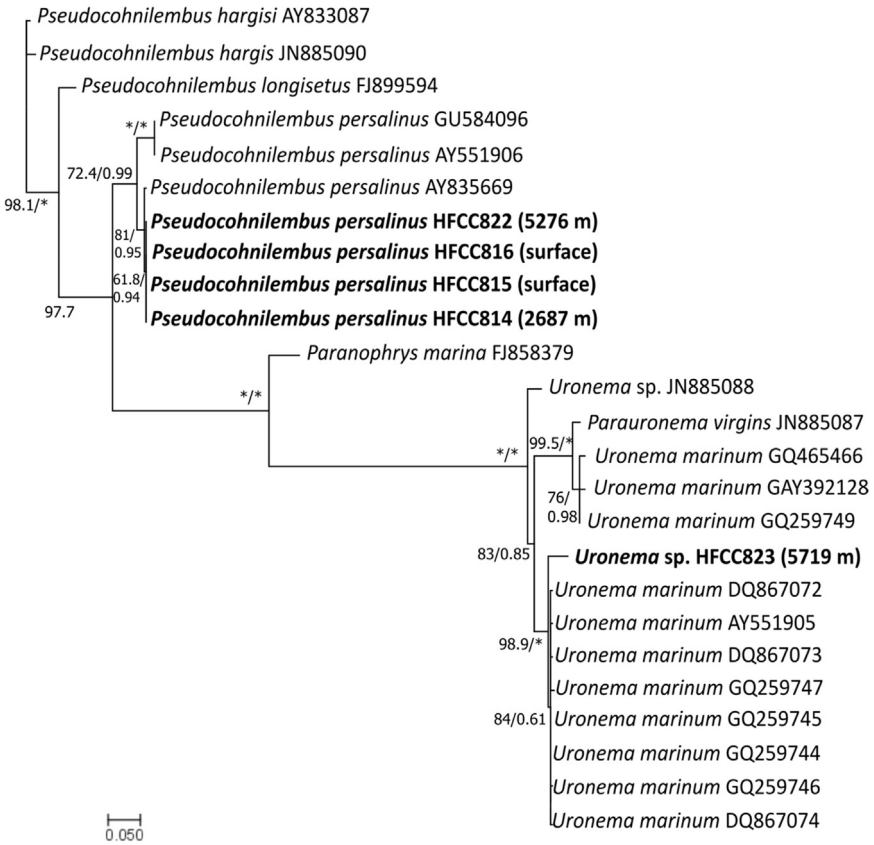

Fig. 4. Maximum likelihood (ml) phylogenetic tree of SSU rDNA of *Pseudocohnilembus persalinus* and *Uronema* sp. (sequences of this study in bold). Bootstrap values represent ml values (1000 replicates) and BI values (1,000,000 generations). 1.00 BI posterior probabilities and 100% ml bootstrap percentage support are denoted by *. Additional sequences of ciliates were downloaded from GenBank database.

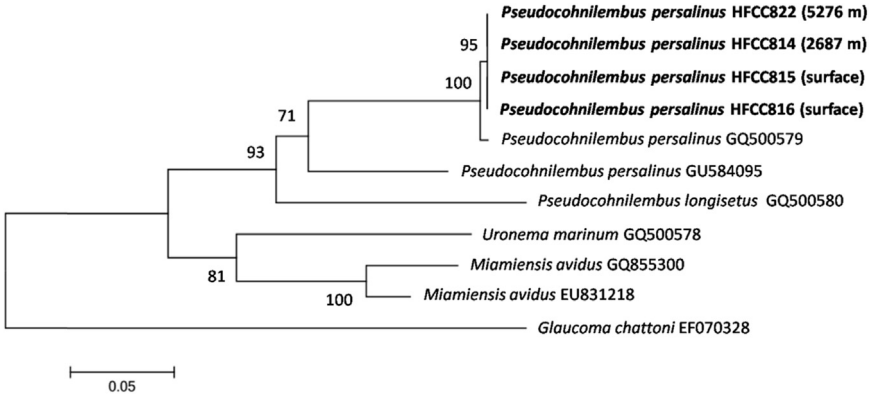

Fig. 5. Neighbor-joining (NJ) phylogenetic tree of *cox1* sequences of *Pseudocohnilembus persalinus* (sequences of this study in bold). The number at each of the nodes represents the bootstrap percentages of 1000 replicates. Additional sequences of ciliates were downloaded from GenBank database.

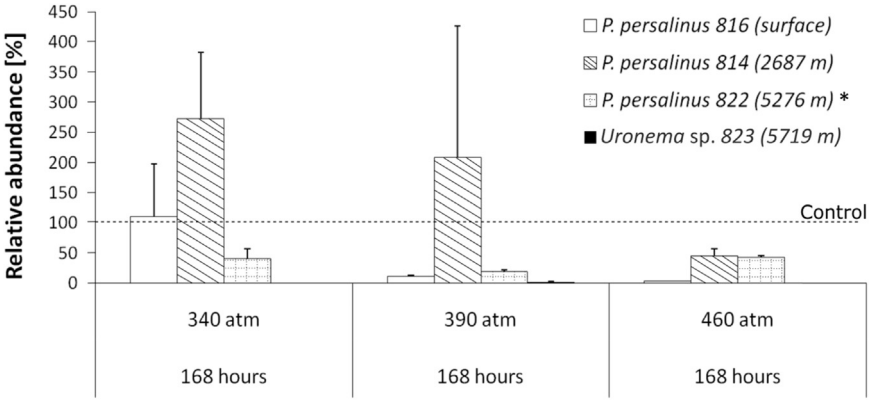

Fig. 6. Direct exposure of ciliates to high hydrostatic pressures of 340 atm, 390 atm and 460 atm at 13 °C. Relative abundance [%] of ciliates in experimental vessels are shown relative to the control at 1 atm (set to 100%, dashed line) vessels (35 PSU artificial seawater at 13 °C). Vertical bars represent ± SD (n = 3). One-way ANOVAs were conducted and significant differences between pressure treatments for each ciliate strain are indicated by '*' ($p < 0.05$).

cysts), control and start values see Table S3. *Pseudocohnilembus persalinus* HFCC 814 from 2687 m water depth showed higher relative abundances at 340 atm (3227 ± 1292 ind./ml) and 390 atm (2467 ± 2572 ind./ml) compared to the control at 1 atm (1180 ± 265 ind./ml) (Fig. 5). In comparison to the start abundance (8500 ± 4682 ind./ml) control and pressure abundance values were much lower (max. decrease of 7967 ind./ml at 460 atm). The highest relative abundances (273%) were observed at 340 atm being closest to the environmental sampling conditions. The surface water isolate of *Pseudocohnilembus persalinus* HFCC 816 had also a higher relative abundance (110%, 1523 ± 1195 ind./ml) at 340 atm in comparison to the control with 1180 ± 265 ind./ml. For this isolate, however, decreasing relative abundances were observed with rising pressure (down to 45% at 490 atm) in comparison to the control, while the two isolates from the abyss, *Pseudocohnilembus persalinus* HFCC 822 and *Uronema* sp. HFCC 823, showed lower relative abundances compared to the control for all pressure treatments (Table S3). *Uronema* sp. did not survive pressures > 390 atm. Only for *P. persalinus* HFCC 822 a significant influence of pressure on the relative abundance could be detected (One-way-ANOVA, $p < 0.05$).

In the second set of experiments, ciliates were allowed to adapt to high pressures either at 13 °C or 2 °C. Experiments were conducted with an adaptation time of three days for each stepwise increase at pressures from 170 atm, 340 atm and 550 atm. The different pressure treatments were plotted against the relative abundance resulting from comparisons with the controls of each pressure treatment. Due to general lower abundances and zero countings of the abyss isolates *P. persalinus* HFCC 822 and *Uronema marinum* HFCC 823 under hydrostatic pressure, standard deviations were quite high (Fig. 6, E, F, G, H). A stepwise increase in hydrostatic pressure at 13 °C allowed a better survival of the *P. persalinus* strain HFCC 822 from 5276 m up to a pressure of 550 atm in comparison to the direct exposure experiment at 13 °C (Fig. 5, Fig. 6 E). Except for *P. persalinus* HFCC 822, all other ciliate strains showed higher relative abundances indicating better survival at 2 °C. For several ciliate strains, one-way ANOVAs with Tukey's HSD post-hoc test showed significant differences within pressure experiments for each of the temperature treatments (Fig. 6 A, B, C, G). For experiments with *P. persalinus* HFCC 816 (surface), a two-way ANOVA revealed significant main effects of temperature ($F = 36.67$, $p < 0.001$) and pressure ($F = 9.45$, $p < 0.001$) as well as for the interaction effect of both factors ($F = 27.80$, $p < 0.001$). There was no evidence for a significant interaction of pressure and temperature for the other ciliate strains. However, for *P. persalinus* HFCC 814 (2687 m) the test for the main effect of treatment showed a significant pressure effect on the relative ciliate abundance ($F = 4.51$, $p < 0.05$). Results of the two-way ANOVA for the abyss species *P. persalinus* HFCC 822 (5276 m) and *Uronema* sp. HFCC 823 (5719 m) was significantly influenced by temperature ($F = 6.98$, $p < 0.5$; $F = 5.85$, $p < 0.5$; respectively) (Fig. 7).

4. Discussion

Our literature survey regarding the diversity of ciliates based on morphological studies and genotypes recorded from molecular studies of deep-sea samples indicated the potential existence of a very large variety of ciliate taxa. Nearly all large taxonomic groups were represented including karyorelictids, heterotrichs, hypotrichs, oligotrichs, armorphorids, litostomatids, suctorians, nassophoreans, prostomatids, peritrichs, which almost all can also be found in shelf coastal sediments (< 200 m) (e.g. Forster et al., 2015; Massana et al., 2015; Meng et al., 2012). However, Rhynchodia (Phyllopharyngea) and Protocruziidia (Spirotrichea), also found in shelf sediments (Meng et al., 2012), could not be detected for deep-sea sediments within our literature review, while the newly described class Cariacotrichea might be presumably restricted to anoxic marine environments (Orsi et al., 2012b).

A surprising result was the fact that among the 429 sequences and 16 morphological records obtained from the literature survey only 10 deep-

sea ciliate sequences of our alignments could be linked to species (with our set species threshold of > 99.7% similarity) known from surface waters. One of these sequences (DQ918623; Countway et al., 2007) showed a 100% similarity to two species, *Sinistrostrombidium cupiformum* and *Strombidium* sp., probably due to a short available sequence length (379 bp). *Sinistrostrombidium cupiformum* was described from the littoral zone in China (Liu et al., 2015). Two other deep-sea sequences, EU446379 and FN598351, resulted both with a similarity of 99.73% in the named blast hits *Cyclidium varibonneti* (KF256817) from mangrove wetlands in China (Gao et al., 2014) and *Parauronema virginianum* (JN885087) isolated from the littoral zone of China (Gao et al., 2012). Three other sequences (SRR2480516.90234, SRR2480516.90340, SRR2480517.38866) from a study of Zhao and Xu (2016) also showed a 99.73% similarity to *Amphisiella annulata* (GU170843), isolated from surface waters in the Incheon harbor of Korea, *Tunicothrix brachysticha* (GU574811), isolated from sediment in the intertidal zone of Qingdao Bay, and *Ancistrum crassum* (HM236340), isolated from the mantle cavity of the marine bivalve *Ruditapes philippinarum* bought from a market in Qingdao. Furthermore, one sequence (SRR2480518.52836) resulted with a similarity of 99.71% in the named blast hit *Trichodina sinonovaculae* (FJ499386). With a 100% similarity one sequence (SRR2480517.38848) from Zhao and Xu (2016) could be assigned to *Madsenia indomita* (JQ956550) detected from the marine coast in China. Within their study they even found with a 100% similarity (SRR2480516.91203) *Pseudocohnilembus persalinus* (blast hit AY835669). Our own isolated scuticociliate species *Pseudocohnilembus persalinus* seems to be the first ciliate species based on morphological and genotypical characterization which may occur in both habitats, shallow water and deep sea. *Pseudocohnilembus persalinus* was also detected in the deep sea from environmental samples (SRR2480516.91203) at depths of Within our own isolates of *P. persalinus*, a genetic analysis of the 18 S rDNA and *cox1* gene revealed no genetic differences (no p-distance). Our sampled deep-sea strain of *Uronema* sp. HFCC823 showed a sequence similarity of only 99.41% to NCBI deposited *Uronema marinum* sequences (GQ259745/GQ259744). One sequence (SRR2480518.51323) from the deep sea within our literature review could be assigned to *Uronema marinum* (GQ465466) from coastal waters off Qingdao with a sequence similarity of 99.73%. Because of the unresolved species concept (Boenigk et al., 2012; Caron et al., 2009) we used within our literature review an arbitrary species boundary of 99.7% taking into account the quality score of sequencing technology (between 99.4% (Stoeck et al., 2014), 99.75% (Huse et al., 2007) and ~99.82% (Niklas et al., 2013)) and the expected Pfu polymerase error rate (2.6×10^{-6}; Thermo Scientific) during PCR (Huse et al., 2007; Stoeck et al., 2010). The ≥ 97% pairwise identity level was used as a conservative proxy level to separate genera (Caron et al., 2009; Venter

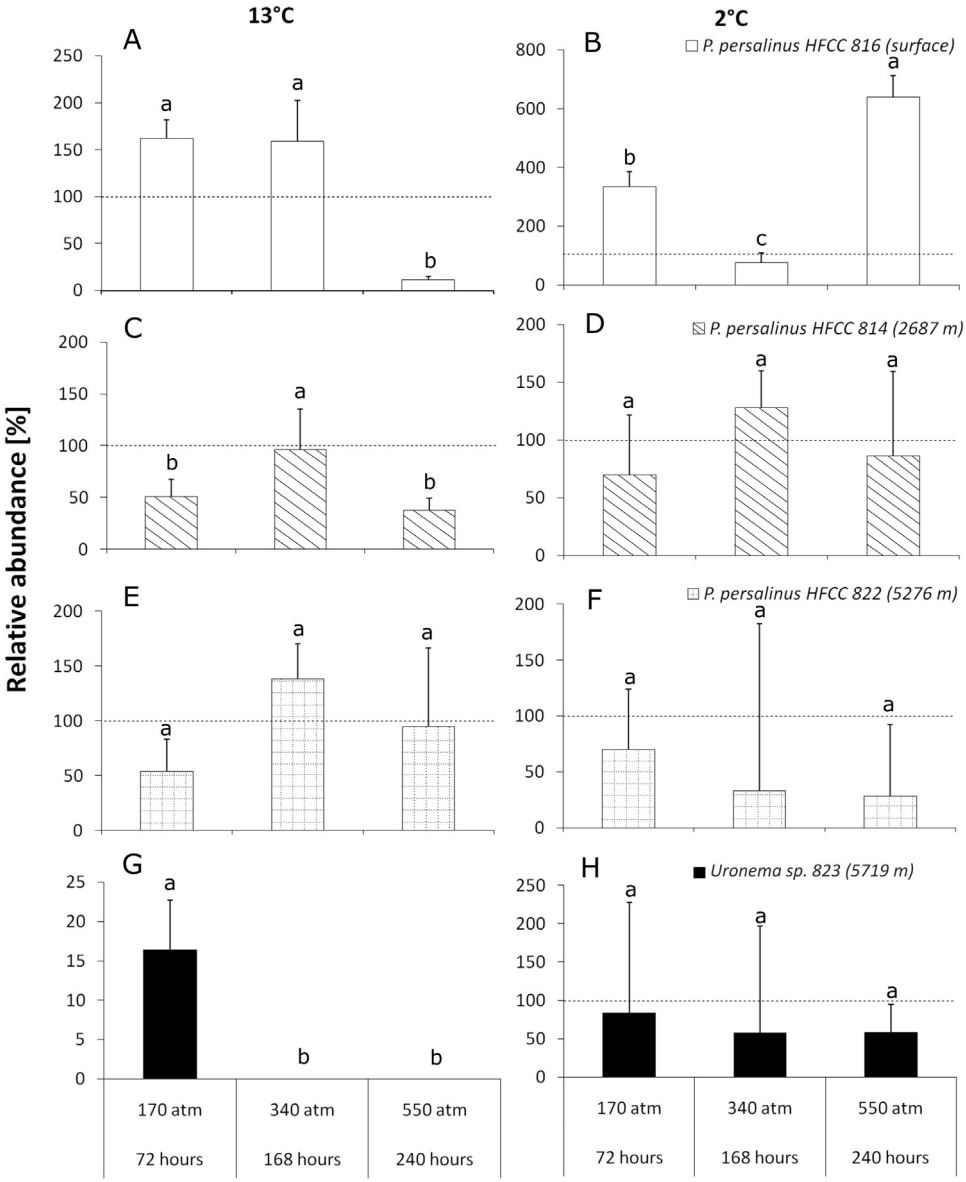

Fig. 7. Exposure of ciliates to stepwise increasing hydrostatic pressure. Relative abundance [%] of four ciliate strains in experimental vessels are shown relative to the control at 1 atm (set to 100%, dashed line) vessels (35 PSU artificial seawater) for 13 °C (left: A, C, E, G) and 2 °C (right: B, D, F, H). Each experiment lasted 240 h. For the first 72 h, the experimental cultures were kept at 170 atm, followed by an exposure to 340 atm for another 72 h and 550 atm for the last 72 h. Controls were kept at 1 atm. One-way ANOVAs were conducted with Tukey's HSD test as post-hoc analysis. Vertical bars represent ± SD (n = 5). Different letters on top of bars indicate significant differences ($p < 0.05$).

et al., 2017). Nevertheless, one has to keep in mind that a species level threshold of 99.7% sequence similarity might not be applicable for all ciliate genera.

Considering the limited access to the deep sea, undersampling is a severe problem in estimating the diversity of deep-sea ciliates. Nevertheless, comparative studies of deep-sea gene sequences showed that most sequences are often very different from genotypes identified from surface waters pointing to a unique biodiversity in the abyss (e.g. Pawlowski et al., 2011a, 2011b; Sauvadet et al., 2010; Scheckenbach et al., 2010). The relatively large genetic distances of nearly all individual sequences recorded from the deep sea compared to sequences from well-known ciliates of surface waters support this idea. Thus, we have to expect an extremely large undersampled diversity of ciliated microfauna in the deep sea. It is very likely that multiple processes related to water masses, water depth, sedimentation rates, habitat structure, temperature and the number of prokaryotes operate at the same time structuring communities of deep-sea protists (Bochdansky et al., 2017; Caron and Countway, 2009; Salani et al., 2012).

Direct microscopic observations of sediment samples (live-counting method) did neither recover *P. persalinus* or *U. marinum* nor cysts resulting in the lack of knowledge concerning the original state of these species in the deep sea. Nevertheless, we only investigated 100 µl sample volume per Multi-Corer under the microscope, because live-counting has to be conducted within a short time frame. We have observed that flagellated died under the microscope during counting, probably caused either by rising temperatures due to microscopic light exposure or exposure to low atmospheric pressure (1 atm). However, we could isolate and cultivate some protists at atmospheric pressure. Due to a closing mechanism at the top and bottom of the cores of a Multi-Corer, the risk of contamination with organisms and cysts from upper water layers is reduced, while being raised through the water column up to surface waters. Otherwise, on the way down to the deep sea, the cores of the Multi-Corer are open and, thus, a risk of contamination with protists from upper water layers being attached to the inner side of the cores cannot be completely excluded. We have three scenarios for the origin and original state of our ciliate samples. First, they might be actually living and reproducing in the abyss and can handle the pressure difference, thus, being active at high atmospheric pressure. Within our pressure experiments we could not prove, that the ciliates are definitely from the abyssal zone. There might be the possibility that ciliates originate from a deep-sea population, but their tolerance with regard to high hydrostatic pressures got lost due to selective cultivation at surface pressure or the tolerance was underestimated because the species might need a longer period of adaptation to higher pressures than offered in the experiments and, therefore, could be the reason, why we only observed barotolerant and no barophilic behavior. The limitation of the stepwise increasing pressure in experiments is the intervening return to 1 atm before we can increase the pressure again. Future experiments will give us the opportunity to investigate the ciliates and flagellates under pressure directly under the microscope and increase the pressure gradually without the intervening return to 1 atm. Second, they only originated from sedimented cysts from surface waters which never hatch in the deep and cultivating at atmospheric pressure resulted in reproduction of the ciliates. Third, ciliates might be a contaminant in deep-sea sediment cores due to contact with surface waters during sampling and, thus, might not exist as cysts or living organisms in the deep. We could isolate *P. persalinus* from surface waters and the deep sea, but *Uronema marinum* was only detected in deep-sea sediment. Until sediment samples can be obtained under pressure from the deep sea allowing us the direct observation of the original state of protists at these depths, ecological experiments under deep-sea conditions (temperatures < 4 °C and high hydrostatic pressure) need to be conducted to investigate their potential to live in the deep sea. We could not observe typical barophilic behavior (significantly higher growth rates at higher pressures) within our experiments. Nevertheless, all four ciliate strains of our study isolated either from abyssal depth or surface waters survived as active ciliates and/or cysts hydrostatic pressures larger than 500 atm at 2 °C (Table S3). This barotolerance of the four ciliate strains was recorded despite the sudden decrease of pressure to 1 atm due to methodological reasons between the different exposures (stepwise pressure experiment) to different hydrostatic pressures. A similar tolerance of protists was observed by Atkins et al. (1998) during investigations of some heterotrophic flagellates. Hemmingsen and Hemmingsen (1983) found that *Tetrahymena* cells stayed relatively unaffected when exposed to pressures of up to 150 atm – a pressure that was tolerated by all of our strains. However, greater pressures led to a significant loss in their experiments. In experiments with *Pseudocohnilembus persalinus* we observed increasing cyst formation (Table S3) at higher hydrostatic pressures (> 300 atm) which may support survival of ciliates when transported by oceanic currents or sedimenting detritus into the deep. At least for bacteria it is known that an increase of fluidity of membranes by incorporation of unsaturated fatty acids plays a role in the survival at high hydrostatic pressures (Allen et al., 1999; DeLong and Yayanos, 1985; Kamimura et al., 1992; Wang et al., 2014). A higher fluidity of membranes has also a positive effect on survival at low temperatures (DeLong and Yayanos, 1986). This might explain the higher survival of three of the four strains when exposed to pressures at 2 °C (deep-sea temperature in the Pacific) compared to exposure at 13 °C (deep-sea temperature of the Mediterranean).

With one exception, growth rates of ciliates in our experiments were negative when exposed to high hydrostatic pressure and prior cultivated at surface pressure for several weeks. One of the four strains (*P. persalinus* HFCC814 from 2687 m depth) reached growth rates of about 0.07 (± 0.05) d^{-1} at 340 atm at 2 °C. Although this is rather low compared to ciliates of the same size cultivated under surface water conditions at 20 °C (Müller and Geller, 1993), this growth rate would be much higher than those of most other eukaryotes under deep-sea conditions. In the vicinity of organic debris or carcasses, sufficient bacteria should be present in the abyss to allow for relatively high growth rates. The two isolated scuticociliates are widely distributed and seem to be extremely euryoecious not only regarding their barotolerance but also regarding the dwelling of different habitats, they even occur as parasites of fish (Jee et al., 2001; Kim et al., 2004).

5. Conclusions

Metagenome data indicated that ciliates may form a very diverse component of deep-sea communities, which seem to have little overlap with communities of shelf regions. Our experimental studies indicated that also ubiquitous species may occur in the deep sea. It is not known which part of the genotypes recorded in deep-sea metagenome data bases is belonging to organisms really living and reproducing in the abyss or only originating from sedimented cysts which never hatch in the deep. RNA data would help to solve this problem, though fixation of samples should be done in the deep to prevent hatching of cysts in the course of sample uptake (Schoenle et al., 2016). Nevertheless, also cysts have RNA and, thus, mRNA analysis (metatranscriptomics) could give more inside. Obtaining unfixated sediment samples under pressure would help to microscopically observe the original state of the protists, active or inactive (cysts). Ciliate strains investigated in the present study were barotolerant. Future studies have to show whether this is restricted to the investigated species or a general feature of many ciliates.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dsr.2017.08.015.

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2.2 New Phagotrophic Euglenids from Deep Sea and Surface Waters of the Atlantic Ocean (*Keelungia nitschei, Petalomonas* gen. nov., *Ploeotia costaversata*

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New phagotrophic euglenids from deep sea and surface waters of the Atlantic Ocean (*Keelungia nitschei, Petalomonas acorensis, Ploeotia costaversata*)

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Abstract

New phagotrophic euglenoid species from marine surface waters and the deep sea were isolated and described by light and scanning electron microscopy and 18S rDNA sequencing: *Keelungia nitschei*, *Petalomonas acorensis* and *Ploeotia costaversata*. The morphological characteristics of *Keelungia nitschei* agree with *Keelungia pulex* besides the slightly truncated anterior front of the cell of our strain. Phylogenetic analysis indicated low sequence similarity between *K. nitschei* and *K. pulex* (87.3%). *Ploeotia costaversata* clustered within the *Ploeotia costata* clade with a sequence similarity of 96.1% to *P. costata* strain Tam. Ultrastructural characteristics of our strain revealed helically twisted strips towards both poles of the protoplast. 18S rDNA phylogenies showed that *Petalomonas acorensis* is related to the clade of *Petalomonas cantuscygni/Scytomonas saepesedens* with the highest sequence similarity of 81.2% to *P. cantuscygni*. Six pellicle strips are visible, while two of them reach only the middle of the cell and four (two longitudinal, two helically twisted) join at the posterior front of the cell. Pressure experiments showed that the deep-sea strain *K. nitschei* was better adapted to high hydrostatic pressures (up to 500 bar) at 4 °C than the two surface water strains. All three strains increased the database (18S rDNA) of the underrepresented group of phagotrophic euglenids.

Keywords: Abyssal; Behavior; Euglenozoa; Phylogeny; Pressure adaptation; 18S rDNA

Introduction

The diverse phylum of Euglenozoa is represented by three major subgroups, kinetoplastids, diplonemids and euglenids. Euglenids have evolved diverse modes of nutrition including photoautotrophy and phagotrophy. Phagotrophic euglenids include bacteriovorous and eukaryovorous taxa. The cell flexibility of the larger eukaryovorous species (peranemids and anisonemids) might be possible due to more pellicular strips (20–56) compared to bacteriovorous taxa (petalomonads and ploeotiids), which are smaller rigid cells with 4–12 pellicular strips (Leander et al. 2007, 2001). However, several euglenids species are capable of ingesting algae (*Ploeotia* cf. *vitrea*, Lax and Simpson 2013) or yeast (*Serpenomonas*, Linton and Triemer 1999) besides bacteria or protists, indicating that rigid pellicles might not be barriers

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to eukaryovory. There is the assumption that the ancestral euglenids fed on both bacteria and protists instead of being solely bacteriovorous (Cavalier-Smith 2016). Besides using ribosomal nucleotide sequences (e.g. 18S rDNA), morphological data and some nucleus-encoded protein genes have been used for analysing the deep-level phylogenetic relationship of phagotrophic euglenids suggesting that rigid phagotrophs form the earliest diverging branches within euglenids (Breglia et al. 2007; Cavalier-Smith 2016; Leander et al. 2001). The secondary structure information and helix numbering were considered by Paerschke et al. (2017) for the 18S rDNA alignment to reduce ambiguities and maximize recognition of homologoues positions. In addition, they used the existence of paramylon (ultrastructural and immunocytochemical) for a deeper evaluation of the phylogenetic relationship of euglenids. Their combination of methods resulted in the identification of the uncertain phylogenetic position of *Entosiphon sulcatum* (Chan et al. 2013; Lax and Simpson 2013; von der Heyden et al. 2004) as the sister taxon of the newly termed monophyletic crown clade Helicales (Paerschke et al. 2017). An increased taxon sampling and cultivation of additional euglenids from different taxonomic groups is necessary for a better resolution of phylogenetic relationships.

Phagotrophic euglenids are known to be typical components of freshwater and marine sediments in shallow waters (Arndt et al. 2000; Lee and Patterson 2000; Lee 2012), but have seldom been reported, and not yet cultivated from deep-sea samples (Buck et al. 2000; Yubuki et al. 2009). Many flagellates determined on a morphospecies level from live samples from the deep sea have been reported from shallow waters as well (Arndt et al. 2003; Atkinson 1998; Patterson et al. 1993; Patterson and Lee 2000). In some cases, even similar genotypes of heterotrophic flagellates had been isolated from the deep sea and surface waters (Scheckenbach et al. 2005). On the other hand, clone libraries and metabarcoding studies indicate the existence of specific deep-sea heterotrophic protist communities (López-García et al. 2001; Scheckenbach et al. 2010; Schoenle et al. 2017). While kinetoplastids and diplonemids have commonly been recorded with high species numbers from environmental sequencing datasets of marine surface waters and the deep sea, phagotrophic euglenids have rarely been recorded in many environmental sequencing datasets from sediments (Countway et al. 2007; de Vargas et al. 2015; Flegontova et al. 2018, 2016; Salani et al. 2012; Stoeck and Epstein 2003; von der Heyden et al. 2004; Zuendorf et al. 2006). This might be due to the lack of the availability of the divergent and expanded 18S rRNA gene sequences for euglenids in databases and the problems with suitable primers to recover the highly divergent sequences of euglenids. Whether euglenids and other protists are actually active under deep-sea conditions or might only be resting as cysts in deeper waters is still unknown. Until sediment samples can be obtained under pressure from the deep sea allowing us the direct observation of the original state of protists at these depths, ecological experiments under deep-sea conditions (temperatures <4 °C and high hydrostatic pressure) need to be conducted. Such pressure experiments with cultured isolates can, thus, be used to verify the potential deep-sea origin of euglenids and might indicate their potential to be active in the deep sea. Representatives of very different phylogenetic groups comprising choanoflagellates, ancyromonads, kinetoplastids, bicosoecids, chrysomonads, and cercozoans isolated from surface waters and the deep sea have been shown to be able to tolerate high hydrostatic pressures (Atkins et al. 1998; Turley et al. 1988; Turley and Carstens 1991; Živaljić et al. 2017).

In our search for potentially specific deep-sea communities of heterotrophic flagellates, we isolated flagellate strains from abyssal depths (>5000 m water depth) of the southern North Atlantic. We could isolate a deep-sea strain looking similar to a recently discovered euglenid genus *Keelungia* Chan et Moestrup 2013. To compare the adaptation to hydrostatic pressure of this deep-sea strain, we isolated two euglenid strains of the genera *Petalomonas* and *Ploeotia* from Atlantic surface waters. For the first time, we were able to directly observe the behavioural response of heterotrophic flagellates to an exposure to high hydrostatic pressure and compare the behaviour of surface water and deep-sea euglenids exposed to high pressure. Molecular and morphological studies indicated that all three strains belonged to new species, namely *Keelungia nitschei*, *Ploeotia costaversata* and *Petalomonas acorensis*. Because the sequence of the type species *Ploeotia*, *Ploeotia vitrea* Dujardin 1841, is still missing, the taxonomic affiliation of *P. costaversata* and *P. costata* to the genus may require revision when more data are available.

Material and Methods

Sampling, isolation and culture conditions

The euglenid *Keelungia* strain HFCC 166 was obtained from sediment samples taken with a Multi-Corer system (MUC) in the southern North Atlantic Ocean (10°25.12′N, 31°04.62′W; 5117 m, R/V *Sonne* SO237). Samples of the Multi-Corer system were taken from the surface sediment layer. A closing mechanism at the top and bottom of the cores reduces the risk of contamination with organisms and cysts from upper water layers and, thus, a contamination should be negligible. Subsamples were cultivated in tissue-culture flasks (Sarstedt, Nümbrecht, Germany) filled with autoclaved sea water. All culture flasks were supplied with sterilized quinoa grains as an organic food source for autochthonous bacteria. The euglenid *Petalomonas* strain HFCC 220 was obtained from sediment on a beach near Feteira, Faial, Azores (38°31.362′N, 28°40.446′W). The euglenid *Ploeotia* strain HFCC 1508 was obtained from biofilms grown at 15 PSU and 28 °C in a shrimp recirculating aquaculture system grow out facility nearby Munich (CrustaNova GmbH, Langenpreisingen, Germany). The process water in Munich was stocked

with the first batch of post larvae of *Litopenaeus vannamei* grown up in Atlantic seawater.

Besides the application of the liquid aliquot method (Butler and Rogerson 1995), a micromanipulator (Patchman MP2, Eppendorf, Germany) was used to isolate the euglenids to obtain monocultures. All three euglenids were further cultivated in 50 ml tissue-culture flasks (Sarstedt, Nümbrecht, Germany) filled with 30 ml 35 PSU Schmaltz-Pratt-medium (per liter: 28.15 g NaCl, 0.67 g KCl, 5.51 g $MgCl_2 \times 6\,H_2O$, 6.92 g $MgSO_4 \times 7\,H_2O$, 1.45 g $CaCl_2 \times 2\,H_2O$, 0.10 g KNO_3, 0.01 g $K_2HPO_4 \times 3\,H_2O$) for strains HFCC 220 and HFCC 166 and 15 PSU Schmaltz-Pratt-medium (per liter: 12.06 g NaCl, 0.29 g KCl, 2.36 g $MgCl_2 \times 6\,H_2O$, 2.97 g $MgSO_4 \times 7\,H_2O$, 0.62 g $CaCl_2 \times 2\,H_2O$, 0.10 g KNO_3, 0.01 g $K_2HPO_4 \times 3\,H_2O$) for HFCC 1508 containing one sterilized wheat grain to ensure growth of ambient bacteria of the original sample.

Light microscopy

For the morphological characterization all three euglenid strains were analyzed with the help of an Allen Video Enhanced Contrast (AVEC) system consisting of an analogous Hamamatsu C6489 camera with noise suppression and contrast amplification (Argus-20, Hamamatsu, Japan) to record high resolution stills of the three strains. An inverted microscope (Zeiss Axio Observer A1) equipped with a 100x/1.4 NA oil immersion objective (DIC) and a water immersion condenser was used. Cultures were observed in petri dishes with a coverslip base. Pictures were taken with VirtualDub-1.10.4 and were edited by ImageJ. Morphological characteristics (cell length, cell width, length of flagella, diameter of nucleus) of specimens were measured with Axio Vision Rel. 4.8 (Zeiss, Germany).

Scanning electron microscopy (SEM)

Samples of the three euglenid cultures were fixed with glutaraldehyde (3%) and 1% osmiumtetroxide (final concentration) buffered with 0.2 M sodium cacodylate buffer at 4 °C and pH 7.4 for 30 min. Samples remained in the culture flask (Sarstedt 50 ml culture flasks) and were dehydrated in an ethanol series comprising 30%, 50%, 60%, 80%, 90%, 96% and pure ethanol. Samples were washed two times with the corresponding ethanol concentration and finally remained for 10 min in each solution. After this procedure, a 1:1 hexamethyldisilazane (HMDS)-ethanol solution was applied for 15 min followed by pure HMDS for 15 min as a substitute for critical point drying. Afterwards, the samples were allowed to dry. The bottom of each flask was cut to appropriate size and stuck to a sample holder. SEM samples were sputter coated with a 120 Å layer of gold before examination by SEM (Hitachi S-520 and FEI Quanta 250 FEG).

DNA extraction, purification and sequencing

Euglenids were concentrated by centrifugation (4000 × *g* for 20 min at 4 °C, Megafuge 2.0 R, Heraeus Instruments). The genomic DNA was extracted using the Quick-gDNA™ MiniPrep (Zymo Research, USA). For the amplification of the 18S rDNA of *Keelungia nitschei* HFCC 166 and *Ploeotia costaversata* HFCC 1508 following primers were used: forward 18S-For (5′-AACCTGGTTGATCCTGCCAGT-3′, Medlin et al. 1988) and reverse 18S-Rev (5′-TGATCCTTCCGCAGGTTCACCTAC-3′, Medlin et al. 1988). The 18S rDNA of *Petalomonas acorensis* HFCC 220 was amplified with the following primers: forward AP7F (5′- GTCATATGCTTYKTTCAAGGRCTAAGC-3′, Busse et al. 2003) and reverse AP8R (5′-TCACCTACAGCWACCTTGTTACGAC-3′, Busse et al. 2003). The PCR reactions were performed in 50 µl PCR reaction mixtures containing 13 µl ddH_2O, 1.5 units TAQ (Mastermix, VWR Germany), 2 µl DNA and 5 µl of each primer (forward and reverse) at a final concentration of 1.6 nM. The PCR conditions were as follows: pre-denaturation at 98 °C for 2 min, 35 cycles of 98 °C for 30 sec, 55 °C (for primer combination: 18S-For and 18S-Rev)/57 °C (for primer combination: AP7F and AP8R) for 45 s and 72 °C for 2 min; final extension at 72 °C for 10 min. The 28S rDNA of *Keelungia nitschei* HFCC 166 was amplified using the following primers: NLF184/21 (5′-ACCCGCTGAAYTTAAGCATAT-3′, Van der Auwera et al. 1994), NLR1126/22 (5′-GCTATCCTGAGGGAAACTTCGG-3′, Van der Auwera et al. 1994), D3For (5′-GACCCGTCTTGAAACACGCA-3′, Wylezich et al. 2007) and NLR2098/24 (5′-AGCCAATCCTTWTCCCGAAGTTAC–3′, Van der Auwera et al. 1994). The PCR reactions were performed as for the 18S rDNA with following PCR conditions: pre-denaturation at 98 °C for 2 min, 35 cycles of 98 °C for 30 s, 55 °C for 45 s, 52 °C for 45 s and 72 °C for 2 min 30 s; final extension at 72 °C for 10 min. The amplified products were analyzed by electrophoresis on 1% agarose gel and fragment sizes were determined by comparison with 100 bp DNA ladder. The amplicons were directly purified from PCR products using a PCR Purification Kit (Jena Bioscience, Jena, Germany) and sequenced with the corresponding amplification primers. Sequences were deposited in the GenBank under the accession numbers MK564753, MK564754, MK564755 (18S rDNA) and MK567808 (28S rDNA).

Phylogenetic analysis

For the phylogenetic 18S rDNA analysis available sequences were downloaded from the GenBank database. We included all significant groups of euglenids, but excluded the genus *Entosiphon* due to its highly divergent sequences (Lax and Simpson 2013). Alignments were carried out using MAFFT v7.311 (Katoh and Standley 2013) within UGENE

version 1.28.1 (Okonechnikov et al. 2012); manual corrections to optimize the alignment were done with BioEdit version 7.2.6 (Hall 1999) previously to the phylogenetic analysis. The 88-taxon alignment contained 1108 well-aligned nucleotide positions of the 18S rDNA that were determined for the phylogenetic analyses. The Maximum likelihood (ML) analysis was carried out using RaxML version 8.2.10 (Stamatakis 2014) on the CIPRES Gateway (Miller et al. 2010) employing the GTR (General Time Reversible) plus Γ model of nucleotide substitution as suggested by MrAIC (GTR + I + Γ) (Nylander 2004). Statistical support was estimated with 1000 bootstrap replicates. Bayesian inference (BI) analysis was run with Mr. Bayes v3.2.6 (Ronquist et al. 2012) using a GTR + I + Γ model and a four-category gamma distribution to correct for among site rate variation. The search consisted of two parallel chain sets run at default temperatures with a sample frequency of 10 and run so that the average standard deviation of split frequencies dropped below 0.01. The analysis consisted of 500,000 generations, with a burnin of 125,000 (25%), before calculating posterior probabilities. Although we recovered the 28S rDNA of *Keelungia nitschei*, we did not construct a 28S rDNA tree due to the low availability of 28S rDNA sequences for euglenids.

Analysis of behavior at increased hydrostatic pressure

We constructed a modified version of the high pressure systems developed by Koyama et al. (2001) for real-time observations. The observation chamber has a 4 mm-thick glass window of 4 mm diameter for direct microscopic observation under high hydrostatic pressure. A manual hydraulic pump allows a gradual increase of pressure up to 600 bar. Cultures of *Keelungia nitschei* (HFCC 166, 5117 m), *Ploeotia costaversata* (HFCC 1508, surface) and *Petalomonas acorensis* (HFCC 220, surface) were used to analyze the motion of specimens under different hydrostatic pressures. Cultures of *K. nitschei* and *P. acorensis* were pregrown in 50 ml-tissue culture flasks filled with 35 PSU Schmaltz-Pratt (SP) medium and stored at 10 °C. *Ploeotia costaversata* was pregrown in 15 PSU SP-medium at 20 °C, because movement stopped after one night at 10 °C. Thus, the experiment with *P. costaversata* was conducted only at 20 °C, while experiments with *P. acorensis* and *K. nitschei* were additionally conducted at 4 °C. Cells were scraped using cell scrapers and 200 µl transferred into the chamber with an air bubble to ensure the availability of oxygen. During experiments pressure was gradually increased in steps of 50 bar every seven minutes until 500 bars were reached. In each single experiment one individual was tracked to observe the effect of pressure on species' behavior. For each strain five replicates (corresponding to five specimens) were carried out in the course of one day to ensure similar culture conditions of specimens. As control euglenid strains (five replicates per strain) were exposed and analyzed in chambers at atmospheric pressure (1 bar).

Recordings were carried out for 22 s with high-speed video-recording at 500 frames per second. The behavioral studies were performed with a motion analyzing microscope (Keyence, VW-6000) consisting of a controller and camera unit. The camera unit was attached to an inverted microscope (Zeiss Primovert, Germany) and observation was done with the help of a 20× LD-objective. Data were analyzed using the routine "moving target analysis" and results were obtained for the displacement in µm (defining how far the individual was distant from the start position) and velocity in µm/sec (the change of the position of the individual between starting and final position with time). Two-way ANOVAs and post-hoc Tukeýs test were conducted with R v.3.5.0 (http://www.r-project.org/) to determine the effect on species' behavior (displacement and velocity) by both experimental factors, hydrostatic pressure and temperature. Furthermore, we categorized the motion of each recorded individual under the different pressure steps at 4 °C for *K. nitschei* HFCC 166 and *P. acorensis* HFCC 220 and 20° for *P. costaversata* HFCC 1508 into five movement behaviors: normal movement, barely moves, circling, twitching and not moving.

Results

Morphological characterization

Microscopic studies of *Keelungia nitschei* HFCC 166

Keelungia nitschei HFCC 166 had an oblong to ovoid cell with a mean cell length of 9.8 ± 0.9 µm (8.3–11.6 µm, n = 19) and mean width of 7 ± 0.8 µm (5–8.3 µm; n = 19). A large flagellar pocket extended to about two fifth of the cell length (Fig. 1 F). The two flagella originated at the anterior front of the cell (Fig. 1 H) from the flagellar pocket (Fig. 1 A). The anterior flagellum had a mean length of 10.2 ± 1.5 µm (8.2–12.3 µm, n = 13) and was directed forward used for grazing on individual bacteria of the biofilm, whereas the posterior flagellum was 2–3 times longer (25.9 ± 4.7 µm, 17.5–33.4 µm, n = 13) than the cell itself (Fig. 1 B, C, F, H). The posterior flagellum was extended in a posterior direction and was used for gliding on the substratum and (Fig. 1 B). The anterior flagellum, commonly kept in an S-shape (Fig. 1 C), moved fast and was used for orientation. The pharynx reached almost the posterior end of the cell and was very well visible in light microscopy by its two rods (Fig. 1 E). We observed ten longitudinal pellicular strips leading across the whole cell congregating at the posterior end (Fig. 1 A, G). The frontal part of the cell was slightly truncated (Fig. 1 F, I).

Microscopic studies of *Ploeotia costaversata* HFCC 1508

The biflagellated protoplasts of *Ploeotia costaversata* HFCC 1508 were oblong to ovoid. The cell size of the strain was 15.6 ± 1.4 µm (13–19.1 µm, n = 23) in length and 8.5 ± 0.8 µm (7–10.5 µm, n = 23) in width. The hook-shaped

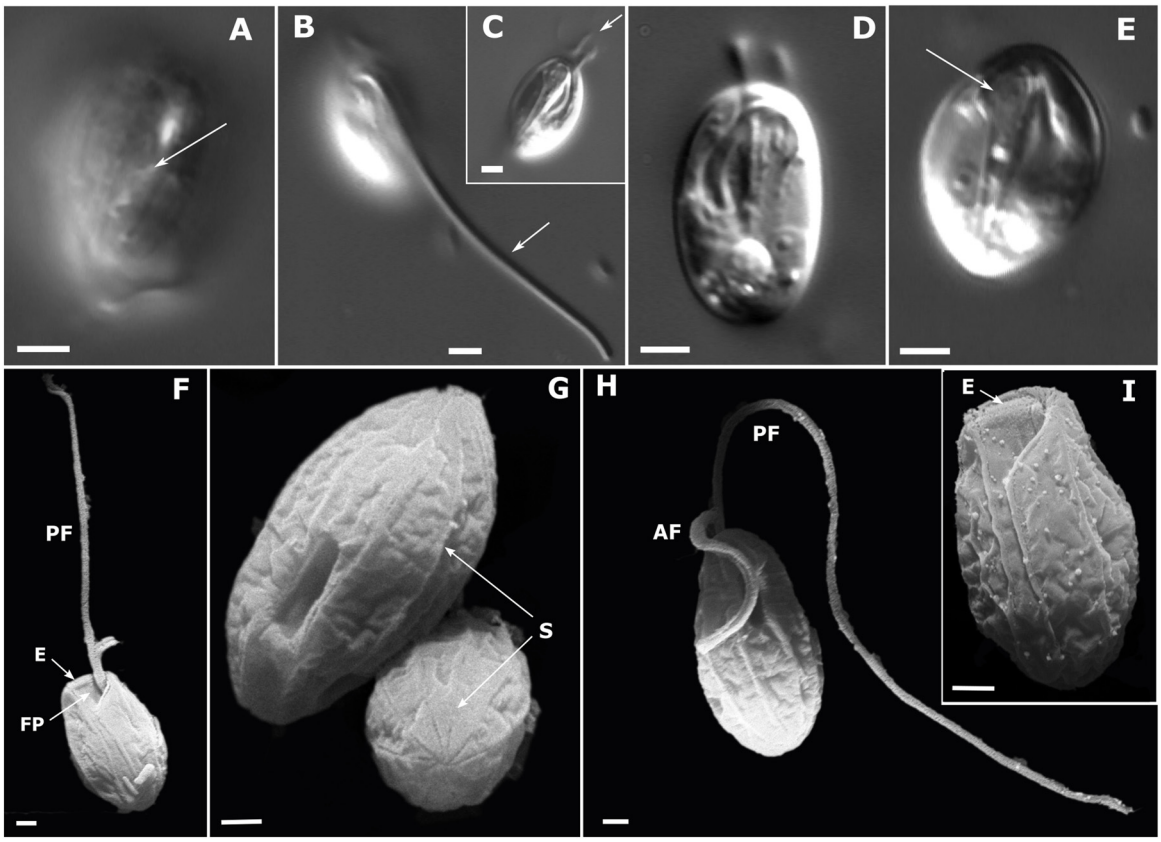

Fig. 1. Light (A–E, scale bars 2 μm) and scanning electron (F–I, scale bars 1 μm) micrographs of *Keelungia nitschei* strain HFCC 166. A: Strips on cell surface visible (white arrow). B: Posterior flagellum emerges from the flagellar pocket (white arrow). C: Ventral view of the cell with anterior flagellum (white arrow) and flagellar pocket. D: Ventral view of the cell. E: Hook-shaped pharynx (white arrow), ventral view. F: Cell in ventral view with long posterior flagellum (PF), flagellar pocket (FP) and dorsoventrally flattened anterior end (E). G: Ten strips (S) visible on the distal pole of the cell on the dorsal side of the cell. H: Dorsal view of the cell with posterior flagellum (PF) and anterior flagellum (AF). I: Dorsoventrally flattened posterior end of the cell (E).

pharynx was 10.9 ± 1.2 μm (8.7–12.9 μm, n = 11) long and 2.5 ± 0.5 μm (1.9–3.4 μm, n = 11) wide (Fig. 2 D). Ten strips joined at the posterior front of the cell and were helically twisted towards both poles of the protoplast (Fig. 2 A, H, I), resulting in the appearance of five grooves in the middle of the cell (Fig. 2 G, H). A lip was located at the anterior end (Fig. 2 F). The two flagella were of unequal length (Fig. 2 C, E). The short anterior flagellum (Fig. 2 C) was 8.0 ± 0.4 μm (7.4–8.7 μm, n = 10) long and the longer posterior flagellum (Fig. 2 E, F, G) had a length of 30.5 ± 5.5 μm (20.0–42.2 μm, n = 17). Up to four vacuoles could be observed with a size of 3.8 ± 0.6 μm (2.5–4.6 μm, n = 29) (Fig. 2 B).

Microscopic studies of *Petalomonas acorensis* HFCC 220

The pyriform shaped *Petalomonas acorensis* strain HFCC 220 had a cell length of 5.8 ± 0.6 μm (4.7–6.9 μm, n = 38) and a cell width of 3.6 ± 0.4 μm (2.8–4.2 μm, n = 38). There was a single flagellum at the anterior end, which was inserted into a deep flagellar reservoir (Fig. 3 A). The length of the single flagellum was slightly longer than the cell length with 6.2 ± 0.5 μm (5.2–7.0 μm, n = 28). The flagellum was directed anteriorly and used for gliding on substratum. In addition, the distal part of the flagellum was beating actively to draw bacteria to its mouth. Cells were ventrally flattened (Fig. 3 E). Six pellicle strips were visible starting from the ventral flagellar pocket (Fig. 3 C–E). While two lateral strips were helically twisted leading from the ventral flagellar pocket to the dorsal posterior front of the cell (Fig. 3 C–E, strip No. 3 and No. 6), two other strips were oriented longitudinally in the middle of the dorsal (Fig. 3 C, strip No. 1) and ventral (Fig. 3 E, strip No. 5) side of the cell. All of these four strips joint at the posterior end of the cell (Fig. 3 C). Two strips only reached the middle of the cell, one lateral/dorsal longitudinal strip (Fig. 3 C, strip No. 2) and one ventral longitudinal strip (Fig. 3 E, strip No. 4).

Phylogenetic analysis

The maximum likelihood (ML) and Bayesian analyses of the 18S rDNA data yielded phylogenetic trees with identical topologies (Fig. 4). Euglenids cluster as sister to kinetoplastids and diplonemids. Phototrophs/mixotrophs (Euglenales, Eutreptiales), primary osmotrophs (Aphagea)

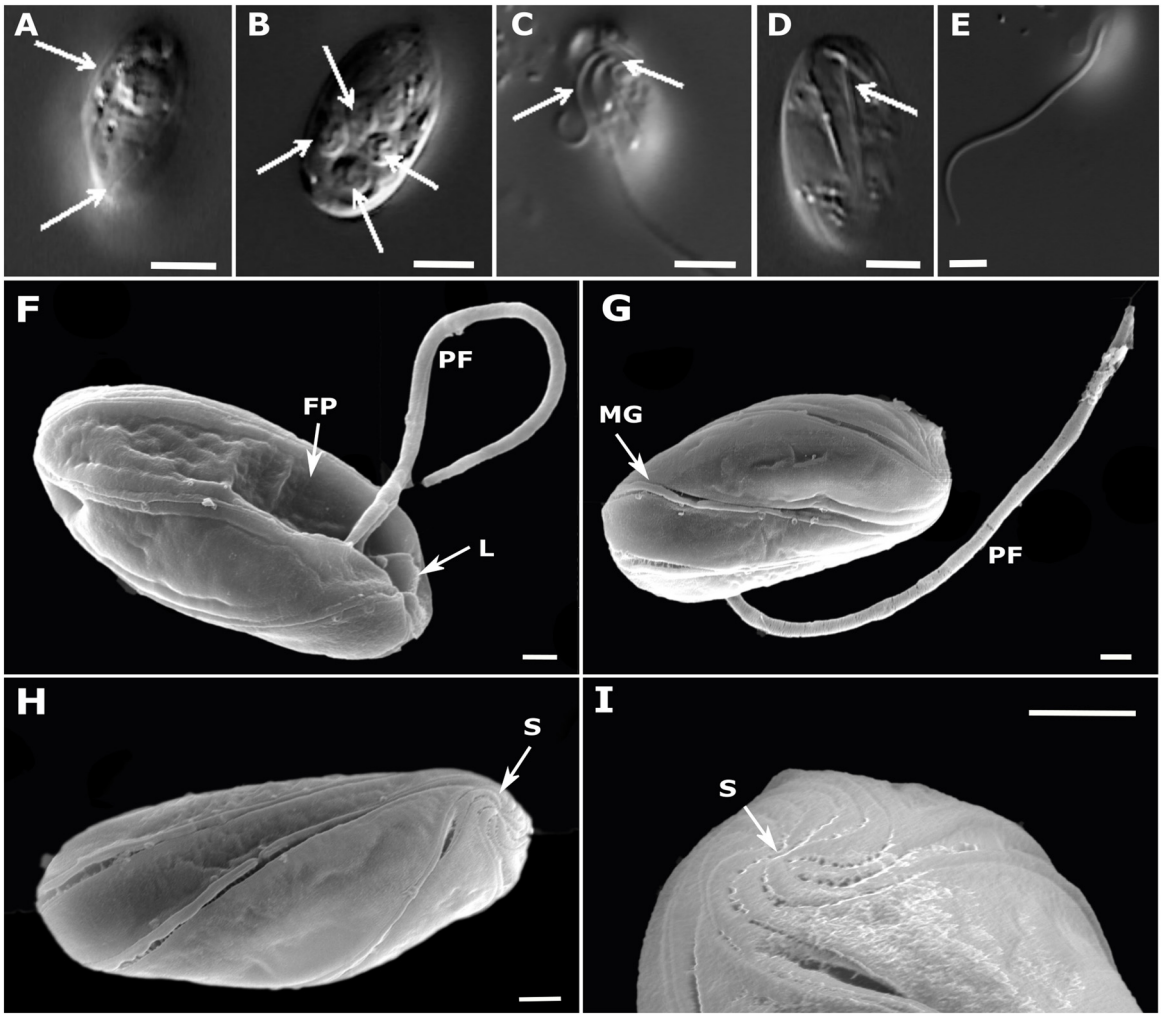

Fig. 2. Light (A–E, scale bars 5 μm) and scanning electron (F–I, scale bars 1 μm) micrographs of *Ploeotia costaversata* strain HFCC 1508. A: Helically twisted strips towards both poles of the protoplast (white arrows). B: Four vacuoles within cell body (white arrows). C: Anterior flagellum and flagellar pocket (white arrows). D: hook-shaped pharynx with two rods (white arrow). E: Posterior flagellum (white arrow). F: Dorsal view of the cell with flagellar pocket (white arrow), lip structure (L, white arrow) and posterior flagellum (PF). G: Side view of the cell with posterior flagellum (PF) and major grooves (white arrow). H, I: Ten helically twisted pellicle strips (S) towards both poles of the protoplast (white arrow).

and phagotrophic euglenids with a high number of pellicle strips (anisonemids, peranemids) were recovered as strongly supported group (mlBP 99%, biPP 1.00, Fig. 4). Other euglenids formed a very poorly resolved basal radiation including (i) a maximally (mlBP 100%, biPP 1.00) supported group of Petalomonadida (*Petalomonas, Scytomonas* and *Notosolenus*), (ii) a maximally supported clade of *Ploeotia*, (iii) a maximally supported Symbiontida clade, (iv) a highly supported group of *Decastava* and *Keelungia* (mlBP 96%, biPP 1.00) (Fig. 4).

Within the ploeotiids the *Keelungia* strain HFCC 166 formed a maximally supported clade (mlBP 96%, biPP 1.0) with *Decastava edaphica* (KY963138) and *Keelungia pulex* (HM044218). Within this clade, *Keelungia nitschei* HFCC 166 clustered closest with *Keelungia pulex*, supported by full bootstrap values (mlBP 100%, biPP 1.00, Fig. 4). The sequence of HFCC 166 differed from that of *K. pulex* in 244 bp within a comparable sequence length of 1955 bp (p-distance 12.7%). The p-distance of HFCC 166 to *Decastava edaphica* was much higher (25.6%).

The sequenced species *Ploeotia costaversata* (HFCC 1508) clustered together with *P. costata* strain Pac (KF586332) and *P. costata* strain Tam (KF586333) with high support (mlBP 100%, biPP 1.00); however, *P. costata* CCAP 1265/1 (AF525486) clustered on a separate branch. Strain HFCC 1508 had the highest sequence similarity of 96.1% to *P. costata* strain Tam (KF586333). The similarity to CCAP 1265/1 was much lower with 90%.

Petalomonads (including the genera *Petalomonas, Scytomonas, Notosolenus*) formed a maximally supported cluster within our phylogenetic analysis (mlBP 100%, biPP 1.00). *Petalomonas sphagnophila* species clustered in a maxi-

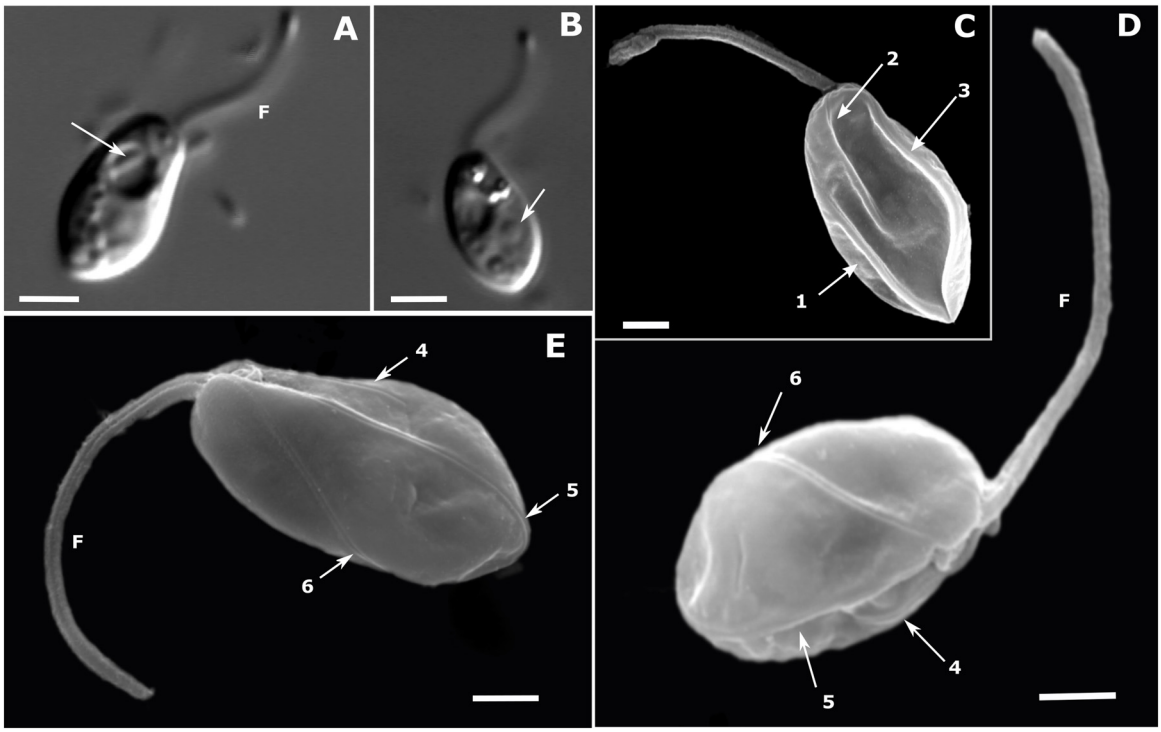

Fig. 3. Light (A–B, scale bars 2 μm) and scanning electron (C–E, scale bars 1 μm) micrographs of *Petalomonas acorensis* strain HFCC 220. A: Cell in dorsal view with single flagellum (F) emerging from a deep flagellar pocket (white arrow). B: Dorsal view of the cell, nucleus visible (white arrow). C: Cell in lateral/dorsal view with three out of six pellicular strips (1–3). D, E: Cell in lateral/ventral view with flagellum (F) and three out of six pellicular strips (4–6). Strips start at the ventral anterior end of the cell: two longitudinal strips (1, 5) reaching the posterior end of the cell, two helically twisted lateral strips (3, 6) reaching dorsal posterior end of the cell, one lateral/dorsal longitudinal strip (2) until the middle of the cell, one ventral longitudinal strip (4) until the middle of the cell.

mally supported branch (mlBP 100%, biPP 1.00), while the clade comprised of *Petalomonas cantuscygni*, *Scytomonas saepesedens*, several environmental sequences and our *Petalomonas* HFCC 220 strain was moderately supported (mlBP 75%, biPP 0.84, Fig. 4). *Petalomonas* HFCC 220 showed a p-distance of 22% to *Scytomonas saepesedens* (KP306755) and 20.8% to the environmental sequence AY821957, while the genetic distance to *Petalmonas cantuscygni* (U84731) was lower with 18.8%.

Pressure experiments

All three euglenid strains (*Keelungia nitschei* HFCC 166, *Petalomonas acorensis* HFCC 220, *Ploeotia costaversata* HFCC 1508) were exposed to hydrostatic pressure in steps of 50 bar (until 500 bar) at temperatures of 4 °C (deep-sea temperature) and 20 °C (surface-water temperature). For *Ploeotia costaversata* HFCC 1508, experiments could only be conducted at 20 °C, because movement of the specimens already stopped at 10 °C. We categorized the movement at 20 °C (HFCC 1508) and 4 °C (HFCC 166, HFCC 220) and measured the speed and travelled distance of all five individuals of each strain at each pressure step. The high standard deviations (Fig. 5) were the result of individual differences in behaviour. Four out of the five observed *Keelungia nitschei* individuals showed a normal movement until 200 bar. None of the investigated *Petalomonas acorensis* specimens moved at pressures higher than 200 bar, while only one or two out of the five observed individuals of the surface water strain *Ploeotia costaversata* (HFCC 1508) barely moved at pressures higher than 300 bar. *Keelungia nitschei* specimens still moved, though barely, or circled at 500 bar; only one specimen did not move at all. In control treatments at 1 bar *K. nitschei* and *P. acorensis* were faster and moved further at 20 °C than at 4 °C. Furthermore, the deep-sea strain *K. nitschei* moved generally slower than the surface strains *P. acorensis* and *P. costaversata* (Fig. 5). While for all three strains movement (displacement and velocity) seemed to level off at 20 °C from 250/300 bar onwards, they seemed to move normally at pressures up to 200 bar. While *Petalomonas acorensis* HFCC 220 showed a similar movement pattern at 4 °C and 20 °C at pressures higher than 200 bar, the deep-sea isolate *Keelungia nitschei* HFCC 166 moved faster at 4 °C compared to 20 °C when pressure was increased from 250 bar until 500 bar, but results showed no significant difference. Furthermore, *K. nitschei* was the only strain that had a similar displacement and velocity over all pressure steps at 4 °C than the control at 1 bar ($p > 0.05$). Overall, pressure had a significant effect on the displacement and velocity of all three euglenid strains (two-way ANOVA; HFCC 166: $p < 0.001$;

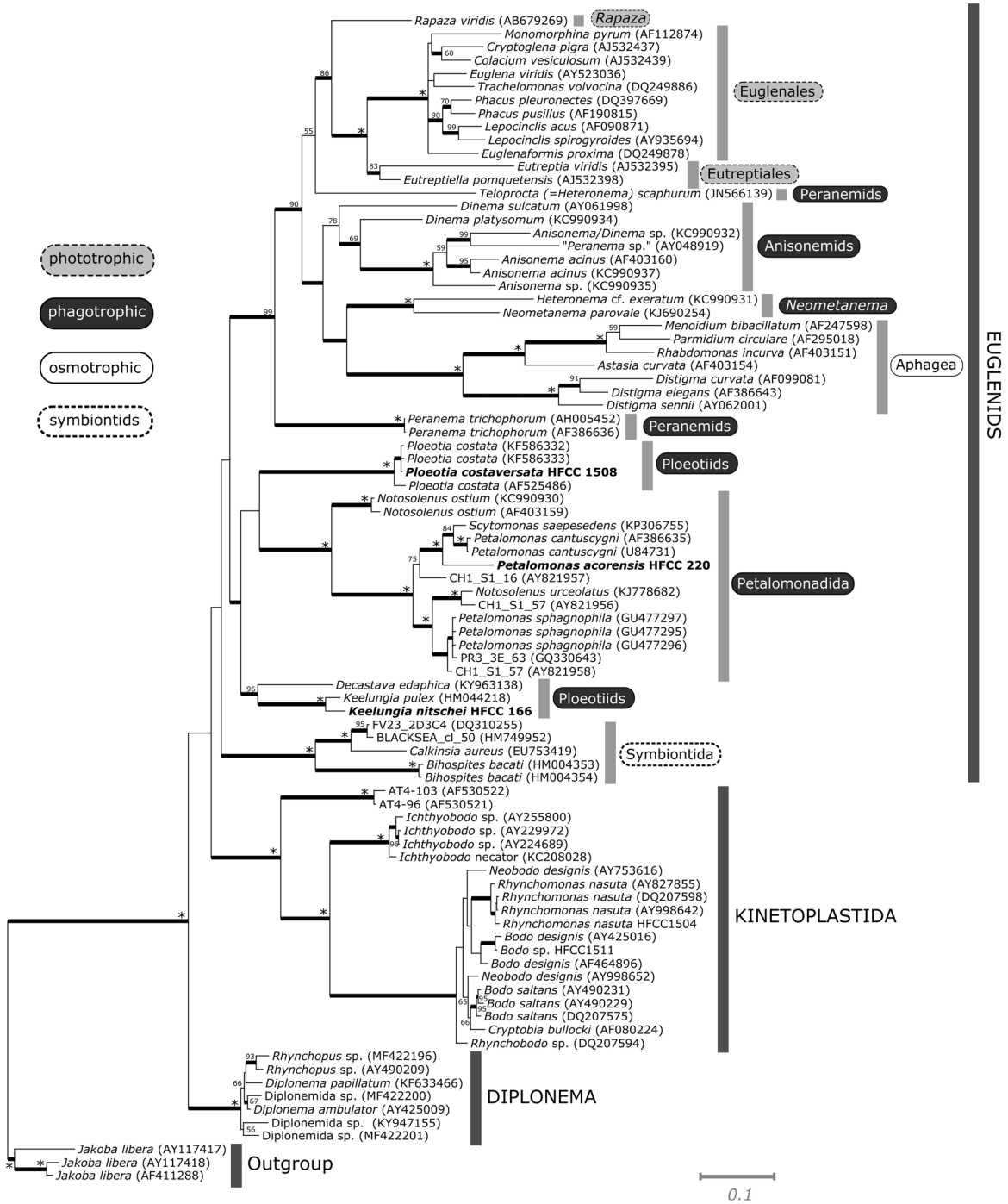

Fig. 4. Phylogenetic tree of euglenids based on 18S rDNA sequences. A Bayesian Inference (BI) tree shown for the analysis. A Maximum likelihood analysis (ML; GTR + gamma model) under the same model yielded an identical topology. Number on edges represent ML bootstrap percentages (RaxML, 1000 replicates); asterisk (*) indicate bootstrap percentages of 100%; values <50% are not shown. Nodes supported by Bayesian posterior probabilities (MrBayes) ≥0.95 are shown by thick lines. Scale bar (bottom right) represents 0.1 expected substitutions/site in the ML analysis. The tree is outgroup-rooted, with Jakobids as outgroup.

HFCC 220: $p < 0.001$; HFCC 1508: $p < 0.001$). Temperature alone only significantly affected the behavior of *Petalomonas acorensis* HFCC 220 ($p < 0.001$). The interaction of pressure and temperature had a significant effect on *Keelungia nitschei* HFCC 166 ($p < 0.001$) and *Petalomonas acorensis* HFCC 220 ($p < 0.001$).

Taxonomic summary

Keelungia nitschei sp. nov. Arndt, Schoenle et Voss
Euglenozoa Cavalier-Smith 1981, emend. Simpson 1997
Euglenida Bütschli 1884 (ICZN), emend. Simpson 1997

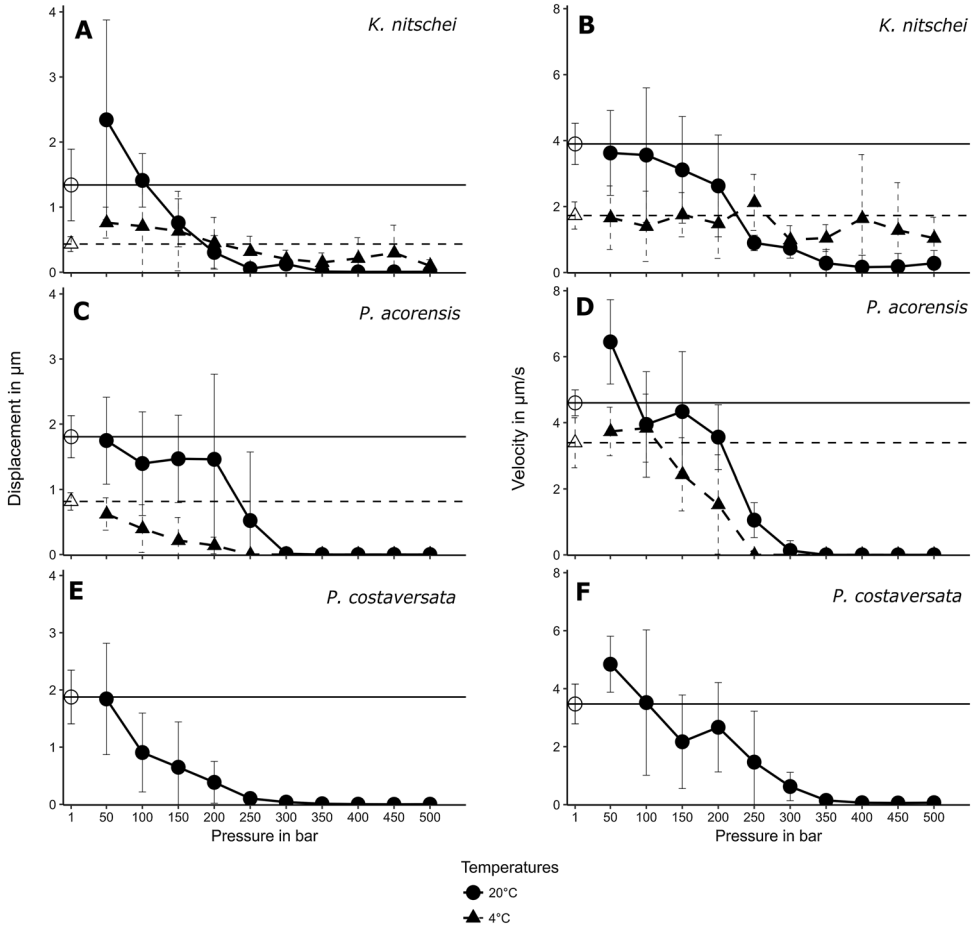

Fig. 5. Exposure of HFCC 166 *Keelungia nitschei* (A, B), HFCC 220 *Petalomonas acorensis* (C, D) and HFCC 1508 *Ploeotia costaversata* (E, F) to stepwise increasing pressure in steps of 50 bar with maximum pressure exposure of 500 bar. Displacement in µm (A,C,E) and Velocity in µm/s (B, D, F) of the three euglenid species are shown for each pressure step. Pressure experiments were conducted at 4 °C (triangles) and 20 °C (circles) in 35PSU Schmaltz-Pratt medium. Controls were kept at 1 bar (4 °C: white triangle and dashed line; 20 °C: white circle and black line). For controls the mean over all pressure steps is shown (n = 50). Vertical bars represent ± SD (pressure: n = 5, control: n = 50).

Heteronematina Leedale 1967

Genus *Keelungia* Chan et Moestrup 2013 (ICZN)

Holotype: Specimen illustrated in Fig. 1 F.

Diagnosis: Marine, free-living and substrate dwelling euglenid, biflagellated oblong to ovoid cell with a mean length of 9.8 ± 0.9 µm (8.3–11.6 µm) and mean width of 7 ± 0.8 µm (5–8.3 µm). Hook-shaped pharynx visible. Ten longitudinal pellicular strips, four ventral with two median ridges more closely spaced, two lateral and four dorsal. Short anterior flagellum is 10.2 ± 1.5 µm (8.2–12.3 µm) long and longer posterior flagellum of 25.9 ± 4.7 µm (17.5–33.4 µm) in length. The anterior flagellum commonly kept in an S-shape, used for orientation, moving fast. The posterior flagellum used for gliding over substratum. The anterior front of the cell is truncated. Closest sequence in public databases is *Keelungia pulex* (HM044218), p-distance 12.7% (18S rDNA).

Gene sequence: The 18S rDNA sequence of HFCC 166 has the Genbank Accession Number MK564755. The 28S rDNA sequence has the GenBank Accession Number MK567808.

Etymology: *nitschei,* dedicated to Dr. Frank Nitsche for his huge commitment in the molecular and morphological characterization of protists.

Type locality: Sediment of southern North Atlantic, depth 5117 m (10°25.12′N, 31°04.62′W)

Type material: A filter with cells of strain HFCC 166, prepared for electron microscopy, was deposited with the Collection of microscopic slides of the Biology Centre of the Upper Austrian Museum, Linz, Austria (deposition # 2019-5).

Remarks: Comparison of *K. nitschei* with *K. pulex* Chan, Moestrup et Chang 2013 shows a high similarity in morphological features, but the anterior front of the cell is truncated, this is the main morphological feature distinguishing *K. nitschei* from *K. pulex* (Chan et al. 2013). *K. nitschei* is smaller than the species *Ploeotia longifilum* (12–20 µm) Larsen and Patterson 1990. The length of the posterior flagellum of *Ploeotia longifilum* is 4-5 times larger than the cell itself,

while for *K. nitschei* the flagellum was only 2-3 times longer than its cell length, as described for *P. punctata* Larsen and Patterson 1990 and *P. azurina* Patterson and Simpson, 1996 (Larsen and Patterson, 1990; Patterson and Simpson 1996). The length of the anterior flagellum was slightly longer than the cell itself for all three species, *P. azurina*, *P. punctata* (Patterson and Simpson 1996) and *K. nitschei*. The pharynx of *K. nitschei* has the same shape (hook-shaped) as described for *P. longifilum*, with "two widely separated rods anteriorly, tapering towards the posterior end" (Larsen and Patterson, 1990; and their Fig. 37 e). The number and orientation/direction of the strips resembles that of *Ploeotia vitrea* Dujardin 1841 emend. Farmer and Triemer 1988 (Farmer and Triemer 1988; Larsen and Patterson, 1990). In several cells of *P. azurina* two lateral ventral ridges have been reported (Patterson and Simpson 1996) in addition to the seven dorsal ridges, while *K. nitschei* has ten longitudinal strips.

Species incertae sedis in genus *Ploeotia* Dujardin, 1841

Ploeotia costaversata, sp. nov. Prausse et Arndt
Euglenozoa Cavalier-Smith 1981, emend. Simpson 1997
Euglenida Bütschli 1884 (ICZN), emend. Simpson 1997
Heteronematina Leedale 1967
Genus *Ploeotia* Dujardin 1841, emend. Farmer and Triemer, 1988

Holotype: Specimen illustrated in Fig. 2 F.

Diagnosis: Biflagellated oblong to ovoid protoplast, cell length of 15.6 ± 1.4 μm (13–19.1 μm) and cell width of 8.5 ± 0.8 μm (7–10.5 μm). Short anterior flagellum 8.0 ± 0.4 μm (7.4–8.7 μm) in length and longer posterior flagellum 30.5 ± 5.5 μm (20.0–42.2 μm) in length. Ten pellicular strips helically twisted towards both poles of the protoplast, resulting in the appearance of five grooves. The helically twisted strips are the main morphological feature distinguishing *P. costaversata* from *P. costata*. Hook-shaped pharynx 10.9 ± 1.2 μm (8.7–12.9 μm) long and 2.5 ± 0.5 μm (1.9–3.4 μm) wide. Up to four vacuoles with a size of 3.8 ± 0.6 μm (2.5–4.6 μm). Closest sequence in public databases KF586333 (*Ploeotia costata* strain Tam) and KF586332 (*Ploeotia costata* strain Pac), p-distance 3.9% (18S rDNA).

Gene sequence: The 18S rDNA sequence of HFCC1508 has the GenBank Accession Number MK564753.

Etymology: 'versari' = to twist (Latin), refers to the ten helically twisted pellicular strips.

Type locality: Biofilm at 15 PSU and 28 °C in a shrimp recirculating aquaculture system grow out facility nearby Munich (CrustaNova GmbH, Langenpreisingen, Germany). The process water in Munich was stocked with the first batch of post larvae of *Litopenaeus vannamei* grown up in Atlantic seawater.

Remarks: The ribosomal sequence of the type species *Ploeotia, Ploeotia vitrea* Dujardin 1841, is still missing. We assign our newly defined species to the genus *Ploeotia,* together with two closely related strains of *P. costata*. However, these species may require establishment of a new genus when more data are available. *Ploeotia costaversata* is smaller than the originally described *P. costata* (formerly *Serpenomonas costata*, Triemer, 1986) (Farmer and Triemer 1988). The cell size of *P. costaversata* lies in the lower size range of *P. costata* (16–22 μm) described by Larsen and Patterson (1990). Cells of both species are slightly flattened. The main difference to *P. costata* is the orientation of the pellicle strips. *P. costata* has five longitudinal grooves (Chan et al. 2015; Larsen and Patterson, 1990; Patterson and Simpson 1996; Triemer, 1986) as does *P. oblonga* Larsen and Patterson 1990, while *P. costaversata* has ten pellicle strips being helically twisted towards both poles joining at the posterior front of the cell resulting in the appearance of five grooves in the middle of the cell. The strips of *P. costaversata* are more helically twisted compared to that of *P. costata* (Triemer, 1986). The length of the flagella resembles the ones from *P. costata* (Larsen and Patterson, 1990; Patterson and Simpson 1996).

Petalomonas acorensis sp. nov. Schoenle et Arndt
Euglenozoa Cavalier-Smith 1981, emend. Simpson 1997
Euglenida Bütschli 1884 (ICZN), emend. Simpson 1997
Heteronematina Leedale 1967
Petalomonas Stein 1859

Holotype: Specimen illustrated in Fig. 3 E.

Diagnosis: Pyriform cell with mean length of 5.8 ± 0.6 μm (4.7–6.9 μm) and mean width of 3.6 ± 0.4 μm (2.8–4.2 μm). Cells ventrally flattened. Only one anterior directed flagellum with 6.2 ± 0.5 μm length (5.2–7.0 μm) originating from a flagellar pocket at the anterior front of the cell. Cell with six pellicular strips starting from the ventral flagellar pocket: two longitudinal and two helically twisted lateral strips joining at the posterior end of the cell, one lateral/dorsal longitudinal strip and one ventral longitudinal strip until the middle of the cell. Substrate mediated gliding, feeding on bacteria with the distal part of the flagellum actively beating. Closest sequence in public databases U84731 (*Petalmonas cantuscygni*), p-distance 18.8% (18S r DNA).

Gene sequence: The 18S rDNA sequence of HFCC 220 has the GenBank Accession Number MK564754.

Etymology: With respect to its sampling location, Acores

Type locality: Sediment on a beach near Feteira, Faial, Azores (38°31.362′N, 28°40.446′W).

Type material: A filter with cells of strain HFCC 220 prepared for electron microscopy, was deposited with the Collection of microscopic slides of the Biology Centre of the Upper Austrian Museum, Linz, Austria (deposition # 2019-4).

Remarks: *Petalomonas acorensis* HFCC 220 formed a clade together with the species *Petalomonas cantyscygnii* and *Scytomonas saepesedens*. Around the flagellum at the anterior end of the cell of *S. saepesedens* a pronounced collar is formed, more extended anteriorly on the ventral flattened

side during gliding. This was not observed for our *P. acorensis* strain HFCC 220. *P. acorensis* has a rigid flattened cell shape with one emergent flagellum and no visible mouth, as has been described for the genus *Petalomonas*. *P. acorensis* has a similar cell size (5–7 μm) than the small petalomonads *P. poosilla* Larsen and Patterson 1990 and *P. minuta* Hollande 1942 (Larsen and Patterson, 1990; Patterson and Simpson 1996). Reported size ranges for *P. poosilla* varied between 4–12 μm (Al-Qassab et al. 2002; Lee and Patterson 2000; Lee et al. 2003). While some studies did not report the existence of visible surface structures for *P. poosilla* (Larsen and Patterson, 1990; Skuja, 1948), more recent studies found fine longitudinal ridges visible on some cells (Al-Qassab et al. 2002; Patterson and Simpson 1996; Lee, 2008; Lee et al. 2003). *P. poosilla* was described by Al-Qassab et al. (2002) to may have up to three fine ridges on the dorsal side and two distinct ridges on the ventral side. Lee and Patterson (2002) described two populations of *P. poosilla* differing in cell size, shape and visibility of ridges. While our light microscopy of *P. acorensis* did not reveal any surface structures, REM pictures showed six strips of which four were longitudinal oriented. In addition, two strips of *P. acorensis* were helically twisted which has not been reported for *P. poosilla* yet. Two helically arranged strips forming a constant ventral channel were observed in *Petalomonas cantuscygni* (Cann and Pennick 1986; Larsen and Patterson, 1990). While *P. cantuscygni* possesses more than six dorsal ribs, *P. acorensis* only had six strips, of which two only reached until the middle of the cell.

Discussion

Our phylogenetic analysis of euglenid 18S rDNA sequences incorporating sequences of our three strains showed that osmotrophic genera formed a single clade, while phagotrophic genera fell into several clades. Eukaryovorous euglenids (peranemids and anisonemids) are paraphyletic, whereas the phylogenetic position of potentially bacterivorous euglenids (ploeotiids and petalomonads) is still uncertain (Leander et al. 2017). However, due to several exceptions the hypothesis that phagotrophic euglenids can be separated into bacteriovorous and eukaryovorous taxa needs to be further investigated.

Ploeotiids are very diverse at a molecular level and, thus, phylogenetic analysis indicated them to be not monophyletic. This is in accordance with Cavalier-Smith (2016), Cavalier-Smith et al. (2016) Chan et al. (2013) and Lax and Simpson (2013). Within the ploeotiids two new genera have been described including e.g. *Decastava* and *Keelungia* (Cavalier-Smith 2016; Chan et al. 2013). We assigned our strain HFCC 166 to the genus *Keelungia*, but as new species (*Keelungia nitschei*) based on morphological and molecular data. Both species, *K. nitschei* and *K. pulex*, possess a pellicle (Leander et al. 2007) with ten longitudinal pellicular strips. However, SEM studies of *K. nitschei* revealed a truncated end at the anterior end of the cell as a distinguishable morphological feature to *K. pulex*. Furthermore, the pairwise sequence divergence was relatively high (12.7%) corresponding to a typically higher 18S rDNA diversity in euglenids as compared to kinetoplastids and diplonemids (e.g. Preisfeld et al. 2001).

Many *Ploeotia* species have been morphologically described so far (e.g., Larsen and Patterson, 1990). In 1986, Triemer described a new euglenid genus, *Serpenomonas costata*, which was reassigned two years later to *Ploeotia costata* due to ultrastructural features being nearly identical with *Ploeotia vitrea* (Farmer and Triemer 1988). *Ploeotia costata* (formerly *Serpenomonas costata*) was originally described as a cell with five prominent longitudinal grooves (Farmer and Triemer 1988). Since then, different orientations of the five grooves have been mentioned for the species *P. costata*, from the originally longitudinal orientation (Chan et al. 2015; Larsen and Patterson, 1990) to slightly twisted (Al-Qassab et al. 2002; Ekebom et al. 1996; Lee et al. 2003; Patterson and Simpson 1996; Tong et al. 1998; Triemer, 1986). On a molecular level, three different sequences assigned to *P. costata* exist so far. While the *P. costata* strain CCAP 1265/1 (Busse and Preisfeld 2003) possessed a group I intron, the other two sequences of strains Tam and Pac (Chan et al. 2015) lacked a group I intron, but had three additional insertions. Our strain HFCC 1508 also lacked a group I intron, but had similar insertions like the strains Tam/Pac with one insertion being 79 bp longer. Despite a low sequence similarity with 92% between CCAP 1265/1 and strains Tam/Pac, Chan et al. (2015) placed their strains Tam/Pac to *P. costata* CCAP 1265/1 based on the similar morphology of pellicle strips suggesting that their *P. costata* strains might represent a cryptic species. Phylogenetic analysis of our strain HFCC 1508 indicated a sequence similarity of 96.1% to the strain Tam and 90% to CCAP 1265/1. Morphological characteristics of our strain HFCC 1508 revealed five grooves (ten pellicular strips), which were much more helically twisted towards both poles of the protoplast, than the pellicular strips of *P. costata* CCAP 1265/1 (Busse and Preisfeld 2003), longitudinal orientated strips of strains Tam and Pac (Chan et al. 2015) and the originally described longitudinal grooves of *P. costata* (Farmer and Triemer 1988). Based on the differences in molecular and morphological characteristics, we assigned our *Ploeotia* strain (HFCC 1508) to a new species, namely *P. costaversata*. A modern molecular redescription of the type species, *Ploeotia vitrea* Dujardin 1841, is still missing. More morphological and molecular studies of *Ploeotia*-like euglenids are necessary for a thorough redescription of the genus. Therefore, the three existing sequences of *P. costata* and our own strain of *P. costaversata* may require a new genus in the future.

While petalomonads are probably monophyletic, some genera (*Notosolenus* and *Petalomonas*) are known to currently represent non-monophyletic groupings (Cavalier-Smith 2016; Lee and Simpson 2014). Petalomonads retained several possibly ancestral characters (few pellicle strips,

bacterivorous modes of nutrition, MtR pocket, kDNA-like mitochondrial inclusions). Recent phylogenetic analyses placed petalomonads as a derived group within euglenids, rather than a basally branching group (Breglia et al. 2013; Chan et al. 2013; Kim et al. 2010; Lax and Simpson 2013; Yamaguchi et al. 2012). Our strain formed a clade together with the species *Petalomonas cantyscygnii* and *Scytomonas saepesedens*. The closest genetic relative to our *Petalomonas acorensis* strain HFCC 220 was *Petalomonas cantyscygni* (U84731) with a p-distance of 18.8%. *Scytomonas* proved to be a phylogenetically sister to *Petalomonas* (Cavalier-Smith et al. 2016). Although the p-distance to the nearest described *Petalomonas* species (*P. cantuscygni*) was quite high with 18.8% we decided to assign our species to the genus *Petalomonas* due to its morphological similarities (rigid, flattened cell, one emergent flagellum, no visible mouth) instead of establishing a new genus. In addition, the type species of *Petalomonas*, *P. abscissa*, has not been sequenced yet. Future discoveries of petalomonad species and sequencing of morphologically already described species might result in a solid justification or reorganisation of this clade. Morphological comparison with non-sequenced petalomonads showed a similar size of our cells of strain HFCC 220 as for the small petalomonads *P. poosilla* Larsen & Patterson 1990 and *P. minuta* Hollande 1942 (Larsen and Patterson, 1990, Patterson and Simpson 1996). A different number of strips has been reported for *P. poosilla*. Al-Qassab et al. (2002) suggested that the presence or visibility of ridges might be the result of environmental conditions including osmotic pressure or food availability, a hypothesis that still needs to be tested. We observed six strips, longitudinally and helically twisted, on the surface of HFCC 220. Due to these morphological and genetic differences to already described petalomonad species we assigned our strain HFCC 220 to a new species, *Petalomonas acorensis*.

While *Petalomonas acorensis* HFCC 220 and *Ploeotia costaversata* HFCC 1508 were isolated from surface waters, *Keelungia nitschei* HFCC 166 was recovered from the Atlantic deep sea. While diplonemids and kinetoplastids are known to contain many deep-sea specific taxa, euglenids have seldom been reported, and not yet cultivated from deep-sea samples (Buck et al. 2000; Yubuki et al. 2009). Because of the distinct isolation habitats of our strains, we compared their behavioural motion to increasing hydrostatic pressure. Such ecological experiments under deep-sea conditions might verify the potential deep-sea origin of protists and might indicate their potential to be active in the deep sea. Furthermore, pressure experiments with surface isolates might underline a possible exchange between surface water and deep-sea communities. Survival of heterotrophic flagellates isolated from the deep sea to high hydrostatic pressures has already been recorded (Atkins et al. 1998; Atkinson 1998; Turley et al. 1988; Živaljić et al. 2017). But to our knowledge this was the first time that the behaviour of heterotrophic flagellates could be directly observed at high hydrostatic pressures. Pressure and temperature had both a significant effect on the movement of our three strains. Furthermore, the deep-sea strain *Keelungia nitschei* HFCC 166 was the only strain still moving at high hydrostatic pressures up to 500 bar at 4 °C. Velocity and displacement of *K. nitschei* under pressure at 4 °C was similar to the movement of species kept at 1 bar (control). This indicates the possible adaptation of *K. nitschei* to the deep-sea environment, while the surface strain *P. acorensis* and *P. costaversata* did not move at high hydrostatic pressures. Genetic approaches like Next-Generation Sequencing (NGS) and clone libraries have turned out to be reliable tools in identifying previously unknown protistan lineages in surface waters and in the deep sea (de Vargas et al. 2015; Edgcomb et al. 2002; Flegontova et al. 2018, 2016; López-García et al. 2003; Pawlowski et al. 2011; Stoeck et al. 2010). Nevertheless, the understanding of phenotypic variation in protistan physiology and behaviour is critical to derive the role they play in ecosystems (Worden et al. 2015). We are not able to reconstruct cellular structures and behaviour from sequence data. One prerequisite is still the traditional cultivation of species allowing for the application of more comprehensive investigations of one and the same protist strain as well as ecological experiments to analyse their role in the deep sea. For a solid justification of the phylogenetic relationship within the Euglenozoa a combination of molecular (including secondary structures) and morphological characteristics (e.g. paramylon, pellicle structure, feeding apparatus) in addition to an increased taxon sampling and establishments of cultures might be necessary (Paerschke et al. 2017).

Conclusions

Although Euglenozoa are important for understanding the eukaryotic early evolution, knowledge about the overall diversity and phylogenetic relationships of phagotrophic euglenids is still poor. Based on morphological (LM, REM) and molecular characteristics we described three new phagotrophic euglenid species belonging to the petalomonads (*Keelungia nitschei, Petalomonas acorensis*) and ploeotiids (*Ploeotia costaversata*). Phylogenetic analysis based on the 18S rDNA showed similar topologies when compared to previous studies. To our knowledge, *K. nitschei* is the first euglenid, which had been cultivated from abyssal depths (5000 m). In addition, autecological experiments under deep-sea conditions (low temperature, high pressure) showed that the deep-sea strain *K. nitschei* was better adapted to high hydrostatic pressure at lower temperatures than the two isolated surface water euglenids, *P. acorensis* and *P. costaversata*. Thus, the survival at high hydrostatic pressures indicates that the phagotrophic euglenid *Keelungia nitschei* might be active in the deep-sea microbial food webs. The deep sea may harbour many additional taxa of this bacterivorous group being active in deep-sea food webs and not only symbiontid euglenozoans which have been described from deep oxic/anoxic layers. We need much more

sequencing data to get a better resolution of the phylogenetic relationships. An increased taxon sampling might influence the phylogenetic position of the relatively few up to now sequenced euglenids, especially within the petalomonads and ploeotiids when the type species of *Petalomonas* and *Ploeotia* will be sequenced in future.

Author contributions

All authors were involved in the sampling and cultivation of protists. S.Z. conducted the pressure experiments and performed the statistical analyses. D.P., J.V. and A.S. conducted the molecular and morphological characterizations. A.S. performed the phylogenetic analyses. A.S. and H.A. wrote the manuscript, and H.A. supervised the studies. All authors reviewed the manuscript.

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2.3 Global Comparison of Bicosoecid *Cafeteria*-like Flagellates from the Deep Ocean and Surface Waters, with Reorganization of the Family Cafeteriaceae

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Global comparison of bicosoecid *Cafeteria*-like flagellates from the deep ocean and surface waters, with reorganization of the family Cafeteriaceae

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Abstract

Cafeteria is one of the most common and ecologically significant genera of heterotrophic nanoflagellates in marine plankton. We could isolate and cultivate 29 strains morphologically similar to *Cafeteria* obtained from surface waters and the deep sea all over the world's ocean. Morphological characterization obtained by high resolution microscopy revealed only small differences between the strains. Sequencing the type material of the type species *C. roenbergensis* (CCAP 1900/1) and molecular analyses (18S rDNA, 28S rDNA) of newly isolated strains resulted in a revision and separation of the Cafeteriaceae into two known species (*C. roenbergensis*, *C. mylnikovii)* and six new species (*C. maldiviensis*, *C. biegae*, *C. loberiensis*, *C. chilensis*, *C. graefeae*, *C. burkhardae*). Many deposited *Cafeteria* sequences at GenBank and most of our own sequences clustered within one clade (*C. burkhardae*) with a p-distance of 5% to strain CCAP 1900/1. Only *C. maldiviensis* clustered together with the type species *C. roenbergensis*. While *C. burkhardae* seems to have a cosmopolitan distribution, the distribution of the other species seems to be more restricted. A strain from the Angola Basin had a p-distance of 10% to *Cafeteria* species and clustered separately within the Anoecales requiring the erection of a new genus, *Bilabrum* gen. nov., with *B. latius* sp. nov. as type species.

Keywords: Bicosoecida; *Bilabrum*; *Cafeteria*; Deep sea; Global distribution; rDNA

Introduction

Belonging to the monophyletic protist lineage of Stramenopiles Patterson, 1989 (heterokonts), bicosoecids are composed of marine as well as freshwater heterotrophic flagellates. Nearly all bicosoecids are very small (<8 μm) and, with the exception of loricated forms, the cells often lack easily recognizable features. Thus, molecular barcoding techniques became an important tool to aid in species identification. Several new bicosoecid genera and species have been established, revised and re-described in the last decades (Cavalier-Smith 2016; del Campo and Massana 2011; Fenchel and Patterson 1988; Harder et al. 2014; Karpov 2000; Karpov et al. 1998; Park and Simpson 2015; Patterson et al. 1993; Ruggiero et al. 2015; Teal et al. 1998; Yubuki et al. 2015). *Cafeteria roenbergensis* Fenchel and Patterson, 1988, a marine aloricate bicosoecid flagellate, was the first species reported for the genus *Cafeteria* in 1988, being

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isolated from surface waters at the Limfjord in Jutland, Denmark (CCAP 1900/1). Since then, *Cafeteria* has been reported from all over the oceans in surface waters and deep-sea habitats by high-throughput sequencing and cultivation techniques (Atkins et al. 2000; de Vargas et al. 2015; Larsen and Patterson 1990; Patterson et al. 1993). *Cafeteria* species are very common and might be ecologically significant heterotrophic nanoflagellates as bacterial consumers due to its high abundances in the marine environment (Fenchel and Patterson 1988; Larsen and Patterson 1990; Patterson et al. 1993; Vørs, 1993). While cultivation-based methods miss a majority of taxa since most species require specific cultivation conditions (del Campo et al. 2013), *Cafeteria* often occurs in cultivation studies in a frequent co-occurrence with other r-strategists among heterotrophic flagellates such as *Caecitellus*, *Rhynchomonas* and *Neobodo* (Boenigk et al. 2002; Patterson and Lee 2000). The taxonomic identity of *Cafeteria* is generally based on cultivated strains, on which ultrastructural, physiological and molecular studies have been performed. However, the role and relevance of cultured strains within the marine environment remains unknown (del Campo et al. 2013). While molecular surveys frequently recover novel eukaryotic lineages that have not been recorded from cultures so far (del Campo et al. 2013; López-García et al. 2001), a high proportion of reads obtained by next-generation-sequencing could be assigned to *Cafeteria* within a global eukaryotic plankton survey (de Vargas et al. 2015). Studies have shown that *Cafeteria* populations can be infected by viruses, including the giant virus CroV (*Cafeteria roenbergensis* virus) (Fischer et al. 2010; Massana et al. 2007). A diverse assemblage of heterotrophic flagellates, including *Cafeteria*, lives in association with marine detritus, feeding directly or indirectly on it (Arndt et al. 2003; Bass et al., 2007; Danovaro, 2010; Baker et al. 2018; Caron et al. 1982; Patterson et al. 1993). As an efficient suspension feeder, *Cafeteria* preys on bacteria by creating a current with its anterior flagellum and jerkily movement of the cell being attached with the tip of its posterior flagellum to substratum (Fenchel and Patterson 1988).

Based on morphological characteristics three *Cafeteria* species have been described in the past by Larsen and Patterson (1990) besides *C. roenbergensis* including *C. ligulifera* Larsen and Patterson, 1990, *C. marsupialis* Larsen and Patterson, 1990 (now *Cantina marsupialis* (Larsen and Patterson, 1990) Yubuki et al., 2015) and *Cafeteria minuta* (Ruinen, 1938) Larsen and Patterson, 1990 (original combination *Pseudobodo minuta*). The *Cafeteria*-sequence hits (in terms of 18S rDNA sequences) on the GenBank (in total 39 sequences, accessed 5/19) can be assigned to two described species (14 *Cafeteria roenbergensis* sequences, two *C. minima* sequences (combination not published, see Cavalier-Smith and Chao 2006 for more details) and several *Cafeteria*/bicosoecid clones (23 sequences). Our survey showed that there was no gene sequence from the type species *C. roenbergensis* strain CCAP 1900/1 available at GenBank. Atkins et al. (2000) uploaded the first *Cafeteria* 18S rDNA sequences (*Cafeteria roenbergensis* AF174364, *Cafeteria* sp. AF174365, *Cafeteria* sp. AF174366) on NCBI GenBank (available since 1992) in 1999, which they isolated from deep-sea hydrothermal vents. More sequences assigned to *C. roenbergensis* and *Cafeteria* sp. were added in the following years isolated from surface and deep-sea waters (e.g. Boenigk et al. 2007; Rigaut-Jalabert et al. 2007; Weber et al. 2017). In addition, two sequences were assigned to *C. minima* (AY520448, AY520449, uploaded 2004), which have been re-described as *Anoeca atlantica* by Cavalier-Smith and Chao (2006) and were deposited at the Culture Collection of Algae and Protozoa as CCAP1902/1 and CCAP1902/2. Cavalier-Smith and Chao (2006) added a new species to the genus *Cafeteria* by re-describing the misidentified *Pseudobodo tremulans* Griessmann, 1913 (DQ102392, strain A.M., isolated 1986) as *Cafeteria mylnikovii* Cavalier-Smith and Chao, 2006 (strain CCAP 1900/2). Yubuki et al. (2015) isolated three different strains with identical morphological traits resembling those of *Cantina marsupialis*. Those isolates were molecularly not closely related to the *Cafeteria* cluster; therefore, they established the genus *Cantina* (Yubuki et al. 2015). For the strain "HFCC35 *Cafeteria* cf. *marsupialis*" (uploaded at GenBank in 2008 by Wylezich et al. 2010) only the 28S rDNA was available.

During the last two decades, we could isolate and cultivate 29 *Cafeteria*-like strains from surface waters and from the deep sea at different parts of the ocean including the Baltic Sea, Mediterranean Sea, Indian Ocean, Pacific and Atlantic Ocean. This gave us the chance to reanalyze the taxonomy and the phylogenetic relationships within the Cafeteriaceae. Besides morphological characterization by high resolution light microscopy, we increased the phylogenetic resolution of our *Cafeteria*-like strains by amplifying more genes (18S rDNA, ITS-1, 28S rDNA) for a concatenated tree as has been done in several protist diversity studies (Bass et al. 2007; Marande et al. 2009).

Material and methods

Sampling

Sampling of deep-sea sediment and water as well as surface water for cultivation took place during several expeditions with the research vessels R/V Sonne and R/V Meteor (Fig. 1, Table 1). Deep-sea sediment samples were taken with a Multi-Corer system with two exceptions of strains isolated from the surface of stones collected by Agassiz Trawl. Plankton samples were generally taken using a CTD-rosette-system, from detritus collected by a plankton net, or simple water bottles. Subsamples of a few milliliters of the sediment suspension or water were cultivated in 50 ml tissue-culture flasks (Sarstedt, Nümbrecht, Germany). Isolation was carried out using a micromanipulator or microtiter plates (liquid aliquot method, LAM). All cultures were supplied with sterilized quinoa or wheat grains as an organic food source

Table 1. Station list of collected and isolated marine *Cafeteria*-like strains during different cruises from different parts of the ocean and accession numbers for *C. roenbergensis* CCAP 1900/1 and *C. mylnikovii* CCAP 1900/2. If an additional station name is given in brackets below the original station name during expeditions, it indicates that at these stations environmental DNA was extracted from sediment samples for next-generation-sequencing (see Fig. 5).

HFCC No.	Depth [m]	Sampling gear	Sampling region	Station	Lat/Long	18S rDNA (+ITS-1)	28S rDNA
Mediterranean Sea [1–4] R/V Meteor M71.2 Dec 2006–Jan 2007							
HFCC113*	2676	MUC OW	Pliny Plain [1]	963	33°44.55′N/26°08.50′E	MN315594 (x)	MN318351
HFCC114	2691	MUC Sed	Pliny Plain [2]	983	33°42.91′N/26°20.43′E	MN315595 (x)	MN318352
HFCC115	4328	MUC OW	Ierapetra Basin [3]	054	34°30.30′N/26°11.51′E	MN315596 (x)	MN318353
HFCC116	2691	MUC Sed	Pliny Plain [2]	983	33°42.91′N/26°20.43′E	MN315597 (x)	MN318354
HFCC117	4323	MUC OW	Ierapetra Basin [4]	51	34°30.31′N/26°11.50′E	MN315598 (x)	MN318355
West Pacific Ocean[5–8] R/V Sonne SO223−T Sept 2012–Oct 2012							
HFCC803	1259	MUC Stones	Philippine Basin [5]	17006–2 (P2)	14°03.699′N/144°03.699′E	MN315614 (x)	MN318371
HFCC804	2687	MUC OW	Mariana Basin [6]	17009–1 (P4)	13°58.640′N/145°30.719′E	MN315615 (x)	MN318372
HFCC805	2687	MUC OW	Mariana Basin [6]	17009–1 (P4)	13°58.640′N/145°30.719′E	MN315616 (x)	MN318373
HFCC808	5497	MUC Sed	Mariana Basin [7]	17013–1 (P5)	10°34.313′N/148°49.032′E	MN315617 (x)	MN318374
HFCC827	2776	MUC OW	Fiji Basin [8]	17019–2	15°13.023′S/173°31.129′E	MN315618 (x)	MN318375
East Pacific Ocean [9–11] Chile Expedition Mar 2018							
HFCC832*	Surface	Rock Pool CT water	Iquique [9]	–	20°14.107′S/70°09.152′W	MN315619 ()	MN318377
HFCC843*	Surface	Rock Pool CT water	Mejillones Peninsula [10]	–	23°03.229′S/70°32.977′W	MN315620 ()	MN318376
HFCC844*	Surface	Rock Pool CT water	Antofagasta,La Portada [11]	–	23°30.405′S/70°25.433′W	MN315621 ()	MN318378
North Atlantic Ocean [12, 13, 14, 15, 16, 17] + Caribbean Sea [18, 19] R/V Sonne SO237 Dec 2014−Jan 2015							
HFCC167	5771	MUC OW	East Vema FZ [13]	A2 4–3 (NA2)	10°25.11′ N/31°04.61′W	MN315599 (x)	MN318356
HFCC168*	5793	MUC OW	East Vema FZ [14]	A2 4–4 (NA2)	10°25.12′N/31°04.62′ W	MN315600 (x)	MN318357
HFCC184	200	Plankton net	East Vema FZ [15]	A2 4–2 (NA2)	10°27.82′N/31°01.64′ W	MN315601 (x)	MN318358

Table 1 (*Continued*)

HFCC No.	Depth [m]	Sampling gear	Sampling region	Station	Lat/Long	18S rDNA (+ITS-1)	28S rDNA
HFCC187	Surface	*Sargassum*	East Vema FZ [12]	A1	10°43.118′N/25°3.893′W	MN315602 (x)	MN318359
HFCC197	8337	MUC OW	Puerto Rico Trench [16]	C1 12–2 (NA10)	19°46.02′N/66°49.00′W	MN315603 (x)	MN318360
HFCC203	5771	MUC OW	East Vema FZ [13]	A2 4–3 (NA2)	10°25.11′N/31°04.61′ W	MN315604 (x)	MN318361
HFCC205	200	Plankton net	East Vema FZ [15]	A2 4–2 (NA2)	10°27.82′N/31°01.64′ W	MN315605 (x)	MN318362
R/V Meteor M139 Jul 2017−Aug 2017							
HFCC772*	Surface	*Sargassum*	East Vema FZ [17]	A5/6−S8	11°13.548′N/34°46.559′W	MN315613 ()	MN318370
HFCC734	1500	CTD	Caribbean Sea [18]	A1–943	15°53.269′N/ 68°55.650′W	MN315610 (x)	MN318367
HFCC736	2000	CTD	Caribbean Sea [18]	A1–943	15°53.269′N/68°55.650′W	MN315611 (x)	MN318368
HFCC750	4000	MUC OW	Caribbean Sea [19]	A1–938 (NA9)	15°55.892′N/68°53.34′W	MN315612 (x)	MN318369
South East Atlantic Ocean [20–22] R/V Meteor M48.1 Jul 2000–Aug 2000							
†HFCC33*	5388	AT Sed	Angola Basin [20]	347	16°14.991′S/05°26.700′E	MN315607 () (†AY827850)	MN318364 (†EF681906)
†HFCC34	Surface	Plankton net	Angola Basin [21]	344	17°04.935′S/04°40.805′E	MN315608 (x) (†AY827851)	MN318365 (†EF681907)
†HFCC35*	5392	AT Sed	Angola Basin [22]	339	18°24.553′S/04°43.854′E	MN315515 ()	MN315517 (†EF681904)
Indian Ocean [23] Expedition Mar 2016							
HFCC661*	Surface	CT Sed	Nalaguaridhoo, Maldive Islands [23]	–	3°28.928′N/72°47.780′E	MN315609 ()	MN318366
Baltic Sea [24] Inst. of Ecology Univ. Greifswald							
†HFCC32	Surface	CT Sed	Hiddensee, Baltic Sea [24]	–	54°34.726′N/13°07.112′E	MN315606 (x) (†AY827849)	MN318363 (†EF681905)
CCAP							
CCAP 1900/1		*C. roenbergensis*				MN334557 ()	MN334555
CCAP 1900/2		*C. mylnikovii*				MN334558 ()	MN334556

Samples were obtained from deep-sea sediment samples as well as surface waters and littoral sediments. Superscripts behind ocean parts and sampling regions correspond to sampling stations indicated on the map (Fig. 1). Success of sequencing (18S rDNA (with ITS-1 marked with (x)), 28S rDNA) is indicated by accession numbers. HFCC: heterotrophic culture collection cologne; MUC: multi-corer; OW: overlaying water; Sed: sediment; FZ: fracture zone; AT: Agassiz trawl; CT: collection tube; Lat/Long: latitude/longitude; †: published on Genbank (Scheckenbach et al. 2005; Wylezich et al. 2010) before this study, but were re-sequenced within this study.

*Strains of the name-bearing types are in good condition and available as cultures at the manuscript submission date.

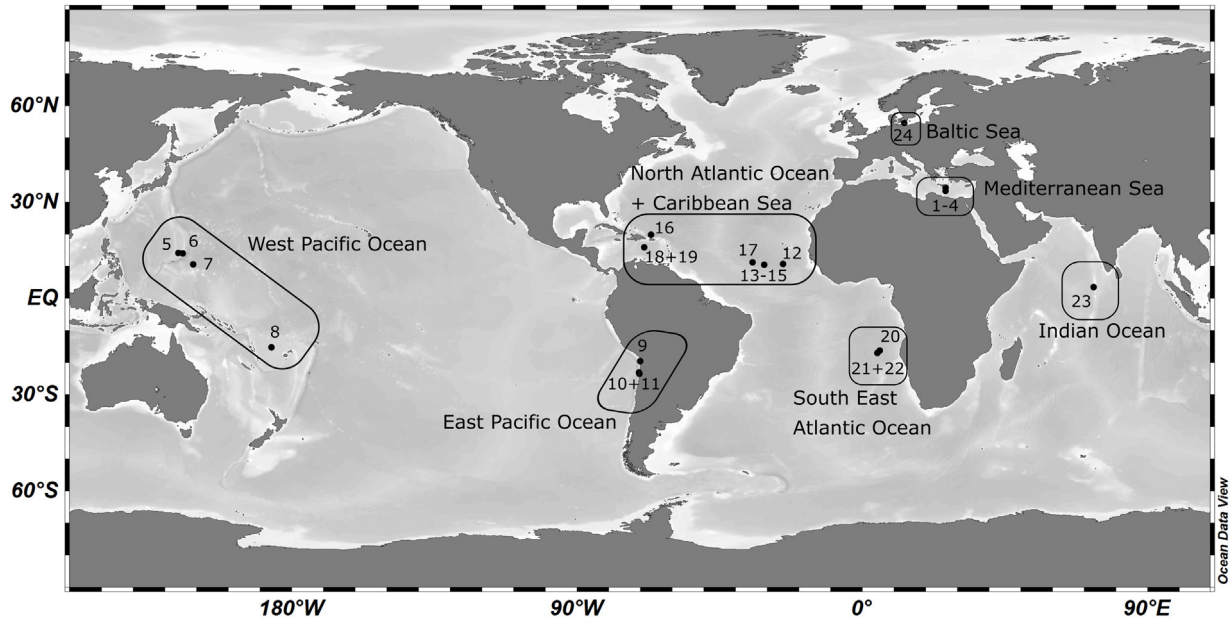

Fig. 1. Station map of collected and isolated marine *Cafeteria*-like strains during different cruises from different parts of the ocean. Map was created using Ocean Data View (Schlitzer 2012).

for autochthonous bacteria. In total, we could isolate 29 *Cafeteria*-like strains from 24 different habitats (Table 1).

After isolation, the strains were cultivated in 50 ml tissue-culture flasks (Sarstedt, Nümbrecht, Germany) filled with 30 ml Schmaltz–Pratt medium (probably Goryatcheva 1971, mentioned in Frolov et al. 2001) containing for 35 PSU per liter 28.15 g NaCl, 0.67 g KCl, 5.51 g $MgCl_2 \times 6\ H_2O$, 6.92 g $MgSO_4 \times 7\ H_2O$, 1.45 g $CaCl_2 \times 2\ H_2O$, 0.10 g KNO_3, 0.01 g $K_2HPO_4 \times 3\ H_2O$. Two quinoa grains were added to the cultures. The cultures were stored at 13 °C in the dark.

Light microscopy

For the morphological characterization one representative strain of each of the seven newly described species was analyzed with the help of an Allen Video Enhanced Contrast (AVEC) system consisting of an analogous Hamamatsu C6489 camera with noise suppression and contrast amplification (Argus-20, Hamamatsu, Japan) to record high resolution stills of the different strains. An inverted microscope (Zeiss Axio Observer A1) equipped with a 100×/1.4 NA oil immersion objective (DIC) and a water immersion condenser was used. Cultures were observed in Petri dishes with a coverslip base. Pictures were taken with VirtualDub-1.10.4 and were edited by ImageJ. Morphological characteristics (cell length, cell width, length of flagella) of specimens were measured with Axio Vision Rel. 4.8 (Zeiss, Germany).

DNA extraction, amplification and sequencing

The cultured strains were concentrated by centrifugation (4000 × *g* for 20 min at 4 °C, Megafuge 2.0 R, Heraeus Instruments). Genomic DNA was extracted using the Quick-gDNA^{TM} MiniPrep (Zymo Research, USA). For amplification of the 18S and 28S rDNA as well as the internal transcribed spacer 1 (ITS-1) region, several primer combinations were used (Table 2). We amplified a long sequence from the 18S rDNA to the 28S rDNA with the primers 18S-For binding at the beginning of the 18S rDNA and either NLR1126/22 or NLR2098/24 binding at the end of the 28S rDNA. PCR reactions were performed in 25 µl PCR reaction mixtures containing 1.5 units (12.5 µl) TAQ (Red Taq DNA polymerase Mastermix, VWR Germany), 2 µl DNA and 2.5 µl of each primer (forward and reverse, concentration of 1 µM) at a final concentration of 1.6 nM. PCR conditions for amplifying the long products (18S–28S rDNA) were as follows: pre-denaturation at 98 °C for 2 min, 35 cycles of 98 °C for 30 s, 55 °C for 45 s and 72 °C for 4 min 30 s; final extension at 72 °C for 10 min. Internal primers were used for sequencing (Table 2). For several strains a separate PCR amplification of the 28S rDNA had to be conducted. PCR conditions for amplifying the partial 28S rDNA were as follows: pre-denaturation at 98 °C for 2 min, 35 cycles of 98 °C for 30 s, 55 °C for 45 s, 52 °C for 45 s and 72 °C for 2 min; final extension at 72 °C for 10 min. The amplified products were analyzed by electrophoresis on 1% agarose gel and fragment sizes were determined by comparison with 10,000 bp DNA ladder (Genaxxon Bioscience, Germany). The PCR products were purified by PCR Purification Kit (Jena Bioscience, Jena, Germany). While 18S rDNA and 28S rDNA sequences were obtained for all 29 strains, the sequencing of the ITS-1 region was not successful for all strains (see Table 1). Thus, ITS-1 sequences were not included in our phylogenetic analyses, but were deposited in the GenBank.

Table 2. Primers for sequencing the 18S rDNA, 28S rDNA as well as the internal transcribed spacer (ITS-1) of our isolated and cultivated *Cafeteria* cultures.

Primer	Primer sequence (5′−3′)	Reference
18S rDNA/ITS-1		
18S–For	AACCTGGTTGATCCTGCCAGT	(Medlin et al. 1988)
1280F	TGCATGGCCGTTCTTAGTTGGTG	(Wylezich et al. 2002)
18S–1480F	TAACAGGTCTGTGATGCCC	This study
1389F	TTGTACACCCGCCC	(Amaral-Zettler et al. 2009)
1630F	TTGTACACACCGCCCGTC	(Frothingham and Wilson, 1993)
18S–Rev	TGATCCTTCTGCAGGTTCACCTAC	(Medlin et al. 1988)
18S–Rev–1	CGTAACAAGGTTTCCGTAGGT	(Nitsche et al. 2007)
28S rDNA/ITS-1		
ITS1–For	TAACAAGGTTTCCGTAGGTG	(Armbruster et al. 2000)
28S−184R	ATATGCTTAARTTCAGCGGGT	(Van der Auwera et al. 1994)
NLF184/21	ACCCGCTGAAYTTAAGCATAT	(Van der Auwera et al. 1994)
D3−For	GACCCGTCTTGAAACACGCA	(Wylezich et al. 2007)
NLF1105/22	CCGAAGTTTCCCTCAGGATAGC	(Van der Auwera et al. 1994)
NLR1126/22	GCTATCCTGAGGGAAACTTCGG	(Van der Auwera et al. 1994)
NLR2098/24	AGCCAATCCTTWTCCCGAAGTTAC	(Van der Auwera et al. 1994)

Phylogenetic analyses

The phylogenetic analyses of *Cafeteria*-like strains was based on the 18S rDNA sequences of *Cafeteria* species and other bicosoecids retrieved from GenBank. Only sequences longer than 750 bp were included in the 18S rDNA analysis. Alignments were carried out using MAFFT version 7.212 (Katoh and Standley 2013) within UGENE version 1.30.0 (Okonechnikov et al. 2012); manual corrections to optimize the alignment were done with BioEdit version 7.2.6 (Hall 1999) prior to the phylogenetic analysis. Yubuki et al. (2015) kindly provided their corrected alignment and we incorporated our sequences and further sequences from GenBank in their alignment. The 163-taxon alignment contained 1215 well-aligned nucleotide positions that were used for the phylogenetic analyses. Alignments are available upon request. The Maximum likelihood (ML) analysis was carried out using RaxML version 8.2.10 (Stamatakis 2014) on the CIPRES Gateway (Miller et al. 2010) employing the GTR (General Time Reversible) plus Γ model of nucleotide substitution. Statistical support was estimated with 1000 bootstrap replicates. Bayesian inference (BI) analysis was run with MrBayes v3.2.6 (Ronquist et al. 2012) using a GTR + I + Γ model and a four–category gamma distribution to correct among site rate variation. The search consisted of two parallel chain sets run at default temperatures with a sample frequency of 10 and run so that the average standard deviation of split frequencies dropped below 0.01. The analysis consisted of 3,000,000 generations, with a burn-in of 750,000 (25%), before calculating posterior probabilities.

For the concatenated tree, 18S and 28S rDNA sequences of 28 of our HFCC strains as well as own sequences obtained from CCAP 1900/1, CCAP 1900/2 and two *Caecitellus* species (downloaded from Genbank) were aligned using MAFFT version 7.212 (Katoh and Standley 2013) within UGENE version 1.30.0 (Okonechnikov et al. 2012) resulting in an alignment with 3300 positions. The Maximum likelihood (ML) analysis was carried out using MEGA7 employing the GTR model. Statistical support was estimated with 500 bootstrap replicates. Bayesian inference (BI) analysis was run with MrBayes v3.2.6 (Ronquist et al. 2012) using a GTR + I + Γ model and a four-category gamma distribution to correct for among site rate variation. The search consisted of two parallel chain sets run at default temperatures with a sample frequency of 10 and run so that the average standard deviation of split frequencies dropped below 0.01. The analysis consisted of 50,000 generations, with a burn-in of 7500, before calculating posterior probabilities.

Next-generation-sequencing of deep-sea sediments

Deep-sea sediment samples (upper 2 mm of sediment) were collected with a Multi-Corer (MUC) at 20 different deep-sea stations during four different expeditions in the Pacific and Atlantic Ocean with the research vessels R/V Sonne and R/V Meteor (SO237, SO223T, M79/1, M139) from bathyal, abyssal and hadal regions. DNA was extracted by pre-washing the samples with three washing solutions (Danovaro 2010) followed by the DNeasy Power Lyzer Power Soil DNA isolation kit (Qiagen) used accordingly to the manufacturer's protocol. The Phusion® High-Fidelity DNA Polymerase (ThermoFisher) and the primer–pair 1389 F and 1510R (Amaral-Zettler et al. 2009) were used for the PCR amplifications of the hyper-variable loop V9 of the 18S rRNA gene. The final 25 μl PCR mixtures contained 5 ng of total

DNA template with 0.35 μM final concentrations of each primer, 3% of DMSO and 2X of GC buffer Phusion Master Mix (Finnzymes). PCR amplifications (98 °C for 30 s; 25 cycles of 10 s at 98 °C, 30 s at 57 °C, 30 s at 72 °C; and 72 °C for 10 min) of all samples were carried out with a reduced number of cycles and conducted in triplicates or six replicates. PCR products were run on a 1.5% agarose gel to check amplicon lengths and then pooled and purified using the Quick-gDNA MiniPrep (Zymo Research). Bridge amplification and paired-end sequencing of the amplified fragments were performed using an Illumina Genome Analyzers IIx system (HiSeq, paired-end, 2 × 150 bp).

The V9 sequences (metabarcodes) were analyzed and filtered (removal of chimeras, quality of sequence, read length <87 bp, p-identity <80%) by using the available bioinformatic pipeline adapted from Frédéric Mahé (https://github.com/frederic-mahe/swarm/wiki/Fred's-metabarcoding-pipeline). Overlapping reads were assembled via VSEARCH v.2.13.4 (Rognes et al. 2016) and only paired reads were retained for downstream analyses if they contained both forward and reverse primers and no ambiguously named nucleotides (Ns). The total reads were dereplicated into strictly identical amplicons with VSEARCH and low abundance metabarcodes with a read abundance of one and two reads were removed from the dataset prior to OTU clustering. Metabarcodes were clustered into operational taxonomic units (OTUs) using Swarm v2.1.5 (Mahé et al. 2015), with the parameter d = 1 and the default fastidious option on. OTUs were taxonomically assigned with a pipeline called Stampa (Mahé 2018) to our reference database (trimmed to the V9 region with Cutadapt (Martin 2011)) using VSEARCH's global pairwise alignment. This reference database for the V9 region contained all sequences from the Protist Ribosomal Reference database PR2 v4.11.1 (Guillou et al. 2012) and 103 sequences of our own marine protist strains of the Heterotrophic Flagellate Collection Cologne including the *Cafeteria* sequences of some of our isolated strains belonging to the species *C. burkhardae* (HFCC32, HFCC34, HFCC116, HFCC117, HFCC167, HFCC168, HFCC184, HFCC187, HFCC197, HFCC203, HFCC205, HFCC734, HFCC750, HFCC803, HFCC804, HFCC805, HFCC808, HFCC827), *C. biegae* (HFCC113, HFCC114, HFCC115), *C. graefeae* (HFCC33, HFCC772), *C. malediviensis* (HFCC661), *C. lobieriensis* (HFCC843), *C. chilenis* (HFCC844), *C. mylnikovii* (CCAP1900/2) and *C. roenbergensis* (CCAP1900/1).

We analyzed the read abundance of OTUs assigned to *Cafeteria* species with a V9 sequence similarity of 100% to investigate the distributional pattern of the different *Cafeteria* species within this deep-sea dataset. We visualized these results in a presence/absence map for all investigated deep-sea stations within this study.

Furthermore, we extracted the representative sequences of each OTU and their abundances assigned to *Cafeteria* (sequence similarity 80–100%) within our deep-sea dataset and the Tara Ocean dataset (Supplement Database W5, de Vargas et al. 2015) to analyze a phylogenetic/evolutionary placement of the *Cafeteria* sequences. This phylogenetic placement calculates the most probable insertion branches for each given query sequence on a reference tree (Czech and Stamatakis 2019). First, we created a reference tree (18S rDNA) containing *Cafeteria*-sequences and *Caecitellus* as outgroup and checked the alignment with the check algorithm (–f c) of RaxML v8.2.10 (Stamatakis 2014). The final alignment contained 19 *Cafeteria* sequences and one *Caecitellus* sequence as outgroup. The reference tree was build using RaxML v8.2.10 with the rapid hill-climbing algorithm (–f o) and 40 alternative runs on distinct starting trees (–N 40) and we selected the best-scoring maximum likelihood tree. The V9 *Cafeteria* query sequences were aligned with PaPaRa v2.5 (Berger and Stamatakis 2011) to the reference sequences. The resulting alignment file contained the aligned reference sequences and the aligned query sequences and was split again into separate files. The aligned query sequences (fasta file), the aligned references sequences (fasta file) and the reference tree itself (newick format) were fed into EPA-ng v0.3.5 (Barbera et al. 2019) to get a phylogenetic placement for all the OTU representative *Cafeteria* sequences. To visualize the results of the phylogenetic placement we used Gappa v0.5.0 (Czech et al. 2019). First, we prepared the EPA-results (.jplace format) by using the module prepare and subcommand unchunkify, so that the abundances of the representative sequences could be taken into account within our tree by creating abundance maps. We then used the module examine and the subcommand heat-tree to visualize the phylogenetic placement of our query sequences on the reference tree (colored branches) according to the placement mass of our samples. We used the absolute mass (–mass-norm) and treated every pquery ("placed query", collection of placements of a single query sequence) as a point mass concentrated on the highest weight placement (–point-mass). The sequential color list (blue, purple, black) was logarithmically scaled (–log-scaling) instead of linearly due to the high differences in read abundances. The resulting tree (shown as cladogram) was further prepared with Inkscape v0.92 (http://www.inkscape.org/).

ZooBank registration

ZooBank registration number of present work: urn:lsid:zoobank.org:pub:A53CC66C-1889-4E2D-97FE-F36525EEB1D3.

Results

Light microscopy

All species within the Cafeteriaceae were attached to substratum with the tip of the posterior flagellum, while

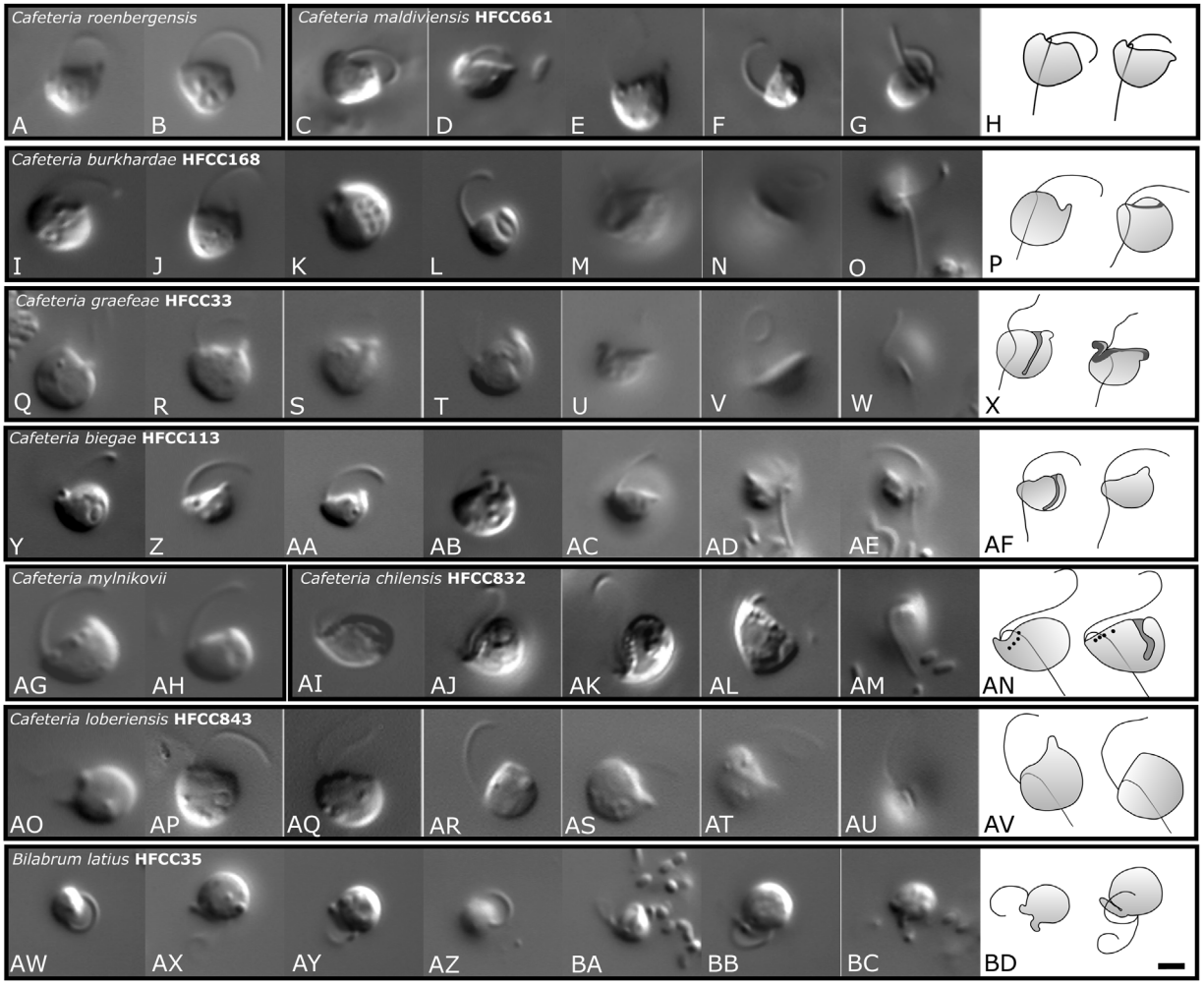

Fig. 2. Differential interference contrast micrographs of *Cafeteria roenbergensis* CCAP 1900/1 (A, B; type population), *C. maldiviensis* HFCC661 (C–H), *C. burkhardae* HFCC168 (I–P), *C. graefeae* HFCC33 (Q–X), *C. biegae* HFCC113 (Y–AF), *C. mylnikovii* CCAP 1900/2 (AG–AH), *C. chilensis* HFCC832 (AI-AN), *C. loberiensis* HFCC843 (AO–AV), *Bilabrum latius* HFCC35 (AW-BD). Scale bar represents 1 μm.

the anterior flagellum was directed upwards towards the right (Fig. 2A, C, J, R, Z, AG, AJ, AR). Both flagella inserted subapically on the posterior portion of the ventral side (Fig. 2G, O, W, AE, AM, AU). While the dorsal side of the species was mainly convex, the ventral side of the cell was flattened or concave. Except for *Cafeteria chilensis* HFCC832, the left side of the cells extended slightly further towards the anterior end than the right side, resulting in a more or less pronounced shelf of the cell (Fig. 2D, J, R, Z, AG, AS). The left side of the cell was mainly roundish (Fig. 2I, Q, Y, AP) or (slightly) flattened (Fig. 2B, C). Cells were sometimes greatly distorted by ingested food. The anterior flagellum was used for drawing bacteria to the ventral furrow (Fig. 2L, AB) whilst beating fast and, thus, creating a current. Due to the fast movement of the anterior flagellum, it was impossible to observe the exact waveform. Detached cells swam with the posterior flagellum trailing behind the cell and the anterior flagellum pointing forward. The nucleus was mostly located in the middle/anterior part of the cell (Fig. 2F, K, Y, AP, AJ). Main morphological differences between the examined strains were the development of the furrow/shelf structure and the overall form of the cell body.

Although good light micrographs for *Cafeteria roenbergensis* (CCAP 1900/1) and *C. mylnikovii* Cavalier-Smith and Chao, 2006 (CCAP 1900/2) already exist, we additionally investigated those species by the same method as used for our strains for comparison (Fig. 2A, B, AC, AD). Our measurements of *Cafeteria roenbergensis* CCAP 1900/1 were slightly different compared to the species description provided by Fenchel and Patterson (1988). The cell length (3.1 ± 0.4 μm, 2.3–4.3 μm, n = 30) and cell width (2.4 ± 0.3 μm, 1.6–2.9 μm, n = 30) were slightly shorter than the original described specimens (5 μm, 4–6 μm long; 4–4.5 μm wide). Flagella measurements (anterior flagellum 5.6 ± 1.2 μm, 4.3–7.4 μm, n = 21; posterior flagellum 6.3 ± 0.7 μm, 5.41–7.45 μm, n = 5) were

within the same range of 5–8 μm (Fenchel and Patterson 1988).

Description of new species

Stramenopiles Patterson, 1989 (emend. Adl et al. 2005)

Bicoecea Cavalier-Smith, 1993

Anoecales Cavalier-Smith, 1997

Cafeteriaceae Moestrup, 1995

Genus *Cafeteria* Fenchel and Patterson, 1988

Cafeteria maldiviensis sp. nov. Schoenle, Rosse and Arndt (Fig. 2C–H)

Diagnosis: Free-living biflagellated bicosoecid, 2.6–4.1 μm long and 1.8–3.8 μm wide. Two flagella of about 4.3–7.5 μm length. Anterior part of the cell truncated.

Etymology: The species-group name *maldiviensis* refers to the type locality (Maldive Islands).

Holotype: Specimen shown in Fig. 2C.

Type strain: Heterotrophic Flagellate Collection Cologne strain HFCC661.

Type sequence data: The 18S rDNA sequence of HFCC661 has the Genbank Accession Number MN315609. The 28S rDNA sequence has the GenBank Accession Number MN318366.

Type locality: Sediment of Nalaguaridhoo, Maldive Islands, depth 1 m (3°28.928′N, 72°47.780′E).

ZooBank registration: urn:lsid:zoobank.org:act:97CBC4BD-B959-4CA9-92C4-1EA198C90724.

Description: Cells D-shaped, 3.2 ± 0.5 μm (2.6–4.1 μm, n = 26) long, 2.7 ± 0.4 (1.8–3.8 μm, n = 26) wide. Highest point of cell width reached in the middle of the axis (Fig. 2C). Mean length of anterior flagellum slightly shorter with 5.6 ± 1.0 μm (4.3–7.4 μm, n = 21) than mean length of posterior flagellum with 6.4 ± 0.7 μm (5.4–7.5 μm, n = 5). Both flagella emerge from anterior portion ventral side (Fig. 2G). Posterior part of cell slightly truncated on left side of cell (Fig. 2C). *Cafeteria maldiviensis* feeds on bacteria whilst creating a current with anterior flagellum. Cells attached with tip of posterior flagellum to substratum. Shelf structure visible on the right side of the cell (Fig. 2D, E). Cells ventrally flattened (Fig. 2E, F). Jerking movement.

Remarks: *Cafeteria maldiviensis* resembles *C. roenbergensis* in shape, position and movement of both flagella (Al-Qassab et al. 2002; Fenchel and Patterson 1988; Larsen and Patterson 1990; Lee 2006; Lee and Patterson 2000; Tong 1997). *Cafeteria maldiviensis* seems to be more truncated on the left side of the cell and has a smaller cell size than the originally described *Cafeteria roenbergensis* (Fenchel and Patterson 1988). However, re–measurement of the originally described strain of *C. roenbergensis* (CCAP 1900/1) resulted in a similar cell size than *C. maldiviensis*. The cell size of *C. roenbergensis* from marine sites worldwide has been reported with a cell length range of 2–10 μm (Al-Qassab et al. 2002; Larsen and Patterson 1990; Lee and Patterson 2000; Tong et al. 1998). The main distinguishable features to the other described *Cafeteria* species are genetic differences in the 18S and 28S rDNA. The closest sequence in public databases (18S rDNA p-distance 1.2%) belongs to an uncultured Cafeteriaceae clone GM1_A5 (FN598450). The closest sequence within our phylogenetic analysis including all our strains belongs to *C. roenbergensis* (CCAP 1900/1), 18S rDNA p-distance 0.4%. However, the p-distance of the 28S rDNA between these two species is much higher (1.2%).

Cafeteria chilensis sp. nov. Filz, Nitsche and Arndt (Fig. 2AI–AN)

Diagnosis: Free-living biflagellated bicosoecid, 3.3–5.1 μm long and 2.4–4.2 μm wide. Two flagella of about 3.6–9 μm length. Raindrop shaped. Anterior flagellum held in a slight S-form.

Etymology: The species-group name *chilensis* refers to the type locality (Chile).

Holotype: Specimen shown in Fig. 2AJ.

Type strain: Heterotrophic Flagellate Collection Cologne strain HFCC832.

Type sequence data: 18S rDNA and 28S rDNA sequences have been deposited at GenBank with the Accession Numbers MN315619 and MN318377.

Type locality: Water sample from a rock pool at Iquique, Chile (20°14.107′S, 70°09.152′W).

ZooBank registration: urn:lsid:zoobank.org:act:044063AD-E58 3-4 929-97F0-6579FDFA2E2.

Description: Cells raindrop-shaped. Mean cell length of 4.4 ± 0.5 μm (3.3–5.1 μm, n = 20) and cell width of 3.4 ± 0.5 μm (2.4–4.2 μm, n = 23, Fig. 2AI). Mean length of anterior flagellum is 5.7 ± 1.5 μm (3.6–9 μm, n = 15) and mean length of posterior flagellum is 7.0 ± 1.0 μm (4.9–8.6 μm, n = 17). Cells attached to substratum with tip of posterior flagellum (Fig. 2AM). Anterior flagellum curved in slight S-form close to cell body (Fig. 2AJ). Extrusomes along right margin of flagellar depression in ordered rows (Fig. 2AJ, AK). Channel at posterior end curved in S-form reaching dorsal side of cell (Fig. 2AL). No pronounced shelf structure.

Remarks: Unlike the arc form of the anterior flagellum of *C. mylnikovii* or *C. loberiensis* the anterior flagellum of *C. chilensis* is held in an S-form. Cells do not have the typical observed D-shaped body like *C. roenbergensis* and *C. mylnikovii* (Cavalier-Smith and Chao 2006; Fenchel and Patterson 1988; Larsen and Patterson 1990), but are rather raindrop-shaped as observed within the drawings of the originally described *Cafeteria minuta* (Larsen and Patterson 1990, their Fig. 49k–m). However, the anterior flagellum of *C. minuta* is 3–4 times the cell length, which was not observed

for *C. chilensis*. The presence of (presumed) extrusomes has also been reported for *C. roenbergensis* and was found in *C. chilensis*. Closest sequence in public databases (18S rDNA p-distance 1.2%) belongs to *C. mylnikovii* (DQ102392). The closest sequence based on the 18S rDNA within our phylogenetic analysis is a strain of *C. chilensis* HFCC844 with no p-distance, followed by *C. loberiensis* (HFCC843) with a p-distance of 0.5%.

Cafeteria loberiensis sp. nov. Filz, Nitsche and Arndt (Fig. 2AO–AV)

Diagnosis: Free-living biflagellated bicosoecid, 3.9–6.7 μm long and 3.2–4.7 μm wide. Two flagella of about 3.9–7.2 μm length. D- to globular shaped cells.

Etymology: The species-group name *loberiensis* refers to the type locality (rock pool at the Loberia point, Mejillones Peninsula, Chile).

Holotype: Specimen shown in Fig. 2AU.

Type strain: Heterotrophic Flagellate Collection Cologne strain HFCC843.

Type sequence data: 18S rDNA and 28S rDNA sequences have been deposited at GenBank with the Accession Numbers MN315620 and MN318376.

Type locality: Water sample from rock pool at Loberia point, Mejillones Peninsula, Chile (23°30.405′S, 70°25.433′W).

ZooBank registration: urn:lsid:zoobank.org:act:20092ED9-D10C-4F15-80C8-DDE91D66E6AF.

Description: D-shaped to globular cells. Cells with mean length of 3.9 ± 0.5 μm (3.0–4.7 μm, n = 23) and mean width of 3.1 ± 0.4 μm (3–4.2 μm, n = 23). Two flagella of equal length with anterior flagellum 5.5 ± 0.7 μm (4.0–6.5 μm, n = 17) and posterior flagellum with 4.7 ± 0.7 μm (3.6–6.5 μm, n = 9). Cells with pronounced shelf structure (Fig. 2AS), while right side more or less roundish shaped (Fig. 2AP, AQ). Cells with two pleat-like thickenings, one reaching from shelf structure at anterior front of ventral side to middle of the posterior front of cell and other parallel to first thickening closer to dorsal side (Fig. 2AJ).

Remarks: Cells slightly more globular than the D-shaped cells of *C. mlynikovii* and *C. chilensis*. Cell length (3–5 μm) is the same as reported for *C. mylnikovii* (Cavalier-Smith and Chao 2006). Anterior flagellum shorter than that of *C. mylnikovii* with a length of 6–10 μm (Cavalier-Smith and Chao 2006), more resemblance to flagella length of *Cafeteria roenbergensis* (5–8 μm long, Fenchel and Patterson 1988). *Cafeteria loberiensis* can be distinguished from *C. minuta* by having flagella of equal length (Larsen and Patterson 1990). No extrusomes visible by light microscopy like in *C. chilensis*. Closest sequence in public databases (18S rDNA p-distance 1.2%) belongs to *Cafeteria mylnikovii* (DQ102392). The closest sequence based on the 18S rDNA within our phylogenetic analysis including all our strains is *C. chilensis* strain HFCC832 with a p-distance of 0.5%.

Cafeteria burkhardae sp. nov. Schoenle and Arndt (Fig. 2I–P)

Diagnosis: Free-living biflagellated bicosoecid, 3.0–4.8 μm long and 2.6–4.5 μm wide. Two flagella of about 5–8 μm length. Concave ventral furrow surrounded by ovoid pleat-like thickening.

Etymology: This species is dedicated to Isabell Burkhard being the inspiration for the work on *Cafeteria*.

Holotype: Specimen shown in Fig. 2I.

Type strain: Heterotrophic Flagellate Collection Cologne strain HFCC168.

Type sequence data: 18S rDNA, ITS-1 and 28S rDNA sequences have been deposited at GenBank with the Accession Numbers MN315600 and MN318357.

Type locality: Deep-sea sediment (overlaying water of Multi-Corer) from the East Vema Fracture Zone, Atlantic Ocean, depth 5793 m (10°25.12′N, 31°04.62′W).

ZooBank registration: urn:lsid:zoobank.org:act:8D8FA6DA-D792-4335-A74C-9A7CC1FCC03A.

Description: Cells D-shaped/globular and ventrally flattened. Biflagellated suspension feeding protists without chloroplast. Cells with mean length of 3.80 ± 0.58 μm (2.9–4.8 μm, n = 22) and mean width of 3.4 ± 0.6 μm (2.6–4.5 μm, n = 22). Highest point of cell width shifted towards posterior end of cell. Anterior flagellum extended laterally, mean length of 5.9 ± 0.7 μm (5.0–7.9 μm, n = 19), used for feeding whilst creating feeding current. Cells attached to substrate with tip of posterior flagellum (Fig. 2O). Mean length of posterior flagellum with 6.5 ± 0.4 μm (6.1–7.4 μm, n = 9). Flagella emerge from ventral, concave side surrounded by ovoid pleat-like thickening, gives cell appearance of a pouch (Fig. 2M, N). Bacteria ingested at rearmost part of ventral furrow (Fig. 2K, L). No channel used for bacterial ingestion observed as shown for some other species. Detached cells swim with recurrent flagellum trailing behind cell and anterior flagellum pointing forward. Jerking movement.

Remarks: The furrow on the ventral side of the cell is surrounded by an ovoid pleat-like thickening, giving the cell the appearance of a pouch as described in *Cantina marsupialis*. However, *C. marsupialis* is much larger (usually 7–10 μm long, flagella length 10 μm) than *Cafeteria burkhardae*. The ovoid pleat-like thickening seems to be the main morphological feature distinguishing *C. burkhardae* from the other Cafeteriaceae species. Sequencing of the type species *C. roenbergensis* showed that almost all deposited sequences at GenBank did not belong to *C. roenbergensis* CCAP 1900/1 (18S rDNA p-distance 4.9%). Thus, we recommend a re-naming of the deposited GenBank sequences concerned to *C. burkhardae*. Closest sequences in public databases (18S rDNA no p-distance) belong to *Cafeteria* Accession numbers KT860979, KT861042, EU106847, EU106804, KX431446, KX431447, KX431450, KX431454, KX431455, FN598363.

Cafeteria graefeae sp. nov. Schoenle and Arndt (Fig. 2Q–X)

Diagnosis: Free-living biflagellated bicosoecid, 2.8–4.7 μm long and 2.6–4.2 μm wide. Two flagella of about 2.3–5.5 μm length. Cells laterally ventral depressed with a channel curved towards the dorsal middle side of the cell, used for bacterial ingestion.

Etymology: This species is dedicated to Brigitte Gräfe for her invaluable efforts in cultivation, storage and morphological characterization of protists.

Holotype: Specimen shown in Fig. 2R.

Type strain: Heterotrophic Flagellate Collection Cologne strain HFCC33.

Type sequence data: 18S rDNA (+ITS-1) and 28S rDNA sequences have been deposited at GenBank with the Accession Numbers MN315607 and MN318364.

Type locality: Deep-sea sediment (overlaying water of Multi-Corer) from the Angola Basin, South East Atlantic Ocean, depth 5388 m (16°14.991′N, 05°26.700′E).

ZooBank registration: urn:lsid:zoobank.org:act:A5BCAE2 F-E451-452D-9587-8521906E122C.

Description: Cells D-shaped. Mean cell length with 3.7 ± 0.5 μm (2.8–4.7 μm, n = 20) and mean cell width with 3.2 ± 0.5 μm (2.6–4.2 μm, n = 20). Both flagella of equal mean length (anterior flagellum with 3.8 ± 0.8 μm, 2.3–5.5 μm, n = 19; posterior flagellum with 3.8 ± 0.7 μm, 2.5–4.7 μm, n = 18, Fig. 2W). Viewed from right side, cells globular at anterior end (Fig. 2Q) and depressed on ventral side. Pronounced shelf structure visible from left side of cell together with pleat-like thickening (Fig. 2R, U, V). Bacteria ingested into channel. Channel curved from ventral furrow towards dorsal middle side of cell (Fig. 2S, T).

Remarks: A pronounced furrow as observed in *Cafeteria burkhardae* was not visible. Feeding on bacteria is the same as described for *Cantina marsupialis* (Larsen and Patterson 1990). Bacteria are drawn into the furrow and ingested at the rearmost part of the furrow via a channel. The channel of *C. graefeae* is curved towards the dorsal side of the cell as observed in *Cantina marsupialis*. However, the shelf of the cell seems not to be as pronounced as in the drawing in Fig. 49J of *C. marsupialis* in Larsen and Patterson (1990). Closest sequence in public databases (18S rDNA p-distance 2.3%) belongs to *Cafeteria "roenbergensis"* (KX431450). The closest sequence based on the 18S rDNA within our phylogenetic analysis including all our strains is *Cafeteria graefeae* strain HFCC772 with a p-distance of 0.1%.

Cafeteria biegae sp. nov. Schoenle and Arndt (Fig. 2Y–AF)

Diagnosis: Free–living biflagellated bicosoecid, 2.6–4.4 μm long and 2.1–3.4 μm wide. Two flagella of about 5.0–9.7 μm length. Cells laterally ventral depressed with a channel curved towards the dorsal middle side of the cell, used for bacterial ingestion. Cells have a flattened D-shaped form.

Etymology: This species is dedicated to Rosita Bieg for her invaluable efforts in cultivation, storage and sequencing of protists.

Holotype: Specimen shown in Fig. 2AA.

Type strain: Heterotrophic Flagellate Collection Cologne strain HFCC113.

Type sequence data: 18S rDNA (+ITS-1) and 28S rDNA sequences have been deposited at GenBank with the Accession Numbers MN315594 and MN318351.

Type locality: Deep-sea sediment (overlaying water of Multi-Corer) from the Pliny Plain, Mediterranean Sea, depth 2676 m (33°44.55′N, 26°08.50′E).

ZooBank registration: urn:lsid:zoobank.org:act:AB0C963D-250A-4EB5-B81E-06D328D747BA.

Description: Mean cell length of 3.5 ± 0.4 μm (2.6 –4.4 μm, n = 30) and mean width of 2.7 ± 0.4 μm (2.1–3.4 μm, n = 30). Smaller width of cells results in flat D-shaped form (Fig. 2Z, AA). Anterior flagellum length of 6.0 ± 0.9 μm (5.0–8.3 μm, n = 25) and posterior flagellum length of 6.8 ± 1.3 μm (4.9–9.6 μm, n = 11) (Fig. 2AD, AE). Cells with pronounced shelf structure (Fig. 2Z, AC). Right side of cell more or less roundish shaped (Fig. 2Y). Pleat-like thickening from shelf structure at anterior ventral side to middle of posterior front of cell (Fig. 2Z). Bacteria drawn and ingested into channel at anterior end of cell (Fig. 2AB). Channel almost reaches dorsal side going straight up (Fig. 2AA).

Remarks: *Cafeteria biegae* has a smaller width compared to the other Cafeteriaceae strains resulting in a flattened D-shaped form. The anterior flagellum is held in an arc form as generally found for the genus *Cafeteria*. Feeding on bacteria is the same as described for *Cantina marsupialis* (Larsen and Patterson 1990). Bacteria are drawn into the furrow and ingested at the rearmost part of the furrow via a channel. The channel of *Cafeteria biegae* is going straight up and not curved towards the dorsal side of the cell as observed in *Cantina marsupialis* (Larsen and Patterson 1990). Flagella have almost the same length with 5.0–9.7 μm in *C. burkhardae* and 5–8 μm in *C. roenbergensis* (Larsen and Patterson 1990). Closest sequence in public databases (18S rDNA p-distance 2.2%) belongs to an uncultured Cafeteriaceae clone BS16_G10 (FN598365). The closest sequences based on the 18S rDNA within our phylogenetic analysis including all our strains are *C. biegae* strains HFCC114 and HFCC115 with no p-distance.

Bicosoecida incertae sedis

Bilabrum gen. nov. Schoenle and Arndt

Diagnosis: Biflagellated heterotrophic flagellate, shaped almost globularly. Ventral pouch visible with highly developed lips.

Type species: *Bilabrum latius* sp. nov.

Etymology: The genus-group name *Bilabrum* refers to its highly developed lip-structure consisting of two lips. bi = two (Latin), labrum = lip (neuter, Latin).

ZooBank registration: urn:lsid:zoobank.org:act:E3D8F813-A42B-46A9-BAAF-42267F63D2E1.

Species included: *Bilabrum latius* sp. nov.

Bilabrum latius sp. nov. Schoenle and Arndt (Fig. 2AW–BD)

Diagnosis: Globular shaped free-living biflagellated bicosoecid. Mean cell length of 2.2–3.5 μm and mean cell width of 1.9–2.9 μm. Length of both flagella about 2.4–6.9 μm. Isolated from marine habitats. Ventral pouch visible with very pronounced lips.

Etymology: The species-group name*latius* refers to the broad lip-like structures of this species. latius (from latus, Latin, Nominative Singular Neuter Comparative) = broader.

Holotype: Specimen shown in Fig. 2AY.

Type strain: Heterotrophic Flagellate Collection Cologne strain HFCC35.

Type sequence data: 18S rDNA and 28S rDNA sequences have been deposited at GenBank with the Accession Numbers MN315515 and MN315517.

Type locality: Deep-sea sediment (sampled with Agassiz Trawl) from the Angola Basin, South East Atlantic Ocean, depth 5392 m (18°24.553′S, 04°43.854′W).

ZooBank registration: urn:lsid:zoobank.org:act:F4521045-051F-48CD-A9B5-6F0193D1DA9D.

Description: Globular shaped cells with mean length of 2.8 ± 0.5 μm (2.2–3.5 μm, n = 18) and mean cell width of 2.5 ± 0.3 μm (1.9–2.9 μm, n = 18). Extremely pronounced lip (pleat-like thickenings) at ventral pouch, resulting in look of lips (Fig. 2AY, BA, BC). Anterior flagellum length 3.6 ± 1.0 μm (2.4–7.0 μm, n = 16) and posterior flagellum length of 4.1 ± 0.9 μm (2.6–6.2 μm, n = 18). Circling movement around cell axis, while being attached to substratum with posterior tip of posterior flagellum below cell body. Posterior flagellum closely curved to cell body (Fig. 2AW, AZ). Anterior flagellum held quite straight away from cell body (Fig. 2AX, BA). Posterior end of anterior flagellum jerkily moves to draw bacteria to cell.

Remarks: Cells are much smaller than *Anoeca atlantica* having a similar size range as the Cafeteriaceae species. Unlike the flagella of *Anoeca atlantica,* flagella of *Bilabrum* are much shorter, especially the anterior flagellum. While no visible ventral pouch appeared in *Anoeca*, *Bilabrum* had a ventral pouch, more expressed than in several Cafeteriaceae strains. A curved shelf extends around the ventral side at the level of flagella. A similar observation has been made for *Cafeteria ligulifera* (Larsen and Patterson 1990). However, flagella were much longer with 10 μm. Closest sequence in public databases is *Cafeteria* sp. KT861042 (clustering within the *Cafeteria burkhardae* clade), 18S rDNA p-distance 10%.

Phylogenetic analyses

The maximum likelihood (ML) and Bayesian analysis of the 18S rDNA data yielded phylogenetic trees with similar topologies, while bootstrap support (maximum likelihood bootstrap percentages (mlBP) and Bayesian inference posterior probability (biPP)) differed between both trees (Fig. 3). *Cantina marsupialis* was recovered as a highly supported monophyletic group (mlBP 100%, biPP 1.0, Fig. 3). Bicosoecids clustered as a monophyletic group with moderate bootstrap support (mlBP 64%, biPP 1.0). Bicosoecids included (i) a moderately/highly supported group of Borokaceae (mlBP 75%, biPP 1.0), (ii) a lowly supported *Halocafeteria* clade (mlBP <50%, biPP <0.5), (iii) a highly supported group of Bicoecales (mlBP 90%, biPP 1.0), (iv) a lowly/moderately supported group of Pseudodendromonadales (mlBP <50%, biPP 0.85), (v) the lowly supported *Bilabrum latius*, (vi) a highly supported group of Anoecaceae as well as Symbiomonadaceae (mlBP 100%, biPP 1.0), (vii) a moderately supported group of Caecitellaceae (mlBP <50%, biPP 1.0), and (viii) a highly supported group of Cafeteriaceae (mlBP 100%, biPP 1.0). Within the Cafeteriaceae, *Cafeteria roenbergensis* CCAP 1900/1 (MN334557) formed a clade with only one environmental sequence (FN598450) and with strain HFCC661 (mlBP 100%, biPP 1.00). The strain HFCC661 from the Maldive Islands had a sequence similarity of 99.6% to *C. roenbergensis* CCAP 1900/1. Besides the low genetic difference of the 18S rDNA the separation of these two species was clearly supported with high bootstrap support (mlBP 99%, biPP 1.00) in the concatenated tree based on the 18S and 28S rDNA (Fig. 4). The genetic difference of the partial 28S rDNA between *C. roenbergensis* (MN334555) and HFCC661 was 98.7%. Thus, we described this strain as a new species, namely *C. maldiviensis*. Three strains (HFCC832, HFCC843, HFCC844) isolated from Chilean rock pools formed a clade with *C. mylnikovii*. The sequence similarity of the already described *C. mylnikovii* CCAP 1900/2 (MN334558) to the type species *C. roenbergensis* CCAP 1900/1 (MN334557) was 94.8%. Three other HFCC strains (HFCC113, HFCC114, HFCC115) of *Cafeteria biegae* isolated from the Mediterranean Sea clustered together with high bootstrap support (mlBP 99%, biPP 1.00) with an environmental sequence (FN598365) isolated from hydrothermal vents in the deep Pacific Ocean with a sequence similarity of 97.8%. *Cafeteria* sp. strain EPM1 (AF174366) was the closest neighbor to *C. biegae* with a sequence similarity of 95.4%. Two HFCC strains (HFCC33 and HFCC772) from the Atlantic Ocean formed a highly supported separated clade (mlBP 78%, biPP 1.00), *Cafeteria graefeae*. Most of our HFCC strains formed a moderately

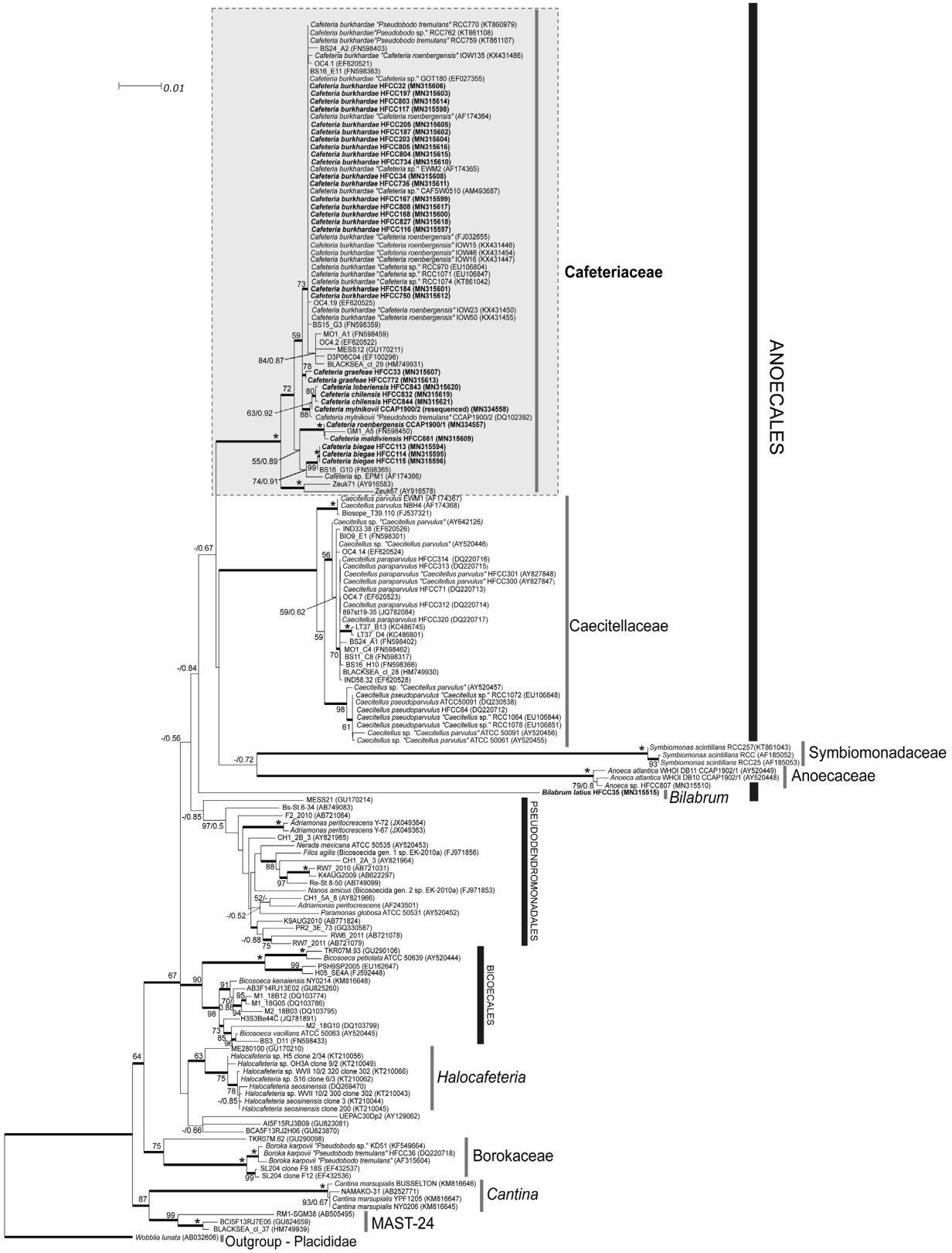

Fig. 3. Phylogenetic tree of bicosoecids based on 18S rDNA sequences. Maximum likelihood tree shown for the analysis (ML; GTR+Γ model). A Bayesian Inference (BI) under the same model yielded an identical topology. Number on edges represent ML bootstrap percentages (1000 replicates); asterisks indicate bootstrap percentages of 100%; values <50% are not shown. Nodes supported by Bayesian posterior probabilities (MrBayes) ≥0.95 are shown by thick lines, otherwise shown behind ML bootstraps values. Scale bar (upper left) represents 0.01 expected substitutions/site in the ML analysis. Species names (Cafeteriaceae) in quotation marks resemble taxonomic assignments from GenBank deposition, those in front is our taxonomic suggestion. The tree is outgroup–rooted, with Placididae (*Wobblia lunata*) as outgroup.

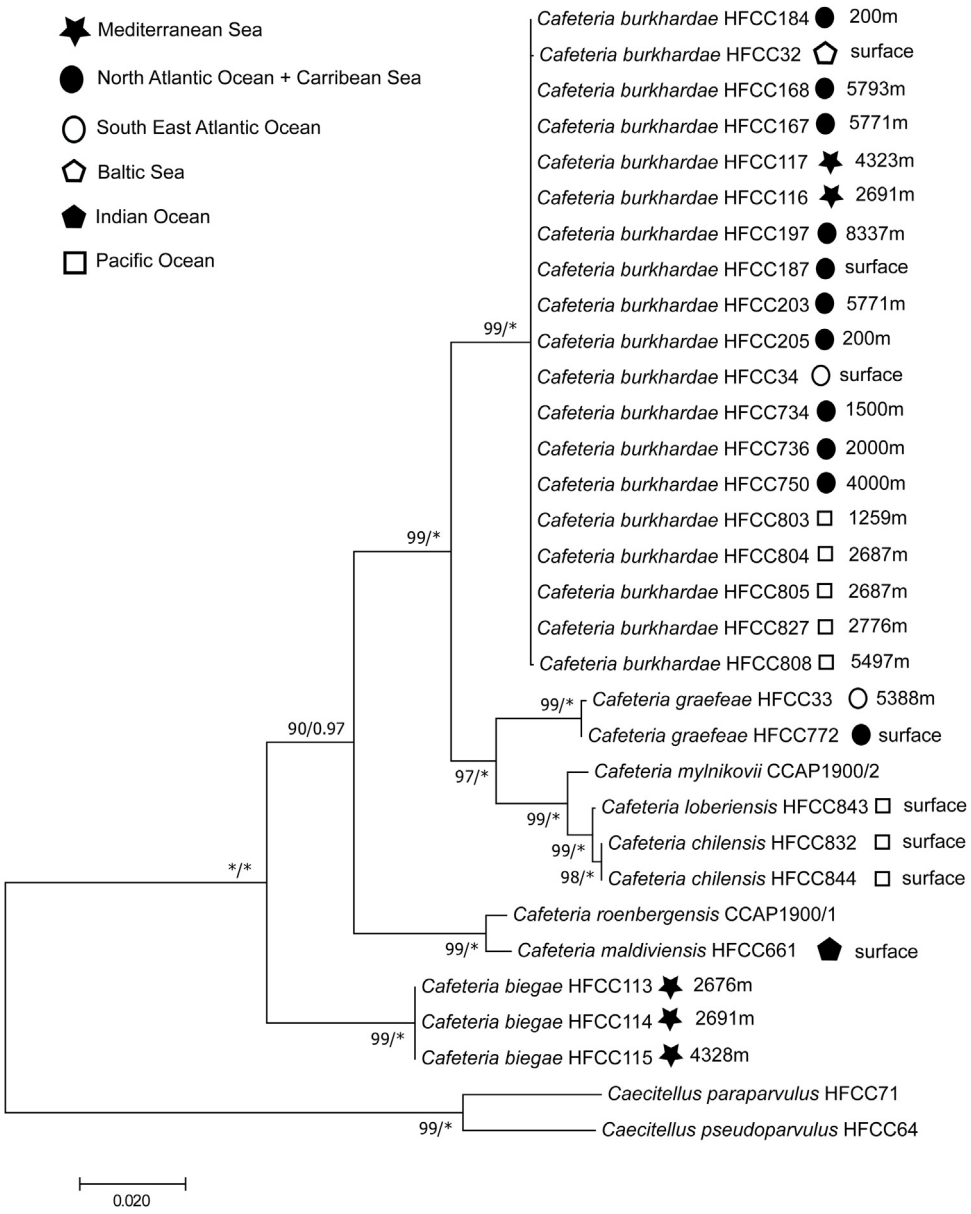

Fig. 4. Maximum likelihood phylogeny (ML; GTR+Γ model, 1000 replicates) of the Cafeteriaceae based on a concatenated dataset of 18S and partial 28S rDNA, 3300 aligned nucleotide positions. The tree includes the 28 HFCC strains, *Cafeteria roenbergensis* CCAP 1900/1 and *C. mylnikovii* CCAP 1900/2. The tree is outgroup-rooted with *Caecitellus*. A Bayesian Inference (BI) under the same model yielded an identical topology. Branches receiving 100% maximum likelihood bootstrap percentages (mlBP) and 1.00 Bayesian inference posterior probability (biPP) support are denoted by an asterisk, values are otherwise given at branches. Scale bar (bottom) represents 0.02 expected substitutions/site in the ML analysis.

supported clade (mlBP 73%, biPP 0.93) with almost all downloaded sequences from GenBank mainly deposited as *C. roenbergensis*. The sequence similarity to either CCAP 1900/1 (95%) or CCAP 1900/2 (97%) was low, thus, we renamed this clade to *Cafeteria burkhardae*. Within this large clade (47 sequences) only two environmental sequences (FN598403, EF620521) and one *Cafeteria roenbergensis* sequence IOW135 (KX431486) isolated from the Baltic Sea clustered separately with a sequence similarity of 99.6–99.9% to *C. burkhardae*. Another cluster within the Cafeteriaceae consisting of only environmental sequences (FN598459, EF620522, GU170211, EF100296, HM749931) putatively represents a new *Cafeteria* species that is capable to thrive in extreme environments. Two uncultured eukaryote clones Zeuk67 and Zeuk71 (AY916578, AY916583) formed a clade outside of the above-mentioned *Cafeteria*-species with high bootstrap support and should be considered as further new species. The *Cafeteria* like strain HFCC35 clustered outside of the family Cafeteriaceae with a long branch requiring the erection of a new genus (*Bilabrum latius*).

The analysis of concatenated 18S and partial 28S rDNA with *Caecitellus* as outgroup revealed a highly supported tree (Fig. 4). We found a similar topology within the Cafeteriaceae compared to the 18S rDNA bicosoecid tree (Fig. 3). All eight *Cafeteria* species were supported by high bootstrap values. *Cafeteria biegae* clustered at the basal branch of this clade. While *C. burkhardae* could be cultivated from surface waters and the deep sea in different parts of the world's ocean (Mediterranean Sea, Atlantic Ocean, Pacific Ocean and Baltic Sea), *C. biegae* only occurred in cultures from the Mediterranean deep sea. *Cafeteria loberiensis* and *C. chilensis* strains were only found in rock pools at the Chilean coast. Only one strain (HFCC661), sampled from surface waters of the Indian Ocean, of the 29 strains clustered closely with *Cafeteria roenbergensis* strain CCAP 1900/1.

Next-generation-sequencing of deep-sea sediments

We generated 222,597,698 raw reads and clustering and filtering led to a final eukaryotic dataset of 46,694 OTUs (70,358,779 reads). We could recover *C. burkhardae*, *C. maldiviensis*, *C. biegae and C. graefeae* with a 100% p-identity to the V9 region of our cultivated strains. In total, 45.63% of our total eukaryotic reads could be assigned to the genus *Cafeteria* with a 100% p-identity. The majority (45.61%) of the reads belonged to *C. burkhardae*, followed by *C. maldiviensis* with 0.02% (Table S1). *Cafeteria burkhardae* and *C. malediviensis* could be recovered in almost all 20 deep-sea sediment stations (20 stations and 19 stations, respectively), while *C. biegae* and *C. graefeae* were recovered in less than 10 stations (4 stations and 8 stations, respectively) (Fig. 5). The phylogenetic placement of all *Cafeteria* associated V9 query sequences (representative sequence of each OTU and the abundance of each representative sequence) within our deep-sea samples and the Tara Ocean dataset revealed that the majority of V9 sequences could be assigned to the *Cafeteria burkhardae* clade (Fig. 6).

Discussion

Morphological and molecular differences

Since 1994 we have been able to establish and maintain 29 cultures of *Cafeteria*-like strains from different parts of the ocean (Baltic Sea, Mediterranean Sea, Pacific, North Atlantic, Caribbean Sea, South East Atlantic, Indian Ocean) sampled during various expeditions. Sequencing of these strains and sequencing of the type species of the genus *Cafeteria, C. roenbergensis* deposited at CCAP, for which no 18S rDNA sequence was available in GenBank, required a revision of the family Cafeteriaceae. Overall, our phylogenetic analysis supports the monophyly of bicosoecids as has been shown in previous studies (Cavalier-Smith and Chao 2006; Harder et al. 2014; Karpov et al. 2001; Yubuki et al. 2015). Several attempts for making a systematic formal revision of bicosoecids have been made, dividing and splitting species into different families and orders (Cavalier-Smith and Chao 2006; Ruggiero et al. 2015). The family Caecitellaceae was well recovered within our phylogenetic analysis. Three species have been described so far within the Caecitellaceae including *Caecitellus parvulus*, *Caecitellus pseudoparvulus* and *Caecitellus paraparvulus* (Hausmann et al. 2006). The assignment of these three species to the genus *Caecitellus* is with a genetic difference of 4–6% (18S rDNA p-distance) within the same range as for our *Cafeteria* strains. Strain HFCC35 had been classified as *Cafeteria* cf. *marsupialis* based on morphological determination. Since only the 28S rDNA was available, it was not compared with other *Cafeteria* strains based on 18S rDNA (Wylezich et al. 2010). However, p-distance calculations using 18S rDNA sequences showed that HFCC35 had p-distances of more than 10% to the other *Cafeteria* species. Thus, we reassigned this strain to a new genus and species, *Bilabrum latius*. *Bilabrum latius* clustered solely on a long branch, separated from the Cafeteriaceae, Caecitellaceae and the *Anoeca/Symbiomonas* clade within our phylogenetic analysis, but within the Anoecales. The branching of the strain HFCC35 might be an artefact of long-branch attraction as has been already mentioned by Harder et al. (2014) for the *Anoeca/Symbiomonas* clade since the taxon sampling for this lineage is still poor.

Morphological characteristics obtained with high-resolution light microscopy are not sufficient to classify the species of the Cafeteriaceae. Four *Cafeteria* species (*C. marsupialis*, *C. minuta*, *C. ligulifera*, *C. roenbergensis*) have been morphologically described prior to this study (Fenchel and Patterson 1988; Larsen and Patterson 1990), while *Cafeteria marsupialis* has been assigned to a new genus *Cantina* (Yubuki et al. 2015). Morphological studies of the newly isolated strains with high resolution video-enhanced microscopy revealed only slight differences between them. A shelf extending the anterior part of the cell was observed for all species but was distinctly pronounced. The re-analysis of the morphological characteristics of *Cafeteria roenbergensis* strain CCAP 1900/1 resulted in distinct differences to the size measurements of the originally described species. This could be due to long-term cultivation, which might influence cell size of the species or due to individual differences in measuring cells. In addition, the cell size of *Cafeteria roenbergensis* has been reported within a size range of 2–10 μm (Al-Qassab et al. 2002; Fenchel and Patterson 1988; Larsen and Patterson 1990; Lee 2006; Lee and Patterson 2000; Tong 1997). The cell size is obviously not suitable as the main distinctive characteristic for *Cafeteria* species. The cell shape within the Cafeteriaceae strains was sometimes greatly distorted due to ingested food as has been shown for *Cantina marsupialis*. However, the raindrop-shaped cell was a morphologically distinct characteristic for

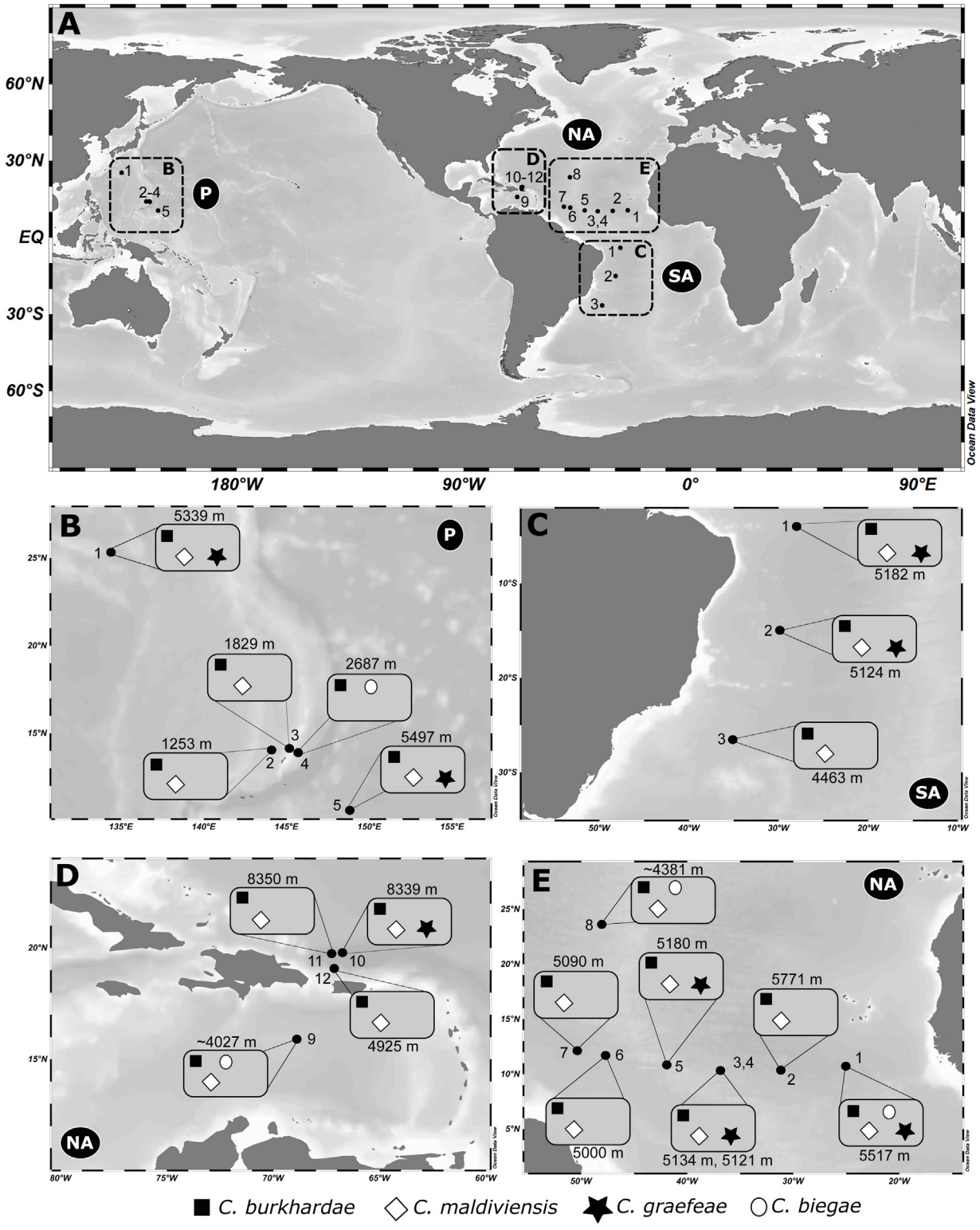

Fig. 5. (A) Station map of collected deep-sea sediment samples from 20 stations (NA1-NA12, P1-P5, SA1-SA3) used for next-generation-sequencing during different expeditions in the Pacific Ocean (P), North Atlantic Ocean (NA) and South Atlantic Ocean (SA). Samples were taken at bathyal (1−3 km), abyssal (3−6 km) and hadal (>6 km) depths (B)–(E) Occurrence of the cultivated and sequenced *Cafeteria* species (indicated by different symbols) within the NGS deep-sea dataset based on the V9 region of the 18S rDNA at the different stations (indicated by dots and numbers) within the Pacific Ocean (B), South Atlantic Ocean (C), Caribbean Sea (D), North Atlantic Ocean (E). Sampling depths are given above/below species occurrence boxes. Maps were created with Ocean Data View using Ocean Data View (Schlitzer 2012).

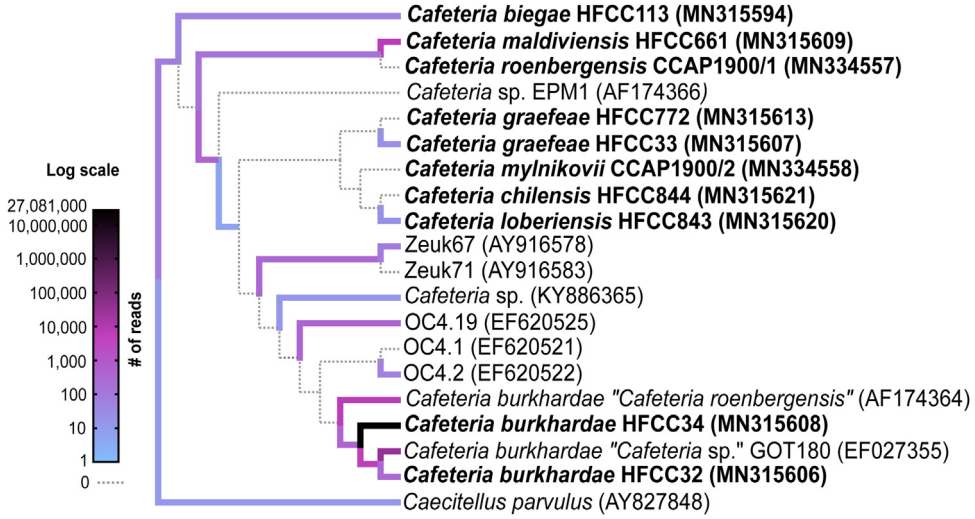

Fig. 6. Phylogenetic placement of deep-sea (this study) and surface water (de Vargas et al. 2015) *Cafeteria* V9 OTU representative sequences (and their abundances) on a reference tree (cladogram) including 19 sequences of *Cafeteria* (strains within this study in bold) and *Caecitellus* as outgroup. Every pquery ("placed query", collection of placements of a single query sequence) within the EPA-analysis was treated as a point mass concentrated on the highest-weight placement. The sequential color code is logarithmically scaled due to high differences in read abundances associated to the different *Cafeteria* species.

Cafeteria chilensis, when compared to the other *Cafeteria* species, which were mainly D-shaped excluding the more globular shape of *Cafeteria minuta* (Larsen and Patterson 1990). While for *Cafeteria roenbergensis* the presence of (presumed) bottle-like shaped extrusomes was reported (Fenchel and Patterson 1988), we only observed extrusomes in *C. chilensis* and not in the other Cafeteriaceae strains. This feature clearly separates the mentioned species from the closely related *C. loberiensis*, although the genetic difference between *C. chilensis* and *C. loberiensis* was low with a p-distance of 0.5% for both, 18S rDNA and 28S rDNA. *Cafeteria roenbergensis* and *C. maldiviensis* showed only small morphological differences and a low genetic difference of the 18S rDNA (p-distance 0.4%), but showed a clear genetic separation within the concatenated tree based on the 18S rDNA and 28S rDNA (p-distance 1.2%). Since our phylogenetic analyses based on the 18S rDNA and concatenated 18S rDNA and partial 28S rDNA resulted in a clear differentiation of the strains, we conclude that species identification of newly isolated and cultivated *Cafeteria*-strains based on molecular analysis could be sufficient for identifying species within the Cafeteriaceae together with high resolution microscopy. More distinct morphological traits might become visible by scanning electron and transmission electron microscopy.

Reorganization of the Cafeteriaceae

The phylogenetic analysis using 18S and partial 28S rDNA showed a clear clustering of the strains into several species with high bootstrap support values. The sequencing of the type species *Cafeteria roenbergensis* CCAP 1900/1 revealed that most of the deposited sequences in GenBank do not belong to the species *Cafeteria roenbergensis,* although often submitted under the name *C. roenbergensis*. The type species *Cafeteria roenbergensis* is the deposited culture at CCAP (CCAP1900/1). Unfortunately, the sequence of the type species has never been deposited at any public database. When we sequenced this type species, we clearly found that it is genetically different to the previously assumed *Cafeteria roenbergensis* sequences. We think, that probably the first deposited *C. roenbergensis* sequence was thought to be the actual *C. roenbergensis* sequence due to morphological observations. With our cultivation approach and sequencing of the type species we now have a clear separation of the *Cafeteria* strains. That is why the first sequence has been published as *C. roenbergensis* although it was not the sequence of the type species. And from that time it was suggested, due to the sequence similarity, that the found isolates belonged to *Cafeteria roenbergensis*. Our isolated strains clustered into six clades within the Cafeteriaceae, most of them separately from *Cafeteria mlynikovii* and *Cafeteria roenbergensis*. We reorganized and renamed the clusters of Cafeteriaceae supported by high bootstrap values into eight species (*C. roenbergensis*, *C. maldiviensis* sp. nov., *C. biegae* sp. nov., *C. loberiensis* sp. nov., *C. chilensis* sp. nov., *C. mylnikovii*, *C. graefeae* sp. nov., *C. burkhardae* sp. nov.). Only one of the sequences available at GenBank clustered with *Cafeteria roenbergensis* and none with *Cafeteria mylnikovii*. We showed that surveys based on either 18S rDNA or 18S rDNA + 28S rDNA retrieved a comparable branching order within the Cafeteriaceae. A phylogenetic tree based on the concatenated 18S rDNA and partial 28S rDNA sequences for the HFCC strains including *Cafeteria roenbergensis* CCAP 1900/1 and *Cafeteria mylnikovii* CCAP 1900/2 showed a better resolution yielding a high support for

the Cafeteriaceae clade (Fig. 4). Although some of our strains showed morphological similarities to the species *C. ligulifera* and *C. minuta,* for that no sequences are available up to now, the overall comparison showed no total morphological overlap to these described species. For example, *Cafeteria chilensis* has a raindrop shaped cell body as observed within the drawings of the originally described *Cafeteria minuta* (Larsen and Patterson 1990), but can be distinguished from *C. minuta* by having flagella of equal length. While both flagella of our strains are almost of equal length and smaller than 10 μm, they can be distinguished from *C. ligulifera* in addition to the different movement of the anterior flagellum (Larsen and Patterson 1990). Furthermore, *C. ligulifera* has an almost globular cell body, while the majority of our strains showed a more D-shaped body. Sequencing of the ITS-1 region was only successful for the *Cafeteria burkhardae* and *Cafeteria biegae* strains and, thus, this marker was not included in the phylogenetic analysis. The comparison of the ITS-1 sequences for all *C. burkhardae* strains within this study showed only one nucleotide difference. This underlines the genetic differentiation as new species besides the 18S rDNA and 28S rDNA analysis. The same observation was made for the *C. biegae* strains. We, thus, suggest renaming the *Cafeteria* sequences deposited at GenBank (KT860979, KT861108, KT861107, KX431486, EF027355, AF174364, AF174365, AM493687, FJ032655, KX431446, KX431454, KX431447, EU106804, EU106847, KT861042, KX431450, KX431455) according to our phylogenetic analysis to prevent further confusions.

Distribution and ecology

Concerning ecology and distribution of the members of the Cafeteriaceae, the clade of *Cafeteria biegae* could only be isolated from the Mediterranean Sea, while the two strains of *C. graefeae* (HFCC33 and HFCC772) were only found in samples from the Atlantic Ocean. While *C. maldiviensis* (HFCC661) could only be isolated from the Indian Ocean, we could recover V9 sequences with 100% sequence similarity to *C. maldiviensis* in 19 out of 20 deep-sea stations. *Cafeteria burkhardae,* on the other hand, seems to have a cosmopolitan distribution in brackish and marine environments, in surface waters as well as in the deep sea, which is underlined by our cultivation approach as well as by our next-generation-sequencing results of sediment at 20 different deep-sea stations. We found that a great proportion of environmental sequence V9 reads from our 20 analyzed deep-sea communities belonged to *Cafeteria burkhardae* with a 100% sequence similarity. A high proportion of reads obtained by next-generation-sequencing (NGS) could also be assigned to *Cafeteria burkhardae* within the Tara Ocean global eukaryotic plankton survey (see Supplement Database W5 in de Vargas et al. 2015). The *Cafeteria* species investigated in the present study showed clear differences within the V9 region, ranging from p-distances of 8% (*C. roenbergensis* CCAP1900/1 to *C. burkhardae* HFCC168) to 4% (*C. graefeae* HFCC33 to *C. mylnikovii* CCAP1900/2). An exception are the two uncultured eukaryote strains Zeuk67 (AY916578) and Zeuk71 (AY916583), clustering separately from *Cafeteria burkhardae*, which have a 100% identical V9-region with *Cafeteria burkhardae*. Our phylogenetic/evolutionary placement analysis (EPA) revealed that most of the OTU representative sequences assigned to *Cafeteria* (sequence similarity 80–100%) within our deep-sea study and the Tara Ocean project could actually be placed within the *C. burkhardae* clade. Comparative analyses of microbial communities of litoral and deep-sea habitats showed differences in protistan assemblages indicating the existence of specific deep-sea heterotrophic protist communities (Countway et al. 2007; López-García et al. 2001; Scheckenbach et al. 2010; Schoenle et al. 2017). However, in some cases even similar genotypes of heterotrophic flagellates had been isolated from the deep sea and surface waters (Scheckenbach et al. 2005), as has been shown for *Cafeteria burkhardae* within this study. Surface water protists including *Cafeteria*, for example, have been found on sinking marine snow (Caron et al. 1982; Patterson et al. 1993), carcasses and macroalgae (such as *Sargassum*, see Baker et al. 2018). The question is whether there is a continuous exchange between deep-sea and surface communities and, if so, whether deep-sea communities can survive and thrive under surface conditions and vice versa. Previous pressure experiments with several of our HFCC strains, showed that *Cafeteria* strains isolated from the deep sea survived high hydrostatic pressures (Živaljić et al. 2018). Four deep-sea strains of *Cafeteria burkhardae* (HFCC167, HFCC804, HFCC 808, HFCC827; Živaljić et al. 2018) survived a pressure increase up to 460 bar at certain conditions. In addition, two surface strains of *C. burkhardae* (HFCC187, HFCC34) survived high hydrostatic pressures up to 520–550 bar. The deep-sea strain *C. biegae* (HFCC115) was able to manage a pressure increase up to 550 bar at 13 °C.

Conclusion

Our phylogenetic analysis resulted in a reorganization of the family Cafeteriaceae with six new species. Sequencing the type species *Cafeteria roenbergensis* CCAP 1900/1 revealed that all deposited *Cafeteria* sequences at GenBank and most of our HFCC strains did neither belong to the species *Cafeteria roenbergensis* nor *Cafeteria mylnikovii* and, thus, were assigned to a new species, *Cafeteria burkhardae*. The rest of our strains were organized into the species *C. loberiensis*, *C. chilensis*, *C. biegae* and *C. graefeae*. While *C. burkhardae* seems to be a cosmopolitan, the distribution of the other species seems to be more restricted. The discovery and analysis of new bicosoecids and utilizing more sequences and comparative morphological descriptions as well as multigene analysis are essential to reconstruct a more

robust evolutionary relationship within the bicosoecids and might result in a higher resolution e.g. of *Bilabrum latius* strain HFCC35.

Authors' contributions

All authors were involved in the sampling and cultivation of protists. A.S., M.H., P.F., M.R., C.W., F.N. and H.A. conducted the molecular and morphological characterizations. A.S. performed the phylogenetic analyses. M.H. and A.S. conducted the lab analyses of the deep-sea sediment (next-generation-sequencing). A.S. performed the bioinformatics analyses of the NGS data. A.S. and H.A. wrote the manuscript. All authors reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ejop.2019.125665.

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Chapter 3

High and Specific Diversity of Protists in the Deep-Sea Basins

High and Specific Diversity of Protists in the Deep-Sea Basins

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Abstract: Despite their potential as major regulators of benthic deep-sea bacterial communities, knowledge on eukaryotic microbial diversity in deep-sea sediments is scarce, especially in the abyss. We collected sediment from 20 deep-sea basins at the Atlantic and Pacific Ocean to analyze the genotype diversity of benthic protist communities. We found benthic deep-sea assemblages highly diverse differing from littoral and water column protist communities. Kinetoplastid flagellates, ciliates and foraminiferans had much higher genotype diversity in the abyssal deep sea compared to surface water communities. Many genotypes had no close representatives in genetic databases suggesting the presence of an extremely large number of novel taxa in deep-sea sediments. A high percentage of our retrieved deep-sea sequences was affiliated with heterotrophic species. Deep-sea protist communities differed on a small and large spatial scale. This study not only enlarges the limited knowledge about abyssal deep-sea protist communities, but it is also the first record of protist metagenome data from hadal regions (>8 km).

One Sentence Summary: Comparative analyses reveal global differences between Atlantic and Pacific deep-sea basins and distinct protist richness in the deep-sea basins.

Main Text: Marine heterotrophic flagellates are a major component within the microbial food web and are important nutrient remineralizers in biogeochemical cycles in surface waters (1; 2). Considering the order of geographic magnitude of the deep sea and the potentially importance of protists within these vast ecosystem, the ecological function, species-level distribution and diversity of deep-sea protist communities is scant. Genetic approaches like Next-Generation Sequencing (NGS) and clone libraries have turned out to be reliable tools in identifying previously unknown protistan lineages in surface waters and the deep sea (3; 4; 5; 6). Surface water communities have found to be distinct from benthic deep-sea protist communties (7). Most benthic deep-sea studies focused on assumed "hot spots" in the deep-sea like hydrothermal vents, cold seeps and anoxic basins at bathyal depths (8; 9; 10; 11). Investigations on deep-sea protists from the abyss (3-6 km), covering 54% of the Earth's surface, are scarce and non existent from hadal regions at ∼8 km depths. One study showed the existence of large-scale patterns at abyssal depths by comparing three different plains in the South East Atlantic Ocean (12). Global comparisons, as they were made for the eukaryotic plankton community of the euphotic zone (13), are missing for the benthic deep-sea nanofauna.

We conducted a comparative anaylsis from benthic deep-sea protist communities on a global and local spatial scale at different depths (mainly abyssal regions) in the Atlantic and Pacific Ocean (Fig. 1D) by NGS analysis (V9 region, 14). We generated ∼223 million raw reads for all stations and first bioinformatic quality control led to ∼3,9 million distinct reads, hereafter denoted "metabarcodes" (Fig. S1). We then clustered metabarcodes into biologically meaningful operational taxonomic units (OTUs) (15) and assigned a eukaryotic taxonomic path to all OTUs (16) by global similarity analysis with reference sequences from an in-house database, called V9_ DeepSea. Clustering and filtering led to a final eukaryotic dataset of 48,589 OTUs (∼71 million reads) reads of which 91% (41,152 OTUs, ∼59 million reads) could be assigned to protists (excluding ∼3,000 phototrophic protist OTUs). Of our final protists OTUs (hereafter denoted "POTUs") only 2.3% were 100% identical to reference sequences, and on average POTUs had only a 90.6% match with reference sequences indicating the existence of completely new lineages and a specific protist fauna in the deep sea, especially within the Discoba (Fig. 1E). A global marine plankton survey also recovered heterotrophic protists to be the most diverse, with a great proportion of unknown eukaryotic groups (13).

The estimated saturation of all eukaryotic deep-sea species (∼146,000 OTUs, Fig. S2) was nearly the same when compared to the eukaryotic plankton diversity (∼150,000 OTUs). However, while of our total OTUs many were assigned to bacteria (Fig. S1), we recovered three times less of the eukaryotic richness (∼49,000 OTUs) than within the Tara Ocean plankton survey (∼110,000 OTUs). One should keep in mind that within the Tara Ocean Project the sampling size was more than twice as high as within our deep-sea study. While we only recovered 43% of the total estimated eukaryotic richness, our sampling approach was sufficient to reach saturation of deep-sea protist richness at a global scale (∼40,000 POTUs), thus, allowing extraction of general patterns concerning the protist diversity of our data set.

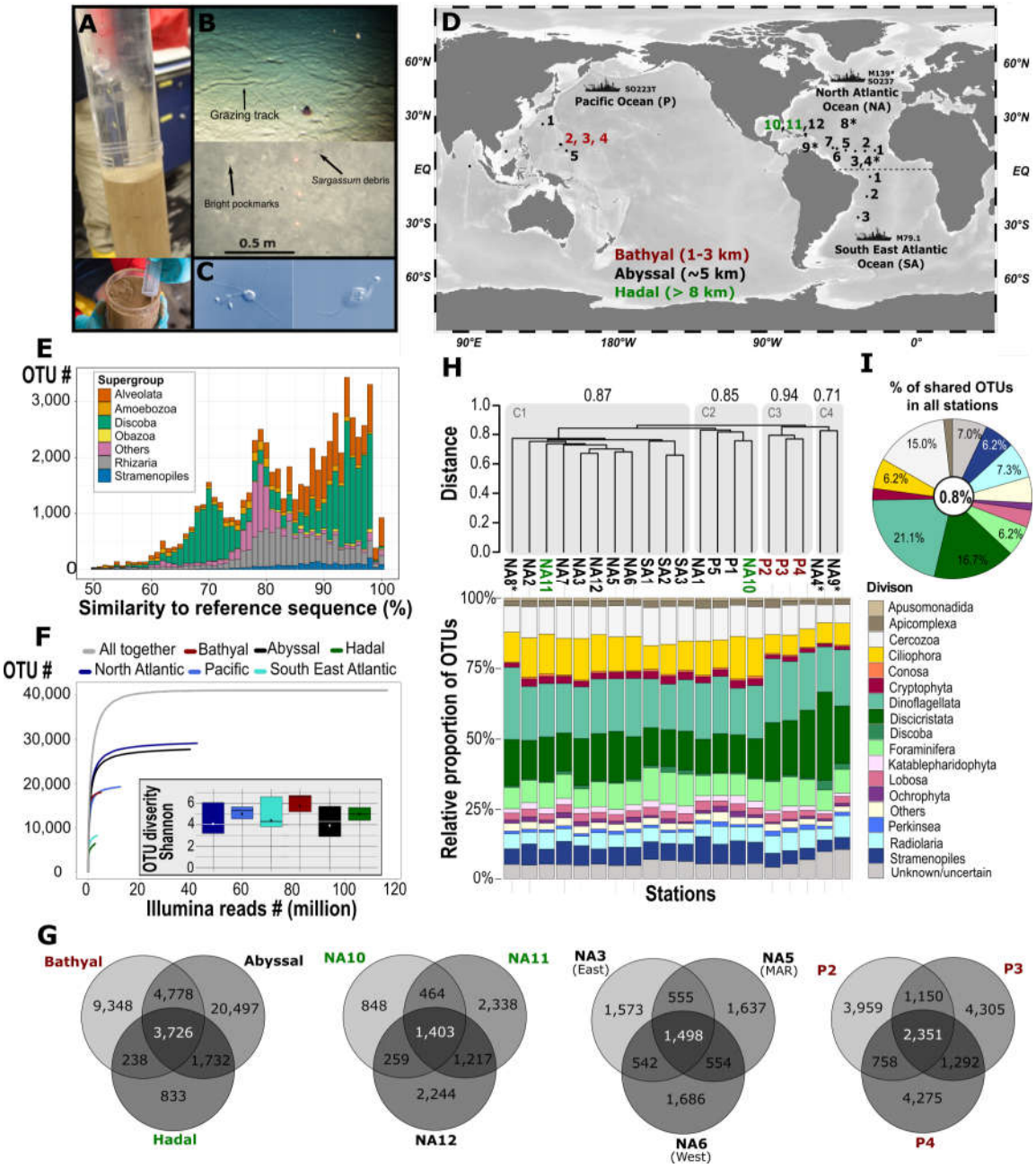

Fig. 1: Deep-sea protist diversity (without phototrophs) and distribution. (**A**) Only the upper 2 mm of undisturbed sediment from cores of the Multi-Corer (MUC) were taken with a sterile syringe. (**B**) Images from the abyssal sea floor showing small scale heterogeneity with signs of grazing, pockmarks and *Sargassum* debris. (**C**) Observed deep-sea flagellates *Massisteria marina* (left) and *Rhynchomonas nasuta* (right). (**D**) Sampling map of the 20 examined deep-sea stations from bathyal (3 stations), abyssal (15 stations) and hadal (2 stations) regions. (**E**) Similarity of rDNA richness to total referenced eukaryotic rDNA divserity in the V9_DeepSea database. Proportion of POTUs per eukaryotic supergroup is color-coded. (**F**) V9 rDNA POTUs rarefaction curves and overall diversity (Shannon index, inset) from either region dependent (North Atlantic, South East Atlantic and Pacific Ocean) or depth dependent (bathyal, abyssal and hadal) clustering of all stations. (**G**) Number of shared and unique POTUs between a global depth profile, two hadal and one abyssal station located in the Puerto Rico Trench, three abyssal stations from the East and West North Atlantic Ocean and the Mid-Atlantic Ridge and bathyal regions in the Pacific Ocean, (from left to right, respectively). (**H**) Dendrogram of UPGMA clustering based on the Jaccard index (presence/absence matrix) of the total POTUs (upper graph). Relative proportion of POTUs within the 20 deep-sea stations related to the major taxonomic protist groups (lower graph). (**I**) Pie chart of the 0.8% of total POTUs occuring in all 20 deep-sea stations related to the major taxonomic protist groups.

Only 0.8% of the total protist richness (number of POTUs) were shared among all twenty stations with a majority of shared POTUs belonging to the Dinoflagellata (21.1%), Discicristata (16.7%) and Cercozoa (15.0%) (Fig. 1I). About 54% of the POTUs detected in this study were unique to one station indicating the existence of large-scale patterns of protist diversity in deep-sea sediments. 50% of our POTUs could only be found in abyssal depths, followed by 23% in bathyal and 2% in hadal regions. Only 9% of POTUs where shared within the depth profiles. The present study included only a limited number of bathyal (3 locations) and hadal (2 locations) samples, thus, an overall conclusion regarding distribution patterns by a depths gradient should be considered with care. In addition, three-quarters of our examined deep-sea samples were taken from abyssal depths. POTU richness was on average highest at station NA9* located in the Carribean Sea (∼15,000 OTUs) and lowest at station P5 (∼2,500 OTUs) located in the Pacific Ocean. Although comparisons of the Shannon-Wiener indices revealed no significant differences between the oceanic and depth profiles regarding the species richness, a PERMANOVA analysis detected significant differences between oceanic regions and depth gradients ($p<0.01$). The UPGMA clustering resulted into a four cluster solution mainly supported by high bootstrap values and significant differences between cluster C1 and the other three clusters (PERMDISP $p<0.05$, Fig. 1H). The bathyal stations in the Pacific Ocean formed a separate cluster (high boostrap support 93%) and the three stations from the abyssal South Atlantic Ocean clustered separately within the large cluster C1, comprised of hadal and abyssal regions from the North Atlantic and Pacific Ocean. Otherwise no region or depth dependent pattern was observed. In all twenty stations Discicristata and other Discoba, Dinoflagellata, Foraminifera and Ciliophora showed the highest protist richness (Fig. 1H).

For a more detailed view on the distribution of deep-sea protist communities we investigated shared and unique POTUs within several subsamples (Fig. 1G). Comparison of the two hadal stations and one abyssal station within the Puerto Rico Trench in the North Atlantic Ocean showed, that the two hadal samples ∼30 km apart shared much less POTUs than the hadal station NA11 and abyssal station NA12 which had been further apart from each other with ∼65 km apart (for a detailed location map see (17)). The MAR is assumed to be a potential barrier which is regularly interrupted by Fracture Zones, as e.g. the Vema Fracture Zone. One of our deep-sea sediment samples (NA5) was taken within the Vema fracture zone, which is unique in its flat valley, theoretically providing a continious habitat in terms of depths and sediment cover (17). Distinct assemblages of isopods could be found east and west of the Mid-Atlantic Ridge at the Vema fracture zone (18), while other studies concluded that the MAR is not a dispersal barrier. While ∼20% of our POTUs in the comparison of the East, Mid Atlantic Ridge and West Northern Atlantic Ocean were unique to each of the three stations, the same proportion was shared within all three stations indicating that several protists might use this Fracture Zone as dispersal while others do not.

These results suggest and show differences among the communities of the 20 deep-sea basins, emphasizing patterns of protist diversity at a large spatial scale. Grouping all examined stations by their depths profile in bathyal, abyssal and hadal regions or oceanic

regions, our results suggest that some taxa can maintain eurybathic ranges and cosmopolitan deep-sea distributions, but the majority of species appear to be regionally restricted. For deep-sea ciliates, for instance, no significant correlation with geographic distance was observed (19), while in coastal sediments community composition of protists are driven by water depth overriding both geographic distance and environmental heterogeneity (20). Within our study we can say, that we observed distinct communities within oceanic regions and depths gradients, but whether depths on a global scale might be a mechanism driving community patterns would need to be verified by a larger sampling abundance of hadal and bathyal regions.

We already observed distinct protist communities on a small spatial scale in abyssal regions. Compared sediment samples were taken from two cores of the same Multi-Corer, being separated by ∼1 m. Abyssal plains are not flat or featureless but are strongly influenced both by the underlying plate geology and subsequent sedimentary processes (17). For the sediment station sampled during the SO237 expedition bathymetric features and images were recovered from each of the sampling sites. While e.g. at station NA6 a field of manganese nodules was found, on the easterly site of the North Atlantic Ocean more *Sargassum* debris could be detected. Sediment grain-size analysis shows that the silt-clay fraction dominated at all sites, but differed within other sediment components (17).The sediment patchiness at the abyssal sea-floor is extremely high, as indicated with many grazing tracks, pockmarks and *Sargassum* debris (Fig. 1B). Thus, environmental factors might already impact and shape deep-sea protist communities on a small scale and it is likely that multiple processes might operate at the same time structuring communities. If all protist species are affected in the same manner or if the abundant and rare proportion of protists is affected differently, still needs to be answered.

The total 48,589 eukaryotic OTUs (including Metazoa, Streptophyta and Fungi) covered the full spectrum of cataloged eukaryotic diversity amongst the eight recognized supergroups and multiple lineages of uncertain placement (Fig. 2). Twelve hyperdiverse lineages contained > 1000 OTUs. Ten of these lineages could be assigned to protists including Discoba (Diplonemea, Kinetoplastea and others), Rhizaria (Foraminifera, Endomyxa), Alveolata (Dinophyceae, MALV-I, MALV-II, Ciliophora) and Cryptista. Together they accounted for 70% of all OTUs, while only 38% of the reads could be assigned to these 12 groups. Particularly striking was the extremely high read abundance of stramenopiles within the Bicocoeca group (46% of the total reads) consisting of only 300 OTUs. The largest OTU with regards in abundance (number of reads) belonged with a pairwise identity of 100% to the *Cafeteria burkhardii* species including 55% of our total protist read abundance. This coincides with our observation of the dominance of *Cafeteria* species during cultivation of deep-sea protists from several deep-sea expeditions. Even within the eukaryotic plankton 75% of the reads were assigned to the genus *Cafeteria* (13). Comparing our Shannon-Wiener indices for the twenty stations, the diversity was highest at sampling site P3 (Pacific) and lowest at sampling site NA1 (North Atlantic) due to extremely high read abundances associated to the one OTU assigned to *Cafeteria burkhardii*. The most hyperdiverse lineage in regard to species richness were the diplonemids. Diplonemids are

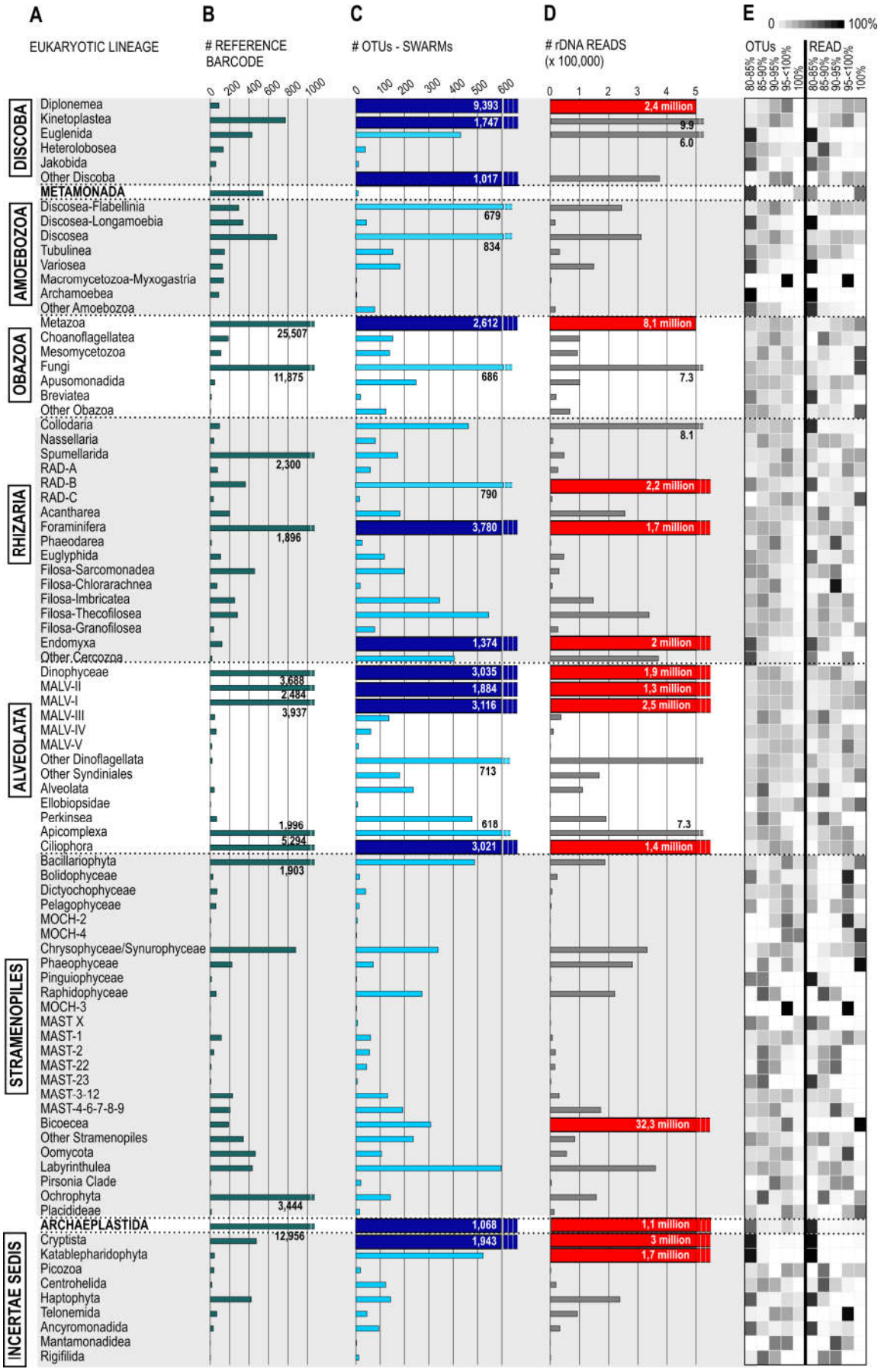
A
EUKARYOTIC LINEAGE
B
REFERENCE BARCODE
C
OTUs - SWARMs
D
rDNA READS (x 100,000)
E
OTUs
READ
DISCOBA
Diplonemea
Kinetoplastea
Euglenida
Heterolobosea
Jakobida
Other Discoba
METAMONADA
AMOEBOZOA
Discosea-Flabellinia
Discosea-Longamoebia
Discosea
Tubulinea
Variosea
Macromycetozoa-Myxogastria
Archamoebea
Other Amoebozoa
OBAZOA
Metazoa
Choanoflagellatea
Mesomycetozoa
Fungi
Apusomonadida
Breviatea
Other Obazoa
RHIZARIA
Collodaria
Nassellaria
Spumellarida
RAD-A
RAD-B
RAD-C
Acantharea
Foraminifera
Phaeodarea
Euglyphida
Filosa-Sarcomonadea
Filosa-Chlorarachnea
Filosa-Imbricatea
Filosa-Thecofilosea
Filosa-Granofilosea
Endomyxa
Other Cercozoa
ALVEOLATA
Dinophyceae
MALV-II
MALV-I
MALV-III
MALV-IV
MALV-V
Other Dinoflagellata
Other Syndiniales
Alveolata
Ellobiopsidae
Perkinsea
Apicomplexa
Ciliophora
STRAMENOPILES
Bacillariophyta
Bolidophyceae
Dictyochophyceae
Pelagophyceae
MOCH-2
MOCH-4
Chrysophyceae/Synurophyceae
Phaeophyceae
Pinguiophyceae
Raphidophyceae
MOCH-3
MAST X
MAST-1
MAST-2
MAST-22
MAST-23
MAST-3-12
MAST-4-6-7-8-9
Bicoecea
Other Stramenopiles
Oomycota
Labyrinthulea
Pirsonia Clade
Ochrophyta
Placidideae
ARCHAEPLASTIDA
INCERTAE SEDIS
Cryptista
Katablepharidophyta
Picozoa
Centrohelida
Haptophyta
Telonemida
Ancyromonadida
Mantamonadidea
Rigifilida

Fig. 2: (Previous page.) **Phylogenetic distribution of the complete assignable eukaryotic deep-sea ribosomal diversity (V9 SSU rDNA) from 20 deep-sea basins at the North- and South Atlantic and Pacific.** (**A**) Deep-branching eukaryotic lineages in the deep sea, ordered by (21). (**B**) Number of reference barcodes used to annotate the metabarcoding data set. (**C**) Eukaryotic deep-sea OTU richness. Dark blue thicker bars indicate the twelve hyperdiverse lineages containing more than 1,000 OTUs. (**D**) Eukaryotic deep-sea abundance expressed as numbers of rDNA reads (the red bars indicate the thirteen most abundant lineages with >1 million reads). (**E**) Percentage of reads and OTUs with 80 to 85%, 85 to 90%, 90 to 95%, 95 to <100%, and 100% sequence similarity to the reference sequence.

the most diverse planktonic eukaryotes in the ocean, showing a depth stratification with higher abdundances and diversity occuring the deep ocean. In the pelagic ocean, they had no clear biogeographic structuring (22). We had a much higher diversity in kinetoplastid flagellates, foraminiferans, ciliates and Cryptista than found in surface waters (13).

There exists an extremely high diversity of protists in the deep sea with large local differences between individual deep-sea basins. This indication of a potentially high global diversity of protists with a major fraction of heterotrophic protists might make them good indicators of changes in environmental conditions in the abyss. In addition, there is an overlap of barotolerant species between surface and deep-sea communities (23), and certainly also between littoral and deep-sea communities. Nevertheless, the deep-sea protistan communities are quite specific. The abyssal sea floor seems to be a mosaic of semi-isolated habitats (17), shaped and maintained on larger as well as small scales by diverse environmental gradients. Nevertheless, the abyssal sea floor might to be a contiguous habitat for microbial eukaryotes on regional scales. Investigating the diversity and distribution of natural microbial communities in Earth's largest habitat is critical to our understanding of global biogeochemical cycles. Unique techniques and large-scale studies, as well as long-term surveys/time series, may further elucidate the diverse composition of deep-sea communities over both space and time.

Acknowledgements

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Supplementary materials

Materials and methods:

Sampling procedure. Sediment samples were collected with a Multi-Corer (MUC) from 20 deep-sea stations during four different expeditions in the Pacific and Atlantic Ocean with the research vessels R/V Sonne and R/V Meteor (SO237, SO223T, M79/1, M139). Samples of the Multi-Corer system were taken from the upper 2 mm sediment layer by means of a sterile syringe. Only tubes with undisturbed sediment and overlaying water were used for further analyses. For 17 stations taken during expeditions SO237, SO223T and M79/1, three replicate samples from three MUCs (corresponds to one core per MUC) were taken in total. Samples were fixated with 70% molecular biology graded ethanol and stored at -80°C for further analyses. For the three stations from the expedition M139, two to four replicates from three MUCs (corresponds to one to two cores per MUC) per station were taken and deep frozen at -80°C.

DNA extraction, PCR amplification, and sequencing of 18S V9 rDNA metabarcodes. Ethanol preserved sediments were put in a speed vac for 45 minutes at 45°C to evaporate the ethanol. Environmental DNA was extracted from 0.5 g sediment of each replicate sample (a total of 1.5 g per stations) using the DNeasy Power Lyzer Power Soil DNA isolation kit (Qiagen) according to the manufacturer's protocol. Prior to the kit, sediment samples were pre-washed with three washing solutions to improve the success of DNA amplification by PCR in marine sediments (24). Total DNA was quantified using a Nanodrop Spectrophotometer. For sediment samples taken during the expeditions SO237, SO223T and M79/1, DNA of the three replicates per station were pooled in same concentrations prior to PCR amplifications. Sediment samples from the expedition M139 were separately PCR amplified without prior pooling of DNA per station to investigate small scale patterns of protist diversity. PCR amplifications of the hyper-variable loop V9 of the 18S rRNA gene was performed with the Phusion® High-Fidelity DNA Polymerase (ThermoFisher) and the forward/reverse primer-pair 1389F (5'- TTG TAC ACA CCG CCC -3') and 1510R (5'- CCT TCY GCA GGT TCA CCT AC -3') (14). The PCR mixtures (25 μL final volume) contained 5 ng of total DNA template with 0.35 μM final concentration of each primer, 3% of DMSO and 2X of GC buffer Phusion Master Mix (Finnzymes). PCR amplifications (98°C for 30 sec; 25 cycles of 10 sec at 98°C, 30 sec at 57°C, 30 sec at 72°C; and 72°C for 10 min) of all samples were carried out with a reduced number of cycles to avoid the formation of chimeras during the plateau phase of the reaction, and in triplicate in order to smooth the intra-sample variance while obtaining sufficient amounts of amplicons for Illumina sequencing. PCR products were run on a 1.5% agarose gel to check amplicon lengths. Amplicons were then pooled and purified using the Quick-gDNA MiniPrep (Zymo Research). Bridge amplification and paired-end sequencing of the amplified fragments were performed using an Illumina Genome Analyzers IIx system.

Reference database. Due to the lack of reference sequences for the V9 region in common databases (NCBI, PR2), we generated a data set consisting of the V9 region of 84 marine protist strains of our Heterotrophic Flagellate Collection Cologne (HFCC) (Table S3). Protistan cultures were concentrated by centrifugation (4000 x g, 20 min at 4°C, Megafuge

2.0 R, Heraeus Instruments). Genomic DNA of each isolated protist strain was extracted using the Quick-gDNA^{TM} Mini Prep Kit (Zymo Research, USA). We amplified a long sequence from the 18S rDNA to the 28S rDNA with the primers 18SFor (5'-AAC CTG GTT GAT CCT GCC AGT-3') binding at the beginning of the 18S rDNA and either NLR1126/22 (5'- GCT ATC CTG AGG GAA ACT TCG G-3') or NLR2098/24 (5'- AGC CAA TCC TTW TCC CGA AGT TAC-3') binding in the 28S rDNA. PCR reactions were performed in 25μl PCR reaction mixtures containing 5.5μl ddH2O, 1.5 units TAQ (Mastermix, VWR Germany), 2μl DNA and 2.5μl of each primer (forward and reverse) at a final concentration of 1.6 nM. The PCR conditions for amplifying the SSU-ITS-LSU region were as follows: pre-denaturation at 98°C for 2 min, 35 cycles of 98°C for 30 s, 55°C for 45 s and 72°C for 4 min 30 sec; final extension at 72°C for 10 min. For bodonid strains, a different primer combination was used: 18SForBodo (5-CTG GTT GAT TCT GCC AGT AGT-3) + NLR1126/22 (5-GCT ATC CTG AGG GAA ACT TCG G-3). Internal primers were used for sequencing (Table S2).

We established a new reference database for the V9 region by combining the Protist Ribosomal Reference database PR2 v4.10.0 (25), the Tara Ocean V9 database (13)), the SILVA_ 132_ SSURef_ Nr99 database (26) as well as the 84 sequences of marine protist strains of the Heterotrophic Flagellate Collection Cologne. Using Cutadapt (27), the reference database was trimmed to the V9 region. As the PCR primers used in this study amplify also prokaryotic taxa, also prokaryotic sequences from the databases were included. Furthermore, we included the trophy levels were possible.

Taxonomic assigment. OTUs were taxonomically assigned to the reference database using VSEARCH's global pairwise alignment with our reference database. Amplicons were assigned to their best hit, or co-best hits in the reference database, using a pipeline called Stampa (16).

Sequence data cleaning, filtering and clustering. Our bioinformatics pipeline allowed filtering of high-quality V9 rDNA sequences (metabarcodes) and their clustering into operational taxonomic units (OTUs, Fig. S1). Overlapping reads were assembled via VSEARCH v.2.6.2 (28) using fastq_ mergepairs. Paired reads were retained for downstream analyses if they contained both forward and reverse primers and no ambiguously named nucleotides (Ns) using cutadapt (27) and VSEARCG. Reads were then dereplicated into strictly identical amplicons with VSEARCH. Metabarcodes were clustered into biologically meaningful OTUs, using Swarm v2.1.5 (15), with the parameter d=1 and the fastidious option on. The most abundant amplicon in each OTU was searched for chimeric sequences with the chimera search module of VSEARCH, and their OTUs were removed even if they occurred in multiple samples. Low abundance OTUs were removed from the combined dataset only if they included > 4 reads. Sequences with a quality value lower than 0.002 were discarded. In addition, OTUs were discarded, when a phylogenetic placement within the kingdom level was not possible. Only OTUs with a pairwise identity of ≥ 80 % to a reference sequence were used for downstream analyses.

Statistical analyses. Stampa plots were produced to visualize our taxonomic coverage assessment to the reference database sequences. A high proportion of environmental

reads assigned with a high similarity to references indicates a good coverage, while low similarity values indicate a lack of coverage (16). Statistical analysis was conducted with R v.3.5.1. The R package vegan, was used to analyse frequency count data derived from OTU clustering. Different functions of vegan were called to randomly subsample our samples (rrarefy function, normalized to the smallest sample size) and to estimate and compare species compositions, using the Jaccard index on presence/absence data. The jaccard distance values were then used for the unweighted pair-group method with arithmetic means (UPGMA) cluster analyses (hclust function). Rarefaction curves were used in order to investigate the degree of sample saturation. Alpha diversity of the stations and grouping of stations was assessed based on OTU richness and the Shannon-Wiener-Index. Figures were made with ggplot2.

Data availability.The data analysed in this study will be available at GenBank's Sequence Read Archive under BioProject number XYZ. The 18S rDNA sequences from our 84 HFCC strains will be deposited at GenBank under the Accession numbers xyz-xyz.

Table S1 -S2

Table S 1: List of sequenced (SSU rDNA V9-region) protist strains from the Heterotrophic Flagellate Culture Collection (HFCC), ordered by classification of eukaryotes from (21). Sequences isolated from samples of the expedition M139 are marked (*). Given are taxonomy, HFCC numbers, expedition name, region, sampling station, coordinates, sampling depth and sampling gear

Species	HFCC	Expedition	Region	Station	Lat/long	Depth [m]	sampled with/from
STRAMENOPILES							
Caecitellus parvulus	118	M71.2	MS	1B	N.A.	N.A.	MUC OW
Caecitellus sp.	812	SO233T	PO	17013-1	10°34.300'N 148°49.000'E	5497	MUC Sed
Caecitellus sp.	811	SO233T	PO	17012-1	10°37.800'N/ 148°37.800'E	5719	MUC Sed
Caecitellus sp.	810	SO233T	PO	17012-1	10°37.800'N/ 148°37.800'E	5719	MUC OW
Caecitellus sp.	742	M139	NAO	978	10°20.382'N/ 36°57.760'W	5134	MUC OW
Caecitellus sp.	761	M139	NAO	S8	11°13.548'N/ 34°46.559'W	surface	*Sargassum*
Cafeteria biegii	113	M71.2	SAO	963	33°44.550'N/ 26°08.500'E	2676	MUC OW
Cafeteria biegii	114	M71.2	MS	983	33°42.910'N/ 26°20.430'E	2691	MUC Sed
Cafeteria burkhardii	187	SO237	NAO	2-3	10°43.112'N/ 25°03.886'W	surface	*Sargassum*
Cafeteria burkhardii	203	SO237	NAO	4-3	10°25.110'N/ 31°04.610'W	5771	MUC OW
Cafeteria burkhardii	167	SO237	NAO	4-3	10°25.110'N/ 31°04.610'W	5771	MUC OW
Cafeteria burkhardii	168	SO237	NAO	4-4	10°25.120'N/ 31°04.620'W	5759	MUC OW
Cafeteria burkhardii	197	SO237	NAO	12-2	19°46.020'N/	8337	MUC OW

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Table S 1 – continued from previous page

Species	HFCC	Expedition	Region	Station	Lat/long	Depth [m]	with/from
					66°49.000′W		
Cafeteria burkhardii	184	SO237	NAO	4-2	10°25.110′N/ 31°04.610′W	surface	Plankton net
Cafeteria burkhardii	205	SO237	NAO	4-2	10°25.110′N/ 31°04.610′W	200	Plankton net
Cafeteria burkhardii	808	SO233T	PO	17013-1	10°34.300′N/ 148°49.000′E	5497	Sediment
Cafeteria burkhardii	803	SO233T	PO	17006-2	14°03.700′N/ 144°03.200′E	1259	Stones
Cafeteria burkhardii	804	SO233T	PO	17009-1	13°58.600′N/ 145°30.720′E	2687	MUC OW
Cafeteria burkhardii	827	SO233T	PO	17019-2	15°13.000′S/ 173°31.100′E	2776	MUC OW
Cafeteria burkhardii	805	SO233T	PO	17009-1	13°58.640N/ 145°30.719E	2687	MUC OW
Cafeteria burkhardii	806	SO233T	PO	17009-1	13°58.640N/ 145°30.719E	2687	MUC OW
Cafeteria burkhardii	734	M139	NAO	937	15°53.980′N/ 68°54.430′W	1500	CTD
Cafeteria burkhardii	736	M139	NAO	937	15°53.980′N/ 68°54.430′W	2000	CTD
Cafeteria burkhardii	750	M139	NAO	938	15°55.980′N/ 68°53.280′W	4000	MUC OW
Cafeteria burkhardii	34	M48.1	SAO	344	17°04.935′S/ 04°40.805′E	surface	Plankton net
Cafeteria burkhardii	116	M71.2	MS	983	33°42.910′N/ 26°20.430′E	2691	MUC Sed
Cafeteria burkhardii	117	M71.2	MS	51	34°30.310′N/ 26°11.500′E	4323	MUC OW
Cafeteria burkhardii	32	Hiddensee	BS	N.A.	N.A.	surface	Sediment
Cafeteria graefii	33	M48.1	SAO	347	16°14.991′S/ 05°26.700′E	5388	AT
Cafeteria malediviensis	661	Maldives	IO	House Reef	03°28.696′N/ 72°74.180′E	1	Biofilm
Ciliophyrs sp.	198	SO237	NAO	43168	11°41.37′ N/ 47°57.36′W	0	*Sargassum*
Developayella sp.	215	Azores	NAO	N.A.	N.A.	N.A.	Littoral
Halocafeteria sp.	768	M139	NAO	S8	11°13.548′N/ 34°46.559′W	surface	*Sargassum*
Paraphysomonas sp.	221	Azores	NAO	N.A.	N.A.	N.A.	Littoral
Placidia cafeteriopsis	745	M139	NAO	940	15°53.210′N/ 68°55.740′W	4033	MUC OW
Placidia cafeteriopsis	748	M139	NAO	940	15°53.210′N/ 68°55.740′W	4033	MUC OW
Pseudobodo sp.	169	SO237	NAO	43163	10°25.110′ N/ 31°04.610′W	5771	MUC OW
Pseudobodo tremulans	820	SO223T	PO	17013-1	10°34.300′N/ 148°49.000′E	5497	MUC OW

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Table S 1 – continued from previous page

Species	HFCC	Expedition	Region	Station	Lat/long	Depth [m]	with/from
Pseudobodo tremulans	813	SO223T	PO	17012-1	10°37.800′N/ 148°37.800′E	5719	MUC OW
Bicosoecide	772	M139	NAO	S8	11°13.548′N/ 34°46.559′W	surface	*Sargassum*
Bicosoecide	743	M139	NAO	940	15°53.210′N/ 68°55.740′W	4033	MUC OW
Bicosoecide	657	Maldives	IO	Beach bar	3°28′59.5′N/ 72°48′21.5E	0.5	Sediment
Stramenopile	769	M139	NAO	S8	11°13.548′N/ 34°46.559′W	surface	*Sargassum*
Stramenopile	214	Azores	NAO	N.A.	N.A.	N.A.	Littoral
Stramenopile	218	Azores	NAO	N.A.	N.A.	N.A.	Littoral
CERCOZOA							
Massisteria marina	194	SO237	NAO	43161	10°43.112′N/ 25°03.886′ W	surface	*Sargassum*
Massisteria marina	176	SO237	NAO	43163	10°25.110′N/ 31°04.610′W	5771	Sediment
Cercozoa	177	SO237	NAO	43106	10°21.010′ N/ 36°57.580′W	5138	MUC OW
Cercozoa	195	SO237	NAO	43143	19°46.020′N/ 66°49.000′W	surface	*Sargassum*
DISCOBA							
Bodonidae	174	SO237	NAO	43194	10°25.120′N/ 31°04.620′W	5759	MUC OW
Keelungia nitschei	166	SO237	NAO	43381	10°42.580′N/ 42°40.990′W	5117	MUC OW
Rhynchomonas nasuta	171	SO237	NAO	43324	19°46.010′N/ 66°49.990′W	8340	MUC OW
Rhynchomonas nasuta	196	SO237	NAO	43143	19°46.020′N/ 66°49.000′W	8337	MUC OW
Rhynchomonas nasuta	173	SO237	NAO	43324	19°46.010′N/ 66°49.990′W	surface	*Sargassum*
Rhynchomonas nasuta	656	Maldives	IO	House Reef	3°29′23.200′N/ 72°47′59.900′E	12	Sediment
Neobodo designis	828	SO233T	PO	17019-2	15°13.000′S/ 173°31.100′E	2776	
Neobodo designis	817	SO233T	PO	17013-1	10°34.300′N/ 148°49.000′E	5497	Sediment
Neobodo designis	141	M79.1	SAO	551	035°13.150′W/ 26°34.070′S	4474	Sediment
Neobodo designis	142	M79.1	SAO	548	035°13.180′W/ 26°34.050′S	4479	MUC OW
Neobodo designis	143	M79.1	SAO	548	35°13.180′W/ 26°34.050′S	4479	Sediment
Neobodo sp.	731	M139	NAO	943	15°53.269′N/ 68°55.650′W	1500	CTD
Neobodo sp.	751	M139	NAO	978	10°20.382′N/ 36°57.760′W	5134	MUC OW

Continued on next page

Table S 1 – continued from previous page

Species	HFCC	Expedition	Region	Station	Lat/long	Depth [m]	with/from
Neobodo sp.	216	Azores	NAO	N.A.	N.A.	N.A.	Littoral
Bodonidae	729	M139	NAO	943	15°53.269′N/ 68°55.650′W	600	CTD
Bodonidae	217	Azores	NAO	N.A.	N.A.	N.A.	Littoral
Bodonidae	654	Maldives	IO	House Reef	3°29.387′N/ 72°47.998′E	11	Sediment
Bodonidae	655	Maldives	IO	House Reef	3°29.387′N/ 72°47.998′E	11	Sediment
Excavata	670	Maldives	IO	Water bunga-low	3°28.837′N/ 72°48.035′E	1.5	Sediment
ALVEOLATA							
Aristerostoma sp.	202	SO237	NAO	43194	10°25.120′N/ 31°04.620′W	5759	MUC OW
Aristerostoma sp.	193	SO237	NAO	43168	11°41.370′N/ 47°57.360′W	4996	MUC OW
Aristerostoma sp.	201	SO237	NAO	43199	11°41.360′N/ 47°57.340′W	5000	MUC OW
Protocruzia contrax	186	SO237	NAO	43293	19°46.020′N/ 66°49.000′W	surface	*Sargassum*
Pseudocohnilembus persalinus	814	SO233T	PO	17009-1	13°58.600′N/ 145°30.720′E	2687	MUC OW
Pseudocohnilembus persalinus	816	SO233T	PO	17013-17014	N.A.	N.A.	Rain
Pseudocohnilembus persalinus	822	SO233T	PO	17004	25°22.700′N/ 134°22.514′E	5276	CTD
Uronema sp.	744	M139	NAO	953	23°33.234′N/ 48°5.0377′W	4296	Sediment
Ciliat	766	M139	NAO	S8	11°13.548′N/ 04°46.559′W	surface	*Sargassum*
OBAZOA							
Amastigomonas sp.	180	SO237	NAO	43168	11°41.370′N/ 47°57.360′W	surface	*Sargassum*
Choanoflagellata	164	SO237	NAO	43194	10°25.120′N/ 31°04.620′W	5767	MUC OW
Choanoflagellata	165	SO237	NAO	43165	10°21.030′N/ 36°57.590′W	5138	MUC OW
Fabomonas tropica	175	SO237	NAO	43168	11°41.370′N/ 47°57.360′W	surface	*Sargassum*
Ministeria vibrans	191	SO237	NAO	43168	11°41.370′N/ 47°57.360′W	4996	MUC OW
Ministeria vibrans	178	SO237	NAO	43163	10°25.110′N/ 31°04.610′W	surface	Plankton net
CRYPTOPHYTA							
Goniomonas sp.	157	Azores	NAO	N.A.	N.A.	N.A.	Littoral

Table S 2: Primers used for this study for sequencing the 18S rDNA of our isolated and cultivated deep-sea and surface water strains.

Primer	Primer sequence (5′-3′)
18S-For	AACCTGGTTGATCCTGCCAGT
18SF-1280	TGCATGGCCGTTCTTAGTTGGTG
18S_1480F	TAACAGGTCTGTGATGCCC
1389F	TTGTACACCCGCCC
1630F	TTGTACACACCGCCCGTC
18S-Rev	TGATCCTTCCGCAGGTTCACCTAC
Rev-1-for-ITS	CGTAACAAGGTTTCCGTAGGT

Figures

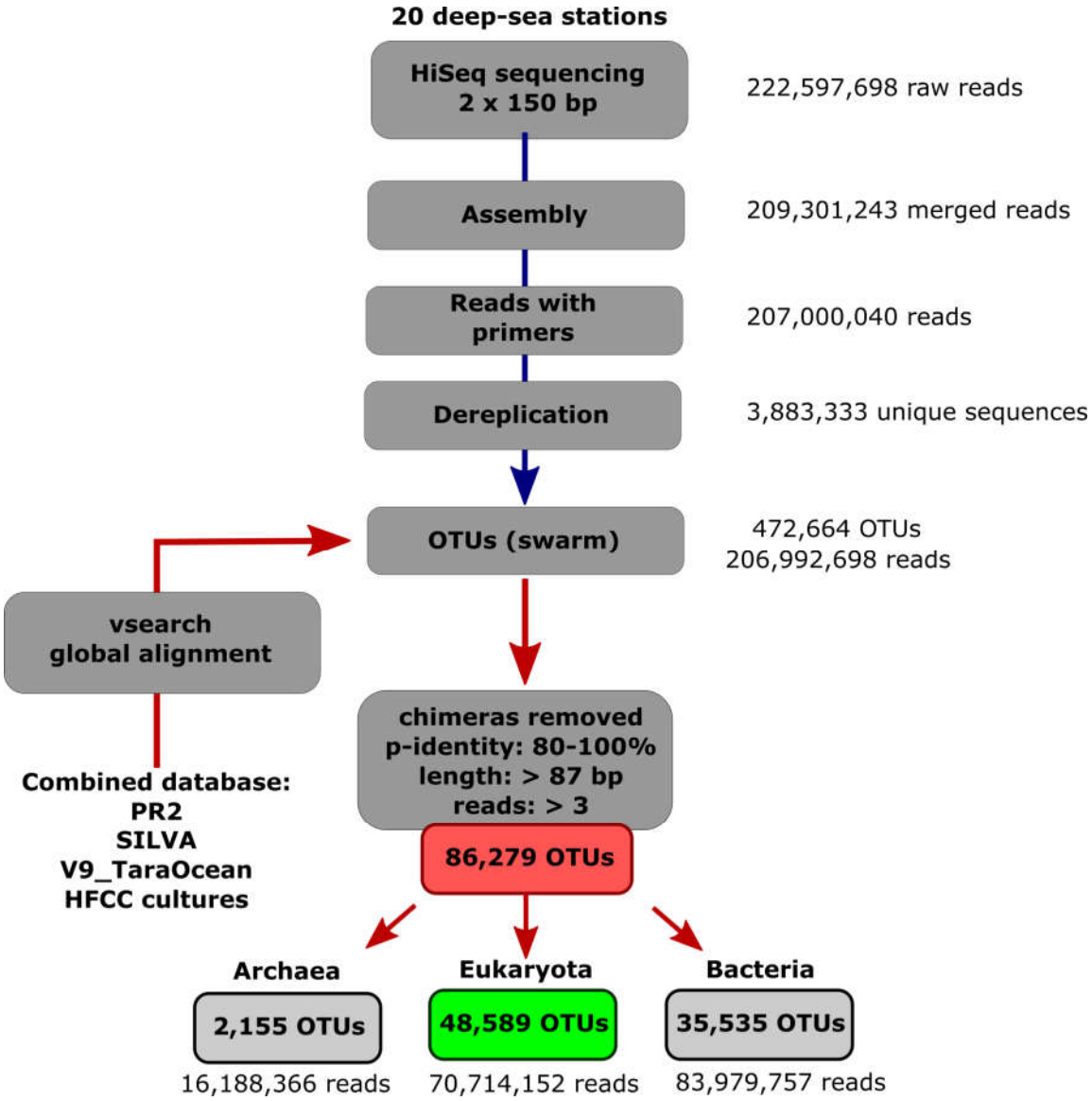

Fig. S 1: Bioinformatics pipeline. Raw V9 rDNA reads from the 20 deep-sea stations were merged and only used for downstream analyses, when they contained both primers and no ambigious N's. After dereplications, unique sequences (metabarcodes) were clustered into operational taxonomic units (OTUs) using the Swarm algorithm. A taxonomic path of the OTU representatives was assigned using Stampa. After removal of chimeras, OTUs with less than 80% p-identity and smaller than 87 bp and low abundant OTUs (read abundance <4), 86,279 meaningful OTUs were left including Bacteria, Archaea and Eukaryota.

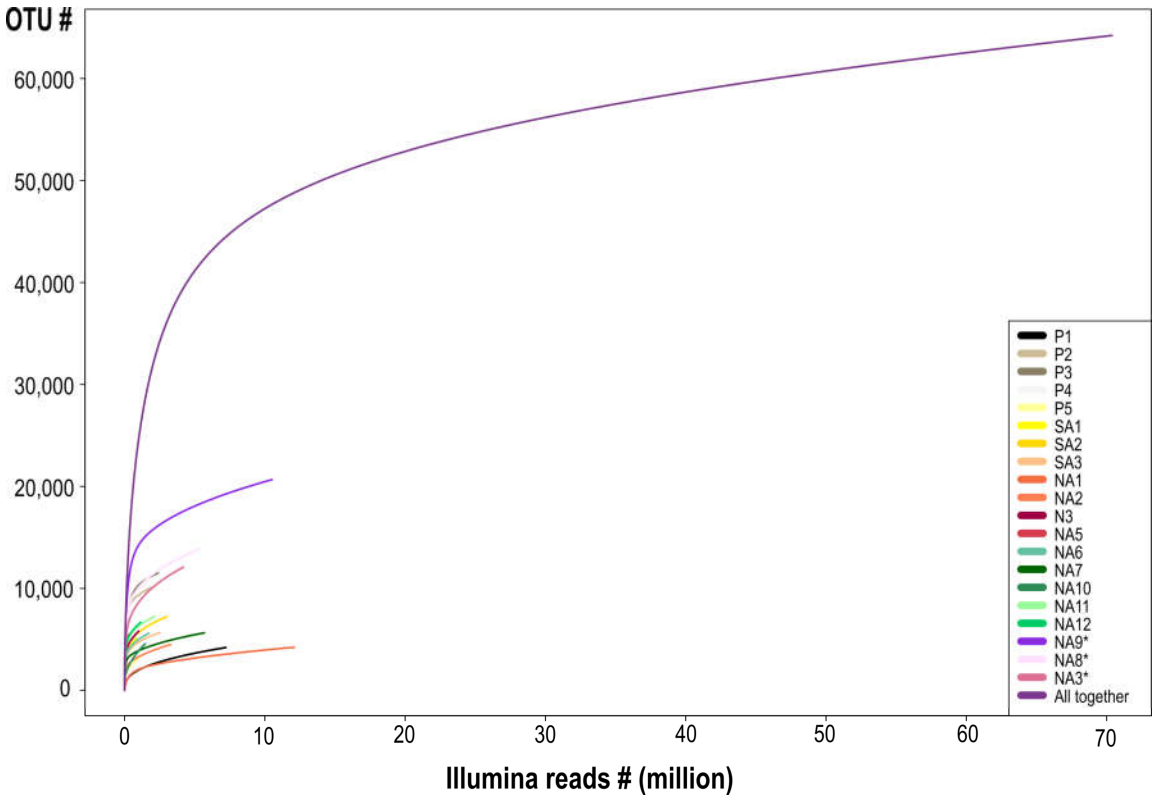

Fig. S 2: Rarefaction curve. Saturation curves for eukaryotic metabarcodes richness from the 20 stations and all samples together.

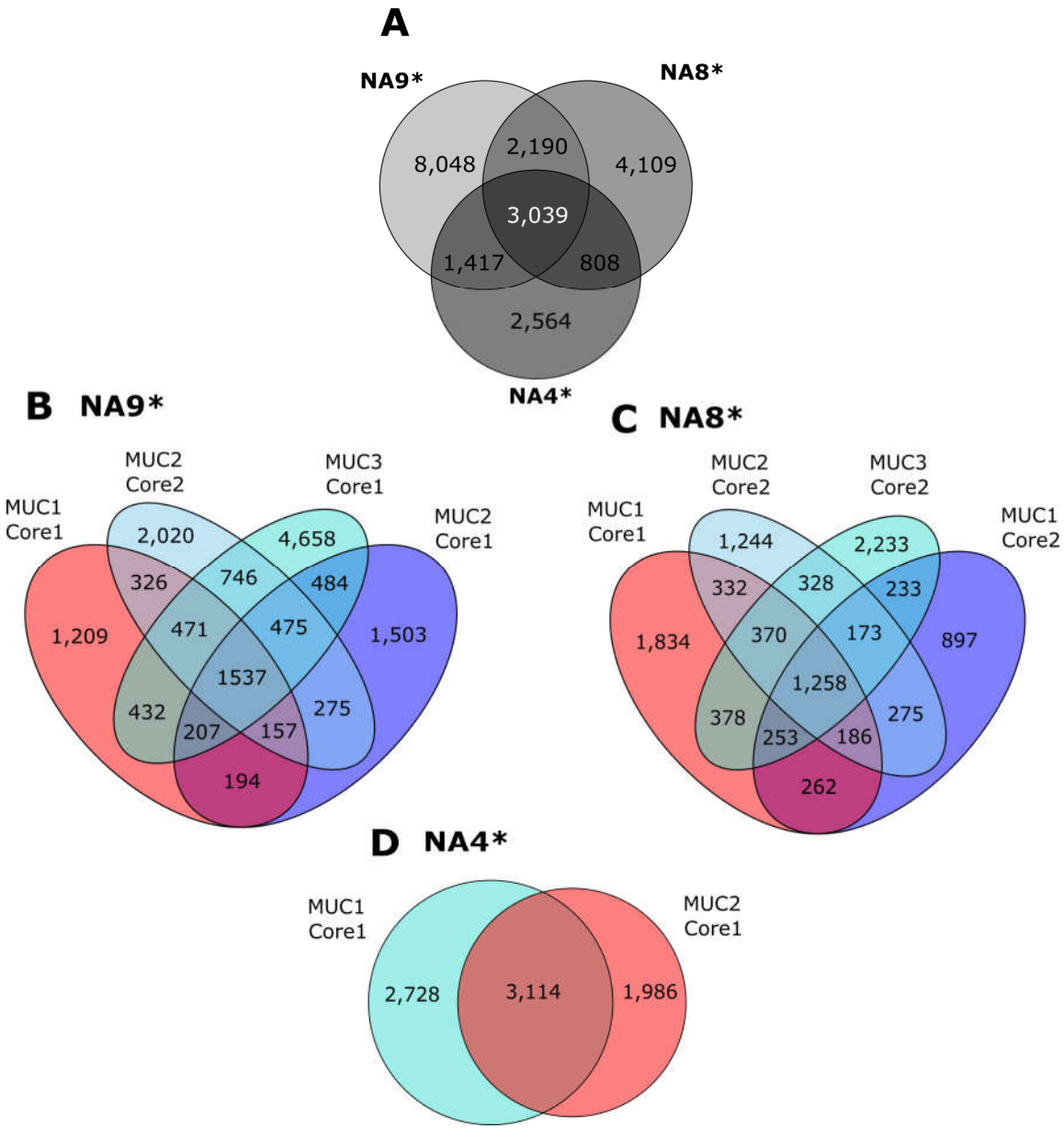

Fig. S 3: Small scale pattern of deep-sea heterotrophic protists. Number of shared and unique OTUs between (**A**) all three stations (inter-similarity) and per station (intra-station similarity) for (**B**) station NA9* (total richness = 14,694), (**C**) NA8* (B, total richness = 10,146) and (**D**) NA3* (C, total richness = 7,828) sampled during the deep-sea expedition (M139).

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Chapter 4

Potential Contribution of Surface-Dwelling *Sargassum* Algae to Deep-Sea Ecosystems in the Southern North Atlantic

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Potential contribution of surface-dwelling *Sargassum* algae to deep-sea ecosystems in the southern North Atlantic

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ABSTRACT

Deep-sea ecosystems, limited by their inability to use primary production as a source of carbon, rely on other sources to maintain life. Sedimentation of organic carbon into the deep sea has been previously studied, however, the high biomass of sedimented *Sargassum* algae discovered during the VEMA Transit expedition in 2014/2015 to the southern North Atlantic, and its potential as a regular carbon input, has been an underestimated phenomenon. To determine the potential for this carbon flux, a literature survey of previous studies that estimated the abundance of surface water *Sargassum* was conducted. We compared these estimates with quantitative analyses of sedimented *Sargassum* appearing on photos taken with an autonomous underwater vehicle (AUV) directly above the abyssal sediment during the expedition. Organismal communities associated to *Sargassum fluitans* from surface waters were investigated and *Sargassum* samples collected from surface waters and the deep sea were biochemically analyzed (fatty acids, stable isotopes, C:N ratios) to determine degradation potential and the trophic significance within deep-sea communities. The estimated *Sargassum* biomass (fresh weight) in the deep sea (0.07–3.75 g/m^2) was several times higher than that estimated from surface waters in the North Atlantic (0.024–0.84 g/m^2). Biochemical analysis showed degradation of *Sargassum* occurring during sedimentation or in the deep sea, however, fatty acid and stable isotope analysis did not indicate direct trophic interactions between the algae and benthic organisms. Thus, it is assumed that components of the deep-sea microbial food web form an important link between the macroalgae and larger benthic organisms. Evaluation of the epifauna showed a diverse nano- micro-, meio, and macrofauna on surface *Sargassum* and maybe transported across the Atlantic, but we had no evidence for a vertical exchange of fauna components. The large-scale sedimentation of *Sargassum* forms an important trophic link between surface and benthic production and has to be further considered in the future as a regular carbon input to the deep-sea floor in the North Atlantic.

1. Introduction

Oceans cover roughly 70% of the earth's surface and two third of their volume is deeper than 1000 m. Therefore, the deep sea can be considered the largest biome on earth. For a long time it was considered to be a single, featureless and stable environment, lacking barriers for the pelagic dispersal of species. Due to the absence of light, most of the deep-sea life is assumed to be heterotrophic and dependent on the production and sinking of organic matter from the surface waters to act as a carbon source (Johnson et al., 2007). Carbon flux decreases exponentially with depth (Suess, 1980). This creates a carbon-limited ecosystem, which leads to a low abundance of benthic organisms (Rex

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et al., 2006). Long-term studies have investigated surface primary production as well as sedimentation to the deep sea (e.g. Rowe and Staresinic, 1979; Krause-Jensen and Duarte, 2016). Trap measurements of carbon flux showed the importance of vertical energy transport and its resulting effect on deep-sea benthic ecosystems (e.g. Smith et al., 2006).

During the Vema-TRANSIT cruise with R/V Sonne in January 2015 we were especially interested in the potential dispersal of benthic organisms along the Vema Fracture Zone and the comparison of benthic communities between the Eastern and Western Atlantic basins. Since the potential dispersal of a large number of individuals over wide geographical ranges leads to a high gene flow, it was assumed that mainly cosmopolitan species exist (Gage and Tyler, 1991) and allopatric speciation was supposed to be a rare event (Palumbi, 1994). During the last four decades, different studies showed that the species diversity in deep-sea environments is high, but the underlying mechanisms are poorly understood (White, 1987; Palumbi, 1994; Rex and Etter, 2010). Today, the importance of water currents in the deep sea as possible pathways for dispersal is basically understood (Dickson et al., 1982; Gage, 1996; Levin et al., 2001). For the tiny protists, the occurrence of similar genotypes has been shown in surface as well as deep-sea samples (e.g. Scheckenbach et al., 2005). Thus, potentially also sedimentation from surface aggregations (e.g. on the surface of floating macrophytes) could influence distribution patterns of some protists in the deep sea.

Sargassum floating mats form a unique ecosystem comprising the accumulations of holopelagic brown algae, *S. natans* (Linnaeus) Gaillon 1828 and *S. fluitans* (Børgesen) Børgesen 1914 (Laffoley et al., 2011; Huffard et al., 2014; Schell et al., 2015). Our interest in the wider importance of these floating systems for the abyssal areas was raised due to the detection of isolated branches of *Sargassum* on the seafloor even in single cores of multicorer samples from abyssal depths. The presence of relatively large clumps of *Sargassum* in photographs of the seafloor had already been reported by Schoener and Rowe (1970) and later in several publications (e.g. Wolff, 1979; Wei et al., 2012) giving clear evidence that *Sargassum* is sedimenting down to the deep sea. Rowe and Staresinic (1979) already estimated that 10% of organic carbon sedimented to the North Atlantic deep sea could be due to *Sargassum*. In their recent review, Krause-Jensen and Duarte (2016) concluded that macroalgae represent an important source of the overall carbon sequestered in the deep ocean. The means by which *Sargassum* sinks is still not completely understood; very little is known on the contribution of epifauna to the sedimentation process of *Sargassum*. The resulting accumulation of sedimenting *Sargassum* represents a potentially large and consistent carbon flux to deep-sea ecosystems. While the particular abundance and nature of holopelagic *Sargassum* is relatively unique to the North Atlantic (particularly the Sargasso Sea), the mechanism of large-scale sedimentation of macrophytes as a carbon flux is an important finding and is translated through the world's oceans (Krause-Jensen and Duarte, 2016). One can easily imagine that accumulations of *Sargassum* might influence distribution patterns of deep-sea fauna which is generally limited in carbon sources.

The importance of organic material as both a food source and habitat for sessile taxa on the ocean's deep-sea floor was highlighted by several authors (e.g. Wolff, 1979; Grassle and Morse-Porteous, 1987; Johnson et al., 2007; Bernardino et al., 2010). Usually, the origin of such organic material is not well defined. Fatty acids (FAs) can be used as biomarkers because they are transmitted between tissues of food and feeders almost without change (Howell et al., 2004; Iverson, 2009) and so allow interpretations about a specimens' diet (e.g. Daalsgard et al., 2003; Peters, 2006; Würzberg et al., 2011). The usage of FAs as a biomarker approach relies on the assumption that some FAs can only be synthesized by certain organisms. They become traceable components of an animals' diet at higher trophic levels. Like the FA approach, stable isotopes can provide information about the long-term diet of organisms (Tieszen et al., 1983; Ponsard and Arditi, 2000; Laakmann and Auel, 2010). Furthermore, it is a method to detect relative trophic levels as well as possible carbon sources of ecosystems through selective metabolic fractionations.

After our attention was attracted during the cruise by findings of leaflets of *Sargassum* in the small areas of MUC corers, we used the unique chance during the Vema-TRANSIT expedition, to get quantitative estimates of the *Sargassum* in the abyssal region using AUV photographing of the seafloor at the respective stations. Nano-, micro-, meio-, and macrofauna that were found on the surface of *Sargassum* were documented and compared within stations. Furthermore, we performed biochemical analysis on the surface and deep-sea samples, including C:N ratios as well as dry and ash-free dry weights to determine degradation that occurs during sedimentation. In addition, stable isotope and fatty-acid analysis of deep-sea macrofauna, *Sargassum* and sediment samples were conducted to investigate relative trophic levels and the potential of *Sargassum* as a direct food source for higher trophic levels.

We hypothesized that sedimented *Sargassum* could reach significant biomasses compared to floating *Sargassum* in surface waters. Derived from the observations that some sedimented *Sargassum* could still contained pigments, we assumed that sedimentation should be relatively high. Epifauna organisms or remains of it might be visible in sedimented *Sargassum*. Further, we presumed that biochemical signals of *Sargassum* can be traced in macrofauna organisms.

2. Material and methods

2.1. Sampling

Samples were collected during the VEMA Transit expedition on R/V Sonne (SO 237; December-January 2014/15) conducted to analyze potential biological and geological differences between the eastern and western basins of the equatorial Atlantic (Brandt et al., 2017; Devey et al., 2017). The cruise followed the southern edge of the westward-directed North Equatorial Current and the northwestward-directed Antilles Current located within the North Atlantic Subtropical Gyre which includes the Sargasso Sea (Fig. 1).

Samples of *Sargassum* were collected from the surface by means of a 10 l bucket and fishing gear, while benthic deep-sea samples were collected by a camera-epibenthic sledge (C-EBS) (C-EBS, Brandt et al., 2013; Table 1). The nano-, micro-, meio- and macrofauna associated with the surface-collected *Sargassum* samples were microscopically investigated immediately after sampling (see below). Parts of the material collected from the surface as well as the deep sea were stored at −80 °C to be used for later biochemical studies. Additionally, sediment sampled with a Multi-Corer (MUC) was used for biochemical studies. Deep-sea macrofauna, sampled by the C-EBS, were also used for stable isotope analysis.

2.2. Biomass estimation of Sargassum

An estimation of the biomass of *Sargassum* in the deep sea was carried out for three stations of the VEMA expedition using photos taken by an automated underwater vehicle (AUV, HYDROID Inc.). The AUV was deployed for mapping purposes of the seafloor, using a Pike camera mounted with a 15 mm Nikkor underwater lens, cropped to a focal length of 22 mm providing a field of view of 41 degrees. All approximately 27,000 photos from three AUV deployments were screened for *Sargassum* (Abyss 163: 1712 photos; Abyss 165: 2153 photos; Abyss 166: 145 photos). Out of these total 4010 *Sargassum*-containing pictures, every randomly chosen tenth picture was quantitatively analyzed using ImageJ (https://imagej.nih.gov/ij/) regarding the surface area covered by *Sargassum* for those pictures. For a minimum estimation, *Sargassum* in the pictures was assumed to be only one layer thick. A program (Tiffy2tiff, GEOMAR, Kiel) was written in order to extract metadata from the photos regarding the coordinates of the AUV, depth,

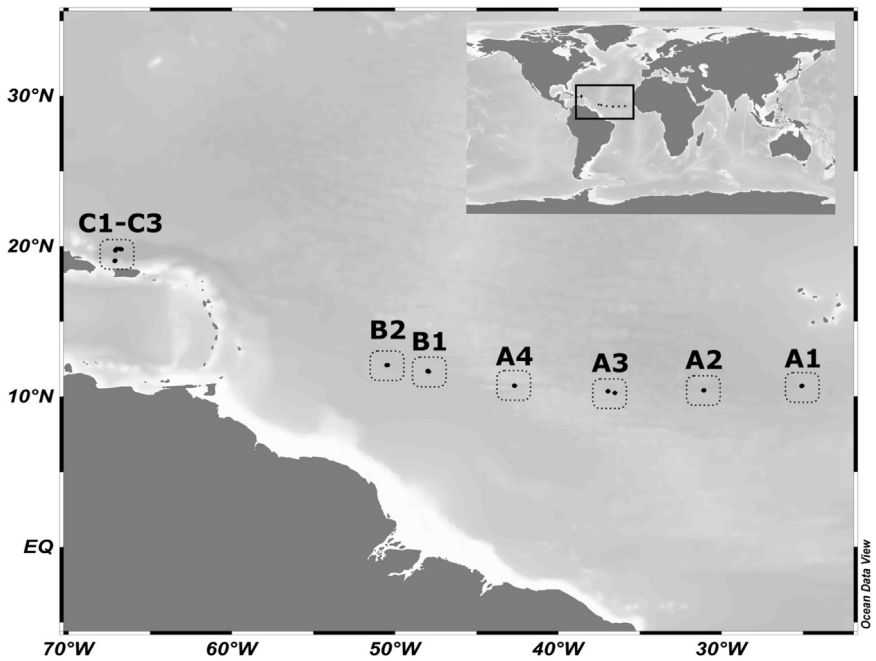

Fig. 1. Sampling stations of VEMA Transit expedition with R/V Sonne (SO 237; December-January 2014/15). The details of sampling gear used at the different stations are given in Table 1. Station map was created with Ocean Data View (Schlitzer, 2012).

altitude above sea floor, and the pitch and roll for each photo. Based on the lens and metadata, the dimensions of each photo were calculated and inserted into ImageJ to determine the overall surface area of the *Sargassum* in each photo. The average surface area coverage per picture was calculated for each site and then compared with the ratio of *Sargassum* containing pictures to total pictures per AUV deployment to determine a total biomass per deployment. Surface collected *Sargassum fluitans* (stored frozen at −80 °C until analysis in the home lab) were used to determine an average weight per centimeter squared (see below), to estimate the biomass of *Sargassum* per m^2 in each inspected photo.

2.3. Sedimentation rates of Sargassum algae

We carried out preliminary studies on the sedimentation of freshly collected *Sargassum fluitans* on board over a depth of only 20 cm revealing first estimates of about 35 s/m sedimentation rate. Since there are very few data on sedimentation rates in the literature, we further analyzed sedimentation rates of *Sargassum*. In the absence of *S. fluitans*, we used a non-holopelagic marine species (tentatively identified as *Sargassum vulgare* C. Agardh 1820) which has a similar morphology to *S. fluitans. S. vulgare* was collected at the coast of the Canary Islands at depth of two meters attached to a rocky substrate. The algae showed no signs of decomposition or deterioration. In order to determine the maximum sedimentation rate, all bladders were cut from the plant. Four replicated experiments were carried out determining the rate of sedimentation over a distance of 10 m close to the sampling area of the algae in the North Atlantic (Puerto de Mogan, Canary Islands). Depths and times were recorded using a Mares Smart dive computer, while free diving.

2.4. Epifauna composition on floating Sargassum

Sargassum surface samples were collected from six stations (Table 1) during the cruise and analyzed to determine associated nano-, micro-, meio- and macrofauna immediately after sampling. For each surface sample, we selected a volume of 570 cm^3 per station for an analysis of meio- and macrofauna. The sessile fauna was separated from the mobile fauna, while the supernatant with the mobile fauna was sieved through three mesh sizes (1000 µm, 500 µm and 100 µm). The samples were qualitatively examined for mobile micro- and macrofauna, prior to fixation of the selected volume in 96% ethanol. Observed species were photo-documented and fixed. Only organisms from 1000 µm and 500 µm have been analyzed so far. Quantitative comparison between the six stations was not possible, thus, for standardization sessile fauna was investigated using a squared container with an area of 91.5 cm^2. Organisms were investigated using a light microscope and binocular. In addition, studies were carried out by scanning electron microscopy (some crustaceans, mollusks).

Nano- and microfauna was investigated in freshly collected samples suspended in sterilized seawater. Two sets of culture flasks at each station were used for qualitative studies. One set of replicates of about 4 cm^3 was transferred into 50 ml tissue culture flasks (Sarstedt, Nümbrecht, Germany) filled with 30 ml autoclaved seawater (35 PSU). In addition, about 10 cm^2 of the sample at each station were transferred to 600 ml tissue culture flasks. Culture flask were inspected under an inverted microscope (Zeiss Primovert with LD objectives of 10–40* magnification) immediately after collection of samples and in intervals of three days after inoculation.

2.5. Biochemical analyses of Sargassum

2.5.1. Fresh, dry and ash-free dry weights and C:N ratios

Frozen samples from both surface and deep-sea collected *Sargassum* were analyzed for fresh, dry and ash-free dry weight, as well as C:N ratios. Immediately after sampling, samples were stored at −80 °C. One "leaf" per sample, with three replicates each, was selected for the measurements. Fresh weights were determined after blotting with filter paper to remove free water droplets accumulated during thawing. For determining dry weights, samples were dried in a compartment dryer at 80 °C until weight became constant (approx. 19 h). For determining the ash-free dry weight, dried samples were burned in a muffle furnace at 500 °C for 3 h. For the C:N analysis, dried samples were ground to a fine powder inside small glass containers and weighed into pressed tin capsules (3 mg). The total content of carbon and nitrogen was determined using an Organic Elemental Analyzer, Flash EA 2000 from Thermo Scientific. In addition, "leafs" of surface water collected *Sargassum* incubated for 18 months at 10 °C were analyzed for C:N ratios and weights to estimate long-term degradation.

Table 1
Station list of collected *Sargassum fluitans* from the surface and deep sea as well as stations sampled for sediment and fauna during the research cruise SO237. A. Surface samples. B. Deep-sea samples taken with the camera-epibenthic sledge (C-EBS) and Multi-Corer (MUC). C. Analysis of the oceanic floor by an automated underwater vehicle (AUV). Explanations of superscripts: ([1]) Samples used for biochemical analyses of *Sargassum* material collected from surface waters and degraded for approx. 1 ½ years under laboratory conditions in culture flasks; ([2]) *Sargassum* samples taken from surface waters; ([3]) sediment samples; ([4]) sampling of deep-sea macrofauna. *Sargassum* samples taken from surface waters (A) and the deep sea (B) were used to analyze associated fauna.

Area/ Site	Date/UTC Time	Depth [m]	Sampling	Latitude/ Longitude
A. Surface samples				
A1/2	19.12.2014/ 11:32	Surface	Bucket	10°43.118'N/ 25°03.893'W
[1]A2/4	26.12.2014/ 12:16	Surface	Bucket	10°25.114'N/ 31°04.617'W
A2/4	28.12.2014/ 16:58	Surface	Bucket	10°24.481'N/ 31°05.318'W
[1, 2]A3/6	03.01.2015/ 14:54	Surface	Fishing gear	10°14.161'N/ 36°31.615'W
[1]A4/8	08.01.2015/ 19:10	Surface	Fishing gear	10°42.645'N/ 42°41.893'W
[1]B1/9	12.01.2015/ 00:51	Surface	Fishing gear	11°41.357'N/ 47°57.334'W
[1]C1/12	19.01.2015/ 00:54	Surface	Bucket	19°43.400'N/ 67°08.010'W
B. Deep-sea samples				
A1/2–6	20.12.2014	5520	C-EBS	10°42.330'N/ 25°05.580'W
A1/2–7	20.12.2014	5514	C-EBS	10°41.370'N/ 25°05.137'W
[3]A2/4–3	26.12.2014	5771	MUC	10°25.110'N/ 31°04.610'W
[2, 4]A2/4–8	27.12.2014	5735	C-EBS	10°24.161'N/ 31°06.205'W
[2, 4]A2/4–9	27.12.2014	5735	C-EBS	10°24.082'N/ 31°04.795'W
A3/6–7	02.01.2015	5085	C-EBS	10°20.659'N/ 36°57.010'W
A3/6–8	02.01.2015	5119	C-EBS	10°21.542'N/ 36°57.236'W
[3]A4/8–6	07.01.2015	5180	MUC	10°43.540'N/ 42°41.580'W
[2, 4]A4/8–4	06.01.2015	5176	C-EBS	10°43.000'N/ 42°39.910'W
B1/9–2	11.01.2015	4995	C-EBS	11°40.299'N/ 48°00.071'W
[2, 4]B1/9–8	12.01.2015	5004	C-EBS	11°39.014'N/ 47°56.168'W
[2, 4]B2/11–1	14.01.2015	5093	C-EBS	12°05.732'N/ 50°30.239'W
[2, 4]B2/11–4	14.01.2015	5130	C-EBS	12°04.753'N/ 50°30.348'W
[3]B2/11–5	14.01.2015	5091	MUC	12°05.400'N/ 50°26.980'W
C1/12–5	20.01.2015	8339	C-EBS	19°49.500'N/ 66°50.970'W
C1/12–6	21.01.2015	8340	C-EBS	19°48.490'N/ 66°45.440'W
C2/13–4	23.01.2015	8329	C-EBS	19°46.730'N/ 67°06.210'W
C2/13–5	23.01.2015	8082	C-EBS	19°49.850'N/ 67°02.910'W
C3/14–1	24.01.2015	4552	C-EBS	19°00.760'N/ 67°10.219'W
C3/14–2	25.01.2015	4930	C-EBS	19°03.044'N/ 67°08.650'W
C. AUV deployment			Dive Number	
A3/6–2	01.01.2015	5136	ABYSS 163	10°20.998'N/ 36°57.616'W
B1/9–6	12.01.2015	4977	ABYSS 165	11°42.58'N/ 47°59.07'W
B2/11–3	14.01.2015	5093	ABYSS 166	12°05.99'N/ 50°28.4'W

2.5.2. Fatty acid analysis

All tissue (including Polychaeta, Amphipoda, Decapoda, Asteroidea, Ophiuroidea) samples and *Sargassum fluitans* samples were lyophilized for 24 h, sediment samples for 48 h. Dry masses were then determined using a microbalance scale (Sartorius ISO 9001 (± 2 μg)), with samples being kept in a desiccator to prevent hydration during measurements. For the extraction, samples were transferred into glass vials with 4 ml of dichlormethane: methanol (DcM:MeOH) (2:1/v:v) for at least one week and stored at −30 °C. Afterwards, the solid parts were stored at −80 °C for the stable isotope analysis. An internal standard solution, Tricosanoic acid (S23:0), with a concentration of 0.1 mg ml^{-1} was added based on the dry weight of the sample. To maintain an equal volume of sample, the standard solution was filled up with DcM:MeOH to a final volume of 1 ml before 1 ml of aqueous KCl solution (0.88%) was added. The samples were then centrifuged for 15 min at 1200 rpm at 0 °C and then vaporized with N_2. Next, the samples were dissolved in 500 μl DcM:MeOH and an aliquot was stored as a backup. The rest was mixed with 1 ml of methanol with 3% of concentrated Sulfuric acid (H_2SO_4) and heated up to 80 °C for 4 h to esterify the FAs into their methyl ester derivatives (FAMEs) (Kattner and Fricke, 1986). For the FAME extraction, 2 ml of distilled water and 1 ml hexane were added. The extraction with hexane, centrifugation for 10 min with 1200 rpm at 0 °C and vaporization with elemental nitrogen (N_2) was repeated three times for each sample. This was followed by dilution with hexane and the analysis in a gas chromatograph. The FAMEs were detected and identified using the retention times compared to those of Marinol, using the software Agilent OpenLab Data analysis, as well as analyzed manually afterwards. Dirt and blurred peaks were excluded.

2.5.3. Stable isotope analysis

Stable isotope analysis was conducted for *Sargassum fluitans* and sediment samples as well as for the sampled macrofauna including Polychaeta, Amphipoda, Decapoda, Asteroidea, Ophiuroidea. The *Sargassum fluitans* samples were roughly cleaned of epibionts and after defatting with DcM:MeOH, all samples were kept in a compartment drier for 24 h at 60 °C and were ground afterwards. To remove inorganic carbon, half of the total amount was treated with diluted Hydrogen chloride (HCl) (2 N) drop by drop until bubbling ceased (provoked by the reaction of HCl with calcium carbonate). The samples were then dried again for 24 h, whereas sediment samples were kept inside for 48 h. The non-acidified samples were used to analyze the $\delta^{15}N$ ratio and the acidified ones to analyze the $\delta^{13}C$ ratio. Aliquots (1–25 mg) were weighted and transferred into silver and tin capsules (HEKAtech, Germany), respectively. If enough material was available, triplicates of the samples were analyzed, using a CNHO- isotope- mass spectrometer (Nu Horizon Stable Isotope Mass Spectrometer, Nu Instruments Ltd., UK) linked to an elemental- analyzer (EURO- EA 3000, Euro Vector, Italy) in continuous flow configuration (set- up by HEKAtech, Germany). N_2 and carbon dioxide (CO_2) were used as standards for nitrogen and carbon respectively. Isotope and mass calibration were conducted by the use of the certified standards like IAEA-600 Caffeine ($\delta^{13}C = -27.771‰$ VPDB, SD 0.043; $\delta^{15}N = +1.0‰$ air N_2, SD 0.2‰), IAEA- NO- 3 Potassium Nitrate ($\delta^{15}N = +4.7‰$ air N_2, SD 0.2‰) and 2,5-bis (5-tert-butyl-2-benzoxazol-2-yl) thiophene (6.51% N; 72.52% C; HEKAtech, Germany). Values of the SIs are represented as δ-notations in per mil (‰) deviation representing the ratio of the heavier to the lighter isotope ($\delta^{15}N = {}^{15}N/{}^{14}N$, $\delta^{13}C = {}^{13}C/{}^{12}C$) (Fry 2006) relative to their international standards (AIR for nitrogen, VPDB for carbon) (Fry and Peterson, 1987; Minagawa and Wada, 1984).

2.5.4. Statistics

One-way analyses of variance (ANOVA) tests were performed to check for significance in variation between the ratios of organic material weight to total weight of the different samples using software StatPlus. Two-way analyses of variances (ANOVAs) including two

factors and an interaction between them were performed to test differences of fatty acid (FA) and stable isotope composition between the taxonomic groups and the three areas using the software R. The Shapiro-Wilk test was conducted to prove normal distribution. The Levene test was used to check for the homogeneity of variances. Outliers were detected using the Cooks distance. To gain specific information about the differences between the factor levels a Tukey's HSD post hoc test was performed. The confidence level was 95%, so the significance level was 5% ($\alpha = 0.05$). If the assumptions were not complied the significance level was raised up to 1% ($\alpha = 0.01$). A principal component analysis (PCA) was performed using the software PASW Statistics 20.0 (SPSS) comparing the FA profiles between the different groups taking the ten most important FAs into account. This test requires a normal distribution, so an arcsine square root transformation was applied for the percentage data of the FAs.

3. Results

3.1. Biomass estimation of sedimented Sargassum

During the expedition, floating *Sargassum* could be observed on the surface in all regions; sedimented *Sargassum* could be detected in all EBS-C sledge samples and AUV recordings (Fig. 2A-I). Biomass of sedimented *Sargassum* was estimated for each of the three AUV deployment areas. An average surface area (m^2) of sedimented *Sargassum* per photo, total surface area (m^2) per site, total biomass (kg) per area, and biomass (g/m^2) was calculated (Table 2). The biomass was calculated using an average mass of 0.65 g/cm^2 taken from the frozen surface sample. *Sargassum* found at area B1 showed evidence of bioturbation (Fig. 2H), both in and around the algae, with large patches missing within the aggregation. This area also had the highest occurrence of macroscopically visible fauna in AUV photos, including fish, shrimp and holothurians. Area A3 had consistently larger patches of *Sargassum*, with little or no evidence of bioturbation. Macrofauna was visible in these photos, but at a much lower frequency.

Area B2 had the least amount of *Sargassum* in the photos. Aggregations were sparse, with only small branches in most photos. There were significant differences in substrate quality noted in AUV photos with soft sediment dominating in the first two AUV deployments while mostly rock with little sediment overlay dominated the last deployment (area B2). The area B2 was at the center of the VEMA transform fault (VTF) and, unfortunately, only a small area could be inspected by this AUV dive. Multiple faults were visible in the photos, which were suggested by the geological survey to be tectonically active.

3.2. Abundance estimation of surface Sargassum from literature

3.2.1. Sedimentation rate of Sargassum

Preliminary experiments carried out during the expedition onboard, revealed an estimated rate of sinking of freshly collected *Sargassum fluitans* (all bladders removed to estimate maximum sinking rate) of 2.5 km per day (undisturbed). With this estimated sinking rate a sample of *Sargassum* (ø 10 cm) would take between 2 and 2.5 days to reach abyssal depths. We investigated the maximum sedimentation rate of *Sargassum vulgare* in more detail at the coast of the Canary Islands. The experiments revealed an average rate of 2 m min^{-1} (± 0.48) estimated during four 10-m runs. Based on these tests at maximum sedimentation, it would take a sample of *Sargassum* approximately 1.7 days to reach a depth of 5000 m, resulting in a sedimentation rate of approximately 2900 m per day.

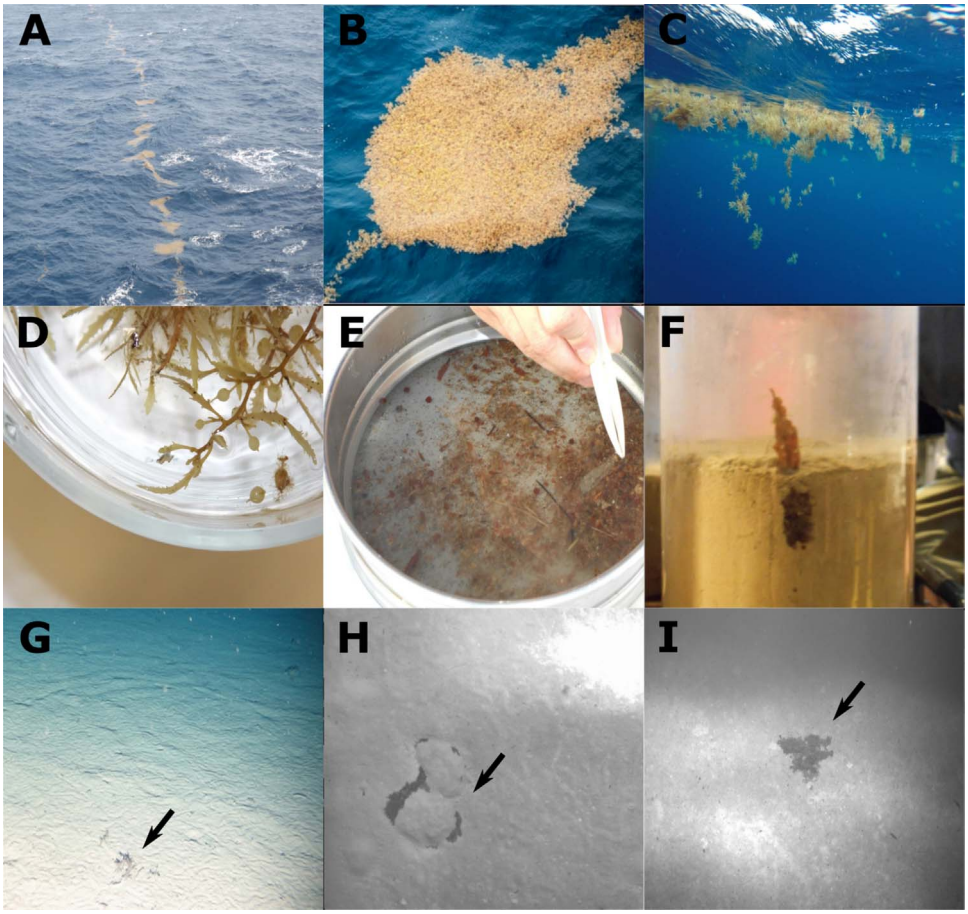

Fig. 2. A, B *Sargassum* algae aligned along Langmuir circulation. **C:** Beginning of sedimentation of *Sargassum* algae. **D:** Collected sample of *Sargassum fluitans* from surface waters. **E:** Pieces of sedimented *Sargassum* algae collected by C-EBS. Samples were sieved (300 µm) to remove sediment. **F:** Thallus of *Sargassum* collected in sediment core by a Multi-Corer (MUC) from the deep sea. **G:** Photo of *Sargassum* on sea floor taken by C-EBS at 5100 m depth (area A3). **H, I:** Photos of sedimented *Sargassum* algae on seafloor taken by AUV from around 5000 m water depth and a nominal altitude of 8 m above the seafloor (areas B1 and A3, respectively).

Table 2
Sargassum biomass (fresh weight) in the abyssal of the southern North Atlantic estimated from photos taken by AUV deployments.

Area/Site/ Dive No.	Depth (m)/ Area (km^2)	No. of photos taken	No. of photos with *Sargassum*/ photos analyzed	Average biomass of *Sargassum* per photo with *Sargassum* (g)	Average surface area per analyzed photo (m^2)	Total surface area (m^2) of photos from a deployment	Total biomass in deployment area (kg)	Biomass per deployment area (g/ m^2)
A3/6–2/ Abyss 163	5136/4.5	9217	1712/171	1161	0.18	306.0	1989.3	3.75
B1/9–6/ Abyss 165	4977/0.2	9576	2153/215	335	0.05	111.1	722.5	1.29
B2/11–3/ Abyss 166	5093/23	8112	145/14	241	0.04	5.4	35.0	0.07

3.3. Epifauna composition on floating Sargassum

3.3.1. Meio- and macrofauna

From the floating *Sargassum* collected at the six stations, individuals belonging to five different phyla were found, including Caenogastropoda (Gastropoda), Cheilostomatida (Bryozoa), Leptothecata (Cnidaria), Polycladida (Plathelminthes) and Sabellida, Phyllodocida, Clitellata (Annelida) (Table 3). Preliminary results indicated the presence of epibiotic bryozoans (genus *Membranipora* de Blainville, 1830), and the annelid worm *Spirorbis* sp. at all sampled stations. There was little variation in the diversity of the sessile and motile fauna between the different stations with the most common sessile organisms being *Spirorbis* sp. and *Jellyella* cf. *tuberculata* (Bosc, 1802). The most common motile organism was the gastropod *Litiopa melanostoma* Rang, 1829, which was found at all sites except for site 8. However, juveniles and veliger stages of this species were only found at the two easternmost stations. Site 8 in area 4 (A4/8) was the only site where we detected the crab *Portunus sayi* (Gibbes, 1850). An undetermined shrimp was found with heavy infections by bopyrid isopods. The copepod *Scutellidium* cf. *longicauda* and the isopod *Carpias minutus* were the most frequent crustaceans.

3.3.2. Micro- and nanofauna

The surface of *Sargassum* was densely populated at all areas investigated. Among protists associated with floating *Sargassum fluitans*, we found representatives of nearly all phylogenetic groups (Table 4). Regarding diversity, heterotrophic flagellates dominated including cryptomonads, apusomonads, thaumatomonads, ancyromonads, choanoflagellates, stramenopiles, cercomonads, bodonids, euglenids and dinoflagellates. Remarkable was the record of the filasterean *Ministeria vibrans*, which was confirmed by molecular studies (18 S rDNA) and high resolution video-enhanced microscopy. Some “rhizopod” species appeared including foraminiferans, labyrinthulids, different forms of heteroloboseans and rhizarians. There were obvious differences in the diversity observed for the different samples, sites 2 (area A1) and 8 (area A4) contained the largest number of taxa, though quantitative estimates were not carried out. After one week of cultivation, ciliates comprising representatives of very different taxonomic groups dominated the community in all raw cultures. A few epiphytic diatoms present in all samples represented the autotrophic fraction of epiphytes.

3.4. Biochemical analysis of Sargassum

3.4.1. Fresh, dry and ash-free dry weights and C:N ratios

Weights were measured from two floating *Sargassum* samples stored at −80 °C (three replicates), five surface material samples that were stored at 10 °C for degradation (three replicates), and seven deep-sea samples stored at −80 °C (three replicates). Values for the fresh, dry and ash free dry weights were averaged between the three sample types (surface material, surface material degraded, deep-sea material). Using the ash-free dry weight, the percentages of organic material to total weight were calculated and then averaged resulting in 64.0 (± 6.2) % for surface material, 46 (± 5.2) % for surface material degraded material, and 54 (± 9.7) % for deep-sea material. The ratio of organic weight to total weight showed significant differences within all the three sample types (one-way ANOVA, $p < 0.001$). The C:N ratios were determined for each sample weighed, and averages were calculated based on sample location (Fig. 3). Due to high variance there was no significant difference between the three sample types (one-way ANOVA, $p > 0.05$).

3.4.2. Fatty acid analysis

Stations were pooled from one area for the analysis. The variability within the samples of *Sargassum* was high and no significant difference between the depths could be detected. The five most abundant fatty acids (FAs) in the *Sargassum* samples were 16:0, 18:0, 18:1(n-9), 20:4(n-

Table 3
Recorded meio-and macrofauna from sampled surface *Sargassum fluitans*. For site 4 at sampling area 2 we collected *Sargassum* twice: on the 26th of December 2014 (*) and on the 28th of December (**).

***Sargassum* community from surface samples**	**Surface stations (area/site)**						
	A1/2	*A2/4	**A2/4	A3/6	A4/8	B1/9	C1/12
Gastropoda - Caenogastropoda							
Litiopa melanostoma Rang, 1829	X	X	X	X		X	X
Gastropoda - Opistobranchia							
Doto sp.	X						
Polychaeta - Sabellida							
Spirorbis sp.	X	X	X	X	X	X	X
Polychaeta - Phyllodocida - Nereididae	X				X		
Clitellata	X	X					X
Gymnolaemata - Cheilostomatida							
Jellyella cf. tuberculata (Bosc, 1802)	X	X	X	X	X	X	X
Rhabditophora - Polycladida							
Gnesioceros sp.		X		X		X	X
Hydrozoa - Leptothecata							
Clytia sp.	X	X					
Aglaopheniidae sp.				X	X	X	X
Crustacea - Maxillopoda - Harpacticoida							
Scutellidum cf. *longicauda* (Philippi, 1840)	X	X	X	X			X
Paralaophonte cf. *congenera* (G.O. Sars, 1908)	X	X		X			
Harpacticus cf. *gurneyi* Jakubisiak, 1933	X	X			X		X
Dactylopusia cf. *tisboides* (Claus, 1863)			X				X
Crustacea - Maxillopoda - Poecilostomatoida							
Macrochiron cf. *sargassi* G.O. Sars, 1916					X	X	X
Crustacea - Malocostraca - Decapoda							
Portunus sayi (Gibbes, 1850)					X		
Hippolyte cf. coerulescens (Fabricius, 1775)	X		X		X		
Latreutes fucorum (Fabricius, 1798)					X	X	X
Crustacea - Malacostraca - Isopoda							
Isopoda sp. 1				X			
Isopoda sp. 2							X
Isopoda sp. 3							X
Carpias minutus (Richardson, 1902)		X	X	X	X	X	

6) and 22:6(n-3) with ranges of 9.9–39.1%, 3.4–11.7%, 4.1–10.9%, 1.3–20.8% and 1.2–10%, respectively (Table S1). Having a closer look at the ten most abundant FAs in all analyzed samples, the *S. fluitans* samples revealed a low and not consistent FA composition neither between the depths, nor between areas (Fig. 4).

3.4.3. Stable isotope analysis

The stable $\delta^{15}N$ values revealed a clustered picture for each sampled group (Fig. 5). Therefore, significant differences between the sediment, the *Sargassum* samples and the abyssal specimens could be detected. The $\delta^{13}C$ values showed a wide range among the groups from −16‰ to −19‰ but were consistent within each taxonomic group (Fig. 5). Only the sediment samples revealed a statistic-supported separation from all other groups. However, no obvious difference between the eastern and western side of the Mid-Atlantic Ridge (MAR) was detectable.

To determine the relative trophic levels of the taxa, sampled in area A2, the mean $\delta^{15}N$ values were compared (Fig. S1). The *Sargassum fluitans* material showed the lowest values (0.31‰ for *Sargassum*_surface/ 0.82‰ for *Sargassum*_deep) followed by the sediment samples (6.68‰). The mean value of the sediment samples (6.7‰) was used as the baseline of the enrichment process, thus, two relative trophic levels scarcely could be identified.

4. Discussion

Mass occurrences of *Sargassum* are well known from the North Atlantic and Gulf of Mexico (e.g. Butler et al., 1983; Gower and King, 2008) and can also form inundation events (Schell et al., 2015). Floating mats of *Sargassum* can be aggregated by Langmuir circulations and in areas of converging currents (Haney, 1986). With increasing biomass, there is an accumulation of organic nutrients and a development of communities within these mats (e.g. Huffard et al., 2014). Earlier studies revealed an increase of diversity of benthic communities in habitats which are enriched with *Sargassum* (e.g. Smith and Hessler, 1987; Grassle and Morse-Porteous, 1987). It was argued that sedimentation of these algal mats offer a potential food source for deep-sea communities (Schoener and Rowe, 1970; Wolff, 1979; Turner and Rooker, 2006). To get an idea regarding the relative importance of sedimented *Sargassum* biomass, we tried to relate our own estimates of sedimented *Sargassum* biomass with that reported for floating *Sargassum*.

4.1. Biomass estimation of Sargassum

4.1.1. Estimation of floating Sargassum biomass from literature

For a comparison with sedimented *Sargassum*, we reviewed available quantitative estimates of floating *Sargassum* in the North Atlantic. Free floating species of *Sargassum*, like that found in the Gulf of Mexico and the North Atlantic, have been studied since at least the 1830's, and have been part of marine lore, as in the naming of the Sargasso Sea (Gower and King, 2008). Various groups, like NOAA (http://www.noaa.gov) and the Sargasso Sea Alliance (http://www.sargassoalliance.org), in conjunction with the Bermuda government, have compiled previous reports, as well as conducted primary research (neuston pulls) to determine abundance, positioning and movement of *Sargassum* algae within the Gulf of Mexico and North Atlantic (Schell et al., 2015). Data were also collected from NOAA, NASA, and ESA satellites using MODIS and MERIS imaging (Stoner, 1983; Butler and A.W. Stoner, 1984; Gower and King, 2008; Siuda, 2011). There are a few abundance estimates available for different years and periods (Table 3). Separate tows conducted and published by Stoner (1983) that occurred between 1977 and 1981 compared the average *Sargassum* biomass of the Sargasso Sea, the Bahamas and the Gulf Stream. These results were compared with tows made by Parr in the 1930's for the same areas, and concluded that

Table 4
Recorded protists from sampled surface *Sargassum fluitans*. Taxonomic groups summarized following Adl et al. (2012).

***Sargassum* community from surface samples**	**Surface stations (area/site)**				
	A1/2	*A2/4	A3/6	A4/8	B1/9
Incertae sedis					
Kiitoksia sp.				X	
Cryptomonadida					
Goniomonas sp.				X	
Apusomonadida					
Amastigomonas sp.	X				
Ancyromonadida					
Ancyromonas sp.	X				
Fabomonas tropica Glücksman & Cavalier-Smith, 2013					X
Amoebozoa					
Undet. amoebid	X	X	X	X	X
Undet. vannellid				X	
Undet. dactylopodid			X		
Thecamoeba sp. 1	X	X		X	
Thecamoeba sp. 2		X		X	
Vahlkampfia			X		
Filasterea					
Ministeria vibrans Tong, 1997		X			
Choanoflagellata					
Monosiga-like	X				
Stephanoeca sp.	X				
Salpingoeca tuba Kent, 1880	X				
Salpingoeca cf. *frequentissima* Zacharias, 1894	X				
Salpingoeca sp.			X	X	X
Excavata - Euglenida					
Anisonema-like 1	X	X	X	X	X
Anisonema-like 2	X	X	X	X	X
Petalomonas sp.			X		
Peranema sp.			X		
Excavata - Kinetoplastea					
NeoBodo designis (Skuja, 1948) Vickerman, 2004	X	X	X	X	X
Neobodo curvifilus (Larsen & Patterson, 1990) Moreira et al. 2004	X				X
Rhynchomonas nasuta (Stokes, 1888) Klebs, 1892	X				
Bodo sp.				X	X
Excavata - Heterolobosea					
Percolomonas cosmopolitus (Ruinen, 1938) Fenchel & Patterson, 1986	X				
Vahlkampfia sp.		X			
Stramenopiles - Dictyochophyceae					
Ciliophrys sp.					X
Pteridomonas danica Patterson & Fenchel, 1985	X		X	X	
Stramenopiles - Actinophyridae					
Actinophrys sp.				X	
Stramenopiles - Bicosoecida					
Caecitellus sp.			X		X
Cafeteria roenbergensis Fenchel & Patterson, 1988	X	X	X	X	X
Pseudobodo sp.	X	X	X	X	X
Stramenopiles - Labyrinthulomycetes					
Labyrinthulids			X		
Rhizaria - Foraminifera					
Rotaliidae	X				
Reticulomyxa-like					X
Globigerina-like	X				
Rhizaria - Thaumatomonadida					
Thaumatomonas sp.	X			X	
Glissomonas-like			X	X	X
Massisteria marina Larsen & Patterson, 1990			X	X	X
Rhizaria - Metromonadea					
Metromonas sp. 1	X	X	X	X	
Metromonas sp. 2	X				
Metopion sp.				X	
Alveolata - Dinoflagellata					
undet. Dinoflagellata	X				
Ciliophora - Hypotricha					
Euplotes sp. 1	X	X	X	X	
Euplotes sp. 2	X	X	X		
Diophrys sp.	X		X	X	
Dysteria sp.	X		X	X	
Aspidisca sp.	X			X	
Ciliophora - Stichotrichia					
undet. Stichotrichid		X	X	X	
Ciliophora - Suctoria					
Suctoria undet.			X		
Ciliophora - *Protocruzia*					
Protocruzia sp.		X	X		
Ciliophora - Scuticociliata					
Cinetochilum sp.	X				
Cristigera setosa Kahl, 1928	X				
Ciliophora - Oligotrichia					
undet. Oligotrichia	X				
Ciliophora - Karyorelictea					
Tracheloraphis sp.	X				
Pleuronema sp.	X				
Litonotus sp.	X				
Blepharisma-like			X		
Ciliophora - Peritrichia					
Cothurnia-like	X				

For site 4 at sampling (area 2) we collected twice *Sargassum:* on the 26th of December 2014 and on the 28th of December. For the protist community we only determined protist communities from the 26th of December 2014 (*). In area C1, protists were present but could not be analyzed in detail due to time constraints onboard.

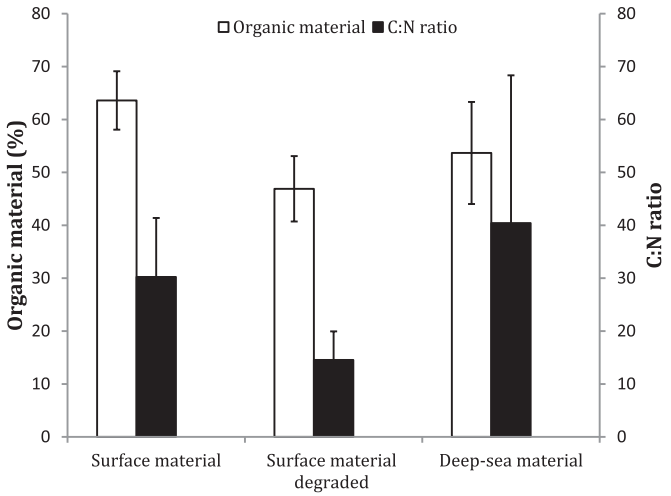

Fig. 3. Percentage of organic material for surface material, surface material degraded, and deep-sea material of *Sargassum* samples (left axis, white columns), and C:N ratio (right axis, black columns).

the overall biomass of pelagic *Sargassum* had decreased over a period of about 50 years (Stoner, 1983). However, a later publication by Butler and A.W. Stoner (1984) questioned this conclusion on the basis that Stoner did not consider seasonal variation of *Sargassum* abundance. Gower and King's (2008) studied the movement of free floating *Sargassum* between the Gulf of Mexico and Atlantic Ocean using satellite-imaging data and Medium Resolution Imaging Spectrometer (MERIS) from the European Space Agency. They calibrated the satellite data with ship measurements from the corresponding months (Gower and King, 2008). The MERIS study also referenced previous studies by Parr in the 1930's of free floating *Sargassum* in the western North Atlantic (Parr, 1939 cited in Gower and King, 2008). During fall, quantities of *Sargassum* were greater in the North Sargasso Sea (average of 0.27 g/m^2) than in the South (0.03 g/m^2), while the reverse was noted for

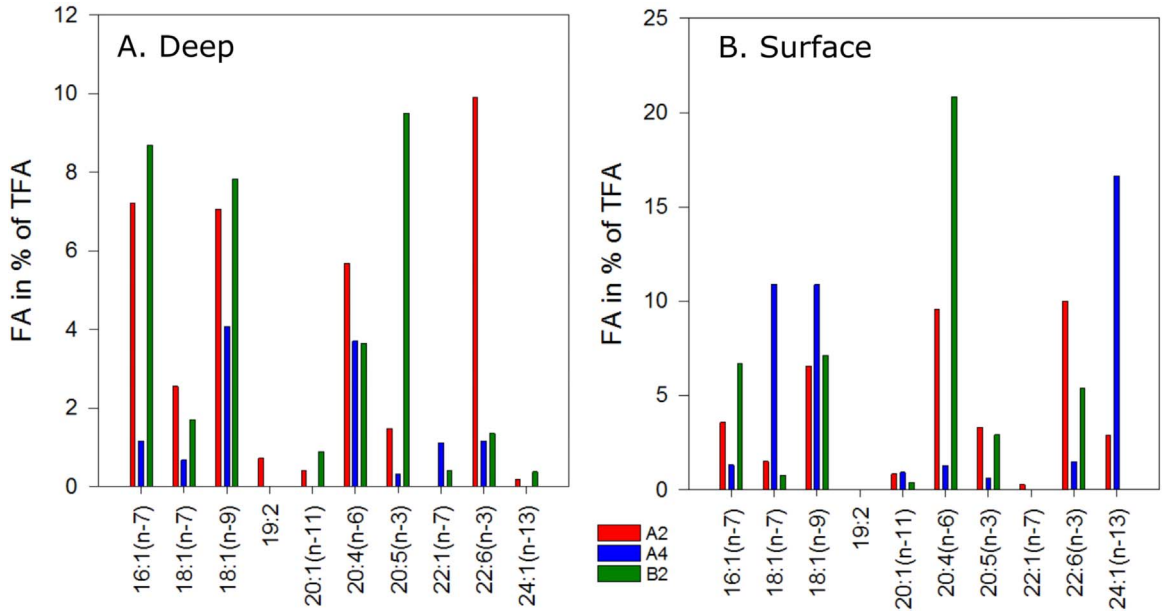

Fig. 4. Mean values of *Sargassum fluitans* sampled in the deep sea (A) and surface (B) for the ten most abundant fatty acid [%] of total fatty acid contents (TFA) from the three different areas.

spring (0.12 and 0.18 g/m^2 respectively). It was also shown that there was an increased overall abundance during fall, with an average biomass of 0.30 g/m^2 throughout the year (Siuda, 2011). Siuda (2011) used 1999 individual neuston-tow pulls conducted from 1973 to 2010 by SEA from Woods Hole Oceanographic Institute to determine local and seasonal differences. A recent review of Schell et al. (2015) clearly showed strong annual fluctuations of floating *Sargassum* biomass determined by neuston tows during autumn studies with peaks in recent years causing inundation events. We summarized all data available to us in Table 5, where we tried to standardize the estimates of *Sargassum* biomass (fresh weight) by the different authors as biomass per m^2.

With the literature reviewed in this study, there are consistent findings of large quantities of holopelagic *Sargassum* throughout the North Atlantic, Caribbean Sea and Gulf of Mexico. Studies looking into the abundance of *Sargassum* have regularly shown these quantities, leading to the assumption that, while seasonal variances occur, there is a consistent supply of *Sargassum* throughout these waters to serve as a potential carbon flux to the deep sea through sedimentation. These findings are supported by the amount and frequency of *Sargassum* noted during the present expedition. Other studies have shown that these floating aggregations of algae can support a large diversity of marine organisms in surface waters (Fine, 1970; Settle, 1993), as they provide feeding grounds, refuge areas and, thus, increase habitat complexity (Fine, 1970; Kingsford, 1995).

The interactions between these organisms could possibly lead to nutrient enrichment of the *Sargassum* mats, as well as an enhanced primary production indicated by findings of 20:5(n-3) as a diatom marker in the sediment. Normally equatorial oceans are generally considered to be oligotrophic and therefore exhibit a lower total primary production rate than coastal or polar areas. In contrast, Uitz et al.

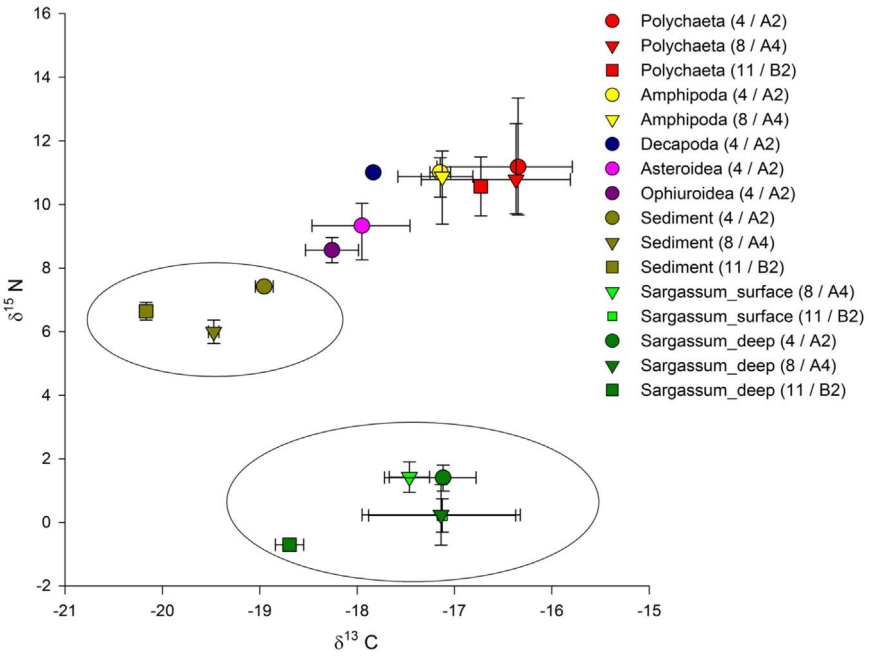

Fig. 5. Biplot overview of stable isotope composition for organisms of different taxa, sediment and *Sargassum fluitans* at different stations (site/area), minimum and maximum values served as error bars. The symbol color indicates the kind of sample, while the shape of symbols indicates the sample origin. Sampling sites and respective sampling area are given in brackets.

Table 5
Biomass estimates (fresh weight) for *Sargassum* recorded from the North Atlantic by different authors.

Source	Biomass	Year	Location	Area for recalculation	Biomass recalculated [g/m^2]
Parr (1939)	7 million tons	1933	North Atlantic	41.5 M km^2	0.169
	11 million tons	1934	North Atlantic	41.5 M km^2	0.265
	4 million tons	1935	North Atlantic	41.5 M km^2	0.096
Stoner (1983)	74 mg/m^2	1977–1981	Sargasso Sea		0.074
	165 mg/m^2	1977–1981	The Bahamas		0.165
	280 mg/m^2	1977–1981	Gulf Stream		0.28
Gower and King (2008)	1 million tons	2002–2008	Gulf of Mexico	1.6 M km^2	0.625
	1 million tons	2002–2008	North Atlantic	41.5 M km^2	0.024
Siuda (2011)	0.39 g/m^2	1977–2010	North Sargasso Sea		0.39
	0.21 g/m^2	1977–2010	South Sargasso Sea		0.21
Schell et al. (2015)	0.17 g/m^2	1995–2013	South Sargasso Sea		0.17
	0.23 g/m^2	2014/2015	South Sargasso Sea		0.23
	0.25 g/m^2	2011/2012	South Sargasso Sea		0.25
	0.0027 g/m^2	1992–2013	West. Trop. North Atlantic		0.0027
	0.07 g/m^2	2011	West. Trop. North Atlantic		0.07
	0.84 g/m^2	2014	West. Trop. North Atlantic		0.84

(2010) found increased microplankton concentrations (diatoms) in the studied area which could explain the higher proportions of 20:5(n-3) found in the organisms and in the sediment. The increased amount of organic matter could be explained by the influence of the NECC transporting nutrients and POM from the coastal areas of the African and American continents. This assumption is also supported by the theory of Oschlies and Garçon (1998) who predicted an increase of primary production in areas of strong eddy kinetics which are found in the studied region. Furthermore, trophic interactions on *Sargassum* might initiate the sedimentation process by removal of the bladders through grazing and degradation (Fig. 2C). The resulting vertical transfer of energy includes not only the *Sargassum* itself, but potentially also the community of organisms on its surface.

4.1.2. Biomass estimates of sedimented Sargassum

Three different AUV deployments during the VEMA Transit expedition showed the presence of *Sargassum* algae on the seafloor at high abundances, which was additionally confirmed by qualitative samples of *Sargassum* taken with the camera of the C-EBS as well as by the sledge. Analysis of the AUV photos revealed a biomass density of up to 3.75 g/m^2 and the lowest density of 0.07 g/m^2. These results fall within the biomass previously estimated for *Sargassum* at the surface (Table 5). While area B1 showed a 20 times higher biomass density compared to the estimated surface density, one needs to consider that surface communities of *Sargassum* tend to aggregate in large mats, some noted to be over a kilometer in length, and are not evenly distributed through the North Atlantic. Variation in the appearance of *Sargassum* between the different sites and stations could show differences in the role that it plays, depending on the surrounding organisms. The *Sargassum* found at area A3 had a consistently larger biomass per clump than the other two areas (B1, B2), with little signs of bioturbation. However, at area B1, the majority of *Sargassum* found had signs of bioturbation surrounding it (Fig. 2H). We assume that this might be caused by the treatment of *Sargassum* by benthic animals (Schoener and Rowe, 1970; Wolff, 1979), although we had no direct photographic evidence. Schoener and Rowe (1970) presented the first direct evidence for high abundance of *Sargassum* in the abyssal. They found *Sargassum* clumps of varying sizes in varying degrees of degradation at 33 of 150 stations in the western North Atlantic off North Carolina by a camera survey. Here we present the first quantitative data indicating the high potential contribution of *Sargassum* to the matter flux in the southern part of the North Atlantic. We consider AUV studies to be a very useful tool to quantify sedimented macroalgae and to get a better insight into particle flux from the surface to deep-sea communities.

4.1.3. Sedimentation of Sargassum

Sedimentation rates of *Sargassum* determine how viable it could be as a source of carbon for deep-sea ecosystems. If the rate is too slow, there might be a decrease in usable carbon and nutrients, either due to degradation, or consumption by other organisms in the water column. The rate of sedimentation found in this study is in theory a maximum potential sedimentation rate, based on the removing of all bladders (pneumatocysts) from the algae, thus, decreasing the buoyancy. The two different sedimentation tests conducted, the preliminary one on board the R/V *Sonne* cruise with *S. fluitans* and the study at the Canary Islands using *S. vulgare*, showed similar rates (5000 m in 48 h). Both of these tests removed all the bladders from the plants, and while they were done with two different species, we assume that similar results would occur with other morphometrically similar species of algae. It must be noted, however, that a maximum sedimentation rate with all bladders being removed is unlikely to be found naturally. That leaves an important question as to what causes the initial sedimentation of the algae. It is possible that some of the bladders need to be removed before the initiation of sedimentation begins, however, as previously mentioned, the nutrification and development of surface dwelling organisms on the algae can have an effect on the sinking rates, potentially initiating the sedimentation process (Turner and Rooker, 2006). Moreover, the compression of bladders with increasing depth will further reduce the buoyancy of the algae.

Schoener and Rowe (1970) determined sedimentation rates of *Sargassum* preserved in 10% Formalin 3.3 (2.8–4.0) cm/s and calculated a sedimentation to 5000 m depth to occur within 41 h. This value is in the same range as our estimates using live specimens. It is not known what caused a change in its specific gravity. Schoener and Rowe (1970) argued that there could be changes in the integrity of the air bladders at a critical shallow depth which would result in rapid sinking or there might be a high specific gravity by slow degradation. Johnson and Richardson (1977) have shown in experiments with pressure chambers that the depth, at which the whole *Sargassum* plants lost their positive buoyancy and sank, appeared to be an inverse function of the rate of hydrostatic pressure change, indicating that the slower the plant descent the shallower the depth at which sinking occurred due to positive buoyancy loss. They calculated that, once negatively buoyant, *Sargassum* will sink to the sea floor in about 40 h. This value is again in the same range as estimated in the present study. In accordance with Johnson and Richardson (1977) we argue sedimentation may occur via several mechanisms: 1) fragmentation of weed clumps due to wave action with the subsequent sinking of the older parts which are more heavily populated by epibenthic micro- and macroorganisms (Fig. 2C; Tables 3 and 4); 2) diseased *Sargassum* may lose its buoyancy; 3) entrainment of *Sargassum* clumps in the zones of convergence and down

welling associated with Langmuir circulation cells and large scale down welling. For vital *Sargassum*, Woodcock (1993) hypothesized that the algae may be adapted to a cyclic submergence in the wind-induced vertical currents, returning to the surface only when the currents are less than the plant's rise rate. This phenomenon might support the maintenance of their holopelagic life.

The well preserved *Sargassum* clumps in the AUV photos might be explained by a steady sedimentation and replacement of degraded material at relatively high rates or a very slow degradation rate. The biochemical data support this ambivalent view. However, several facts support the first explanation: 1) macrofauna is known to feed on and destroy *Sargassum* in the deep (e.g. Schoener and Rowe, 1970; Wolff, 1979; Grassle and Morse-Porteous, 1987); 2) macrofauna was present at relatively high abundances at the three investigated areas (Brandt et al., 2017; Riehl et al., 2017) and might have fed at least indirectly on *Sargassum* (see below); 3) traces of macrofauna activities around *Sargassum* clumps were recorded (Fig. 2H).

4.2. Epifauna composition on Sargassum

The analysis of floating *Sargassum* revealed a diverse community. Regarding the epiphytic meio- and macrofauna, the random occurrence of the shrimp *Latreutes fucorum*, the polychaete *Platynereis dumerilli*, the turbellarian *Planocera* cf. *pellucida* and the nudibranch gastropod *Doto* sp. fits well with the observations of previous studies (e.g. Weis, 1968; Fine, 1970; Stoner and Greening, 1984; Huffard et al., 2014). It has been shown that many organisms known to be found in the Sargasso Sea also occur further south and east of the Atlantic as previously described, where mats of floating *Sargassum* can still be found. The recorded meio- and macrofauna might well be responsible for the destruction of bladders of *Sargassum* directly or indirectly by feeding and thereby stimulating microbes which destroy the algal cell layers (Johnson and Richardson, 1977).

Regarding the nano- and microfauna, a community structure was found as it is typical for marine sediments (e.g. Arndt et al., 2000). Many taxa we observed were also recorded from oceanic detritus (Patterson et al., 1993; Arndt et al., 2003). Thus, a potential transatlantic distribution of benthic protists by *Sargassum* would be possible. The preliminary studies revealed that most of the recorded taxa belong to ubiquitously distributed protists (e.g. Patterson and Lee, 2000). The protistan fauna obviously contributes to a diverse microbial food web on the *Sargassum* surface. Some peritrichous ciliates feed on pico- and nanoplankton surrounding the *Sargassum,* several choanoflagellate species and stramenopile heterotrophic flagellates feed on the surrounding bacterioplankton. All other protists, however, belonged to surface dwelling forms feeding on attached bacteria and algae stimulating the degradation of *Sargassum* by exerting a strong grazing pressure on bacteria biofilms. Several protist species were found to survive drastic increases in hydrostatic pressure occurring during sinking (Živaljić et al., 2017), thus, the grazing impact might continue during the sedimentation process of *Sargassum*, as several representatives have been found alive on sedimenting detritus from deep-sea samples (Patterson et al., 1993; Arndt et al., 2003).

To check, whether the epiphytic fauna from the surface might give rise to the population of the deep-sea benthos, we did preliminary studies on blades of *Sargassum* collected by the C-EBS (meio- and macrofauna) or the Multi-Corer. Several blades were inspected for meio- and macrofauna and three blades obtained from undisturbed MUC cores revealed no protists in direct observations or cultures. Though we did not find any species identified by our investigations of surface samples of *Sargassum*, at least several nanoprotists we found on the floating *Sargassum* have been recorded from the deep sea (e.g. *Neobodo* spp., *Cafeteria* sp., Patterson et al., 1993; Arndt et al., 2003; Scheckenbach et al., 2010). Thus, a potential contribution of protists sedimenting with *Sargassum* to the oceanic floor cannot be ruled out at least for some nanofauna species. Regarding the epiphytic meio- and macrofauna on sedimented *Sargassum*, we could not record any organism. These finding might be influenced by the sampling technique which has potentially washed away attached organisms. Future studies with more refined methods are necessary to study this matter. At the moment, we have to assume that even though there are high densities of a very diverse epifauna on floating *Sargassum*, only a few organisms might be transferred to the deep-sea communities.

4.3. Biochemical analysis and potential carbon flux from sedimented Sargassum

4.3.1. Fresh, dry and ash-free dry weights and C:N ratios

The analysis of dry and ash-free dry weights showed significant differences between samples collected at the surface and those collected from the deep sea. Samples that were collected from the surface and immediately stored at −80 °C had a higher percentage of organic content than samples collected from the surface and degraded for 18 months at 10 °C and deep-sea samples, stored at −80 °C. It should be expected that degradation is noted between the surface sample immediately frozen and the surface sample stored at 10 °C for 18 months. Furthermore, degradation was also seen in the deep-sea samples that were frozen at −80 °C immediately after sampling. This could be a result of degradation on the way of sedimentation or due to degradation in the abyss. Since the sinking rate is relatively high when bladders are destructed, it has to be assumed that the major part of the degradation takes place in the abyssal sediment. While destruction of *Sargassum* blades at 10 °C for 18 months was very obvious, visual inspection of the *Sargassum* collected directly from the abyss showed only little destruction, and plant structures remained intact (see discussion above).

C:N ratios found in our study were typical of marine plants, however, with high variance even between similar samples. Literature cites values of between 4 and 10 for marine phytoplankton, between 15 and 50 for holopelagic *Sargassum* depending on if it is found in the neritic zone or the open ocean, and a general tendency for higher values with increasing depth (Müller, 1977; Atkinson and Smith, 1983; Meyers, 1994; Lapointe, 1995). Due to high variability, our results of the C:N analysis did not show statistically significant differences between the surface *Sargassum* and deep-sea samples. This has to be addressed in future studies.

4.3.2. Fatty acids (FA) analysis

Macroalgae, like *S. fluitans,* are often characterized by distinct FA compositions affecting organisms of higher trophic levels. This makes them a useful biomarker to reveal information about food webs (Graeve et al., 2001). Unfortunately, the FA composition of the *S. fluitans* samples was not consistent, thus no specific FA or a distinct composition could be detected. This could have various reasons; maybe it is due to seasonal differences in polyunsaturated FAs (PUFAs) signatures for *S. fluitans* (Turner and Rooker, 2006) influenced by light intensity, salinity, temperature and available nutrients (Thompson et al., 1990, 1992). It could also be due to the material biochemically changing during the floating time, aging, and sinking processes (Mintenbeck et al., 2007; Galloway et al., 2012; Galloway, 2013) or an increased microbial abundance (McArthur et al., 1992; Chen et al., 2008). Also the associated epibionts could affect the FA measurements and lead to scattered results. From freshwater systems it is known that bacterivorous protists might change the PUFA signature (Martin-Creuzburg et al., 2005). We found large numbers of bacterivorous protists being active at the surface of *Sargassum* feeding on attached bacteria.

The PCA of the ten most abundant FAs (16:1(n-7), 18:1(n-7), 18:1(n-9), 20:1(n-11), 22:1(n-7), 24:1(n-13), 19:2, 20:4(n-6), 20:5(n-3) and 22:6(n-3)) revealed a clear separation of the different taxa showing a clustered picture of the Opheliidae (Polychaeta), Porcellanasteroidae (Asteroidea) and Ophiuridae (Ophiuroidea), but a scattered image for the seven different Amphipoda families. Different taxonomic groups have distinct FA patterns consistent within the groups. The fatty acid

20:4(n-6) had the biggest proportion followed by 18:1(n-9) and 16:1(n-7). This pattern is also found by Khotimchenko (1991) for *Sargassum*. Furthermore, 20:4(n-6) is discussed to be a biomarker for the entire taxonomic group of brown algae (Hanson et al., 2010). Turner and Rooker (2006) hypothesized that heterotrophs utilizing the *S. fluitans* rely more on the enhanced phytoplankton production and the associated epibionts, and some analyzed organisms showed higher proportions of those FAs. Our own investigation on the components of the microbial food web indicates that the majority of microbes take advantage of the biofilm on the surface of *Sargassum* blades. This leads to the assumption that, at least for some organisms, *S. fluitans* could serve as a food source in the studied region.

4.3.3. Stable isotope (SI) analysis

Considering the SI results in this study, the very low $\delta^{15}N$ values of the *S. fluitans* samples (mean value = 0.5‰) and the relatively large gap before the first analyzed megafaunal group (Ophiuridea = 8.6‰), it is unlikely that the macroalgae serve as a direct food source for higher trophic levels, while it possibly might be consumed by protists. Protists are known to upgrade the food quality for higher trophic levels (Martin-Creuzburg et al., 2005, 2006). The abyssal seafloor as a food limited habitat is suggested to generate mainly opportunistic feeders using every available food source including wood falls (e.g. Becker et al., 2009; Hoyoux et al., 2009). Therefore, a gap of 2.5 relative trophic levels between the possible food source and the small sized Ophiuridae seem to be very unlikely. The sediment with its organic matter (and the microbial food web on *Sargassum*), however, leads to a more justified result considering the SI. FAs are faster incorporated than the heavy isotopes. Therefore, the slightly increased amounts of 20:4(n-6) could be a signal of the algal material whereas no sign could be detected in the SI patterns. However, microbes (bacteria and protists) in the upper sediment layers can rapidly alter FAs during deposition via biodegradation and/or chemical degradation as a consequence of the created surrounding (Eclinton, 1973). Regardless of whether *S. fluitans* is directly or indirectly used as a food source for metazoans, the floating algae have likely a very significant impact on the environment, on the surface as well as in the deep sea.

5. Conclusion

Deep-sea ecosystems are generally limited by carbon availability. We add quantitative estimates suggesting that floating *Sargassum* could act as an important input of organic carbon to the deep sea in the southern North Atlantic. While previous studies had looked at carbon flux into the deep sea, the sedimentation rates and potential for large quantities of macrophytes to significantly contribute to this flux has been rarely studied (for a recent review see Krause-Jensen and Duarte, 2016). We show that there is a large biomass of sedimented *Sargassum* algae on the seafloor which can be in the same range as that at the surface and should play an important role for benthic production – sinking rates of *Sargassum* are probably high (~ 2 days to 5000 m water depth) allowing little time for degradation or consumption. While these are only estimates, the results show that a significant part of the production of *Sargassum* at the surface waters eventually reaches the deep sea, at least in the area of the southern North Atlantic covered during the Vema-TRANSIT expedition. Analysis of fatty acids and stable isotopes indicate that macrofauna might not directly consume *Sargassum* in the abyssal, but probably via the components of the abyssal microbial food web including bacteria and protists (probably several trophic levels) as it is known already for macrofauna making use of wood falls (Becker et al., 2009; Hoyoux et al., 2009). The particular phenomenon of large-scale sedimentation of *Sargassum* as a regular carbon input has to be much more considered. Further studies are needed to determine to what extent deep-sea organisms rely on this source of energy.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dsr2.2017.10.002.

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Further reading

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Conclusive Summary and Perspectives

The general aim of this dissertation was to provide a global comparison of biodiversity and biogeography of deep-sea protists. This thesis aimed to add significant contributions to facts on benthic deep-sea protist communities, especially from the marginally researched abyssal regions. First results of protist communities sampled from hadal regions (8 km depths) are presented within this thesis. We provide deep-sea protist patterns on a global scale, which significantly differs from those of other marine habitats. The conducted work within this thesis gives insight into a new understanding and the potential importance of the role of nano- and microprotists in deep-sea ecosystems.

While no standardized protocol for sampling deep-sea protists exist, we developed a protocol combining available culture-dependent and culture-independent methods to gain detailed quantitative and qualitative information on benthic deep-sea protist communities (Chapter 1). This proposed combination helped to mitigate limitations of each method. While high-throughput sequencing methods such as metagenome analyses are an appropriate way to investigate the biodiversity of deep-sea-protist communities, one has to consider that the sequencing results include the active as well as inactive fraction of protist communities in the deep sea. It is uncertain, if benthic protist communities detected by metagenomics are actually thrieving in the deep sea or are rather an artifact by depsoited cells from the upper water column, encysted cells or extracellular DNA (Stoeck et al., 2007). As DNA is well preserved in this environment and protists are able to form cysts to overcome harsh conditions, one should consider data from metagenomic analysis as seed bank analysis. Several studies rely on RNA analysis in microbial communities to recover only the active fraction in deep-sea samples. This assumption would suggest that RNA would only be derived from active organisms. However, recent studies have shown that RNA can remain in the environment and can also be recovered from cysts (inactive cells) (Mahé et al., 2017). Collection and storage of environmental samples, which is a main concern in large-scale studies (Mahé et al., 2015a), is easier for DNA than RNA, supporting the DNA analysis of environmental samples. Future investigations of deep-sea protist communities could include mRNA analysis to try to retrieve the actually active fraction of deep-sea protists. In addition, genes involved in pressure adaptation could be investigated with transcriptome analyses from surface water and deep-sea protist strains cultivated at atmospheric pressure and under high hydrostatic pressure. Using the hotspots of the up- and down-regulated genes, the involved protein domains could be examined to interpret their role in pressure adaptation. On the basis of such data, metatranscriptome analysis

should target those genes that are influencing the pressure adaptation. With the help of such marker genes, indigenous deep-sea protists might be identified. For now, pressure experiments under deep-sea conditions within Chapter 2 (see also Appendix A, Zivaljić et al., 2018) were conducted to validate the potential deep-sea origin of cultivated protists sampled from deep-sea sediment. In several cases also surface water protists were exposed to high hydrostatic pressure to investigate their capability to survive under deep-sea conditions to underline the potential of an exchange between the surface and deep-sea habitats.

We extracted the DNA of 20 sediment samples from deep-sea stations at bathyal, abyssal and hadal depths located in the Atlantic and Pacfic Ocean for next-generation sequencing to allow for the first global comparison of deep-sea protist communities (Chapter 3). While we only recovered 43% of the total estimated eukaryotic richness, our sampling approach was sufficient to reach saturation of deep-sea protist richness at a global scale. Overall, few dominant protist OTUs (high read abundances) were globally dispersed. Most protist populations were rare and exhibited a high degree of endemism, explaining the substantial differences in community composition observed over large spatial scales. A high percentage (91%) of of our retrieved operational taxonomic units (OTUs) were affiliated with heterotrophic protists. Many OTUs had no close representative to our self-created in-house reference database suggesting the existence of a specific and highly diverse nanofauna in deep-sea sediments. Less than 1% of protist OTUs occured in all 20 examined deep-sea stations. Around 50% of protist OTUs detected within this study were unique to one station. Ten hyperdiverse lineages including the Discoba, Alveolates and Rhizaria contained more than 1000 OTUs. We recovered a much higher diversity in kinetoplastid flagellates, foraminiferans, ciliates and Cryptista than found in surface waters. Detected differences in the community structure among the investigated deep-sea basins may reflect a shift in the protist community composition due to environmental gradients, indicating the existence of large scale patterns of protist diversity. Grouping all examined stations by their depths profile in bathyal, abyssal and hadal regions or oceanic regions, our results suggest that some taxa can maintain eurybathic ranges and cosmopolitan deep-sea distributions, but the majority of species appear to be regionally restricted. Within our study we can say, that we observed distinct communities within oceanic regions and depths gradients, but whether depths on a global scale might be a mechanism driving community patterns would need to be verified by a larger sampling abundance of hadal and bathyal regions. The sediment patchiness at the abyssal sea-floor is extremely high, as indicated with many grazing tracks, pockmarks and *Sargassum* debris (Devey et al., 2018). Thus, environmental factors might already impact and shape deep-sea protist communities on a small scale and it is likely that multiple processes might operate at the same time structuring communities. If all protist species are affected in the same manner or if the abundant and rare proportion of protists is affected differently, still needs to be answered.

While downstream analyses of protist communities completely depend on the reference database, a appropriate taxonomic description of deep-sea protists and protists in

general by means of molecular and morphological characzerization are of paramount importance. Thus, we added 84 sequences of cultured marine protists from surface waters and the deep sea from our Heterotrophic Flagellate Collection Cologne (HFCC) to the reference sequences obtained from public databases (PR^2, SIlVA an V9 TaraOcean database). Many, but not all, of our HFCC references were recovered in ∼2% (669 protist OTUs) of the total protist richness (41,152 POTUs). The HFCC reference sequences resulted in an 0.5% increase of recovery of assigned protists, meaning that 217 POTUs could solely be assigned to our HFCC protists, not to any other reference sequence of public databases. Of these 217 HFCC POTUs, 15 were recoverd with a 100% pairwise identity covering species within the Stramenopiles, Discoba, Cryptista, Obazoa and Alveolata. Some of these POTUs belonged to globally deep-sea distributed species, occurring in all 20 examined deep-sea basins, including for example the newly revised species *Cafeteria burkhardae* (Chapter 2.1), *Ministeria vibrans* and the ciliate *Aristerostoma*.The largest OTU with regards in abundance (number of reads) belonged with a pairwise identity of 100% to the *Cafeteria burkhardae* (Chapter 2.3) species including 55% of our total protist read abundance (Chapter 3). This *Cafeteria* POTU occured in all examined deep-sea stations indicating a global distribution of *Cafeteria burkhardae* in the deep sea. These findings could be supported by our sequencing results of cultivated *Cafeteria* strains showing that *Cafeteria burkhardae* could be cultivated from marine surface waters and the deep sea in the Pacific and Atlantic Ocean (Chapter 2.3). Strains of this species isolated from both surface waters and the deep sea survived the exposure of high hydrostatic pressure (∼520 bar) (Zivaljić et al., 2018). Three of the other newly described *Cafeteria* species *C. biegii, C. graefii, C. maledivіensis*, which had been included in our reference database, were recovered with a 100% pairwise identity within our NGS results, however, with low read abundances. The low abundant POTU of *Cafeteria biegii* might be reasonable, because this species was isolated and cultivated solely from the Mediterranean Sea, which has not been included in our NGS analysis. The included *Cafeteria graefii* HFCC sequence (HFCC33) was isolated from deep-sea sediment recovered with an Agassiz Trawl. Thus, contamination with surface waters cannot be ruled out, as for example sediment sampled with a Multi-Corer, and thus, could explain the low abundance in our NGS dataset. The other available *C. graefii* strain HFCC772, isolated from surface floating *Sargassum* algae, was not included in our V9 Deep Sea database due to time constraints. Those results could imply that *C. graefii* might be a marine surface water *Cafeteria* species. Overall, regarding the abundance of OTUs (read abundance), next-generation-sequencing studies should be interpreted carefully due to the fact that some species' barcode gene may be biased by PCR amplification leading to a possible overrepresentation of certain OTUs (Stoeck et al., 2014; Geisen et al., 2015). However, with our cultivation of the *Cafeteria* strains we could underline the observed trends and interpretations of *Cafeteria* species within our NGS output.

Besides the similar genotypes within the bicosoecids (*Cafeteria*) occurring in surface waters and the deep sea, we could investigate the genotype distribution also for ciliates. We could isolate two scuticociliate species (*Pseudocohnilembus persalinus* and *Uronema* sp.)

from the surface and the deep ocean (2687 m, 5276 m, 5719 m) of the Pacific. Direct microscopic observations of sediment samples (live-counting method) did neither recover *P. persalinus* or *U. marinum* nor cysts resulting in the lack of knowledge concerning the original state of these species in the deep sea. Pressure and temperature experiments were used to investigate their adaptation to deep-sea conditions. Although the results did not show a general barophilic behavior, all four isolated strains survived the highest experimental hydrostatic pressure. A better survival at 550 bar could be observed at lower temperatures. Within our NGS results ciliates belonged to one of the ten hyperdiverse lineages (>1,000 POTUs) with 3,021 POTUs (Chapter 3). This high diversity of deep-sea ciliates was also recovered within our literature review of deep-sea ciliates (>1,000 m). Representatives of the major phylogenetic ciliate groups could be documented from deep-sea regions (Chapter 2.1). We suggest that among microbial eukaryotes, ciliates should be considered as a diverse and potentially important component of deep-sea microeukaryote communities.

We could also recover and cultivate three new phagotrophic euglenoid species from marine surface waters (*Petalomonas acorensis* gen et sp. nov, *Ploeotia costaversata* sp. nov.) and the deep sea (*Keelungia nitschei* sp. nov.) (Chapter 2.2). Belonging to the Euglenozoa, phagotrophic euglenids are important for understanding the eukaryotic early evolution. Although molecular barcoding studies have identified euglenozoans (comprising kinetoplastids, euglenids and diplonemids) as a specifically diverse group among deep-sea protists, only a very few euglenids have been described up to now. Phagotrophic euglenids are known to be typical components of marine sediments in shallow waters, but have not yet been cultivated from deep-sea samples. With our morphological and molecular characterisation of the three phagotrophic euglenids, we enlarged the taxon sampling of an underrepresented group of euglenids. In addition, we analyzed the the species reaction towards increasing hydrostatic pressure directly under the microscope and found that the deep-sea strain *Keelungia nitschei* was better adapted to high hydrostatic pressure ate lower temperatures as compared to the two examined surface water euglenid strains. Phagotrophic euglenids have rarely been recorded in many environmental sequencing datasets from sediments This might be due to the lack of the availability of the divergent and expanded 18S rRNA gene sequences for euglenids in databases. The *Keelungia nitschei* strain could not be recovered with a 100% pairwise identity within our study. The majority of euglenid POTUs had a p-distance of 80-85% to reference sequences indicating the lack of knowledge concerning this group in deep-sea sediments (Chapter 3). Kinetoplastids and diplonemids belonged to the ten hyperdiverse lineages within our NGS results. Within our metagenomic dataset diplonemids were recovered with the highest diversity (>9,000 POTUs) being consistent with previous deep-sea sediment analyses.

Within the North Atlantic Ocean we investigated the potential of sedimented *Sargassum* algae as a carbon source in the deep sea (Chapter 4). The estimated *Sargassum* biomass in the deep sea was higher than that of surface waters in the North Atlantic. Biochemical analyzes showed degradation of *Sargassum* occurring during sedimentation or in the deep sea, fatty acids and stable isotope analyzes showed no direct trophic interactions between the algae and macrozoobenthos organisms. Therefore, we assume that components of the

microbial food web in the deep sea form an important link between the macroalgae and the larger benthic organisms. The evaluation of epifauna on surface *Sargassum* showed a diverse nano-, micro-, meio- and macrofauna and may be transported across the entire North Atlantic. Among the protists associated with floating *Sargassum fluitans*, we found representatives of almost all phylogenetic protist group, including e.g. the bicosoecid *Cafeteria*.

Our comparison of NGS results (Chapter 3) with our cultivation approach including pressure experiments (Chapter 2), Appendix A, Zivaljić et al., 2018) showed, that a possible exchange of surface water and deep-sea protists might occur for at least several genotypes. The large biomass of the approximated biomass of *Sargassum* to the deep-sea floor indicate the possibility of *Sargassum* as a transportation vehicle into the deep-sea ecosystem. In addition, *Sargassum* might be used as additional carbon source for protist communities, linking the algae to higher trophic levels via protists. Furthermore, our investigations point to the lack of our understanding regarding the components of deep-sea microbial food webs. We found nearly the same extremely high diversity of protist OTUs in the upper 2 mm of deep-sea sediment when compared to the global survey of productive surface waters, which investigated up to 2.5 times more stations having twice as many OTUs (de Vargas et al., 2015). However, comparing the hyperdiverse lineages from our global deep-sea approach with the global plankton survey showed, that surface water and deep-sea protist communities differ on a large scale. Overall, our metagenomic investigations of the 20 deep-sea basins located in the Atlantic and Pacific Ocean showed, that the different deep-sea basins harbour a specific protist community with less than 1% of protist OTUs occuring at all sampled stations. Due to the result of deep-sea protist communities being $\sim$ 50 cm apart, biotic environmental factors seem to already shape deep-sea protist communities on small spatial scales. It is very likely that multiple processes operate at the same time structuring communities of deep-sea protists including bathymetric features, depths and biotic environmental factors may all play a role.

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Subpuplications and Records of Achievement

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Chapter 1

Schoenle A., Jeuck A., Nitsche F., Venter P., Prausse D., Arndt H., **2016**. Methodological studies on estimates of abundance and diversity of heterotrophic flagellates from the deep-sea floor. Journal of Marine Science and Engineering 4 (1), 22. doi: 10.3390/jmse4010022

The conception and writing was conducted by the author under guidance of Prof. Dr. Hartmut Arndt. Results were achieved by the author and coauthors Paul Venter, Alexandra Jeuck and Dennis Prausse.

Chapter 2

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The study was designed and planned together with Prof. Dr. Hartmut Arndt. Pressure experiments were carried out by the author and coauthor Jennifer Werner. Molecular analyses and the literature review were carried out by the author. The article was mainly written by the author under guidance of Prof. Dr. Hartmut Arndt.

Schoenle A., Živaljić S., Prausse D., Voß J., Arndt H., **2019**. New phagotrophic euglenids from deep sea and surface waters of the Atlantic Ocean (*Keelungia nitschei*, *Petalomonas* gen. nov., *Ploeotia costaversata*). European Journal of Protistology 68, 102-116. doi: 10.1016/j.ejop.2019.02.007

The study was designed and planned together with Prof. Dr. Hartmut Arndt. Pressure experiments were carried out by Suzana Živaljić. Electron and light microscopy were performed by Dr. Frank Nitsche, Brigitte Gräfe, Dennis Prausse and Janine Voß. Molecular analyses were carried out by the author, Janine Voß and Dennis Prausse. Phylogenetic analyses were performed by the author. The article was mainly written by the author under guidance of Prof. Dr. Hartmut Arndt.

Schoenle A., Hohlfeld M., Rosse M., Filz P., Nitsche F., Arndt H., **2020**. Global comparison of bicosoecid *Cafeteria*-like flagellates from the deep ocean and surface waters, with

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The conception, the bioinformatic evaluation, the statistical analyses were carried out by the author. The molecular biological work was conducted mainly by the author, together with Manon Hohlfeld, Mona Rosse and Paulina Filz. Light microscopy was performed by Brigitte Gräfe. The article was mainly written by the author under guidance of Prof. Dr. Hartmut Arndt.

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Chapter 4

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The author was partly involved in the writing of the article and in performing and assisting in several laboratory experiments.

Further publications

Živaljić S., **Schoenle A.**, Nitsche F., Hohlfeld M., Piechocki J., Reif F., Shumo M., Weiss A., Werner J., Witt M., Voss J., Arndt H., **2017**. Survival of marine heterotrophic flagellates isolated from the surface and the deep sea at high hydrostatic pressure: Literature review and own experiments. Deep Sea Research Part II: Topical Studies in Oceanography 148, 251-259. doi: 10.1016/j.dsr2.2017.04.022

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Appendix A

Survival of Marine Heterotrophic Flagellates Isolated from the Surface and the Deep Sea at High Hydrostatic Pressure: Literature Review and Own Experiments

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Survival of marine heterotrophic flagellates isolated from the surface and the deep sea at high hydrostatic pressure: Literature review and own experiments

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ABSTRACT

Although the abyssal seafloor represents the most common benthic environment on Earth, eukaryotic microbial life at abyssal depths is still an uncharted territory. This is in striking contrast to their potential importance regarding the material flux and bacteria consumption in the deep sea. Flagellate genotypes determined from sedimentary DNA deep-sea samples might originate from vital deep-sea populations or from cysts of organisms sedimented down from surface waters. The latter one may have never been active under deep-sea conditions. We wanted to analyze the principal ability of cultivable heterotrophic flagellates of different phylogenetic groups (choanoflagellates, ancyromonads, euglenids, kinetoplastids, bicosoecids, chrysomonads, and cercozoans) to survive exposure to high hydrostatic pressure (up to 670 bar). We summarized our own studies and the few available data from literature on pressure tolerances of flagellates isolated from different marine habitats. Our results demonstrated that many different flagellate species isolated from the surface waters and deep-sea sediments survived drastic changes in hydrostatic pressure. Barophilic behavior was also recorded for several species isolated from the deep sea indicating their possible genetic adaptation to high pressures. This is in accordance with records of heterotrophic flagellates present in environmental DNA surveys based on clone libraries established for deep-sea environments.

1. Introduction

Although the deep sea covers over half of the Earth's surface (Gage and Tyler, 1991), it remains one of the most unknown and unexplored habitats on Earth due to limited access caused by expensive and time-consuming ship time as well as extreme environmental conditions. The abyssal sea floor extends between 3000 and 6000 m (Bruun, 1956). Deep-sea organisms have to cope with extreme environmental conditions including low food resources, lower temperatures, darkness and high pressure, making life more challenging in the deep sea in comparison to surface waters. Despite the vastness of this biotope, the most studies of marine ecosystems are based on protists inhabiting the euphotic zone, while deep-sea protistan assemblages remain largely uncharacterized (Countway et al., 2007; Schoenle et al., 2016). However, there are some exceptions, such as deep-sea foraminiferans, who have received considerable attention because of their geological significance and morphological diversity (Countway et al., 2007; Pawlowski et al., 2011; Gooday and Jorissen, 2012).

Flagellated protists play an important role in microbial food webs (Jürgens and Massana, 2008). Heterotrophic flagellates (HF) are known as important grazers of bacteria in many aquatic ecosystems (Arndt et al., 2000; Boenigk and Arndt, 2002) with capabilities of regenerating nutrients and other inorganic molecules, enhancing the bioavailability of these compounds to other organisms (Fenchel, 1982; Sherr et al., 1983; Caron and Goldman, 1990). In shallow benthic and pelagic marine ecosystems, the importance of protozoan associations in energy transfer through aquatic food webs has been well established (Azam et al., 1983; Alldredge et al., 1986; Patterson et al., 1993). Although primary production is limited to the euphotic zone, delivery of fixed carbon to the deep sea via sinking detritus and carcasses provides a link between surface-associated and deep-sea detritus based microbial food webs (Caron et al., 1982; Gooday, 2002; Arndt et al., 2003). Whether all protists from euphotic surface waters can grow under deep-sea conditions is still unknown. According to Morgan-Smith et al. (2013), some

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surface isolates of *Cafeteria roenbergensis* and *Neobodo designis* were able to survive after exposure to 2°C and 500 bar (50 MPa) and even positive growth rates were recorded under these conditions. Turley et al. (1988) found a barophilic (better growth at high pressure) bodonid flagellate indicating an adaptation to deep-sea conditions (450 atm; 2 °C). Another flagellate (*Neobodo curvifilus*) was shown to have a wide barotolerance and its reproduction was recorded at pressures of 300 atm; a *Cercomonas*-like species isolated from the deep sea only grew at pressures of ≥300 atm (Turley and Carstens, 1991). Furthermore, Atkins et al. (1998) observed that deep-sea isolates of *Caecitellus parvulus* and *Rhynchomonas nasuta* had a higher growth rate at higher pressures (up to 300 atm) than their shallow-water counterparts. Protists can also form cysts in adverse conditions. A choanoflagellate isolate (*Monosiga* sp.) was observed to encyst at pressures greater than 50 atm (Atkins et al., 1998).

Environmental DNA surveys based on clonal libraries and next generation sequencing have revealed an enormous genotypical diversity of heterotrophic flagellates collected from the deep sea (Lopez-Garcia et al., 2001; Edgcomb et al., 2009; Scheckenbach et al., 2010; Pawlowski et al., 2011; Salani et al., 2012). However, for most genotypes it is not clear whether they originate from vital deep-sea populations or from cysts of organisms sedimented down from surface waters, never being active in the deep sea. Thus, a combination of ecological studies together with molecular surveys is necessary to understand the function of deep-sea heterotrophic flagellate communities. Here, we present ecological studies on pressure tolerance of different flagellate strains isolated from different marine habitats at different depths to check for their ability to survive exposure to high hydrostatic pressures. These data sets expand the few available studies (e.g., Turley and Carstens, 1991; Atkins et al., 1998; Morgan-Smith et al., 2013). We aimed to summarize available knowledge on pressure tolerance of HF and to add a significant number of additional experiments on strains belonging to a very wide range of taxonomic groups surviving hydrostatic pressure of up to 670 bar.

2. Material and methods

We summarized available studies from literature (Turley et al., 1988; Turley and Carstens, 1991; Atkins et al., 1998; Morgan-Smith et al., 2013) and compared these with our own studies. The conditions in the experiments were principally similar, though some basic parameters differed (Table 1).

In general, literature and own studies were carried out in a similar way: deep-sea and surface water samples were subsampled and cultivated in sea-water medium (≈34–35 PSU). Monocultures (except for Turley and Carstens (1991), who used mixed cultures) were established and stored for long-term cultivation at atmospheric pressure. Before performing pressure experiments, cultures were pre-cultivated at the respective experimental temperature until flagellates reached the exponential growth phase. Except in experiments done by Morgan-Smith et al. (2013), cultures were not acclimated to the experimental temperature and they used a continuous-flow chemostat to grow the flagellates in high density. Experimental cultures were enriched by autochthonous bacteria using additions of organics (cereal grains or glucose) or direct additions of bacteria obtained from cultures (*Pseudomonas putida*, *Holomonas halodurans*). In all experiments, it was assumed that food concentrations for flagellates were high enough to ensure ad libitum conditions. To determine whether heterotrophic flagellates (HF) were able to survive or even grow at experimental conditions, HF abundance was determined in subsamples taken from the experimental vessels and control vessels at the beginning, daily and at the end of experiments. The number of replicates (experimental vessels) varied between 3 and 14. Exposure to the experimental hydrostatic pressure was conducted in two ways: experimental vessels were exposed either directly to the final experimental pressure (end point; EP, see Table 1) or via different steps (time point; TP), and HF abundance and survival were analyzed after each step. In the latter, experimental vessels were released from pressure and exposed to the next higher pressure immediately after subsampling. Atkins et al. (1998) showed that time point experiments revealed a pressure tolerance of HF lying in the same range of end point experiments. In most experiments (see Table 1), end point sampling was used. Daily sampling, with minimum loss of pressure, was done only in experiments from Morgan-Smith et al. (2013). Except for Turley et al. (1988) and Morgan-Smith et al. (2013) live-counting was performed. Control vessels were investigated which were left at atmospheric pressure (all studies except for those by Morgan-Smith et al., 2013). Morgan-Smith et al. (2013) exposed control vessels at 0.8 MPa (8 bar).

2.1. Origin and cultivation of heterotrophic flagellates (HF)

We collected surface water samples from the Atlantic, Pacific Ocean, and the Baltic Sea. Deep-sea samples were obtained from different depths using a Multicorer system (Table 1). Samples were collected during different cruises with the research vessels R/V *Sonne* (SO223T, 2012; SO237, 2014/2015), R/V *Meteor* (ME 71/2, 2007; ME79/1, 2009), and R/V *Polarstern* (PS 62, 2002). Only corers with undisturbed sediment and overlaying water were used for further analyses. Once on deck, cores were immediately processed. Defined volumes of sediment (2 ml) or surface water (1 ml) were transferred into 50 ml-tissueculture flasks (Sarstedt, Nümbrecht, Germany) filled with 30 ml autoclaved seawater (35 PSU) and two quinoa grains to ensure growth of autochthonous bacteria. In the home laboratory, monocultures of different species were established by serial dilution or with the help of a micromanipulator (PatchMan NP 2 from Eppendorf, Germany) under an inverted microscope (ZEISS Axiovert 25). Isolated species were cultivated in 50 ml-tissue-culture flasks (Sarstedt, Nümbrecht, Germany) filled with 30 ml of autoclaved 35 PSU Schmaltz-Pratt medium (a liter contained 28.15 g NaCl, 0.67 g KCl, 5.51 g $MgCl_2$ x 6 H_2O, 6.92 g $MgSO_4$ x 7 H_2O, 1.45 g $CaCl_2$ x 2 H_2O, 0.10 g KNO_3, and 0.01 g K_2HPO_4 x 3 H_2O) and supplied with quinoa or wheat grains as a carbon source for autochthonous bacteria. Long-term cultivation conditions were 10 °C at 23-35 PSU.

2.2. Pressure experiments

Experiments on pressure tolerance of HF were carried out with a pressure generating system at pressures of up to 670 bar. The system consisted of three stainless steel pressure chambers (ø 30 mm, depth 50 mm) and a pressure pump with a transfer ratio of 1:400 bar (SITEC M 189-2 L, AMATIC-DIETRICH GmbH). Experimental cultures were adapted for two weeks either to 2 °C (deep sea of Atlantic), 13 °C (deep sea of the Mediterranean) or 20 °C (room temperature). To ensure unlimited food conditions, cultures were provided with quinoa, wheat or canola grains (long-term experiments) or with a glucose solution (3 ml of 2 g l^{-1} glucose added to 30-ml-batch culture; short-term experiments) to support growth of coexisting bacteria. One set of experiments (undetermined choanoflagellate HFCC 824) was carried out with a direct addition of bacteria (*Pseudomonas putida*).

We exposed isolated flagellate strains either directly (end point; EP) or stepwise (time point; TP) to different hydrostatic pressures. In experiments exposed to a direct increase of hydrostatic pressure (EP), all samples were observed at the beginning and after a period of 1 to 7 days of exposure to different pressures ranging from 50 to 670 bar. The pressure was established within a few minutes. In the other sets of experiments, a stepwise increase of pressure (TP) was applied (Table 1). Flagellates were exposed to pressure in total for 6 or 7 days and were decompressed every two days for taking subsamples and were then exposed to higher pressure again. Survival of the flagellates was recorded when the active movement of flagella was observed after exposure to a given hydrostatic pressure.

All experiments were run with 3-10 replicates. The same number of

Table 1
List of species (literature and own studies) used for pressure experiments. Strain number, origin of strains and experimental conditions are given. Experimental conditions included experimental temperature, exposure time, applied pressure as well as the kind of pressure experiment (end point (EP) or time point (TP)). Number of replicates (N) in experiments, the experimental volume and an indication which type of food was provided is also given. The pressure at which survival could be observed is marked in bold. Strains which were studied at different experimental conditions are marked with one to three asterisks (*). Underlined strains were investigated by Turley et al. (1988), Turley and Carstens (1991), Atkins et al. (1998) and Morgan-Smith et al. (2013). For further information on the sampling location and depth etc. see Table S1.

Taxonomy	Strain	Isolate origin (depth [m])	Exp. temp. [°C]	Exp. time [h]	Pressure [bar]	EP/TP	N	Exp. volume [mL]	Added food	Author
Choanoflagellate										
Undet. choanoflagellate	HFCC[h] 164	Atlantic (5756)	20	20	**500**	EP	4	2.16	+ gluc[j]	This study
Undet. choanoflagellate	HFCC 199	Atlantic (0)	20	20	**460**	EP	4	2.16	+ gluc	This study
Monosiga sp.[a]	BSZ[c] 6	Pacific (2500)	20	144	**50**, 100, 150, 200, 250, 300	EP	3	10	+ *H.h.*[k]	Atkins et al., 1998
Undet. choanoflagellate	HFCC 824	Pacific (5276)	13	144	**250**,**350**, **450**, **550**	EP	8	0.6	+ grain[l]	This study
Undet. choanoflagellate	HFCC 824*	Pacific (5276)	13	144	**250**, **350**, **550**	EP	4	0.6	+ *P.p.*[m]	This study
Undet. choanoflagellate	HFCC 824**	Pacific (5276)	13	144	**350**, **550**	TP	4	0.6	+ *P.p.*	This study
Undet. choanoflagellate	HFCC824***	Pacific (5276)	13	144	**350**, **450**, **550**	TP	8	0.6	+ grain	This study
Ancyromonads										
Ancyromonas sp.[a]	BRM[b] 2	Pacific (2500)	3.5	144	**50**, **100**, **150**, 200, 250, 300	EP	3	10	+ *H.h.*	Atkins et al., 1998
Euglenids										
Keelungia sp.	HFCC 166	Atlantic (5117)	20	20	540	EP	4	2.16	+ gluc	This study
Kinetoplastids										
Neobodo curvifilus	III	Atlantic (2665)	5	72	**50**, **100**, **200**, **300**, **450**	EP	2	0.35	+ grain	Turley and Carstens, 1991
Bodo sp.	I	Atlantic (4500)	2	528	**450**	TP	14	160	–	Turley et al., 1988
Neobodo designis	SCCAP[i] V	? (0)	2	120–360	**500**	EP	6	5	+ grain	Morgan-Smith et al., 2013
N. designis	HFCC 8	Baltic (0)	2	168	**50**, **150**, 250	EP	3	0.2	+ grain	This study
N. designis	HFCC 8*	Baltic (0)	13	1.5	**50**, **100**, **150**, 200, 250, 300	EP	3	0.2	+ grain	This study
N. designis	HFCC 8**	Baltic (0)	13	72	**50**, **150**, **250**	EP	3	0.2	+ grain	This study
N. designis	HFCC 8***	Baltic (0)	13	72	**50**,**150**,**250**, **350**,**450**,550	EP	10	0.2	+ grain	This study
N. designis	HFCC 110	Mediterranean (2670)	2	168	**50**, **150**, 250	EP	3	0.2	+ grain	This study
N. designis	HFCC 110*	Mediterranean (2670)	13	1.5	**50**, **100**, **150**, **200**, 250, 300	EP	3	0.2	+ grain	This study
N. designis	HFCC 110**	Mediterranean (2670)	13	72	**50**, **150**, **250**	EP	3	0.2	+ grain	This study
N. designis	HFCC110***	Mediterranean (2670)	13	72	**50**,**150**,**250**, **350**,**450**,**550**	EP	10	0.2	+ grain	This study
N. designis	HFCC 140	Atlantic (5121)	13	1.5	**50**, **100**, **150**, **200**, **250**, 300	EP	3	0.2	+ grain	This study
N. designis	HFCC 144	Atlantic (4474)	13	1.5	**50**, **100**, **150**, **200**, **250**, 300	EP	3	0.2	+ grain	This study
Rhynchomonas nasuta	BSZ[c] 1	Pacific (2500)	20	144	**50**, **100**, **150**, **200**, **250**, 300	EP	3	10	+ *H.h.*	Atkins et al., 1998
R. nasuta	CBR[f] 1	Atlantic (0.5)	20	144	**50**, **100**, **150**, **200**, 250, 300	EP	3	10	+ *H.h.*	Atkins et al., 1998
R. nasuta	HFCC 18	Atlantic (0)	2	168	**50**, **150**, 250	EP	3	0.2	+ grain	This study
R. nasuta	HFCC 18*	Atlantic (0)	13	1.5	**50**, **100**, **150**, 200, 250, 300	EP	3	0.2	+ grain	This study
R. nasuta	HFCC 18**	Atlantic (0)	13	72	**50**, **150**, **250**	EP	3	0.2	+ grain	This study
R. nasuta	HFCC 18***	Atlantic (0)	13	72	**50**,**150**,**250**, **350**,**450**,550	EP	10	0.2	+ grain	This study
R. nasuta	HFCC 99	Atlantic (2484)	13	1.5	**50**, **100**, **150**, **200**, 250, 300	EP	3	0.2	+ grain	This study
R. nasuta	HFCC 147	Atlantic (5121)	2	168	**50**, **150**, 250	EP	3	0.2	+ grain	This study

(continued on next page)

Table 1 *(continued)*

Taxonomy	Strain	Isolate origin (depth [m])	Exp. temp. [^{0}C]	Exp. time [h]	Pressure [bar]	EP/TP	N	Exp. volume [mL]	Added food	Author
R. nasuta	HFCC 147*	Atlantic (5121)	13	1.5	**50**, **100**, **150**, **200**, **250**, 300	EP	3	0.2	+ grain	This study
R. nasuta	HFCC 147**	Atlantic (5121)	13	72	**50**, **150**, **250**	EP	3	0.2	+ grain	This study
R. nasuta	HFCC147***	Atlantic (5121)	13	72	**50**,**150**,**250**, **350**,**450**,**550**	EP	10	0.2	+ grain	This study
R. nasuta	HFCC 149	Atlantic (4478)	13	1.5	**50**, **100**, **150**, 200, 250, 300	EP	3	0.2	+ grain	This study
R. nasuta	HFCC 171	Atlantic (8346)	20	20	**660**	EP	3	2.16	+ gluc	This study
R. nasuta	HFCC 173	Atlantic (0)	20	20	**670**	EP	4	2.16	+ gluc	This study
Bicosoecids										
Caecitellus parvulus	EWM[d] 1	Pacific (2500)	20	144	**50**, **100**, **150**, **200**, **250**, 300	EP	3	10	+ *H.h.*	Atkins et al., 1998
C. parvulus	NBH[g] 4	Atlantic (2.5)	20	144	**50**, **100**, **150**, **200**, **250**, 300	EP	3	10	+ *H.h.*	Atkins et al., 1998
Cafeteria roenbergensis	SCCAP[i] VI	? (0)	2	168–192	**500**	EP	6	5	+ grain	Morgan-Smith et al., 2013
C. roenbergensis	HFCC 34	Atlantic (0)	2	168	**50**, **150**, 250	EP	3	0.2	+ grain	This study
C. roenbergensis	HFCC 34*	Atlantic (0)	13	1.5	**50**, **100**, **150**, **200**, **250**, 300	EP	3	0.2	+ grain	This study
C. roenbergensis	HFCC 34**	Atlantic (0)	13	72	**50**, **150**, **250**	EP	3	0.2	+ grain	This study
C. roenbergensis	HFCC 34***	Atlantic (0)	13	72	**50**,**150**,**250**, **350**,**450**,**550**	EP	5	0.2	+ grain	This study
Cafeteria sp.	HFCC 114	Mediterranean (2670)	2	168	**50**, **150**, 250	EP	3	0.2	+ grain	This study
Cafeteria sp.	HFCC 114*	Mediterranean (2670)	13	1.5	**50**, **100**, **150**, **200**, **250**, 300	EP	3	0.2	+ grain	This study
Cafeteria sp.	HFCC 114**	Mediterranean (2670)	13	72	**50**, **150**, **250**	EP	3	0.2	+ grain	This study
Cafeteria sp.	HFCC 115	Mediterranean (4318)	13	1.5	**50**, **100**, **150**, **200**, **250**, 300	EP	3	0.2	+ grain	This study
Cafeteria sp.	HFCC 115*	Mediterranean (4318)	13	72	**50**,**150**,**250**, **350**,**450**,**550**	EP	5	0.2	+ grain	This study
C. roenbergensis	HFCC 187	Atlantic (0)	20	20	**520**	EP	4	2.16	+ gluc	This study
C. roenbergensis	HFCC 803	Pacific (1259)	2	168	**200**, **260**	TP	6	0.2	+ grain	This study
C. roenbergensis	HFCC 803*	Pacific (1259)	13	168	**150**, **250**, 460	TP	7	0.2	+ grain	This study
C. roenbergensis	HFCC 804	Pacific (2687)	2	168	**200**, **260**	TP	6	0.2	+ grain	This study
C. roenbergensis	HFCC 804*	Pacific (2687)	13	168	**150**, **250**, **460**	TP	7	0.2	+ grain	This study
C. roenbergensis	HFCC 808	Pacific (5497)	13	168	**150**, **250**, **460**	TP	7	0.2	+ grain	This study
C. roenbergensis	HFCC 827	Pacific (2776)	2	168	**200**, **260**	TP	6	0.2	+ grain	This study
C. roenbergensis	HFCC 827*	Pacific (2776)	13	168	**150**, **250**, **460**	TP	7	0.2	+ grain	This study
C. roenbergensis	HFCC 167	Atlantic (5667)	20	20	**460**	EP	4	2.16	+ gluc	This study
Chrysomonads										
Undet. Chrysomonad	DA[e] 2	Atlantic (1500)	3.5	144	**50**, **100**, **150**, **200**, 250, 300	EP	3	10	+ *H.h.*	Atkins et al., 1998
Undet. Chrysomonad	DA[e] 3	Atlantic (1500)	3.5	144	**50**, **100**, **150**, 200, 250, 300	EP	3	10	+ *H.h.*	Atkins et al.,1998
Paraphysomonas butcheri	IV	Atlantic (2665)	5	72	**50**, **100**, **200**, 300, 450	EP	2	0.35	+ grain	Turley and Carstens, 1991
Spumella sp.	HFCC 29	Pacific (0)	2	168	**50**, **150**, 250	EP	3	0.2	+ grain	This study
Spumella sp.	HFCC 29*	Pacific (0)	13	1.5	**50**, **100**, **150**, **200**, **250**, 300	EP	3	0.2	+ grain	This study
Spumella sp.	HFCC 29**	Pacific (0)	13	72	**50**, **150**, **250**	EP	3	0.2	+ grain	This study
Spumella sp.	HFCC 29***	Pacific (0)	13	72	**50**,**150**,**250**, **350**,**450**,**550**	EP	5	0.2	+ grain	This study
Spumella sp.	HFCC 130	Atlantic (4604)	2	168	**50**, **150**, 250	EP	3	0.2	+ grain	This study
Spumella sp.	HFCC 130*	Atlantic (4604)	13	1.5	**50**, **100**, **150**, **200**, **250**, 300	EP	3	0.2	+ grain	This study
Spumella sp.	HFCC 130**	Atlantic (4604)	13	72	**50**, **150**, **250**	EP	3	0.2	+ grain	This study
Spumella sp.	HFCC130***	Atlantic (4604)	13	72	**50**,**150**,**250**, **350**,**450**,**550**	EP	5	0.2	+ grain	This study

(continued on next page)

Table 1 *(continued)*

Taxonomy	Strain	Isolate origin (depth [m])	Exp. temp. [^{0}C]	Exp. time [h]	Pressure [bar]	EP/TP	N	Exp. volume [mL]	Added food	Author
Cercozoans										
Undet. Cercomonad	II	Atlantic (2665)	5	72	50, 100, 200, **300**, **450**	EP	2	0.35	+ grain	Turley and Carstens, 1991
Massisteria sp.	HFCC 192	Atlantic (4997)	20	20	**520**	EP	4	2.16	+ gluc	This study

[a] tentative identification (see Atkins et al., 1998).
[b] BRM-Biovent *Riftia* and Mussel Bed.
[c] BSZ- Biovent Serpulid Zone.
[d] EWM-East Wall Mussel Bed.
[e] DA- Deep Atlantic Shelf.
[f] CBR- Chesapeake Bay Isolate obtained from the culture collection of David Caron (Woods Hole Oceanographic Institution).
[g] NBH- New Bedford Harbor, MA. USA.
[h] HFCC- Heterotrophic Flagellate Culture Collection Cologne.
[i] SCCAP- Scandinavian Culture Collection of Algae and Protozoa, University of Copenhagen, Denmark.
[j] Gluc- glucose.
[k] H.h. – Halomonas holodurens.
[l] Grain- wheat, barley, rice.
[m] P.p. – Pseudomonas putida.

control samples was exposed at the same conditions (temperature, food supply) at atmospheric pressure (1 bar). Samples were either placed into 200 µl microcentrifuge tubes or small cuvettes (2.16 ml) avoiding the formation of any air bubble. HF were counted by the living droplet method (Massana and Güde, 1991; Gasol, 1993) under an inverted microscope (Zeiss Axiovert 100, Germany, magnification 200-630x) placing 1-10 µl on a microscope slide or in a miniaturized version of a Sedgewick-Rafter chamber (Arndt et al., 2000). Each subsample was counted 3 to 5 times.

Growth rates (r) per day (assuming exponential growth) were calculated using the following equation: $r = (\ln N_{t2} - \ln N_{t1}) / (t_2 - t_1)$, where N is the abundance [cell/µl] of organisms at the beginning (t_1) and the end (t_2) of the experiment. We defined the survival of a strain when living organisms were still present in samples (detection limit about 10 individual per ml) after exposure to the respective pressure. Statistical analysis was performed using a one-way ANOVA to test for the influence of pressure on growth rates of heterotrophic flagellates comparing growth rates at incubations with and without (control) pressure.

3. Results

3.1. Pressure resistance of strains from different origin

Almost all deep-sea strains survived the exposure to high hydrostatic pressures up to 300 bar (and sometimes up to 550 bar) at different temperatures (Fig. 1A-C), except for the deep-sea isolate of the euglenid *Keelungia* sp. (HFCC 166; Fig. 1C). In some short-term exposure experiments at 20 °C, flagellates isolated from deep and surface waters survived pressures ranging between 450-670 bar (Fig. 1C). At lower temperatures (2 °C, 3.5 °C, 5 °C) surface strains generally survived only pressures up to 150 bar (Fig. 1A). Exceptions were *Cafeteria roenbergensis* (SCCAP VI) and *Neobodo designis* (SCCAP V) with recorded survival at 500 bar. The pressures at which HF strains survived are indicated in bold in Table 1 (column “Pressure”).

Deep-sea strains of **bicosoecids** were able to survive pressure exposure at all experimental temperatures (Fig. 1). Four deep-sea strains of *Cafeteria roenbergensis* (HFCC 167 at 20 °C; HFCC 804*, HFCC 808, HFCC 827* at 13 °C) survived until the maximum tested pressure (460 bar). The highest maximum pressure of exposure was 550 bar under which the deep-sea strain *Cafeteria* sp. (HFCC 115*, 13 °C) was able to survive after exposure (Fig. 1B). Furthermore, two surface strains of *C. roenbergensis* (HFCC 187 at 20 °C; HFCC 34*** at 13 °C) survived the pressure exposure between 520-550 bar.

An undetermined choanoflagellate (HFCC 164), isolated from the deep sea (5756 m), showed the ability to survive at 500 bar. At the same temperature, the surface strain of another undetermined choanoflagellate (HFCC 199) survived a maximum pressure of 460 bar (Fig. 1C).

Within the **chrysomonads**, the deep-sea strain *Spumella* sp. (HFCC 130) was used for pressure experiments at 2 °C and 13 °C. Better survival (≥250 bar) was recorded at higher temperature (13 °C) and even the maximum pressure was tolerated (550 bar) (Fig. 1B). Similar results were observed for the surface strain *Spumella* sp. (HFCC 29).

Furthermore, among **bodonids** *Bodo* sp. (I; Turley et al., 1988) and *Neobodo curvifilus* (III; Turley and Carstens, 1991) survived an exposure to a maximum pressure of 450 bar at 2 °C. At higher temperatures (13 °C and 20 °C), survival was recorded for several deep-sea strains (*Neobodo designis*, HFCC 110***; *Rhynchomonas nasuta*, HFCC 147***; *R. nasuta*, HFCC 171) until a maximum pressure of up to 650 bar (Fig. 1B; Fig. 1C).

An undetermined **cercozoan** (II; Turley and Carstens, 1991) showed survival between 300450 bar at 5 °C (Fig. 1A). For this strain survival was not recorded at lower pressures. In our experiments, another cercozoan, *Massisteria* sp. (HFCC 192), was able to survive a maximum pressure of 500 bar at 20 °C (Fig. 1C).

One deep-sea strain belonging to **ancyromonads**, *Ancyromonas* sp. (BRM 2, Atkins et al., 1998), showed survival until 150 bar at 3.5 °C, while it was exposed to a maximum pressure of 300 bar (Fig. 1A).

The **influence of surplus food resources** on the survival and growth at high hydrostatic pressures was investigated for several flagellate strains (Fig. 1, AF labelled strains). In general, strains with added bacteria (*Pseudomonas putida, Halomonas holodurens)* did not show better survival at high pressures compared to strains held at normal atmospheric pressure as a control. Some differences were recorded regarding growth rates. Despite additional food, *Monosiga* sp. (BSZ 6; Atkins et al., 1998) formed cysts at a pressure of ≥100 bar.

3.2. Growth at high hydrostatic pressure

Positive growth rates at hydrostatic pressure typical for the deep sea were recorded for a variety of strains belonging to different phylogenetic groups: bicosoecids, cercozoans, choanoflagellates, chrysomonads and kinetoplastids (Fig. 2). Four out of 18 tested strains showed significantly higher growth rates at high hydrostatic pressure compared to exposures at 1 bar (*Cafeteria roenbergensis* strains HFCC167 and HFCC804; *Spumella* sp. HFCC29; *Rhynchomonas nasuta* HFCC147). The *Cafeteria* and *Rhynchomonas* strains were originally isolated from the deep sea, while the *Spumella* strain was isolated in surface waters.

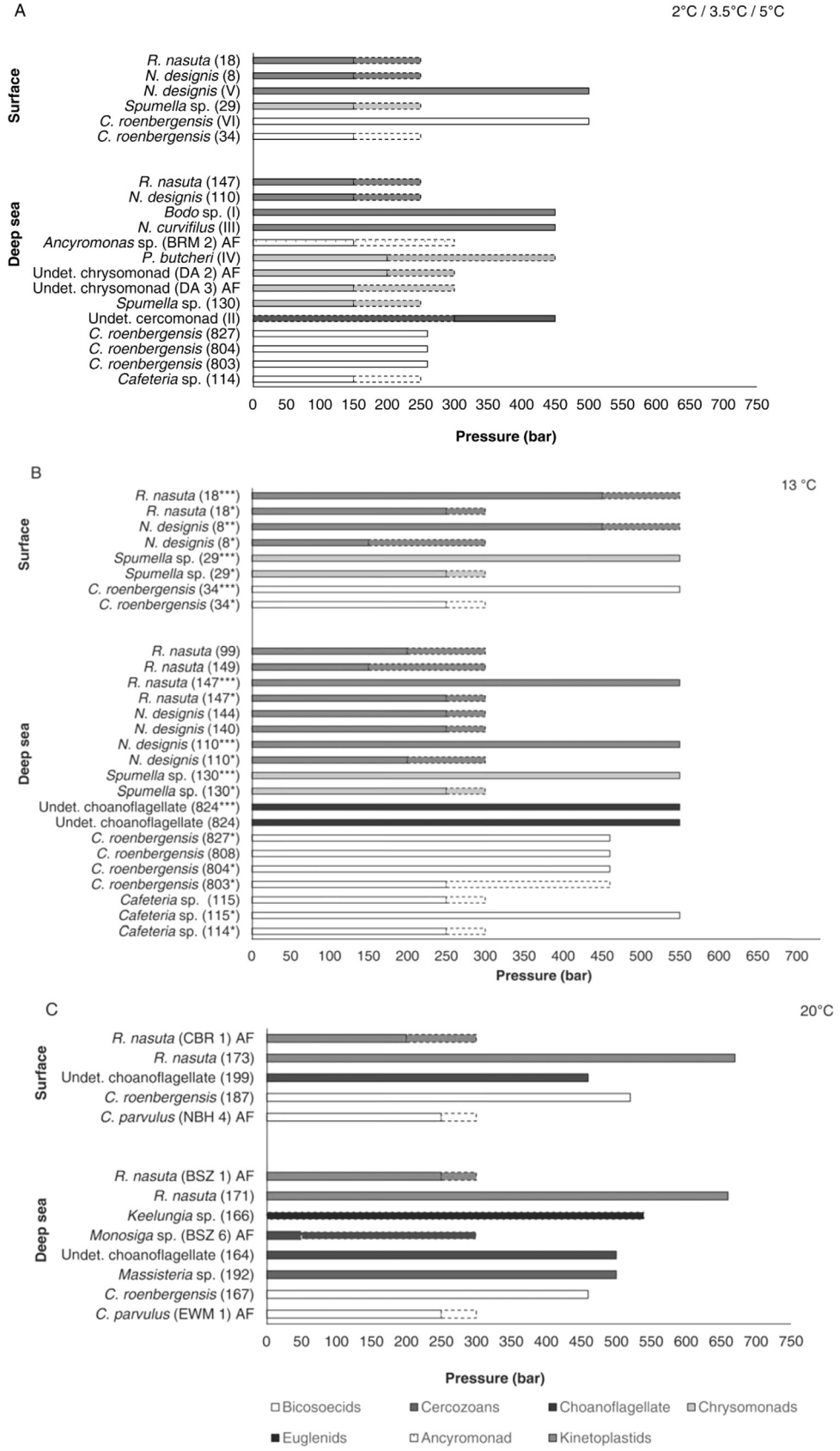

Fig. 1. Survival of marine heterotrophic flagellates isolated from deep-sea and surface waters under high hydrostatic pressures and different temperatures including (A) 2 °C/3.5 °C/5 °C, (B) 13 °C, (C) 20 °C. Experiments were carried out under various conditions: different exposure times (1.5 h – 528 h), different temperatures (2-20 °C), and additions of bacterial food (AF) (*Pseudomonas putida, Halomonas holodurens*)(see Table 1). Filled boxes indicate the range of pressure at which survival was observed. Stippled boxes indicate when the tested range was larger than that survived by the respective strain. Strains which were studied at different experimental conditions are marked with one to three asterisks (*).

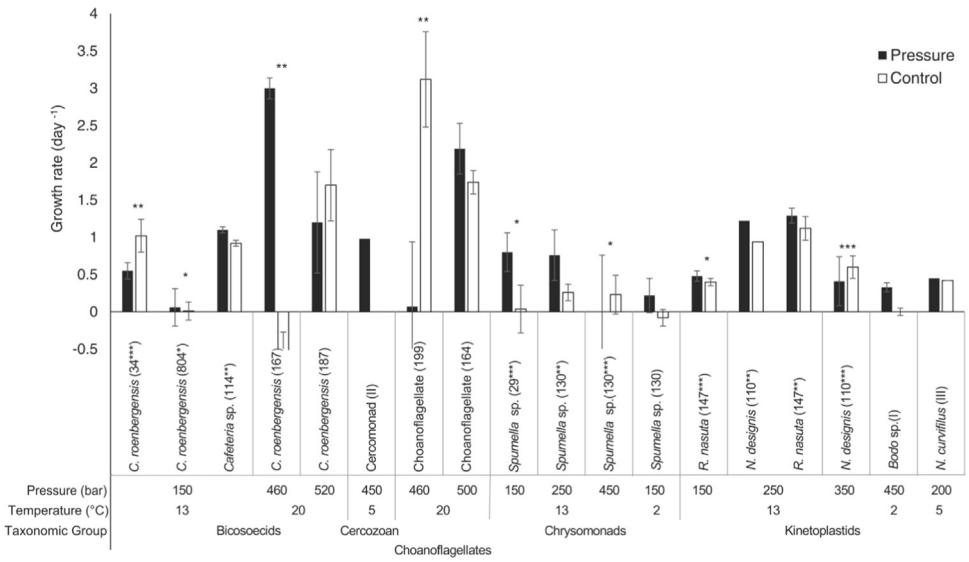

Fig. 2. Growth rates of deep-sea and surface water isolates of marine heterotrophic flagellates exposed to different hydrostatic pressures. Growth rates of flagellates at surface conditions (1 bar) were used as a control. Vertical bars represent ± SD. One-way ANOVAs were used. With the stars (*) above columns are indicated significant differences between pressure treatment and control (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

4. Discussion

Most deep-sea and surface marine heterotrophic flagellates (HF) found in literature (e.g., Turley and Carstens, 1991; Atkins et al., 1998) and in our present study were shown to be barotolerant. Survival was recorded under various pressures, temperatures and other exposure conditions and was independent of the systematic position of the flagellates. At least one strain of bicosoecids, chrysomonads, cercomonads, choanoflagellates, and kinetoplastids were found to survive exposure to very high hydrostatic pressures (> 400 bar). Although flagellate cultures subjected to simulated deep-sea conditions experience a high mortality rate initially, a small portion of the population might remain to reproduce at favorable conditions (Morgan-Smith et al., 2013). In experiments with additional bacterial food supply, deep-sea isolates of *Caecitellus parvulus* and *Rhynchomonas nasuta* had higher positive growth rates than isolates from surface waters which might indicate food limitation at deep-sea conditions (Atkins et al., 1998).

Survival (and sometimes growth) of HF at hydrostatic pressures equal or higher than pressures from depth of isolation indicated that several isolates of HF might have really originated from vital deep-sea populations and were not contaminants from surface waters during sampling. However, this might be considered only for deep-sea isolates as possible proof of their origin. Further studies using RNA-based barcoding at different hydrostatic pressure and/or fixation at the depth of sampling in the deep sea will be necessary to investigate the potential activity of HF in the abyss in future. Since there is a certain detection limit (about 10 individuals per ml), there might have living individuals left in experiments where no survival was recorded. These potentially few remaining cells might remain and reproduce at favorable conditions.

A clear barophilic behavior – a higher growth rate at high hydrostatic pressure compared to growth rates at low hydrostatic pressure - had been reported by Turley et al. (1988). We could add a few further HF strains (*Cafeteria roenbergensis* HFCC167, HFCC804; *Spumella* sp. HFCC29; *Rhynchomonas nasuta* HFCC147) to be potentially barophilic. The long-term cultivation of strains used in the present experiments at 1 bar (sometimes for several years) might have supported the selection for surface adapted genotypes, which have led to an underestimation of barophilic behavior. Furthermore, barophilic behavior of deep sea HF is most probably underestimated since deep-sea samples are generally transported at ambient pressure to the surface neglecting the potential loss of species sensitive to changes in pressure. This is supported by the fact that cultures of deep-sea isolates often get lost a few days after incubation at 1 bar at deep-sea expeditions (Arndt unpubl.). The use of samplers keeping the pressure of the collection site until the transport to the surface might solve this problem in the near future. The occurrence of obvious barophilic behavior of several HF indicates the possible genetic adaptation of HF to high pressures.

Our experiments showed that deep-sea strains were equally successful with handling the pressure at higher temperatures (after adaption in the laboratory during long-term cultivation) compared to lower temperatures. Growth rates obtained from pressure experiments were in the same range or slightly lower than those observed in experiments without pressure (e.g. Fenchel, 1982; Eccleston-Parry and Leadbeater, 1994), however, growth rates are still much higher than in most other eukaryotes under deep-sea conditions. In the vicinity of organic debris or carcasses, sufficient bacteria should be present in the abyss to allow for relatively high growth rates.

Several strains belonging to bicosoecids, chrysomonads kinetoplastids, and choanoflagellates that we isolated from the surface waters showed positive survival (and in some cases growth rates) up to 660 bar at different experimental temperatures. This might indicate that some fractions of sinking protists can survive transport from surface waters to the deep ocean (Arndt et al., 2003) and after a period of acclimation may be able to reproduce and potentially give rise to new populations in the deep (Morgan-Smith et al., 2013). Most protists are able to produce cysts, when the conditions deteriorate. Atkins et al. (1998) have noted several morphological changes among encysting flagellates exposed to increasing pressure. On the other hand, environmental DNA surveys from abyssal depths have shown a HF community structure specific to the deep sea pointing to a unique biodiversity of protists in the abyss (Edgcomb et al., 2002, 2009; Scheckenbach et al. 2010; Pawlowski et al., 2011; Salani et al., 2012). Thus, we have to expect an extremely large hidden diversity of HF in the deep sea.

Heterotrophic flagellates are mainly composed of water which can only slightly be compressed. However, some flagellates and ciliates were shown to be sensitive to hydrostatic pressure (Kitching, 1957; Hemmingsen and Hemmingsen, 1983). For bacteria it is known that an increase of fluidity of membranes by incorporation of unsaturated fatty acids plays a role in the survival at high hydrostatic pressures (Allen et al., 1999; Delong and Yayanos, 1985). Bartlett (2002) gives three possible reasons why mono-unsaturated fatty acids are required for

growth at high pressure. The first possibility is explained with the theory of homeoviscous adaptation, which points out to maintain the membrane within a narrow range of viscosity. The second is that selective pressure will rather maintain the membrane within a liquid--crystalline phase and the third explanation is that permeability to ions (protons or sodium ions) could drive membrane restructuring in response to pressure. A higher fluidity of membranes has also a positive effect on survival at low temperatures, at least for bacteria (DeLong and Yayanos, 1986). In addition to membrane lipids, membrane proteins and enzymes as well as changes in the DNA structure and function have been implicated as playing an important role in growth at high pressure for bacteria (Bartlett, 2002). Whether these effects are important also for protists has not been studied yet, up to our knowledge.

Summarizing, our experiments demonstrated that many species of heterotrophic flagellate species from very different protistan phyla survive drastic changes in hydrostatic pressure. Most species were barotolerant, some barophilic. In accordance with observations based on eDNA metabarcoding showing very specific deep-sea communities of heterotrophic flagellates, our data support the idea that most members of these communities should be able to be active under the high hydrostatic pressure at the deep sea. The discussion of potential endemism for nanoflagellates (< 20 μm in length) compared that for deep-sea metazoans is especially problematic. Benthic nanoflagellates can find habitats on marine snow as well as in/on the sediment. However, also for terrestrial habitats which might be connected by aeroplankton distribution, endemic populations of protists have been recorded (e.g. Foissner, 2006, 2008), and it was also found that marine planktonic foraminiferans have restricted local distributions (de Vargas et al., 1999). The question, whether widespread barotolerance among heterotrophic flagellates should have led to low endemism among littoral and benthic flagellate communities cannot be answered yet. We have not yet enough data on the degree of barophily among deep-sea communities (often these species are difficult to cultivate with present methodologies). Experiments are required to evaluated the competition outcome of heterotrophic flagellate species originating from surface waters and the deep sea exposed under different temperature conditions, food supply and predation pressure.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dsr2.2017.04.022.

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Erklärung gemäß § 4 Absatz 1 Punkt 9 der Prüfungsordnung

“Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzen Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen - , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Hartmut Arndt betreut worden.”

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