Influence of hydrostatic pressure on deep-sea protists

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"What is essential is invisible to the eye."

(Antoine de Saint-Exupéry, The Little Prince)

UNIVERSITY OF COLOGNE

Abstract

Faculty of Mathematics and Natural Sciences

Influence of hydrostatic pressure on deep-sea protists

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. However, deep-sea organisms have to cope with extreme environmental conditions including low food resources, low temperatures, darkness and high pressure, making life more challenging in the deep sea in comparison to surface waters. Despite the vastness of this biotope, most recent studies are based on protists inhabiting the euphotic zone, while deep-sea protistan assemblages remain largely uncharacterized. There are only very few cultures available from protists originating from the deep sea (>3000 m). However, using next generation sequencing technology, a large variety of protist genotypes were determined from deep-sea samples. Whether these protists genotypes originate from vital deep-sea populations or from cysts of organisms sedimented down from surface waters is still an unsolved problem.

To contribute to a solution of this problem, we analysed the principal ability of cultivable surface and deep-sea isolates of 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). Obtained results demonstrated that many different flagellate species isolated from the surface waters and deep-sea sediments survived drastic changes in hydrostatic pressure. Also, barophilic behaviour was recorded for several species isolated from the deep sea indicating their possible genetic adaptation to high pressures. To check the activity of heterotrophic flagellates at high hydrostatic pressures, the movement of three euglenids strains (*Keelungia nitschei*, *Petalomonas acorensis*, *Ploeotia costaversata*) was directly observed under different pressures. These

experiments showed that the deep-sea strain *K. nitschei* was better adapted to high hydrostatic pressures (up to 500 bar) than the two surface strains, *P. acorensis* and *P. costaversata.* Also, the first deep-sea ciliate species, *Euplotes dominicanus*, could be described in the frame of this study based on morphology and molecular phylogeny. To our knowledge, this is the first time that a new ciliate species from abyssal depths of the North Atlantic Ocean (> 4000 m) was isolated, successfully cultivated and morphologically characterized alive. Metabarcoding data demonstrate the presence of *E. dominicanus* in sediments of several deep-sea basins. Further on, the long-term survival experiments were carried out for *E. dominicanus* and two additional deep-sea ciliates, *Aristerostoma* sp. and *Pseudocohnilembus persalinus,* up to 430 bar. Some individuals showed an ability to survive high pressure and to recover (returning to their normal movement) indicating their barotolerance. In addition, the behaviour of these three deep-sea ciliates was directly observed under different pressures. The typical behavioural elements for all three ciliates were observed at least at a pressure of up to 200 bar. This study suggests that ciliates and heterotrophic flagellates inhabit deep-sea waters and should be an active part of deep-sea microbial communities.

UNIVERSITÄT ZU KÖLN

Kurzzusammenfassung

Mathematisch-Naturwissenschaftliche Fakultät

Influence of hydrostatic pressure on deep-sea protists

Obwohl der abyssale Meeresboden den größten benthischen Lebensraum auf der Erde darstellt, ist das eukaryotische mikrobielle Leben in den abyssalen Tiefen noch immer sehr schlecht untersucht. Dies steht im krassen Gegensatz zur potenziellen Bedeutung der einzelligen Eukaryoten (Protisten) für den Stoffffluss und den Bakterienkonsum in der Tiefsee. Tiefseeorganismen sind mit extremen Umweltbedingungen wie niedrigen Nahrungsressourcen, niedrigen Temperaturen, Dunkelheit und hohem Druck konfrontiert, was das Leben in der Tiefsee im Vergleich zu Oberflächengewässern schwierig macht. Trotz der Größe des Lebensraums Tiefsee basieren alle bisherigen Studien an Protisten auf Untersuchungen in der euphotischen Zone, Tiefsee-Protisten blieben bisher weitgehend unberücksichtigt. Von Protisten aus großen Tiefen (>3000 m) gibt es nur sehr wenige Kulturen. Metabarcoding Untersuchungen an Tiefseeproben ergaben eine hohe Diversität an Genotypen von Protisten. Diese können von Protisten, die in der Tiefsee aktiv sind oder von Zysten herrühren, die von Organismen stammen könnten, die sich aus Oberflächengewässern sedimentiert sind.

Im Rahmen der vorliegenden Arbeit wurde zunächst die Fähigkeit heterotropher Flagellaten aus verschiedenen phylogenetischer Gruppen (Choanoflagellaten, Ancyromonaden, Eugleniden, Kinetoplastiden, Bicosoeciden, Chrysomonaden und Cercozoen), die aus Oberflächengewässern und der Tiefsee isoliert wurden, geprüft, ob sie eine Exposition bei hohen hydrostatischen Drücken (bis zu 670 bar) überleben. Die Ergebnisse zeigten, dass viele verschiedene Flagellatenarten drastische Änderungen des hydrostatischen Drucks überstehen. Außerdem wurde für mehrere aus der Tiefsee isolierte Arten barophiles Verhalten festgestellt, was auf eine mögliche genetische Anpassung an hohe Drücke hindeutet. Um die Aktivität heterotropher Flagellaten unter den Bedingungen hohen hydrostatischer Drücke zu überprüfen,

wurde die Bewegung von drei Eugleniden-Arten (*Keelungia nitschei*, *Petalomonas acorensis*, *Ploeotia costaversata*) direkt unter verschiedenen Drücken beobachtet. Diese Versuche zeigten, dass der Tiefseestamm *K. nitschei* besser an hohe hydrostatische Drücke (bis zu 500 bar) angepasst war als die beiden Oberflächenstämme *P. acorensis* und *P. costaversata*.

Außerdem wurde eine neue Tiefsee-Ciliatenart, *Euplotes dominicanus*, basierend auf Morphologie, und molekularer Identität beschrieben. Nach unserer Kenntnis wurde zum ersten Mal eine Ciliatenart aus den Tiefen des Nordatlantiks (> 4000 m) isoliert, erfolgreich kultiviert und morphologisch charakterisiert. Metabarcoding-Daten belegen das Vorkommen von *E. dominicanus* in Sedimenten mehrerer Tiefseebecken. Weiterhin wurden Langzeitexperimente zum Überleben von *E. dominicanus* und zwei weiteren Tiefseeciliaten-Arten, *Aristerostoma* sp. und *Pseudocohnilembus persalinus* (bis zu 430 bar) durchgeführt. Einige Individuen zeigten die Fähigkeit, hohen Druck zu überleben und sich zu erholen (Rückkehr zu ihrer normalen Bewegung), was auf ihre Barotoleranz hinweist. Darüber hinaus wurde das Verhalten dieser drei Tiefseeciliaten bei Exposition bei unterschiedlichem Druck beobachtet. Die typischen Verhaltenselemente konnten für alle drei Ciliatenarten mindestens bis zu einem Druck von 200 bar beobachtet werden. Diese Studie legt nahe, dass Ciliaten und heterotrophe Flagellaten eine wichtige und aktive Komponente des Abyssals darstellen sollten.

Table of Contents

General Introduction

Protists in the deep sea

The deep ocean, usually considered to encompass waters below 200 m, is the largest habitat on our planet by volume and area (Levin et al., 2019). It covers over half of the Earth's surface (Gage and Tyler, 1991). However, the vast majority of oceans remain unseen and unquantified in the face of current and emerging industrial activities and in spite of technologies that have expanded the capability to carry out globally integrated deep-sea observation (Levin et al., 2019). Further deep-sea data limitations are caused by expensive and time-consuming ship time as well as unfavorable environmental conditions which occur there. The environmental conditions in the deep sea are also characterized as extreme ones, as evidenced by low temperature and food resources, permanent darkness and high pressure. In the benthic abyssal, these environmental conditions are quite uniform. The abyssal sea-floor (3000-6000 m depth, Bruun, 1956) is inhabited by a large variety of macroorganisms that have become evolutionary adapted to this environment by changing their morphology and lifestyle. Up to now, less than 1% of the deep-sea area has been investigated despite the recognition of its high biodiversity (Rex and Etter, 2010). Despite the vastness of this biome, most protistological 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., 2017).

Protists are single-celled organisms. They are belonging to the eukaryotes and they possess a true membrane envelope, eukaryotic ribosomal RNA and endoplasmic membranes. Also, they have characteristic eukaryotic organelles (mitochondria, chloroplasts and flagella), histones associated with their chromosomal DNA, the ability to perform phagocytosis and they generally larger than prokaryotic cells (Fenchel, 1987). Usually, their body size ranges from 2 to 200 μm. Protists are very diverse and present in most terrestrial and aquatic environments (Triadó-Margarit and Casamayor, 2012; Bates et al., 2013; del Campo and Massana, 2011; Mahé et al., 2017). They consume bacteria and other protists and they are capable 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) which makes them an important part of the microbial food webs (Jürgens and Massana, 2008). 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). However, there is emerging evidence that protists form also a very significant part of deep-sea microbial food webs (Atkins et al., 1998, Scheckenbach et al., 2010). 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).

Data for protists recorded from the deep sea are mainly available for foraminiferans (Danovaro et al., 2010; Pawlowski et al., 2011; Gooday and Jorissen, 2012). Due to challenges associated with difficulties of sampling and preservation, there is a huge gap of the knowledge about deep-sea flagellated and ciliated protist communities. However, there are some data based on molecular studies indicating their presence in the deep sea. Thus, 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 (López-García et al., 2001; Edgcomb et al., 2009; Scheckenbach et al., 2010; Pawlowski et al., 2011; Salani et al., 2012). Also, a comprehensive analysis of records of genotypes of ciliates from the deep sea (> 1000 m depth) indicated the potential existence of a very large variety of ciliate representatives of all major phylogenetic groups (Karyorelictea, Heterotrichea, Spirotrichea, Armophorea, Litostomatea, Phyllopharyngea, Nassophorea, Colpodea, Prostomatea, Plagiopylea, Oligohymenophorea; Schoenle et al., 2017). A few protists have been isolated from the deep sea, indicating possible adaptations to extreme deep-sea habitats. Whether records of protist genotypes from the deep seafloor might originate from sedimented material from the euphotic surface waters and whether they may grow under deep-sea conditions is still unknown.

The effect of pressure on protists

Up to now, there is not yet enough data on the degree of barophily among deep-sea communities because often these species are difficult to cultivate with present methodologies. Despite these difficulties, there are few cultures successfully obtained from the deep sea and later on used for the experiments. These experiments are mostly performed using closed pressure vessels where a direct observation is not possible (Turley et al., 1988, 1993; Atkins et al., 1998; Morgan-Smith et al., 2013). Turley et al. (1988) found a barophilic (better growth at high pressure) bodonid flagellate isolated from 4500 m depth. This flagellate grew at 450 bar and 2°C and did not grow at 1 bar and 2°C, indicating an adaptation to deep-sea conditions. Most of the present literature data are based on survival ability of the surface and deep-sea isolates of heterotrophic flagellates and ciliates indicating their barotolerance (Kitching, 1957; Schoenle et al., 2017, 2019; Živaljić et al., 2018). According to Morgan-Smith et al. (2013), some surface isolates of *Cafeteria roenbergensis* and *Neobodo designis* were able to survive after exposure to 2°C and 500 bar and even positive growth rates were recorded under these conditions. Another flagellate (*Neobodo curvifilus*) was shown to have a wide barotolerance and its reproduction was recorded at pressures of 300 bar; a *Cercomonas*-like species isolated from the deep sea only grew at pressures of ≥300 bar (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 bar) 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 bar (Atkins et al., 1998). Furthermore, several ciliates belonging to the class Karyorelictea and to the subclasses Suctoria and Peritrichia have been found as epibionts on arthropods collected from the deep sea (Bartsch and Dovgal, 2010; Sedlacek et al., 2013). However, these samples were preserved with ethanol or formalin, not providing the possibility of cultivation and live observation of collected deep-sea ciliates and further ecological experiments. Also, it was shown that deep-sea ciliate strains of *Pseudocohnilembus persalinus* and *Uronema* sp. and one surface strain of *P. persalinus* were able to survive better at 557 bar at lower temperature (2 °C) than at higher temperature (13 °C)

(Schoenle et al., 2017). Some surface ciliates species, belonging to the several different phylogenetic groups of Oligohymenophorea, Spirotrichea, Heterotrichea and Prostomatea, were able to survive and some even to recover their normal activity after the exposure at the pressures up to 953 bar (Kitching, 1957).

Aquatic environments are characterized by gradients of temperature and pressure, both of which affect the functioning of biological membranes (Pond et al., 2014). Aquatic animals are often stimulated to higher activity by pressures of about 100 bar, but they are paralyzed or killed by pressures of from several hundred to 1000 bar (Kitching, 1957). Some flagellates and ciliates were shown to be sensitive to hydrostatic pressure (Kitching, 1957; Hemmingsen and Hemmingsen, 1983). Often, the pressure causes the morphology change of the cells. It starts with the creasing of the body surface, and it is accompanied by an expansion of the pellicle and protoplasmic surface. At higher pressures, the protoplasm later separates from the expanded pellicle with the loss of volume of the protoplasm. When the ciliate was exposed to pressure for a longer period, protoplasm sometimes rounds up. Auclair and Marsland (1958) studied the shape stability of two ciliates, *Blepharisma undulans* and *Paramecium caudatum*, under varying conditions of hydrostatic pressure (up to 689 bar) and temperature (12 °C-25 °C). Cells of *B. undulans* became shorter and rounded at higher pressure (480 bar) and lower temperature (decrease from 25 °C to 12 °C). The same pattern was observed for *P. caudatum* at pressures between 275 and 344 bar and temperatures of 20 °C and 25 °C. The mechanisms in protists that can overcome the effect of the high pressure are still unknown. Still, for bacteria it is known that an increase of fluidity of cell membranes by incorporation of unsaturated fatty acids plays a role in the survival at high hydrostatic pressures (DeLong and Yayanos, 1985; Allen et al., 1999). 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. Nevertheless, it is also known that pressure can cause changes in the movement of the protists. For some ciliates (*Tetrahymena pyriformis*, *Holophrya* sp., *Colpoda cucullus* and *Euplotes* sp.) a moderate pressures (69-207 bar) causes already an increase of their locomotor activity, but in general, high pressure depresses flagellar or ciliary movement, and in most cases, all movements stop at 552-965 bar (Kitching, 1957). Locomotion, the main form of expression of ciliate behaviour, is typically displayed as a trajectory conducted by an individual cell which serves to distribute ciliates in the surrounding space and allows exploration of a new environment (Bohatová and Vďačný, 2018). In the beginnings of studies on the motility and behaviour, an ethogram was used for the detailed description of the behaviour, while today video analysis is mostly used. One of the first scientists, who described the locomotion of several ciliates in details, was Ricci (1990). He described the ciliate behaviour by different elements, but it is not known whether ciliates can perform these elements when exposed to high hydrostatic pressures and what are their potential responses to the stress caused by the increase in pressure.

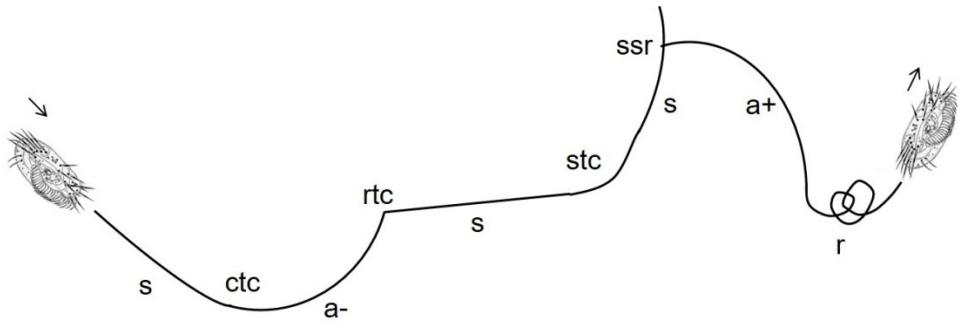

Fig. 1 Schematic drawing of the short lasting elements (continuous trajectory change, ctc; smooth trajectory change, stc; rough trajectory change, rtc; side-stepping reaction, ssr) and the long lasting elements (linear segment, s; rightward arc, a+; leftward arc, a-) according to Ricci (1990). From other elements, the rotation (r) was shown. The arrow indicates the direction of the movement.

The effect of pressure is an important and often overlooked factor in the study of deep-sea ecology (Morgan-Smith et al., 2013). The reason might be because the high hydrostatic pressure is very difficult parameter for imitation in laboratory and laboratory experiments on the effects of hydrostatic pressure on biological organisms are technologically limited. The second reason that there are only a few suitable methods for the isolation and cultivation of deep-sea protists. This lack of knowledge on protists in the largest biome of Earth has encouraged me to start investigations on protists of the deep seafloor to obtain cultures of deep-sea protists and to study their barophilic and/or barotolerant behaviour to get an idea on the potential role of protists in deep-sea ecosystems.

Aims and hypotheses of the study

The general aim of this dissertation was to increase the knowledge on the survival and activity of protists in the deep sea. Protists activity was studied through the survival and behavioural experiments at different temperatures and pressures. It was unclear before, whether protists from the deep sea are active in the deep or originate from cysts sedimenting down from surface waters. A study on the survival ability of very different heterotrophic flagellates isolated from the deep sea and the surface was done. Furthermore, a modified pressure chamber, which can be used at a high hydrostatic pressure, enabled the observation of the behavioural activities at hydrostatic pressures up to 500 bar (Fig. 2B).

For this purpose, we sampled deep-sea sediment from the North Atlantic Ocean and we were able to isolate and successfully cultivate heterotrophic flagellates and three ciliate species. Also, two new species of heterotrophic flagellates were isolated from the surface waters. One ciliate species has been described, using morphological and molecular methods, as a new barotolerant species. Using a modified pressure system (Fig. 2A-D), these protists were exposed at different pressure steps and their behaviour was observed directly under the microscope. Comparison was made between the behaviour of all species conducted under the high pressures and a behaviour at atmospheric pressure (control treatment).

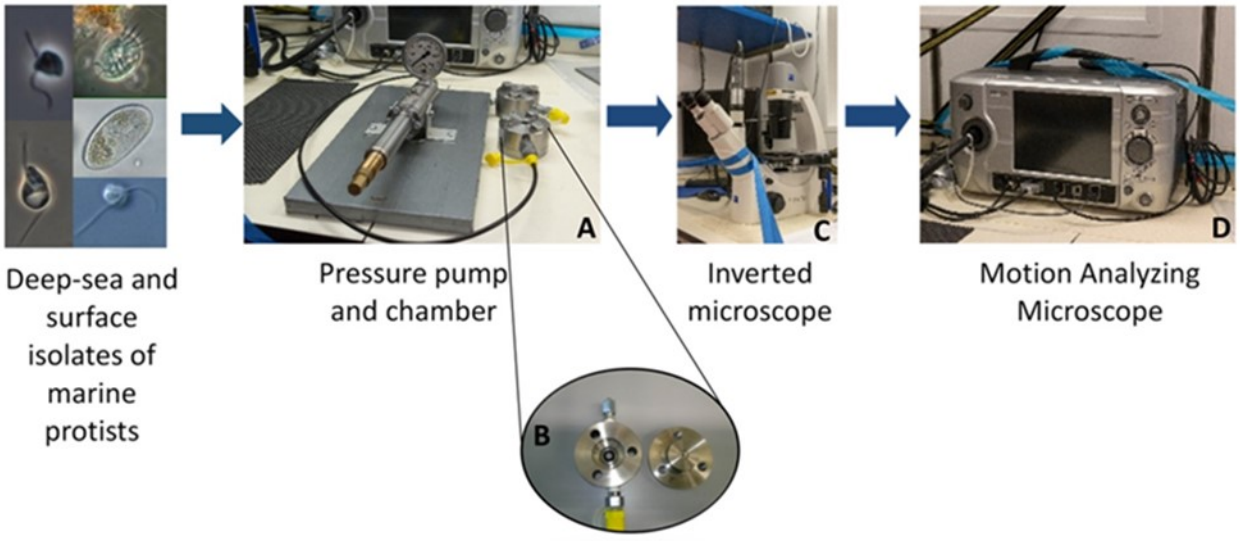

Fig. 2 A-D Workflow of investigations regarding the pressure tolerance of protists. Pressure system consists of: (A) manual hydraulic pump with the ability of gradually increasing the pressure (up to 600 bar), (B) the chamber with a window for direct observation, (C) inverted microscope, (D) Motion Analyzing Microscope (Keyence, VW-6000) consisting of a controller and camera unit.

The main hypothesis of this thesis is that genotypes recordes from abyssal plains belong to organisms that might be active in the deep ocean or originate from cysts sedimenting down from surface waters. In the next four chapters, the possible answers to this hypothesis are discussed.

Chapter 1 - Survival of marine heterotrophic flagellates isolated from the surface and the deep sea at high hydrostatic pressure: Literature review and own experiments.

In this study, we wanted to analyse 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 form literature in pressure tolerance of flagellates isolated from different marine habitats. We aimed to show that many different flagellate species isolated from the surface waters and deep-sea sediments survived drastic changes in hydrostatic pressure. Barophilic behaviour 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.

Chapter 2 - New phagotrophic euglenids from deep sea and surface waters of the Atlantic Ocean (*Keelungia nitschei, Petalomonas acorensis, Ploeotia costaversata*).

This study aimed to increase the knowledge about the overall diversity and phylogenetic relationships of phagotrophic euglenids. Based on morphological and molecular characteristics, three new phagotrophic euglenid species belonging to the petalomonads (*Keelungia nitschei, Petalomonas acorensis*) and ploeotiids (*Ploeotia costaversata*) were described. Also, it was hypothesized whether these euglenids, isolated from the deep-sea and surface habitats, can display behavioural motion at increasing hydrostatic pressure. We aimed to show that the deep-sea strain *Keelungia nitschei* was the only strain still moving at high hydrostatic pressures up to 500 bar at 4 °C, indicating that it is better adapted to high hydrostatic pressure at lower temperatures in comparison with the two isolated surface water strains, *Petalomonas acorensis* and *Ploeotia costaversata*. Thus, the survival at high hydrostatic pressures indicates that the phagotrophic euglenid *K. nitschei* might be active in the deep-sea microbial food webs.

Chapter 3 - A barotolerant ciliate isolated from the abyssal deep sea of the North Atlantic: *Euplotes dominicanus* sp. n. (Ciliophora, Euplotia)

Within this study, we described the new *Euplotes* species, isolated from abyssal depths (>4000 m) of the North Atlantic Ocean, based on morphology, ciliary pattern and molecular data. Phylogenetic analyses inferred from 18S rRNA sequences show that *Euplotes dominicanus* is most closely related to *E. curdsi*, with a sequence similarity of 97.6%. Survival experiments showed that *Euplotes dominicanus* was able to survive hydrostatic pressures up to 500 bar indicating its barotolerance. Additional, metabarcoding data demonstrate the presence of *E. dominicanus* in sediments of several deep-sea basins.

Chapter 4 - Influence of hydrostatic pressure on the behaviour of three ciliate species isolated from the deep-sea floor

In this study, we report on the behaviour of the three ciliate species *Aristerostoma* sp., *Euplotes dominicanus* and *Pseudocohnilembus persalinus* isolated form the deep sea. To our knowledge, these are the first living ciliates isolated from the deep sea (≥4000 m) and observed at high hydrostatic pressure. It was hypothesized that these deep-sea ciliates can display the normal behaviour at high hydrostatic pressure. We showed that all three deep-sea ciliates, performed the typical behavioural elements at least at a pressure of up to 200 bar. Also, long-term survival experiments were carried out with all three ciliates exposed to 200, 350 and 430 bar. Several specimens showed an ability to survive for several days at the highest pressure and to recover from pressure release (returning to their normal movement) indicating their barotolerance. Our results suggest that ciliates are active in the deep sea and might be an important part of the deep-sea microbial food web.

Chapter 1

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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0967-0645/

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. [°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. [°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.

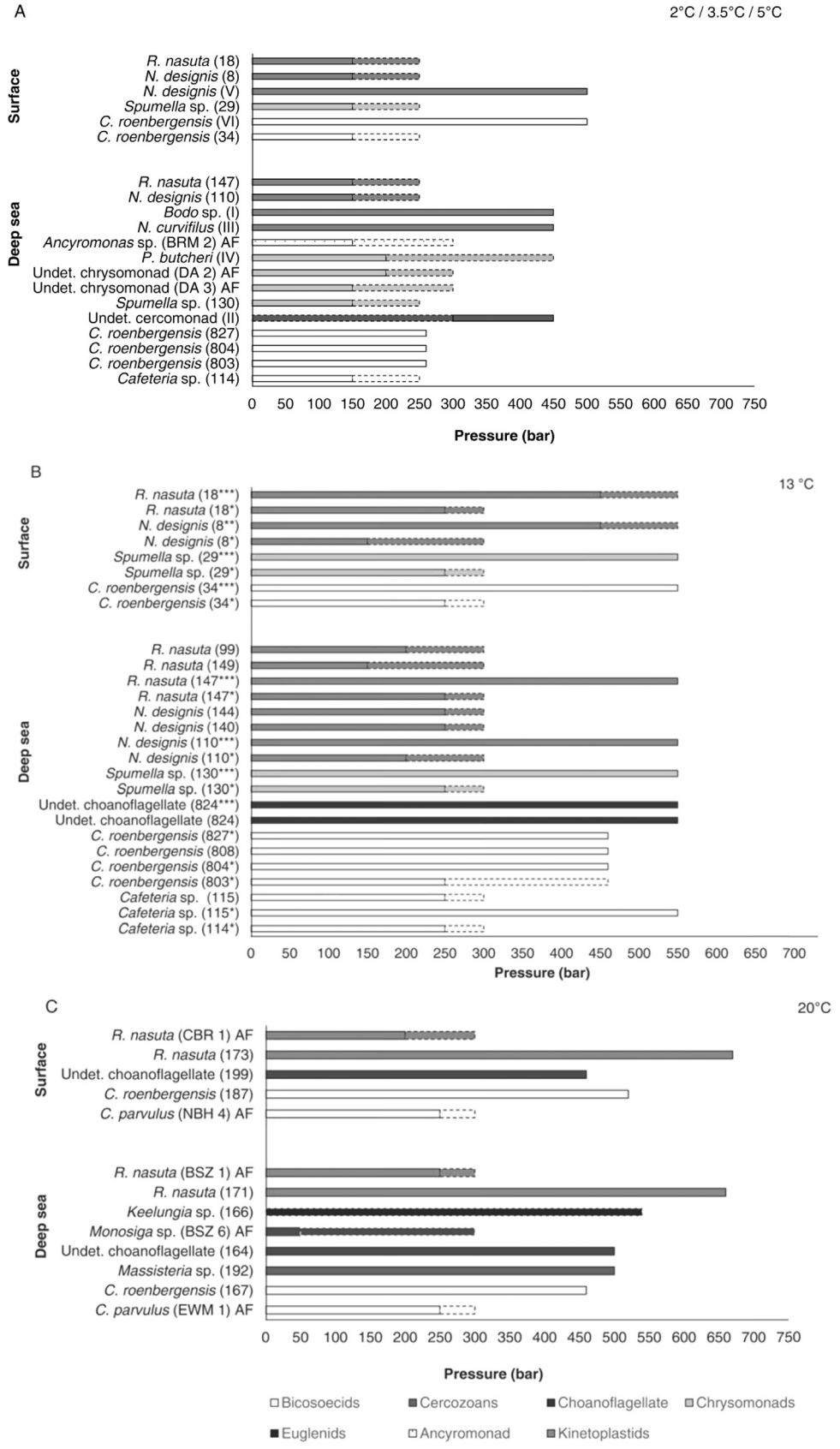

Fig. 1. Survival of marine heterotrophic flagellates isolated from deep-sea and surface waters under high hydrostatic pressures and different temperatures including (A) 2 °C/3.5 °C/5 °C, (B) 13 °C, (C) 20 °C. Experiments were carried out under various conditions: different exposure times (1.5 h – 528 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 (*).

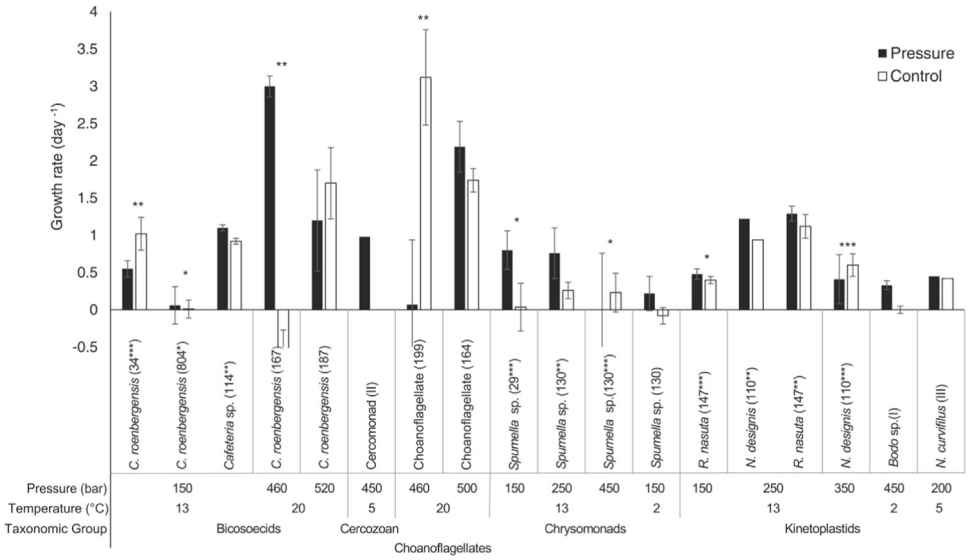

Fig. 2. Growth rates of deep-sea and surface water isolates of marine heterotrophic flagellates exposed to different hydrostatic pressures. Growth rates of flagellates at surface conditions (1 bar) were used as a control. Vertical bars represent ± SD. One-way ANOVAs were used. With the stars (*) above 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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Chapter 2

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

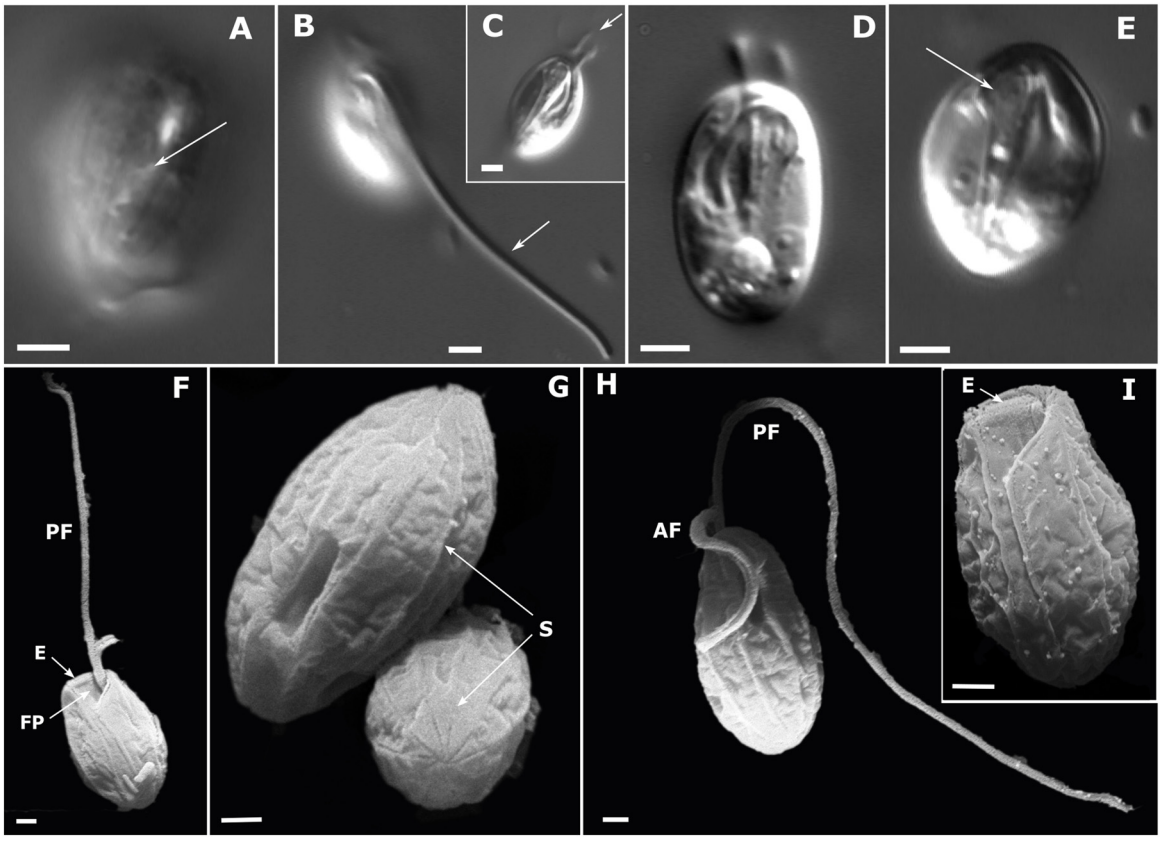

Fig. 1. Light (A–E, scale bars 2 μm) and scanning electron (F–I, scale bars 1 μm) micrographs of *Keelungia nitschei* strain HFCC 166. A: Strips on cell surface visible (white arrow). B: Posterior flagellum emerges from the flagellar pocket (white arrow). C: Ventral view of the cell with anterior flagellum (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)

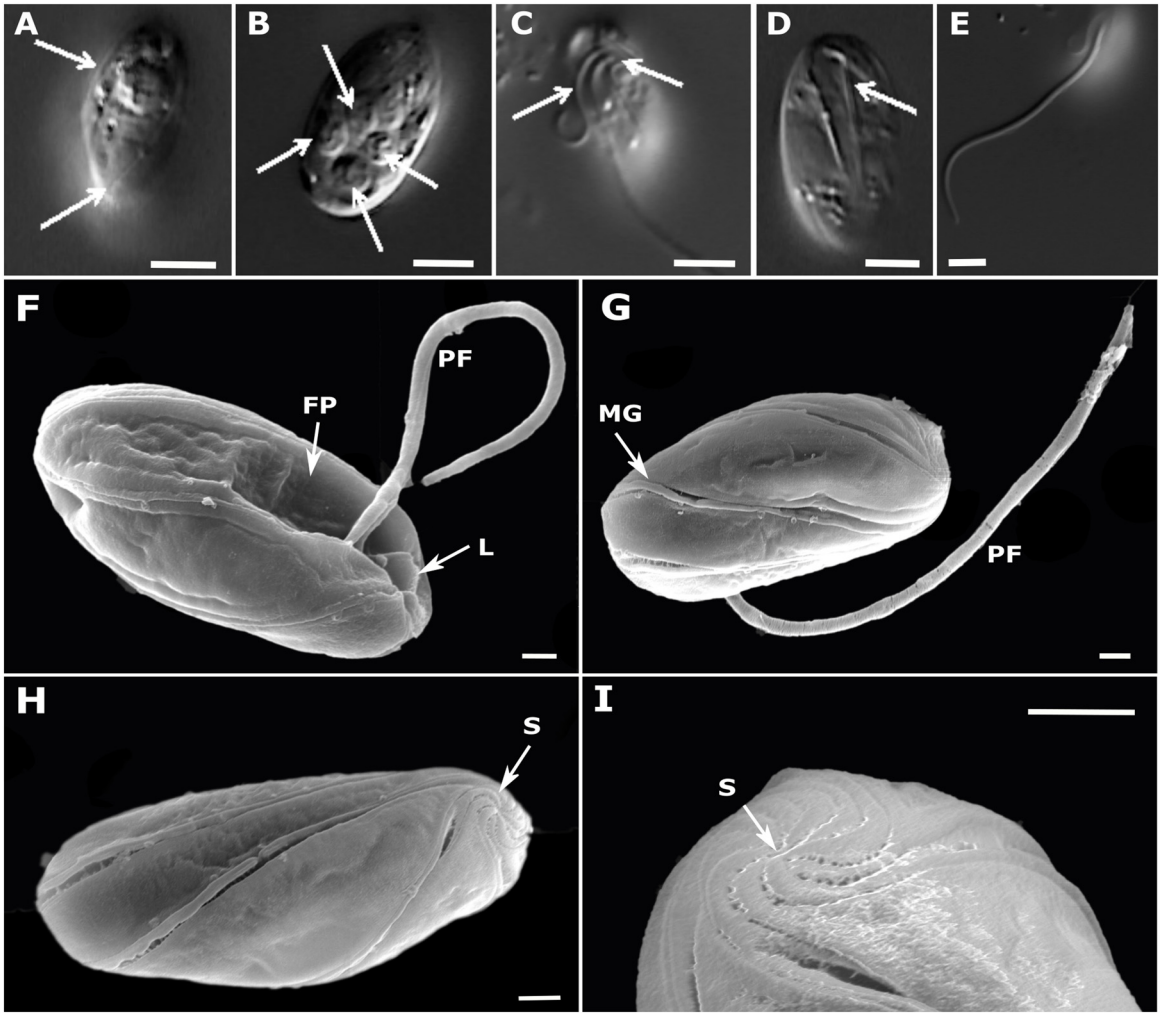

Fig. 2. Light (A–E, scale bars 5 μm) and scanning electron (F–I, scale bars 1 μm) micrographs of *Ploeotia costaversata* strain HFCC 1508. A: Helically twisted strips towards both poles of the protoplast (white arrows). B: Four vacuoles within cell body (white arrows). C: Anterior flagellum and flagellar 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-

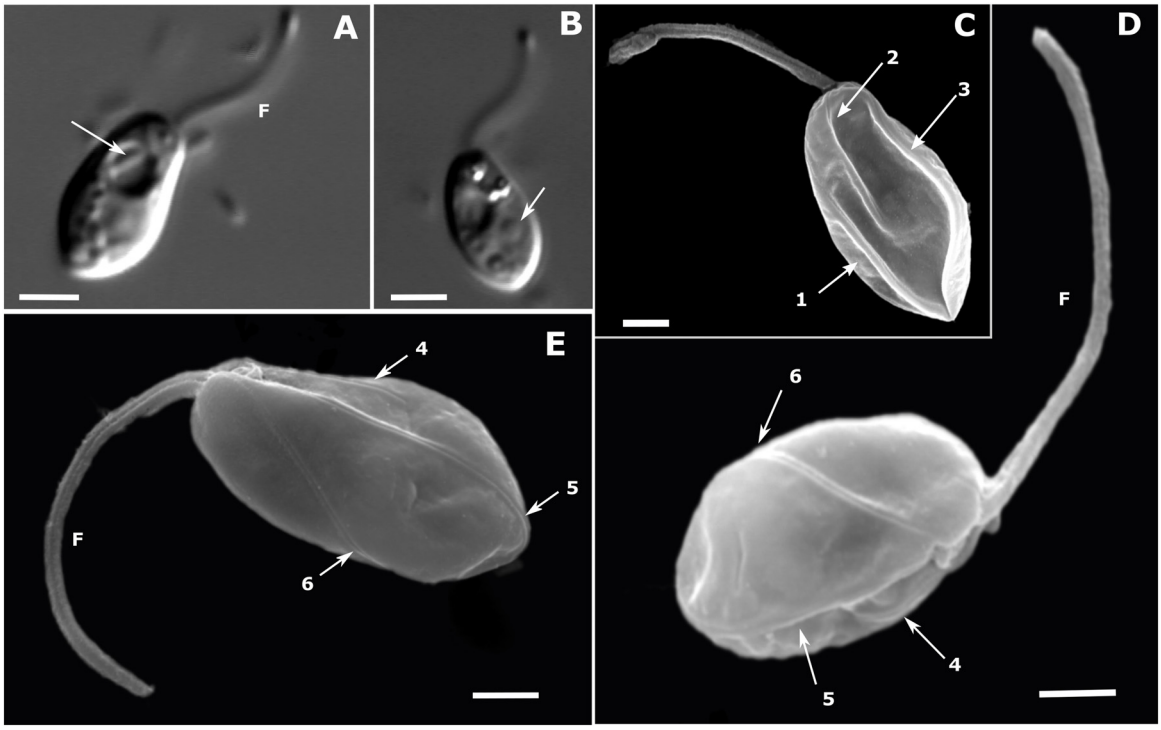

Fig. 3. Light (A–B, scale bars 2 μm) and scanning electron (C–E, scale bars 1 μm) micrographs of *Petalomonas acorensis* strain HFCC 220. A: Cell in dorsal view with single flagellum (F) emerging from a deep flagellar pocket (white arrow). B: Dorsal view of the cell, nucleus visible (white arrow). C: 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: $p < 0.001$;

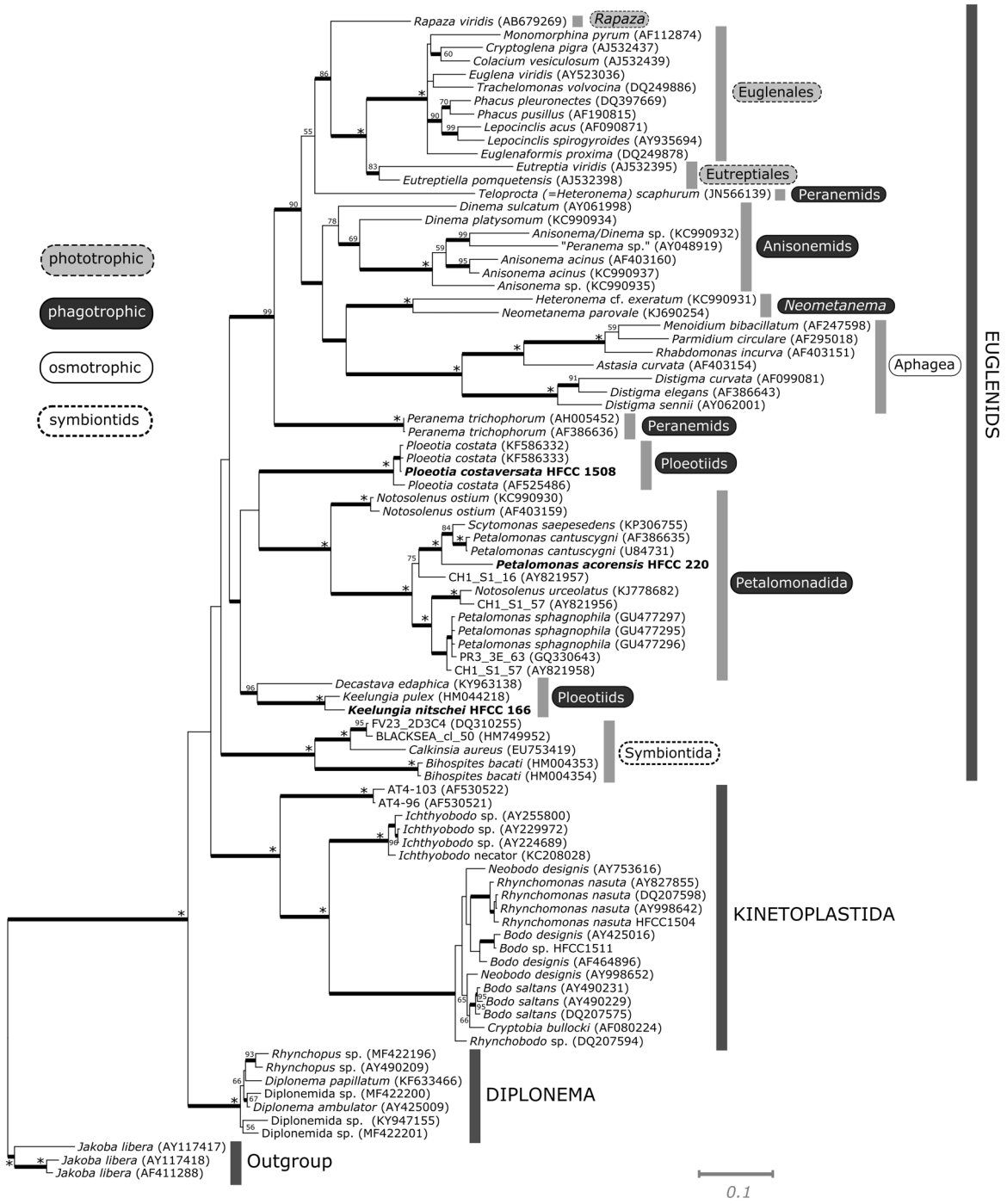

Fig. 4. Phylogenetic tree of euglenids based on 18S rDNA sequences. A Bayesian Inference (BI) tree shown for the analysis. A Maximum likelihood analysis (ML; GTR + gamma model) under the same model yielded an identical topology. Number on edges represent ML bootstrap percentages (RaxML, 1000 replicates); 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

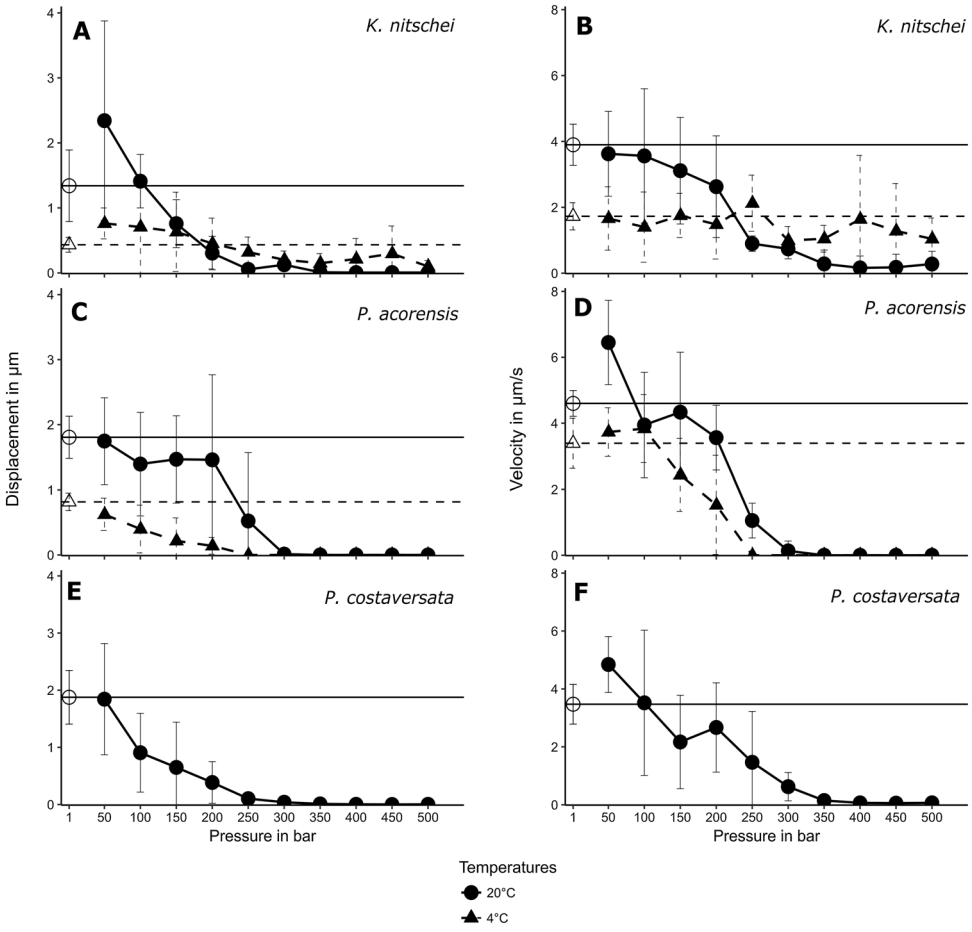

Fig. 5. Exposure of HFCC 166 *Keelungia nitschei* (A, B), HFCC 220 *Petalomonas acorensis* (C, D) and HFCC 1508 *Ploeotia costaversata* (E, F) to stepwise increasing pressure in steps of 50 bar with maximum pressure exposure of 500 bar. Displacement in µm (A,C,E) and Velocity in µm/s (B, D, F) of the 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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Chapter 3

A barotolerant ciliate isolated from the abyssal deep sea of the North Atlantic: *Euplotes dominicanus* sp. n. (Ciliophora, Euplotia)

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A barotolerant ciliate isolated from the abyssal deep sea of the North Atlantic: *Euplotes dominicanus* sp. n. (Ciliophora, Euplotia)

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Abstract

A new *Euplotes* species, isolated from abyssal depths (>4000 m) of the North Atlantic Ocean, was described based on morphology, ciliary pattern and molecular data. *Euplotes dominicanus* sp. n. is characterized by a small body size (29–40 × 17–27 μm in vivo), 18–22 adoral membranelles, 10 frontoventral, five transverse and two left marginal cirri and one caudal cirrus, five or six dorsolateral kineties with 7–9 dikinetids in mid-dorsolateral kinety (DK3), and dorsal silverline system of the double-*eurystomus* type. Phylogenetic analyses inferred from 18S rRNA sequences show that *Euplotes dominicanus* sp. n. is most closely related to *E. curdsi,* with a sequence similarity of 97.6 %. *Euplotes dominicanus* sp. n. was able to survive hydrostatic pressures up to 500 bar indicating its barotolerance. Metabarcoding data demonstrate the presence of *E. dominicanus* sp. n. in sediments of several deep-sea basins.

Keywords: Barotolerance; Ciliates; High hydrostatic pressure; Morphology; Next generation sequencing; Phylogeny

Introduction

A comprehensive analysis of ciliate records from the deep sea (>1000 m depth) indicated the potential existence of a very large variety of ciliate representatives of all major groups (Schoenle et al. 2017). Several genotypes belonging to the genus *Euplotes* were detected from depth ranging from 1998 m to 3744 m. Still, the data about ciliates isolated from the deep sea are very scarce and mostly based on molecular studies. So far, only a few ciliates have been collected from the deep sea and cultivated later successfully in laboratory conditions (Schoenle et al. 2017). The reason for this may be the potentially low abundances of ciliates in the deep sea, or the hydrostatic stress during sampling (deep sea to the surface). Several ciliates belonging to the Karyorelictea, Suctoria and Peritrichia have been found as epibionts on arthropods collected from the deep sea (Bartsch and Dovgal 2010; Sedlacek et al. 2013). However, these samples were preserved with ethanol or formalin, not providing the possibility of cultivation and live observation of collected deep-sea ciliates.

Ciliated protists are a diverse group of microbial eukaryotes being the morphologically very complex and highly differentiated monophyletic taxon among single-celled organisms (Gao et al. 2017; Lynn 2008). Based on the mor-

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phology of living cells, Müller (1773) described several euplotid species (e.g., *Trichoda charon*, *T. patella*). Later on, Ehrenberg (1830) established the genus *Euplotes* with *Trichoda charon* Müller, 1773 as type species. Other genera and subgenera of *Euplotes* were established by Jankowski (1979) and Borror and Hill (1995), however, the establishment of these taxa was not supported by molecular data, and are therefore considered as a part of the single genus *Euplotes* (Gao et al. 2017; Liu et al. 2015; Yi et al. 2009). Within the Euplotia, the genus *Euplotes* is the most species-rich genus, with around 150 different species and sub-species (Berger 2001; Jiang et al. 2010a). They are distributed worldwide, and found in freshwater, terrestrial and marine habitats (Chen et al. 2013; Dai et al. 2013; Di Giuseppe et al. 2014; Fotedar et al. 2016; Gao et al. 2017; Kahl 1932). Because of their distribution and species variety, this genus is usually considered as one of the most complex in comparison to other ciliate groups (Gao et al. 2017; Jiang et al. 2010b; Lian et al. 2019).

Nowadays, it is advised to combine morphological, molecular and other characters such as behavior and environmental data to describe or redescribe ciliated protists (Warren et al. 2017). Despite this, in *Euplotes*, many molecular sequences have been ascribed to morphospecies, without providing additional morphological data which support the identification. Those sequences, however, are frequently included in the datasets used for the molecular identification and phylogeny of euplotid species (Syberg-Olsen et al. 2016). Certain morphological similarities are shared between the species of this genus. Usually, they show similarities in the number of cirri and dorsolateral kineties, body size and shape (Curds 1975; Kahl 1932). Other difficulties for the identification of *Euplotes* species are the low number of unique morphological features, and the lack of detailed information in some original descriptions (Schwarz et al. 2007). It is recommended, whenever possible, to obtain gene sequences from different barcodes (e.g. nuclear ribosomal DNA and mitochondrial markers) to confirm morphological identifications, especially if a complete morphological investigation is not feasible (Boscaro et al. 2018). This will prevent the risk of mistakenly splitting conspecific populations, at least until further detailed morphological data and/or molecular barcodes would allow to increase the resolution at and below the species level. We followed the above mentioned recommendations and propose a new *Euplotes* species, isolated from the deep-sea sediment, based on morphological and molecular characters.

To our knowledge, this is the first time that *Euplotes dominicanus* sp. n., a new ciliate species from abyssal depths (>4000 m) was isolated, successfully cultivated and morphologically characterized alive. The 18S rRNA was analyzed to estimate the phylogenetic position of *E. dominicanus* sp. n. In addition, the survival ability at high hydrostatic pressure (up to 500 bar) of the newly described *Euplotes* species was investigated. We also analyzed its occurrence based on the V9 region of the 18S rRNA in metabarcoding data sets obtained from 12 deep-sea basins of the North-Atlantic, South-West Atlantic and Pacific Ocean.

Material and methods

Sampling, isolation and cultivation

Samples were collected during the deep-sea expedition with the research vessel R/V Meteor (Cruise M139) in 2017. Deep-sea sediment was taken by means of a Multicorer system (MUC). The ciliate *Euplotes dominicanus* sp. n. strain HFCC 757 was isolated from sediment samples, taken at 4296 m depth in the Sargasso Sea (Station A3/4, #953, core 2). After the cores were brought on deck, they were processed immediately. Sediment of the upper 2 mm was transferred into 50 mL-tissue-culture flasks (Sarstedt, Nümbrecht, Germany), filled with 30 mL autoclaved seawater (35 PSU) and one autoclaved wheat grain to promote growth of autochthonous bacteria. Raw cultures of deep-sea protists were established by serial dilution on board, and later in the home laboratory, monocultures were obtained with the help of a micromanipulator (PatchMan NP 2 from Eppendorf, Germany) under an inverted microscope (ZEISS Axiovert 25, Germany). Ciliates 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 $MgCl_2 \times 6H_2O$, 6.92 g $MgSO_4 \times 7\ H_2O$, 1.45 g $CaCl_2 \times 2\ H_2O$, 0.10 g KNO_3, and 0.01 g $K_2HPO_4 \times 3\ H_2O$). To prevent side reactions of the salts contained in the marine water with reagents used for the staining procedures (particularly the silver itself), the salinity of the medium used for cultivation of *E. dominicanus* sp. n. was reduced to 5 PSU. Cultures were supplied with a sterile wheat grain, as a continuous carbon source for autochthonous bacteria, and associated bicosoecid, stramenopiles and cercozoan species, which served as an obligatory additional food source. For *E. dominicanus* sp. n., clonal cultures were set up and used for morphological, molecular and pressure studies.

For the purpose of the next generation sequencing (NGS) data, environmental DNA was extracted from deep-sea sediment samples (in total 27 samples) taken from 20 deep-sea stations during four different expeditions, in the Pacific (five samples) and Atlantic Ocean (19 samples from North Atlantic, three from South Atlantic) with the research vessels R/V Sonne and R/V Meteor (SO237, SO223 T, M79/1, M139). PCR amplifications of the hyper-variable loop V9 on the 18S rRNA gene were performed with the Phusion® High-Fidelity DNA Polymerase (ThermoFisher) and the following primers: 1389 F (5′-TTGTACACACCGCCC-3′) and 1510R (5′-CCTTCYGCAGGTTCACCTAC-3′) (Amaral-Zettler et al. 2009). Sequencing was performed using the

Illumina Genome Analyzers IIx system (HiSeq, paired-end, 2 × 150bp).

Morphological methods

Live cells were observed at magnifications of 40–1000× with bright field, phase contrast and Nomarski differential interference contrast (DIC), using a light microscope (Zeiss Axioplan 2, Carl Zeiss GmbH, Jena, Germany) and an inverted microscope (Zeiss Axio Observer A1, Carl Zeiss GmbH, Jena, Germany). For live observations, modified Petri dishes, i.e. with a portion of the base cut off and replaced by a coverslip, were used under the inverted microscope. This microscope used for video recording was equipped with a 100×/1.4 NA oil-immersion objective (DIC) and a water-immersion condenser and combined with an Allen Video Enhanced Contrast (AVEC) system, consisting of an analogous Hamamatsu C6489 camera with noise suppression and contrast amplification (Argus-20, Hamamatsu, Japan). Single frames from the video recordings were captured with VirtualDub v1.10.4 (www.virtualdub.org) and edited by ImageJ v1.52a (www.imagej.nih.gov/ij/; Schneider et al. 2012). In vivo measurements were conducted at magnifications of 40–1000 ×. Synthesized protargol powder (Pan et al. 2013) and the protargol impregnation technique 'procedure A' described by Foissner (2014) were applied to reveal the infraciliature and nuclear apparatus. Due to the weak impregnation of the nuclear apparatus in our protargol-stained specimens, we additionally used the methyl-green supravital staining method for an accurate characterization of the nuclear apparatus (Foissner 2014). The dry silver nitrate impregnation was used to reveal the silverline systems (Foissner 2014). Counts and measurements on impregnated specimens were performed at a magnification of 1000×. Drawings of live specimens were based on free-hand sketches and photomicrographs, and edited using the programs Inkscape v0.92.3 (www.inkscape.org) and Gimp v2.8.22 (www.gimp.org). Terminology is mainly according to Curds (1975).

Scanning electron microscopy (SEM) was performed using the following procedure. Ciliate samples were fixed with 2.5% of cacodylate buffered glutaraldehyde (final concentration) at 4 °C for approximately 1 h. Additionally, 1% of osmium tetroxide was added and left for 10 min. After this treatment, dehydration of the samples was done with the ethanol series of 30%, 50%, 60%, 80%, 90% and 96%. Samples were washed two times with each ethanol concentration (1 ml), and finally remained for 10 min in each solution. As a substitute for critical point drying, 50:50 hexamethyldisilazane (HMDS; Carl Roth, Germany)-ethanol solution was added and left for 10 min. This was followed by two washing steps with the pure HMDS for 5 min. Then, samples were left to dry and transferred into Petri dishes (Sarstedt, Nümbrecht, Germany). SEM samples were sputter coated with a 12 nm layer of gold before examination by SEM (FEI Quanta 250 FEG).

DNA extraction, PCR amplification and sequencing

For sequencing of *Euplotes dominicanus* sp. n. (HFCC 757) a single-cell PCR was performed. Prior to PCR, single cells were transferred in double distilled water (ddH_2O) and then frozen at −20 °C to disrupt the cells. The 18S rRNA of *E. dominicanus* sp. n. was amplified using following primers: 18S-For (5′-AACCTGGTTGATCCTGCCAGT-3′), 590For (5′-CGGTAATTCCAGCTCCAATAGC-3′), or 1280 F (5′-TGCATGGCCGTTCTTAGTTGGTG-3′), 18S-Rev (5′-TGATCCTTCCGCAG-GTTCACCTAC-3′), and 1300R (5′-CACCAACTAAGAACGGCCATGC-3′) (Medlin et al. 1988; Wylezich et al. 2002). The volume of PCR reaction mixtures was 50 μl, including 5 μl of ddH_2O, 25 μl of Mastermix (VWR Red Taq DNA Polymerase Master Mix by VWR CHEMICALS, USA), 10 μl ddH_2O containing the single cell and 5 μl of forward and 5 μl of reverse primer (10 μM stock concentration). Amplification cycles were as follows: predenaturation 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, and a final extension at 72 °C for 10 min. The PCR products were detected using agarose gel (1%) and fragment sizes were determined by comparison with 250–10000 bp DNA ladder (Genaxxon, Ulm, Germany). The PCR products were purified using the PCR Purification KIT (Jena Bioscience, Germany).

Phylogenetic analyses

For the phylogenetic 18S rRNA analysis of the subclass Euplotia, the initial alignment was kindly provided by Vittorio Boscaro (see Boscaro et al. 2018). Additionally, available sequences were downloaded from GenBank database. Alignments were done using MAFFT v7.311 (Katoh and Standley 2013) within Unipro UGENE version 1.31.1 (Okonechnikov et al. 2012). In total, the 18S rRNA dataset included 68 *Euplotes* sequences and 14 outgroup sequences, containing 1903 unambiguously aligned nucleotide positions. For maximum likelihood (ML) analysis the model GTR + I+Γ was determined by MrAIC (Nylander 2004) and it was computed by RaxML version 8.2.10 (Stamatakis 2014) on CIPRES Gateway (Miller et al. 2010) with 1000 bootstrap replicates. For Bayesian analysis, Mr. Bayes v3.2.6 (Ronquist et al. 2012) was applied using the same model as suggested by MrAIC. The analysis consisted of 100 000 generations with a burnin of 25 % of the total number. The search used two parallel chain sets run at default temperatures.

Next generation sequencing

Established bioinformatic pipeline allowed filtering of high-quality V9 rDNA sequences, and their clustering into

operational taxonomic units (OTUs). First, the paired reads were retained for downstream analyses, if they had both forward and reverse primers and no ambiguously named nucleotides (Ns). One file contained the reads from all stations, which were then dereplicated into strictly identical amplicons with VSEARCH (version 2.13.4; Rognes et al. 2016), keeping the information on their abundance. Low abundance metabarcodes with a read abundance of one or two reads were removed from the dataset before their clustering into operational taxonomic units (OTUs). Clustering into biologically meaningful OTUs was done using Swarm v2.1.5 (Mahé et al. 2015), with the parameter d = 1 and the fastidious option on. For taxonomic assignment a reference database for the V9 region was established by combining the Protist Ribosomal Reference database PR2 v4.11.1 (Guillou et al. 2012) with 101 sequences of marine protists strains of the Heterotrophic Flagellate Collection Cologne (HFCC). OTUs were taxonomically assigned to the reference database using VSEARCH's global pairwise alignment. Amplicons were assigned to their best hit, or co-best hits in the reference database using a pipeline called Stampa (Mahé 2016). The most abundant amplicon in each OTU was searched for chimeric sequences with the chimera search module of VSEARCH, and chimeric OTUs were removed even if they occurred in multiple samples. Sequences with a quality value (min. expected error rate/sequence length) lower than 0.0002 were discarded. Reads shorter than 87 bp were removed from the dataset. Only OTUs with a pairwise identity of ≥80% to a reference sequence were used for downstream analyses. In addition, OTUs were discarded, when a phylogenetic placement on the kingdom level was not possible. Furthermore, OTUs assigned to Metazoa, Fungi and exclusively phototrophic organisms were removed. The analyzed dataset of the four different deep-sea expeditions (SO237, SO223 T, M79/1, M139) was used as reference for a blast search of the deep-sea ciliate *Euplotes dominicanus* sp. n. sequence. Only OTUs with a pairwise identity higher than 95% to a reference sequence were used for analysis.

Furthermore, the metabarcodes (V9 sequences) of the Tara Ocean project (de Vargas et al. 2015; Supplement Database W4) were taxonomically assigned to our reference database using VSEARCH's global pairwise alignment to investigate the occurrence of *E. dominicanus* sp. n. in surface waters in addition to the deep sea. Amplicons were assigned to their best hit, or co-best hits in the reference database using a pipeline called Stampa (Mahé 2016).

Survival analysis

For determination of the survival rates of *Euplotes dominicanus* sp. n. at different hydrostatic pressures, we used a high hydrostatic pressure system modified after Koyama et al. (2001) with the possibility of direct observation. A manual hydraulic pump was used to gradually increase the hydrostatic pressure up to 500 bar. The observation chamber, made of stainless steel with a 4-mm-diameter window of 2 mm-thick glass, allowed the direct observation of the microorganisms under high hydrostatic pressure in an inverted microscope (ZEISS Primovert, Carl Zeiss GmbH, Jena, Germany). *Euplotes dominicanus* sp. n. was exposed to different pressure steps up to a maximum pressure of 500 bar. Prior to the experiment, cultures were adapted for two days to the experimental conditions, i.e., a temperature of 4 °C and darkness. Ten individuals were analyzed separately for the pressure as well as for the control (1 bar) treatments. Pressure was gradually increased in steps of 50 bar, approximately every 7 min, until the maximum pressure was reached. The *Euplotes* cells were considered dead when no movement was observed in both the body and cilia for at least 7 min (Živaljić et al. unpubl.).

ZooBank registration

The ZooBank registration number of the present work is: urn:lsid:zoobank.org:pub:99E6E26E-1B12-4544-85DD-B1D072FDC703.

Results

Family Euplotidae Ehrenberg, 1838

Genus *Euplotes* Ehrenberg, 1830

Euplotes dominicanus sp. n. (Fig. 1A-N, Table 1)

Diagnosis: Marine *Euplotes* about 29–40 × 17–27 μm in vivo, 22–33 × 13–21 μm after protargol impregnation; outline elongate elliptical and about 2:1 dorsoventrally flattened; 5 or 6 inconspicuous dorsal ridges; adoral zone extending about two-thirds (~68 %) of total cell length; adoral zone composed of 18–22 membranelles; 10 frontoventral cirri, 5 transverse cirri, 2 left marginal cirri and 1 caudal cirrus; 5 or 6 dorsolateral kineties; usually about 9 dikinetids in middorsolateral kinety (DK3); macronuleus C-shaped; dorsal silverline system of double-*eurystomus* type.

Type locality: Deep-sea sediment sample from 4296 m depth (23°33.23′ N, 48°5.04′ W), North Atlantic Ocean.

Type material: The slide (registration number: 2019-85) containing the holotype (Fig. 1B, C, L, M), one paratype slide (registration number: 2019-83) with protargol stained specimens, one paratype slide with dry silver nitrate-stained specimens (registration number: 2019-84), and a filter with cells of strain HFCC 757 (Heterotrophic Flagellate Collection Cologne; registration number: 2019-86) prepared for electron microscopy, have been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz (LI). Relevant specimen has been marked by black ink circle on the coverslip.

Etymology: The name *dominicanus* refers to the Dominican Republic. The name is dedicated in honor to the friendly people of the Dominican Republic and their authorities for

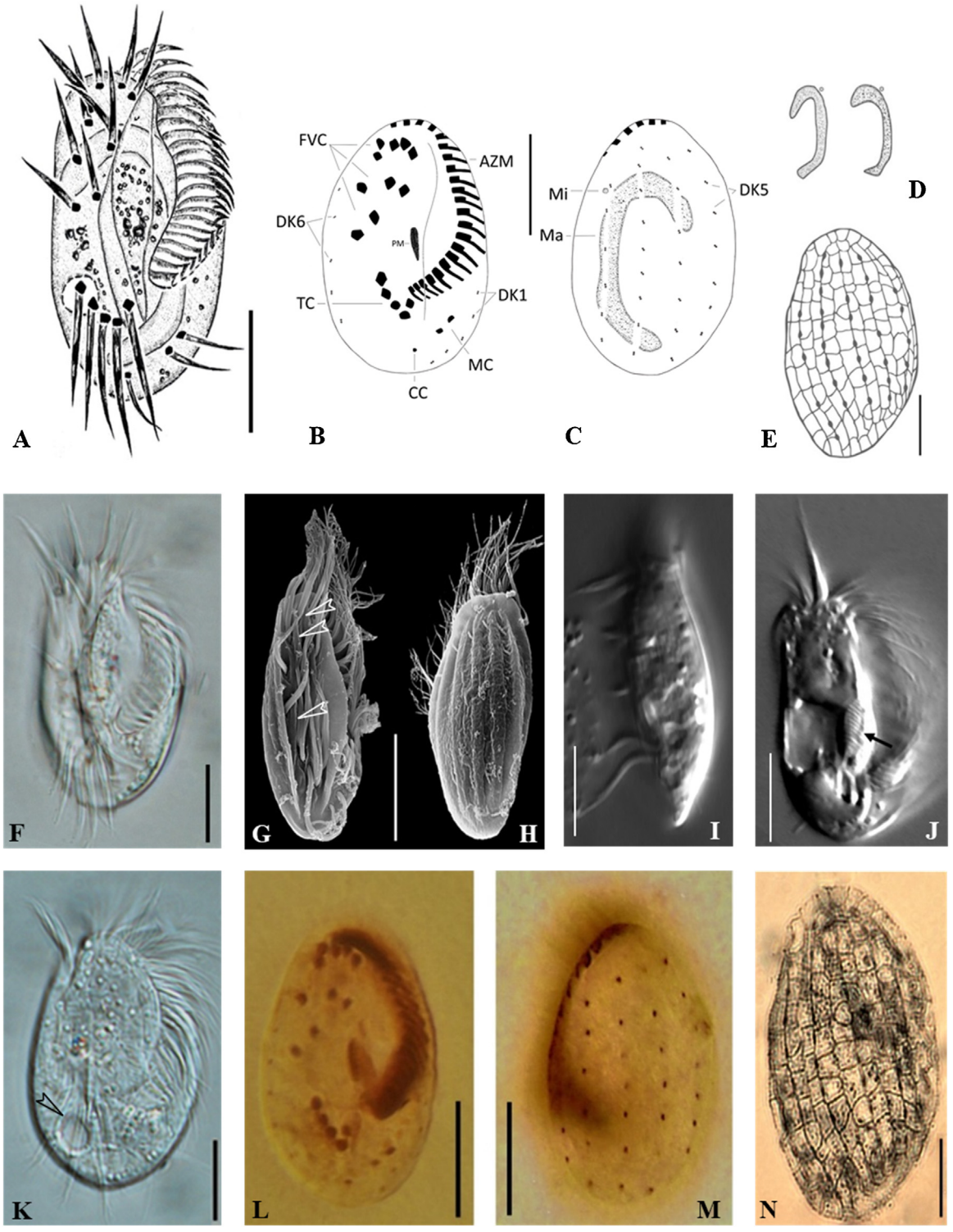

Fig. 1. (A–N). *Euplotes dominicanus* sp. n. in vivo (A, F, I–K), after protargol (B, C, L, M), silver nitrate (E, N) and methyl-green (D) staining and scanning electron microscopy (G, H). **(A, F)** Ventral view of representative cell. **(B, C, L, M)** Ventral (B, L) and dorsal (C, M) view of the holotype specimen, showing the infraciliature. **(D)** Variability of nuclear apparatus after methyl-green staining. **(E, N)** Silverline system on dorsal side (same specimen). **(G, H)** Scanning electron microscopy of ventral (G) and dorsal (H) side, arrowheads in G show ridges on ventral side. **(I)** Left lateral view showing the cirri. **(J)** Ventral view, arrow shows paroral membrane. **(K)** Endoplasm of a squeezed cell; arrowhead points to contractile vacuole. AZM, adoral zone of membranelles; FVC, frontoventral cirri; MC, marginal cirri; CC, caudal cirrus; TC, transverse cirri; DK, dorsolateral kinety; DK 1, dorsolateral kinety 1; Ma, macronucleus; Mi, micronucleus; PM, paroral membrane. Scale bars = 10 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1. Morphometric data of *Euplotes dominicanus* sp. n. based on in vivo (upper line) and protargol-stained (lower line) specimens.

Characteristic	Mean	Med	SD	SE	Min	Max	CV	n
Body, length (μm)	33.4	32.6	3.2	0.6	29.2	39.7	9.5	25
	25.5	25.0	2.3	0.5	22.0	33.0	9.1	25
Body, width (μm)	21.6	21.7	2.7	0.5	16.8	26.6	12.3	25
	16.2	16.0	1.9	0.4	13.0	21.0	11.4	25
AZM, length (μm)	22.3	22.0	1.4	0.3	19.9	24.9	6.2	25
	16.4	16.5	0.9	0.2	14.9	18.1	5.3	25
AZM length: body length (%)	67.1	67.5	5.2	1.0	56.0	78.0	7.8	25
	64.9	65.0	6.9	1.4	51.5	81.8	10.6	25
Adoral membranelles, number[a]	19.8	20.0	1.1	0.2	18.0	22.0	5.5	25
Paroral membrane, length (μm)[a]	3.3	3.3	0.4	0.1	2.4	4.1	12.9	25
Frontoventral cirri, number[a]	9.9	10.0	0.3	0.1	9.0	10.0	2.8	25
Transverse cirri, number[a]	5.0	5.0	0.0	0.0	5.0	5.0	0.0	25
Marginal cirri, number[a]	2.0	2.0	0.0	0.0	2.0	2.0	0.0	25
Caudal cirri, number[a]	1.0	1.0	0.0	0.0	1.0	1.0	0.0	25
Dorsolateral kineties, number[a]	6.0	6.0	1.2	0.2	5.0	6.0	20.0	25
Dikinetids in DK1, number[a]	4.8	5.0	0.4	0.1	4.0	5.0	9.2	25
Dikinetids in DK2, number[a]	7.7	8.0	0.9	0.2	6.0	9.0	11.1	25
Dikinetids in mid-DK3 number[a]	7.8	7.5	2.9	0.6	7.0	9.0	37.4	25
Dikinetids in DK4, number[a]	7.9	8.0	0.9	0.2	6.0	10.0	11.5	25
Dikinetids in DK5, number[a]	7.4	7.0	1.9	0.4	6.0	10.0	25.3	25
Dikinetids in DK6, number[a]	6.2	6.0	1.0	0.2	5.0	8.0	16.8	25

[a] Data based on protargol-impregnated cells. CV, coefficient of variation in %; DK, dorsolateral kinety; AZM, adoral zone of membranelles; Max, maximum; Mean, arithmetic mean; Med, median; Min, minimum; n, number of specimens investigated; SD, standard deviation; SE, standard error of arithmetic mean.

their quick and positive response to take samples in their territory in the frame of the R/V Meteor cruise M139.

Gene sequence: The 18S rRNA gene sequence of *Euplotes dominicanus* sp. n. has been deposited in GenBank under the accession number: MN757874 (1738 bp).

ZooBank registration number of *E. dominicanus* sp. n.: urn:lsid:zoobank.org:act:1BCD5939-145B-4E02-A151-F19B4A171B34.

Morphological description

Cell size in vivo 29–40 × 17–27 μm, usually about 33 × 22 μm; distinctly shrunken in protargol preparations (about 30%); flattened up to 2:1 dorsoventrally, with ventral side plane or slightly convex and dorsal side distinctly arched; body rigid and not contractile. Outline elongated elliptical to oval, with left and right side usually convex in well-fed individuals (Fig. 1A, F). Ventral side with two or three conspicuous ridges extending posteriorly to transverse cirri (Fig. 1G); five or six inconspicuous dorsal ridges extending over entire length of cell (Fig. 1H, I). Buccal field long and deep, extending to approximately 68% of cell length (Fig. 1A, B, L). Cytoplasm colorless, in middle body third usually dark because packed with 1–3 μm-sized lipid droplets and a few food vacuoles (Fig. 1A, K). Contractile vacuole located above right transverse cirri and pulsating at irregular intervals (Fig. 1A). Contractile vacuole pore not observed. Macronucleus C-reverse shaped and about 3 μm in width (Fig. 1C, D). Distance between macronucleus and anterior end of cell around 4 μm. One small micronucleus, about 1 μm in diameter, usually not discernible or faintly stained after methyl-green impregnation, located next to the anterior left area (Fig. 1C, D).

Adoral zone prominent with 18–22 membranelles, bases of membranelles relatively short (up to 3 μm long) (Fig. 1A, B, L). Adoral zone curved, consisting of three or four collar and 15–18 lapel membranelles. Membranelles in lapel zone comprise three rows of basal bodies. Paroral membrane small, about 3 μm in length, relatively straight-shaped but more pointed on the posterior part, and positioned below buccal lip (Fig. 1B, J). Usually 10 thick frontoventral cirri (two out of 25 specimens had only 9 frontoventral cirri), five thick transverse cirri, one thin caudal cirrus located in the midline of the cell and two thin marginal cirri located on left side of cell posterior to buccal field (Fig. 1A, B, L; Table 1). Transverse cirri about 11 μm long and other cirri about 6–8 μm long. Five or six dorsolateral kineties; five dorsolateral kineties, with leftmost (DK1) and rightmost (DK5) kineties, or six dorsolateral kineties, with leftmost (DK1) and rightmost (DK6) kineties, usually positioned on ventral side near body margin (Fig. 1B, C, L, M). Dorsolateral kineties extend for about the entire length of cell, except for leftmost row (DK1),

which starts at about level of proximal area of adoral zone of membranelles (Fig. 1B). Dorsal cilia (bristles), about 1.3 μm in length, present in more anterior and posterior located dikinetids, and lacking from dikinetids located in the middle. Silverline system on dorsal side of double-*eurystomus* type, i.e., two approximately equal rows of alveoli between each two kineties (Fig. 1E, N). No cysts were observed. Locomotion typically consisting of fast crawling on substrate or slight jerking, sometimes staying stationary for short periods.

Phylogenetic position of *Euplotes dominicanus* sp. n.

The SSU rRNA sequence of *E. dominicanus* sp. n. has a GC content of 45.05%. The BLAST analysis showed the closest relation (96% identity) of the *Euplotes dominicanus* sp. n. sequence strain HFCC 757 with several *Euplotes curdsi* strains (Min1, LT615048; AgTo3, KX819312; Wsea4, KX819313; Wsea6, KX819314; 100% identity among them).

ML and BI topologies were identical, and thus node supports from both methods were mapped only onto ML tree (Fig. 2). Our tree can be divided into five *Euplotes* clades (A–E), based on the clustering position originally proposed by Syberg-Olsen et al. (2016). *Euplotes dominicanus* sp. n. nested within the fully supported clade C (100% ML, 1.00 BI), basal to a medium to highly supported (93% ML, 1.00 BI) subclade containing three isolates of the marine species *E. nobilii* (KC599234, GU479382) and *E. raikovi* (EF094974), four marine/brackish strains of *E. curdsi* (LT615048, KX819312, KX819313, KX819314) and one brackish species *E. estuarinus* (MF445655). *Euplotes dominicanus* sp. n. differed from them in 42 nt within a sequence length of 1723 nt (p-distance 2.4%). Other closely related species, belonging to the clade C, were isolated from different habitats such as fresh- (*Euplotes* sp.), brackish (*E. elegans, E. estuarinus*) and hypersaline waters (*E. qatarensis*), and soil (*E. wuhanensis*).

Occurrence of *Euplotesdominicanus* sp. n. sequences in deep-sea samples

We searched our next generation sequencing dataset of the V9 region of the 18S rRNA (119 nt) obtained from the deep-sea expeditions SO237, SO223 T, M79/1 and M139 for *Euplotes dominicanus* sp. n. to check for the occurrence of this ciliate in different deep-sea basins. In ten out of 27 deep-sea samples from different basins taken during different expeditions from the Atlantic and Pacific Ocean, we were able to detect OTUs assigned with 100% identity to *E. dominicanus* sp. n. (Table 3, Fig. 4). Most of the reads were recovered from two abyssal stations (A3/4, A5/6) of the expedition M139 in the North Atlantic Ocean. The strain *E. dominicanus* sp. n. (HFCC 757) was isolated from station A3/4. In the Pacific Ocean in the West Mariana Basin, at only one site (of a total of five stations) nine reads could be assigned to *E. dominicanus* sp. n. with 100% identity (Fig. 4).

In the Tara Oceans database (including samples from surface water and deep chlorophyll maximum layer), no OTUs could be assigned with 100% identity to *E. dominicanus* sp. n.

Survival at deep-sea hydrostatic pressure

To check whether *Euplotes dominicanus* sp. n. is able to withstand the hydrostatic pressure typical for the deep sea, we conducted ten independent survival essays, with one specimen each at increasing hydrostatic pressures. Hydrostatic pressure ranged from 1 to 500 bars, resembling respectively the pressure conditions at the surface and down to an abyssal depth of 5000 m. At atmospheric pressure, the observed locomotion was typically consisting of fast crawling or slight jerking. Only four from the ten individuals of *E. dominicanus* sp. n., exposed separately to the gradual increase of pressure, survived to the maximum exposure of 500 bar (Fig. 3); two individuals survived up to 450 bar, and the remaining three survived up to 400, 350 and 250 bar, respectively. Despite the fact that specimens showed differences in their survival abilities, all of them were able to survive a pressure up to 250 bar, similar to the pressure at 2500 m depth.

Discussion

Morphological differences with related species

We compared the morphology of *Euplotes dominicanus* sp. n. with seven already described species, possessing the double-*eurystomus* silverline pattern, 10 frontoventral cirri and five transverse cirri (Table 2). Only *E. corsica* has been reported to possess one or two caudal cirri, based on statistical data and original drawings of Berger and Foissner (1989). Besides this, these two species are also similar regarding their in vivo body size (40 × 20 μm for *E. corsica* vs. 29–40 × 17–27 μm for *E. dominicanus* sp. n.). However, *E. corsica* differs from *E. dominicanus* sp. n. in: (i) body shape (oval vs. elongated; Fig. 1A, F) and (ii) the number of dorsolateral kineties (7–8 vs. 5–6; Fig. 1B, C, M).

The species *E. curdsi*, which is closely related to *E. dominicanus* sp. n., shows several differences, including (i) the number of adoral membranelles (25–34 vs. 18–22); (ii) bigger body size (52–63 × 27–33 μm vs. 29–40 × 17–27 μm); (iii) number of caudal cirri (invariably 2 vs. 1) and (iv) number of dikinetids in mid-dorsolateral row (10–12 vs. and 7–9; Fig. 1B, C, L, M) (Syberg-Olsen et al. 2016).

As described by Jiang et al. (2010a), *E. parabalteatus* resembles *E. dominicanus* sp. n. in: (i) cell size (30–35 μm in length vs. 29–40 × 17–27 μm); (ii) the number of adoral membranelles (19–23 vs. 18–22) and (iii) the oval-elongated body shape. However, *E. parabalteatus* differs

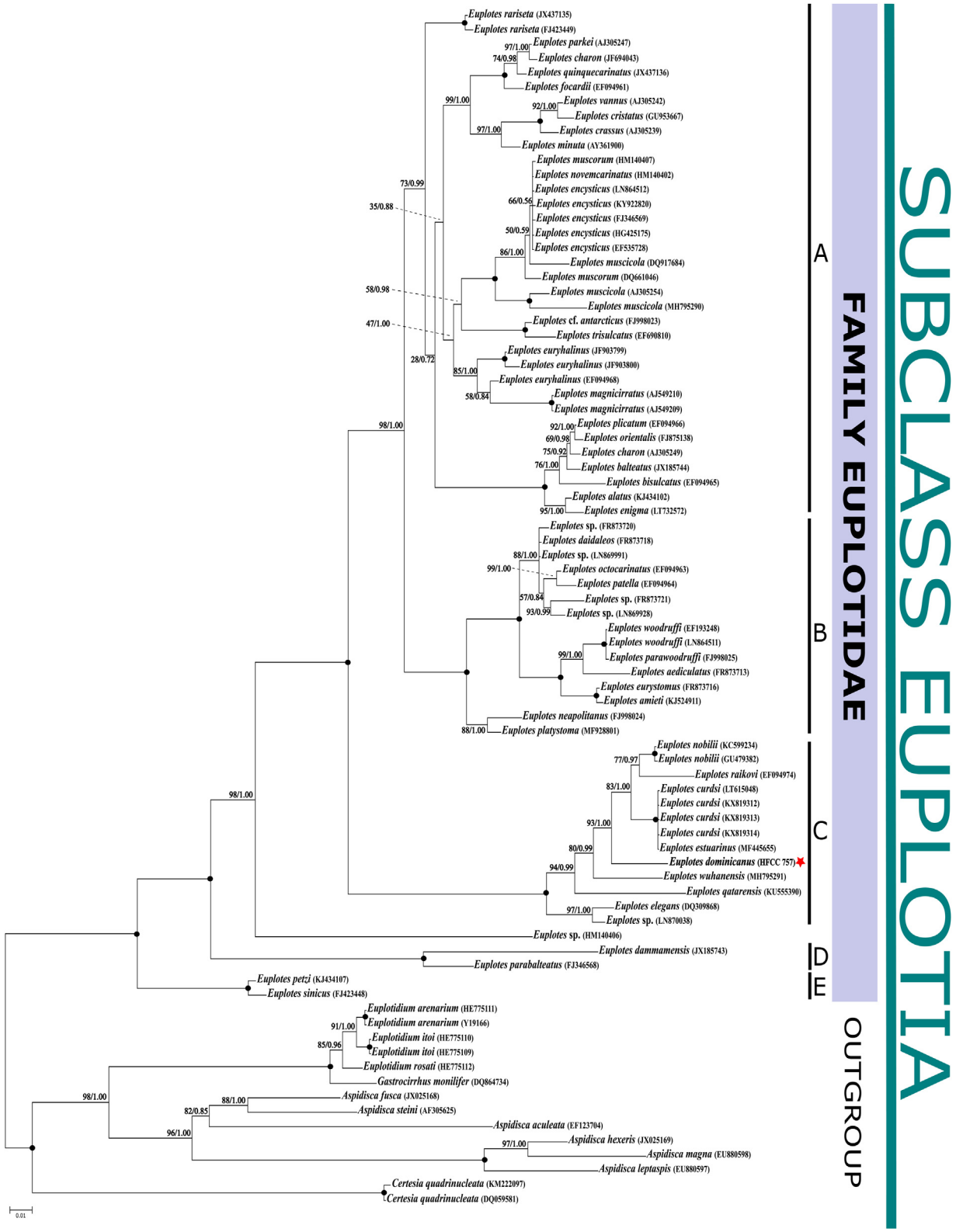

Fig. 2. 18S rRNA sequence genealogy of the subclass Euplotia based on Bayesian analysis. The new sequence is marked with a star. The GenBank accession numbers of 18S rRNA sequences are given behind the species names. Numerical support values are given at the respective nodes as: maximum likelihood (ML) bootstrap percentages (RaxML, 1000 replicates) / Bayesian posterior probabilities (BI). The well supported (100 % ML, 1.00 BI) branches are marked with solid circles. Scale bar represents 0.01 expected substitutions.

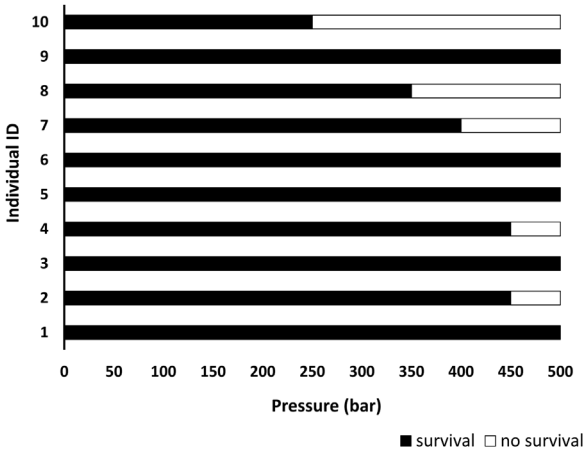

Fig. 3. Survival rates of *Euplotes dominicanus* sp. n. exposed up to a maximum pressure of 500 bar (n = 10). The filled sections of the bars indicate the range of pressure at which survival was observed for the different individuals.

from *E. dominicanus* sp. n. in (i) the number of caudal cirri (invariably 2 vs. 1); (ii) the larger buccal field (~65–75 % vs. ~68 %) and (iii) having 8–11 dikinetids in the mid-dorsolateral row (vs. 7–9).

Borror (1968) confirmed the original description of *E. alatus* given by Kahl (1932) with the description of the silverline system (Curds 1975). *Euplotes estuarinus* has only recently been described (Yan et al. 2018). These two *Euplotes* species can be clearly distinguished from *E. dominicanus* sp. n. by (i) their larger cell size (75–90 × 58–69 μm and 50–75 × 30–50 μm for *E. alatus* and *E. estuarinus* respectively vs. 29–40 × 17–27 μm); (ii) the oval body shape (vs. elongated); (iii) the higher number of adoral membranelles (~26 and 25–33 in *E. alatus* and *E. estuarinus* respectively vs. 18–22); (iv) the higher number of dorsolateral kineties (8 and 7 in *E. alatus* and *E. estuarinus* respectively vs. 5 or 6); (v) the higher number of dikinetids in the mid-dorsolateral row (10–12 and 9–12 in *E. alatus* and *E. estuarinus* respectively vs. 7–9); (vi) number of caudal cirri (2 or 3 in *E. alatus* and invariably 2 in *E. estuarinus* respectively vs. 1) and (vii) the relative length of the adoral zone (~50 % and 60–65 % in *E. alatus* and *E. estuarinus* respectively vs. ~68%).

Euplotes trisulcatus Kahl, 1932 [see redescriptions by Carter (1972) and Curds (1975)] and *E. dominicanus* sp. n. show similarities in body size and shape. However, *E. trisulcatus* has (i) more membranelles (25–36 vs. 18–22); (ii) more

Table 2. Morphological comparison of *Euplotes dominicanus* sp. n. with several related congeners having a double-*eurystomus* type of silverline system, 10 frontoventral cirri and five transverse cirri.

Characteristic	*E. dominicanus* sp. n.	*E. curdsi*	*E. alatus*	*E. trisulcatus*	*E. para-balteatus*	*E. estuarinus*	*E. wuhanensis*	*E. corsica*
Body size (in vivo; μm)	29–40 × 17–27	52–63 × 27–33	75–90 × 58–69	40 × 30	30–35 μm in length	50–75 × 30–50	40–50 × 25–30	40 × 20
Shape	Elongated, elliptical	Oval-ellipsoid; ends rounded	Oval	Elongated, pointed posterior end	Oval-elongated	Oval	Oval	Oval
AZM length: body length (%)	~68	~70	~50	~65	~65–75	60–65	75	~62
Adoral membranelles, number	18–22	25–34	~26	25–36	19–23	25–33	18–24	20–25
Caudal cirri, number	1	2	2–3	2	2	2	2	1–2
Left marginal cirri, number	2	2	2	2	2	2	2–3	2
Dorsal ridge	Inconsp.[a], 5–6	Inconsp.[a], 5–6	Inconsp.[a]	Prom.[b], 3	Inconsp.[a]	Inconsp.[a]	Prom.[b], 4 or 5	Inconsp.[a]
Dorsolateral kineties	5–6	6–7	8	7	6–7	7	7	7–8
Dikinetids in mid-dorsolateral row	7–9	10–12	10–12	up to 11	8–11	9–12	9–13	6–9
Isolation habitat	Marine (deep sea)	Marine and brackish	Marine	Marine	Marine	Brackish	Soil	Soil from a saline pool
References	This study	Syberg-Olsen et al. (2016)	Curds (1975); Kahl (1932)	Carter (1972); Curds (1975)	Jiang et al. (2010a)	Yan et al. (2018)	Lian et al. (2019)	Berger and Foissner (1989)

[a] Inconsp.= Inconspicuous.
[b] Prom.= Prominent.

Table 3. List of the stations sampled during four different deep-sea expeditions. Obtained sediment samples were used for the next generation sequencing data analyses. Station depth, geographical coordinates and number of reads are given. Numbers of OTU reads are assigned to 100 % identity to *E. dominicanus* sp. n.. Each station is marked with a different letter indicating the station ID (see Fig. 4).

Cruise	Sampling station	Depth (m)	Location	Latitude	Longitude	Number of OTU reads	Station ID on map
M139	A1	4004	North Atlantic	15°55.98′N	68°53.28′W	2	a
M139	A 3/4	4482	North Atlantic	23°33.23′N	48°05.04′W	100	b
M139	A 5/6	5121	North Atlantic	10°20.38′N	36°57.76′W	406	c
SO237	A1	5517	North Atlantic	10°43.108′N	25°03.888′W	0	d
SO237	A2	5771	North Atlantic	10°25.11′N	31°04.61′W	0	e
SO237	A3	5134	North Atlantic	10°21.03′N	36°57.59′W	0	f
SO237	A4	5180	North Atlantic	10°43.56′N	42°41.59′W	0	g
SO237	B1	5000	North Atlantic	11°41.36′N	47°57.34′W	9	h
SO237	B2	5090	North Atlantic	12°05.42′N	50°26.98′W	0	i
SO237	C1	8339	North Atlantic	19°46.00′N	66° 49.99W	0	j
SO237	C2	8350	North Atlantic	19°43.817′N	67°09.285′W	0	k
SO237	C3	4925	North Atlantic	19°04.68′N	67°07.77′W	18	l
M79/1	552	4463	South Atlantic	26°34.11′S	35°13.24′W	0	m
M79/1	578	5124	South Atlantic	14°58.66′S	29°57.53′W	0	n
M79/1	600	5182	South Atlantic	03°56.97′S	28°05.18′W	0	o
SO223T	17004	5339	Pacific	25°22.700′N	134°22.519′E	0	p
SO223T	17006	1253	Pacific	14°03.699′N	144°03.699′E	0	q
SO223T	17007	1829	Pacific	14°04.320′N	145°20.592′E	0	r
SO223T	17009	2687	Pacific	13°58.640′N	145°30.719′E	9	s
SO223T	17013	5497	Pacific	10°34.313′N	148°49.032′E	0	t

dorsolateral kineties (7 vs. 5 or 6); (iii) more dikinetids in the mid-dorsolateral row (up to 11 vs. 7–9; Fig. 1A, B, C, L, M) and (iv) less dorsal ridges (3 prominent dorsal ridges vs. 5 or 6 inconspicuous dorsal ridges; Fig. 1H).

Euplotes wuhanensis, recently described by Lian et al. (2019), differs from *E. dominicanus* sp. n. in (i) body shape (oval vs. elongated); (ii) the relative length of the adoral zone (75% vs. ~68%); (iii) number of dorsolateral kineties (7 vs. 5 or 6) and (iv) number of caudal cirri (2 vs. 1).

Phylogenetic position and habitat differences

The results of the phylogenetic analyses based on maximum likelihood (ML) and Bayesian interference (BI) analyses of the 18S rRNA dataset (Fig. 2), were mostly congruent with recently published analyses of the genus *Euplotes* (Boscaro et al. 2018; Lian et al. 2019; Syberg-Olsen et al. 2016). To our knowledge, the newly described *Euplotes* species is the only species belonging to this genus which has been isolated from the deep sea. Other species mentioned above, which share certain similarities with the deep-sea species *E. dominicanus* sp. n., were collected from different surface habitats: *Euplotes alatus*, *E. trisulcatus* and *E. parabalteatus* were isolated from marine waters. *Euplotes estuarinus* was recorded from a brackish habitat. The presence of one species, *E. curdsi*, was reported from marine and brackish waters. *Euplotes wuhanensis* was isolated from the soil and *E. corsica* from the soil of a saline pool. *Euplotes dominicanus* sp. n. was able to survive in medium with low salinity (5 PSU) for several days. However, the long term effect of the reduction of the salinity on the survival of this species was not studied.

Presence of *Euplotes dominicanus* sp. n. in deep-sea sediments

As it has been pointed out previously, there are no other *Euplotes* strains isolated from the deep sea which could be used for morphological and molecular comparison. The deep-sea benthic environment is one of the most diverse and extended habitats on Earth with a high richness of deep-sea micro-eukaryotes as revealed from environmental DNA surveys (Pawlowski et al. 2011; Scheckenbach et al. 2010). Ciliates are considered as a major and active microeukaryote group in microbial mats of cold-seep sediments based on environmental DNA/RNA surveys (Takishita et al. 2010). Still, very little is known about the benthic eukaryotic communities from abyssal depths, though recently published data indicated that ciliates may form a very diverse component of deep-sea communities (Schoenle et al. 2017). The hyper-variable V9 region of the 18S rRNA used to target environmental diversity of microbial eukaryotes (Dunthorn et al. 2012) was used in next generation sequencing studies to search for the presence of *E. dominicanus* sp. n. in sediments of different deep-sea basins. We could confirm that sequences being 100% identical to the V9 region of the 18S rRNA of *E. dominicanus* sp. n. were present in six out of 12 deep-sea basins in the Atlantic and in the Pacific Ocean. A

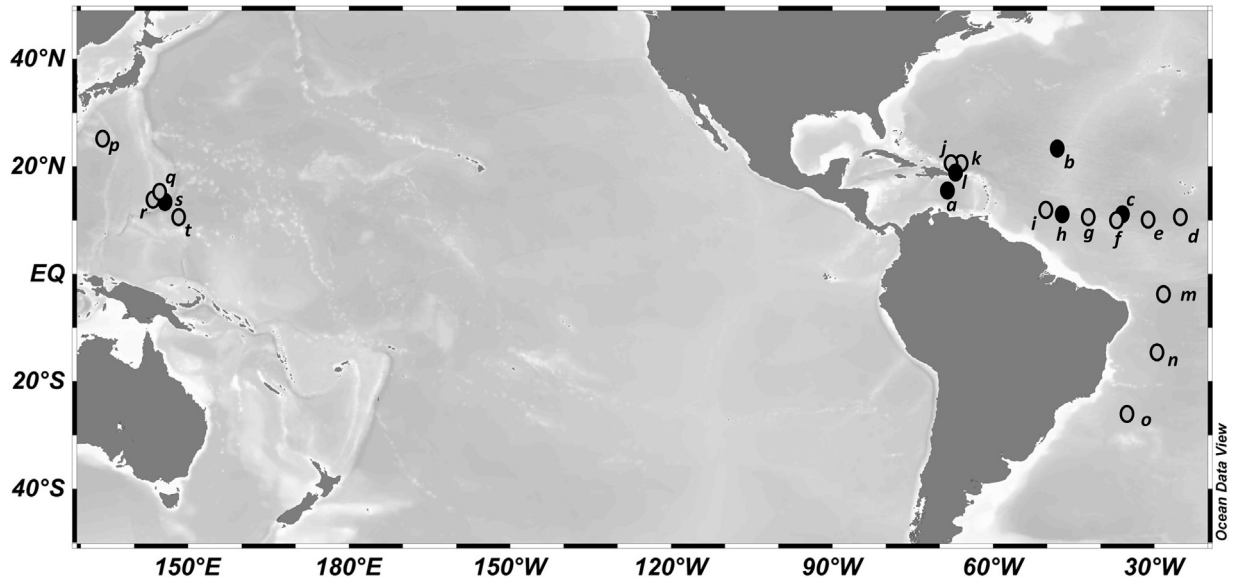

Fig. 4. Geographical positions of 20 stations where deep-sea sediment samples were taken and analyzed for the presence of *Euplotes dominicanus* sp. n. in metabarcoding studies. Each station is marked with a different letter indicating the station ID. Full black dots mark stations where OTU reads (V9 region of 18S rRNA) with 100 % identity to *E. dominicanus* sp. n. were found and empty dots mark the stations where the OTU reads were not found (see also Table 3).

100% identity regarding the V9 region does not mean that sequences belong to exactly the same species, however, our study might indicate that *Euplotes* species at least very similar to *E. dominicanus* sp. n. are not seldom and might be a component of deep-sea microbial communities. In contrast, we did not find sequences being 100% identical to the V9 region of the 18S rRNA of *E. dominicanus* sp. n. in the database of surface-water samples from the Tara Ocean project. We compared the similarity of the V9 region of *E. dominicanus* sp. n. sequence with other *Euplotes* sequences available in GenBank (in total 68 sequences). Our results show that *E. dominicanus* sp. n. has a unique and specific V9 region, only found in this species of all currently available sequences from species within the genus *Euplotes*.

Survival abilities of *Euplotes dominicanus* sp. n. at high hydrostatic pressure

In our pressure experiments, individuals of *E. dominicanus* sp. n. were able to survive exposure to atmospheric pressure as well as to high hydrostatic pressures up to 500 bar. Neither in these pressure experiments nor in cultures grown at atmospheric pressure, we could observe any formation of cysts. This is in contrast to observations on *Pseudocohnilembus persalinus* which showed a stimulated cyst production when exposed to high hydrostatic pressure (Schoenle et al. 2017). Kitching (1957) investigated the effects of pressure on behavior and survival of an unidentified *Euplotes* species isolated from freshwater. He showed that moderate pressures (69−207 bar) caused the increase of the locomotion activity of ciliates and high pressure (>600 bar) depresses ciliary movement. While some individuals failed to recover on release from a pressure of 689 bar, others instantly resume swimming after a few seconds returning to their normal behavior. These results clearly support our observation on potential ability of *Euplotes* species to survive exposure on high hydrostatic pressure. Similar observations were made for different species of heterotrophic flagellates (Živaljić et al. 2018). It is known that bacteria, as an adaptation to high hydrostatic pressures, increase the fluidity of cell membranes by incorporation of unsaturated fatty acids (Allen et al. 1999; DeLong and Yayanos 1985). These mechanisms need still to be investigated for ciliates, as well as the possible reproduction and feeding abilities at high hydrostatic pressure. Furthermore, we encourage the investigation of deep-sea sediments to obtain more living cultures of deep-sea protists, which might help us to get a more comprehensive knowledge on the role of ciliates in deep-sea ecosystems.

Author contributions

A. Schoenle, M. H., A. Scherwass., H. A. and S. Ž. were involved in the sampling and cultivation of the protists. A. Scherwass, P. Q-A., and S. Ž conducted the staining methods. S. Ž. conducted the pressure experiments and performed the statistical analysis. A. Schoenle and S. Ž. performed the phylogenetic analysis. Next generation sequencing data were generated by A. Schoenle and M. H. and analyzed by A. Schoenle and S. Ž. SEM studies were performed by F. N. S. Ž. and H.A. wrote the manuscript, and H.A. supervised the studies. All authors reviewed the manuscript.

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Chapter 4

Influence of hydrostatic pressure on the behaviour of three ciliate species isolated from the deep-sea floor

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ORIGINAL PAPER

Influence of hydrostatic pressure on the behaviour of three ciliate species isolated from the deep-sea floor

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Abstract
Locomotion is considered to be the main form of expression of ciliate behaviour regarding their overall life activity. But how ciliates behave under deep-sea conditions is still unclear. Data on the occurrence of ciliates in the deep sea are scarce and mostly based on molecular studies. We isolated three different ciliates, *Aristerostoma* sp., *Euplotes dominicanus* and *Pseudocohnilembus persalinus* from two stations located in abyssal depths of the North Atlantic Ocean (≥4000 m; 15° 55.89′ N, 68° 53.34′ W; 23° 33.23′ N, 48° 5.04′ W) during the deep-sea expedition with the research vessel R/V Meteor (Cruise M139, 08.07.–08.08.2017). We observed their behaviour directly under high hydrostatic pressures up to 500 bar. The three ciliate species behaved normally up to a pressure of 200 bar, but showed disturbances of the normal behaviour at higher pressures. For all three isolated deep-sea ciliates, additional long-term survival experiments were carried out for 6 days at 200, 350 and 430 bar. Several specimens showed an ability to survive the entire experimental time interval at the highest pressure and to recover from pressure release (returning to their normal movement) indicating their barotolerance. Our results suggest that ciliates are active in the deep sea even in regions deeper than 2000 m and might be an important part of the deep-sea microbial food web.

Introduction

The deep sea is an extreme environment with uniform conditions such as low temperatures, low food resources, permanent darkness and high pressure. Despite these extreme conditions, the deep sea is inhabited by a large variety of organisms which have become evolutionary adapted to this environment. It is well known that in shallow benthic and pelagic ecosystems protists are very important for the energy transfer in aquatic food webs (Azam et al. 1983; Alldredge et al. 1986; Patterson et al. 1993). However, little is known regarding protists in the deep sea and their potential importance within the deep-sea microbial food web (Gooday et al. 2020 in revision). Aside from some heterotrophic flagellates, ciliates and foraminiferans isolated from surface waters and the deep sea were able to survive high hydrostatic pressures (Kitching 1957; Turley et al. 1988, 1993; Morgan-Smith et al. 2013; Schoenle et al. 2017, 2019; Živaljić et al. 2018). Concerning ciliates, it was shown that deep-sea strains of *Pseudocohnilembus persalinus* and *Uronema* sp. and one surface strain of *P. persalinus* were able to survive better at 557 bar at lower temperature (2 °C) than at higher temperature (13 °C) (Schoenle et al. 2017). Data on ciliates isolated from the deep sea are scarce and mostly based on molecular surveys (Schoenle et al. 2017; Živaljić et al. 2020). Whilst abundance estimates from clone library and metabarcoding studies may contain significant methodological biases (e.g., Zhu et al. 2005, Louca et al. 2018, Gutierrez-Rodriguez et al. 2019), several studies indicated that ciliates may form a very diverse component of deep-sea communities (Edgcomb et al. 2002; Countway et al. 2007; Schoenle et al. 2017). There is direct evidence of ciliates from deep-sea hydrothermal vents (~2000 m depth) actively grazing on free-living bacteria, indicating their trophic activity (Pasulka et al. 2019).

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Three species of ciliates isolated from the deep sea (≥ 4000 m) were investigated in the present study: *Pseudocohnilembus persalinus, Euplotes dominicanus* and *Aristerostoma* sp. There are already data about *P. persalinus* isolated from surface waters and deep-sea samples from 1527 and 1598 m depth from the surrounding area of hydrothermal vents in the East China Sea (Zhao and Xu 2016) and from 2687 and 5276 m depth from the North and South Pacific Ocean (Schoenle et al. 2017). This ciliate species has been reported living in saline environments, as an endobiont in a marine olive flounder (*Paralichthys olivaceus*) and in a freshwater adult rainbow trout (*Oncorhynchus mykiss*) (Jones et al. 2010). The ciliate genus *Aristerostoma* was found as a pathogen on gills of farmed Atlantic salmon (*Salmo salar*) (Dyková et al. 2010). Other *Aristerostoma* strains were isolated from surface waters (Dunthorn et al. 2009). The hypotrichous ciliate *Euplotes dominicanus* was described as the first living ciliate isolated from deep waters of the North Atlantic Ocean (> 4000 m) (Živaljić et al. 2020).

So far, the effect of pressure on ciliates has been merely studied for surface water strains. For some ciliates (*Tetrahymena pyriformis*, *Holophrya* sp., *Colpoda cucullus* and *Euplotes* sp.), a moderate pressure increase causes an increase in their locomotor activity, but in general, high pressure depresses flagellar or ciliary movement, and in most cases, all movements stopped at 544–953 atm (552–965 bar) (Kitching 1957). In addition, the pressure may have an influence on the morphology of ciliates: e.g., Auclair and Marsland (1958) studied the shape stability of two ciliates, *Blepharisma undulans* and *Paramecium caudatum*, under varying conditions of hydrostatic pressure (up to 689 bar) and temperature (12–25 °C). Cells of *B. undulans* became shorter and rounded at higher pressure (480 bar) and lower temperature (decrease from 25 to 12 °C). The same pattern was observed for *P. caudatum* at pressures between 275 and 344 bar and temperatures of 20 °C and 25 °C. Most experiments were performed using closed pressure vessels where a direct observation is not possible (Turley et al. 1988, 1993; Atkins et al. 1998; Morgan-Smith et al. 2013). However, there are some pressure systems allowing direct microscopic observations which have been used to study pressure effects on different organisms (Kitching 1954, 1957; Salmon and Ellis 1975; Koyama et al. 2001; Frey et al. 2006; Bao et al. 2010; Nishiyama and Kojima 2012).

Locomotion, which represents the main form of expression of ciliate behaviour, is typically displayed as a trajectory conducted by an individual cell which serves to distribute ciliates in the surrounding space and allows exploration of a new environment (Bohatová and Vďačný 2018). The behaviour should be considered as a complex and variable response of protozoans to adapt activities to constantly changing external conditions (Martin and Bateson 1986). Studies on the behaviour of protists are important to understand vital activities of organisms, such as feeding, reproduction, sexual activity, avoidance of danger and search for safety, and colonisation of new habitats (Ricci 1990). For the interpretation of the behaviour of protists, ethograms based on direct observations or video recordings have been used. One of the first scientists who described the locomotion of several ciliates in detail, was Ricci (1990) describing the ciliate behaviour by different elements. It is not known whether ciliates can perform these elements when exposed to high hydrostatic pressures and what are their potential responses to the stress caused by the increase in pressure.

To our knowledge, we provide the first results on the direct observation of behaviour and locomotion under different hydrostatic pressures of three ciliates (*Aristerostoma* sp., *Euplotes dominicanus, Pseudocohnilembus persalinus*) isolated from the deep sea. Our study was intended to answer the question whether ciliates isolated from the deep-sea can be active there, or whether they exist in the depth only as cysts potentially sedimented from surface waters. In addition, we wanted to test if the behaviour of these organisms changes under abiotic stress like high pressure.

Materials and methods

Isolation and cultivation of deep-sea ciliates

All samples were collected during the deep-sea expedition with the research vessel R/V Meteor (Cruise M139, Cristóbal (Panama)—Mindelo (Cape Verde), 08.07.–08.08.2017; Fig. 1). The deep-sea sediment was taken by means of a Multi-Corer system (MUC). Samples of the Multi-Corer system were taken from the surface sediment layer and 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. After cores were brought on deck, they were immediately processed. The upper 2-mm layers of sediment were transferred into 50-mL tissue-culture flasks (Sarstedt, Nümbrecht, Germany) under sterile conditions and filled with 30 mL autoclaved seawater (35 PSU) and one wheat grain to ensure growth of autochthonous bacteria. On board, raw cultures of ciliate strains were established by serial dilution. Later in the home laboratory, single cells were isolated with the help of a micromanipulator (PatchMan NP 2 from Eppendorf, Germany) under an inverted microscope (ZEISS Axiovert 25, Germany). *Pseudocohnilembus persalinus* (HFCC778) was isolated from sediment taken from depths of 4000 m in the Caribbean Sea (Station A1; Fig. 1; Fig. S1). *Euplotes dominicanus* (HFCC757) and *Aristerostoma* sp. (HFCC744) were isolated from sediment samples taken at 4296 m depth in the North Atlantic (Station A3/4; Fig. 1; Fig. S1). Isolated

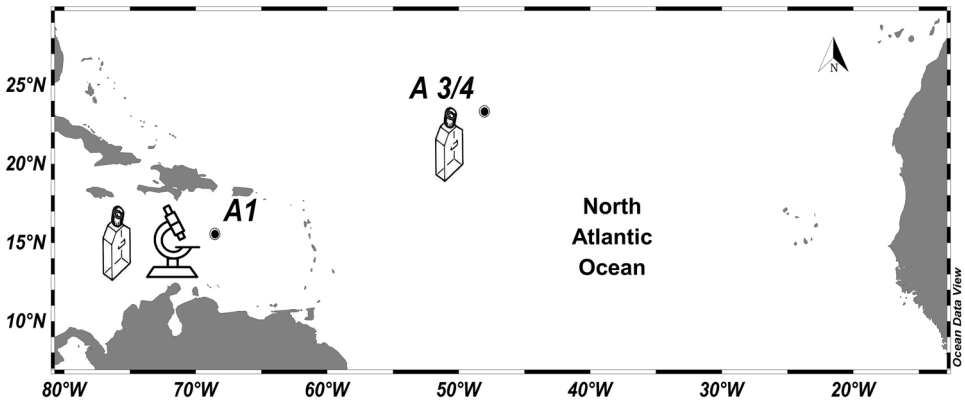

Fig. 1 The geographic locations of stations where the deep-sea ciliates were detected during the expedition M139. Stations are marked with A1 for the isolation site of *Pseudocohnilembus persalinus* (4000 m; 15° 55.89′ N, 68° 53.34′ W) and with A3/4 for the isolation site of *Euplotes dominicanus* and *Aristerostoma* sp. (4296 m; 23° 33.23′ N, 48° 5.04′ W). Culture flasks signify successful cultivation of ciliates. The microscope signifies the first live observation of a deep-sea ciliate. Map created by Ocean Data View (Schnitzler 2012)

specimens were further cultivated in 50-mL tissue-culture flasks (Sarstedt, Nümbrecht, Germany) filled with 30 mL of autoclaved 35 PSU Schmaltz-Pratt medium (a litre contained 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, and 0.01 g $K_2HPO_4 \times 3H_2O$) and supplied with wheat grains as a carbon source for autochthonous bacteria. Only for *Euplotes* cultures, bicosoecid, stramenopiles and an undetermined cercozoan species were added as the food source for the ciliates. Prior to pressure experiments, cultures were stored at least for 2 days at 4 °C in the dark (conditions corresponding to the deep-sea environment, except for pressure).

DNA extraction, PCR amplification and sequencing

To characterise the isolated and cultured organisms, DNA was extracted using the Quick gDNA miniPrep isolation kit (Zymo Research, USA). For *Aristerostoma* sp. and *Pseudocohnilembus persalinus*, PCR was done using the primers: forward 18S-For (5′-AACCTGGTTGATCCT GCCAGT-3′, Medlin et al. 1988) and reverse NLR2098/24 (5′-AGCCAATCCTTWTCCCGAAGTTAC-3′, Van der Auwera et al. 1994). The volume of PCR mixtures was 50 µL, including 12 µL of double distilled water (ddH_2O), 25 µL of Mastermix (VWR Red Taq DNA Polymerase Master Mix by VWR CHEMICALS), 3 µL DNA, and 5 µL of forward and 5 µL of reverse primer (1 µM stock concentration). Amplification cycles 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 and 30 s, and a final extension at 72 °C for 10 min. The 18S rDNA of both ciliates was analysed by Sanger sequencing using the following primers: 18S-For (5′-AACCTGGTTGATCCT GCCAGT-3′, Medlin et al. 1988), 18S-Rev (5′-TGATCC TTCCGCAGGTTCACCTAC-3′, Medlin et al. 1988) and 1280F (5′-TGCATGGCCGTTCTTAGTTGGTG-3′, Wylezich et al. 2002). The 28S rDNA was analysed by Sanger sequencing using the following primers: NLF184/21 (5′-ACCCGCTGAAYTTAAGCATAT-3′, Van der Auwera et al. 1994), NLR1126/22 (5′-GCTATCCTGAGGGAA ACTTCGG-3′, Van der Auwera et al. 1994), D3For (5′-GACCCGTCTTGAAACACGCA-3′, Wylezich et al. 2007) and NLR2098/24 (5′-AGCCAATCCTTWTCCCGAAGT TAC-3′, Van der Auwera et al. 1994). For sequencing of *Euplotes dominicanus* (HFCC757), a single-cell PCR was performed. Prior to PCR, single cells were transferred in double distilled water (ddH_2O) and then frozen at − 20 °C to disrupt the cells. The 18S rDNA of *E. dominicanus* was amplified using following primers: 18S-For (5′-AAC CTGGTTGATCCTGCCAGT-3′), 590For (5′-CGGTAA TTCCAGCTCCAATAGC-3′), or 1280F (5′-TGCATG GCCGTTCTTAGTTGGTG-3′), 18S-Rev (5′-TGATCC TTCCGCAGGTTCACCTAC-3′), and 1300R (5′-CAC CAACTAAGAACGGCCATGC-3′) (Medlin et al. 1988; Wylezich et al. 2002). The volume of PCR mixtures was 50 µL, including 5 µL of ddH_2O, 25 µL of Mastermix (VWR Red Taq DNA Polymerase Master Mix by VWR Chemicals, USA), 10 µL ddH_2O containing the single cell, and 5 µL of forward and 5 µL of reverse primer (10 µM stock concentration). Amplification cycles 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, and a final extension at 72 °C for 10 min. The PCR products were detected using agarose gel (1%) and fragment sizes were determined by comparison with 250–10,000 bp DNA ladder (Genaxxon). The PCR products were purified using the PCR Purification KIT (Jena Bioscience).

Phylogenetic analysis

For the alignment of the phylogenetic 18S rDNA analysis of the family Pseudocohnilembidae, we followed Schoenle et al. (2017). In addition, we included two sequences form GenBank database (AY212806; Z22880) and our own sequence (Accession number MT081565). Alignments were done using MAFFT v7.311 (Katoh and Standley 2013) within Unipro UGENE v1.31.1 (Okonechnikov et al. 2012). In total, the alignment comprised 21 sequences including 8 sequences as outgroup (belonging to the order Philasterida) with the final uncorrected size of 1625 bp. For maximum likelihood (ml) analysis, the model GTR + I + Γ was determined by MrAic (Nylander 2004) and it was computed by RaxML v8.2.10 (Stamatakis 2014) on the CIPRES Gateway (Miller et al. 2010) with 1000 bootstrap replicates. For the Bayesian inference (Bi) analysis with Mr. Bayes v3.2.6 (Ronquist et al. 2012), the same model was used as suggested by MrAic (Nylander 2004). The analysis consisted of 40,000 generations in the Markov chain, with a burn-in of 25% of the total number. The search used two parallel chain sets run at default temperatures.

For the phylogenetic 18S rDNA analysis of the order Crytolophosidida, sequences were downloaded from GenBank database and our own sequence was added (Accession number MT081566). Alignments were carried out as described above. In total, the alignment comprised 17 sequences including 6 sequences as outgroup (belonging to the order Bursariomorphida) with the final uncorrected size of 1620 bp. For further analyses, the same models and programs were used as described above. Bayesian analysis consisted of 200 000 generations in the Markov chain, with a burn-in of 25% of the total number.

Phylogenetic analysis of *Euplotes dominicanus* is detailed described in Živaljić et al. (2020). In total, the 18S rDNA dataset included 68 *Euplotes* sequences and 14 outgroup sequences, containing 1903 unambiguously aligned base pars. Bayesian analysis consisted of 100,000 generations in the Markov chain, with a burn-in of 25% of the total number.

Survival analysis

In long-term survival experiments, a closed type of pressure chamber was connected with a manual hydraulic pump. Three stainless steel pressure chambers (ø 30 mm, depth 50 mm) were used in parallel. Experiments were carried out at 4 °C. Three sets, each containing six cuvettes, were filled with the culture (2 mL). Ciliates were counted at the start and at the end of the experiment using an inverted microscope (ZEISS Axio Vert.A1, Germany) using a 20 × LD objective and an ocular grid. In each of the three pressure chambers, the pressure was increased up to a maximum of 200 bar, 350 bar and 430 bar. Pressure was increased within 72 h and decreased within the next 72 h, respectively. In all three chambers, pressure was gradually increased and decreased in 50-bar steps (Fig. 2). Time interval between each pressure step was 2 h. Parallel experiments at atmospheric pressure served as a control. Active cells and cysts were counted immediately after the pressure release for the determination of the survival rate. To check for the viability of cysts, the same cuvettes were exposed for additional 144 h to atmospheric pressure at 20 °C to stimulate excystment. MS Excel 2010 was used to create graphs and Software R v3.4.4 (https://www.r-project.org/) and MS Excel 2010 for statistical procedures. The normality of the data was checked with the Shapiro–Wilk test. For checking the homogeneity of variance, the Levene's test was used. Statistical analysis was performed using a One-way ANOVA and post-hoc Tukey's test to test the influence of pressure on the abundance of ciliates before and after the pressure exposure. Significance levels were considered at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

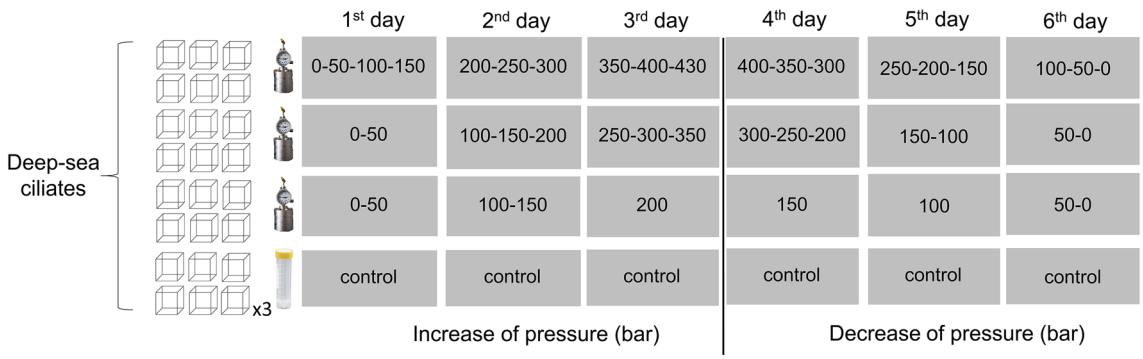

Fig. 2 Schematic view of the long-term survival experiments

Behavioural analysis

Behavioural experiments were done in high-pressure system with windows for direct observation. The system was constructed using the principal idea developed by Koyama et al. (2001). Our pressure system consisted of a chamber with two windows for direct observation and a manual hydraulic pump with the ability of gradually increasing the pressure (up to 600 bar). The construction material of the chamber was stainless steel. The chamber consisted of an upper and a lower lid. In the upper lid, the window was made from acrylic glass (8-mm thick and 20 mm in diameter) for light penetration. The lower lid has a window made of 4-mm-thick (10-mm in diameter) mineral glass leaving an area of 4-mm diameter for microscopic observation. An O-ring served as a spacer for placing approximately 0.2 mL of the sample into the pressure chamber. For each ciliate species, either 10 (*Euplotes, Pseudocohnilembus*) or 12 (*Aristerostoma*) individuals were analysed separately regarding their behaviour at different hydrostatic pressures and at control conditions (1 bar). Pressure was gradually increased in steps of 50 bar every 7 min until maximum pressure was reached (500 bar for *Euplotes dominicanus,* 450 bar for *Aristerostoma* sp. and 350 bar for *Pseudocohnilembus persalinus*). As a control, parallel experiments were performed with individuals exposed at atmospheric pressure (1 bar) in an additional chamber. The behavioural studies were performed with the Motion Analysing Microscope (Keyence, VW-6000; Japan) consisting of a controller and a high-speed camera unit [resolution 640 × 480 pixels at 250 fps (frames per second) and less]. The camera unit was attached to an inverted microscope (ZEISS Primovert, Germany) and observation was done with the help of a 20 × LD objective (with phase contrast). All videos were recorded for 22.4 s with 500 fps and resolution of 640 × 240 pixels (size of one pixel was 4.84 µm/pixel, size of field of view 45.72 mm^2).

The behaviour of the deep-sea ciliates was studied and classified using the long (linear segment, rightward and leftward arc) and short lasting elements (continuous, smooth and rough trajectory change, side-stepping reaction) according to the classification made by Ricci (1990). In addition, other elements that were also observed for the three species were included: backward motion, rotation, flickering, walking, only cilia movement, no movement. For each ciliate, the time used to perform different behavioural elements during pressure and control treatments was analysed. The time spent at a specific behavioural element was calculated as percentages of the total time of observation. MS Excel 2010 was used to create graphs and Software R v3.4.4 (https://www.r-project.org/) for statistical procedures. For the pairwise comparison, the Wilcox two-sided runk sum test was used and for the multiple comparisons, a Kruskal–Wallis test followed by Dunn's post-hoc tests to analyse the influence of pressure on the time ciliates spent at specific behavioural elements during pressure and control treatments. Significance were considered at $p < 0.05$.

Results

Phylogenetic position of investigated deep-sea ciliates

To allow an unambiguous assignment of the experimental results to the respective organisms, we investigated the genotypes of the experimental ciliate species. One deep-sea isolate was morphologically assigned to the marine ciliate genus *Aristerostoma.* The phylogenetic analyses based on maximum likelihood (ml) and Bayesian inference (Bi) analysis of the 18S rDNA data confirmed that our isolate was a morphologically yet undescribed new *Aristerostoma* species. Unfortunately, attempts to morphologically describe the new species using different staining methods failed. As Dunthorn et al. (2009) pointed out for *Aristerostoma marinum*, this was probably due to the cell sensitivity, a high salt concentration in the medium and a mucus shell (mucocysts) covering the organisms. *Aristerostoma* sp. (HFCC744) formed a fully supported clade within the Cyrtolophosidida (Fig. 3; maximum likelihood bootstrap percentages (mlBP) 100%, Bayesian posterior probabilities (BiPP) 1.00) together with *Aristerostoma marinum* (EU264562), *Aristerostoma* sp. (EU264563) and *Aristerostoma* sp. (GQ259748). Within the clade, our *Aristerostoma* sp. (HFCC744) clustered on the same branch with *A. marinum* with a p-distance of 5.2% (Fig. 3; mlBP 87%, BiPP 0.98). The two other available genotypes in GenBank, *Aristerostoma* sp. (EU264563) and *Aristerostoma* sp. (GQ259748), clustered together on a separate branch with high support (Fig. 3; mlBP 99%, BiPP 1.00). Our strain HFCC744 had a p-distance of 3.7% to both *Aristerostoma* sp. sequences (EU264563, GQ259748).

The second deep-sea ciliate was morphologically assigned to the species *Pseudocohnilembus persalinus* within the family Pseudocohnilembidae. The phylogenetic analyses based on maximum likelihood and Bayesian inference analysis of the 18S rDNA data confirmed that our isolate belongs to the morphologically described species *Pseudocohnilembus persalinus*. Our strain (HFCC778) clustered together on a branch with other available sequences of *P. persalinus* with moderate support (Fig. 4; mlBP 79%, BiPP 1.00). Within this clade, two strains of *P. persalinus* strains (GU584096, AY551906) clustered together on a separate branch with a maximum support (Fig. 4; mlBP 100%, BiPP 1.00). The *P. persalinus* strain HFCC778 clustered closely with four other *P. persalinus* strains (MG452732, MG452733, MG452734 and MG452735) isolated from the Pacific Ocean with a support of 92% mlBP and 0.92 BiPP

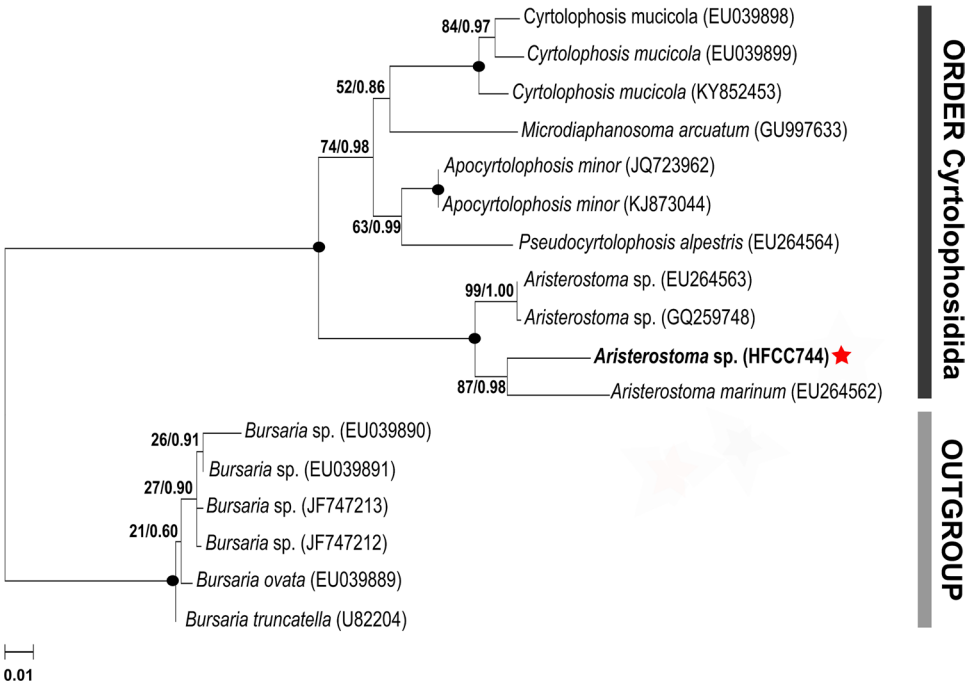

Fig. 3 Maximum likelihood (ml) phylogenetic tree of small subunit (SSU) rDNA of the order Cyrtolophosidida. New sequence is in bold marked with red star. Numerical support values are given at the respective nodes as: maximum likelihood (ml) bootstrap percentages (RaxML, 1000 replicates)/ Bayesian posterior probabilities (Bi) (MrBayes). The well-supported (100% ml, 1.00 Bi) branches are marked with solid circles. Scale bar represents 0.01 expected substitutions. The alignment had a total length of 1620 bp

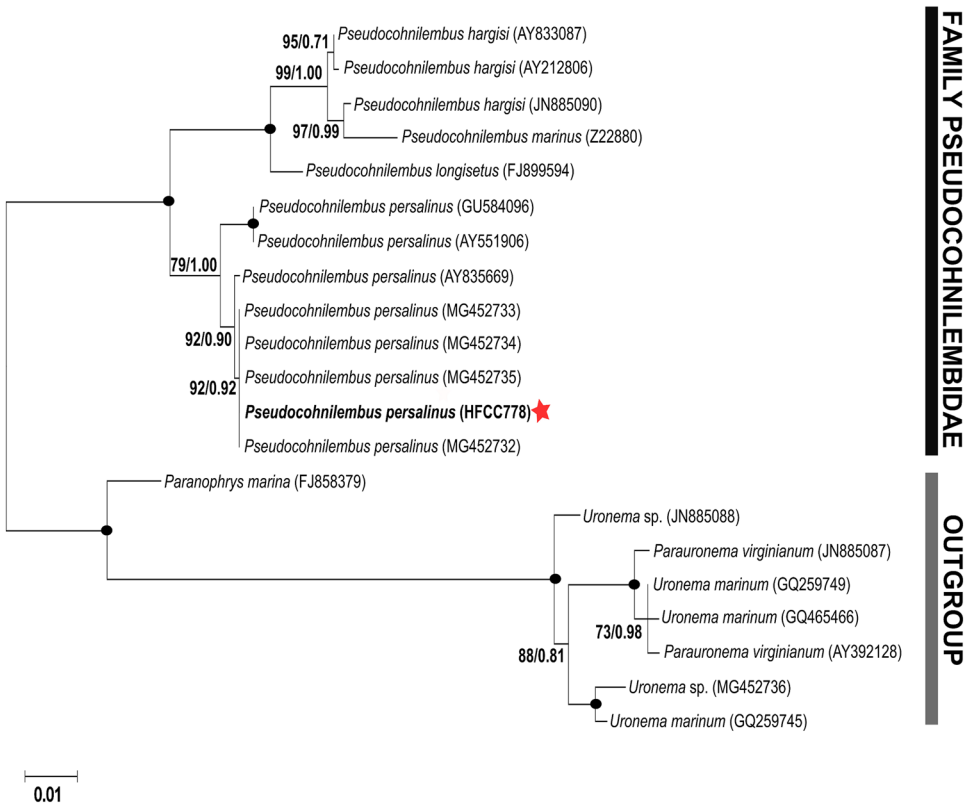

Fig. 4 Maximum likelihood (ml) phylogenetic tree of small subunit (SSU) rDNA of the family Pseudocohnilembidae. New sequence is in bold and marked with red star. Numerical support values are given at the respective nodes as: maximum likelihood (ml) bootstrap percentages (RaxML, 1000 replicates)/Bayesian posterior probabilities (Bi) (MrBayes). The well-supported (100% ml, 1.00 Bi) branches are marked with solid circles. Scale bar represents 0.01 expected substitutions. The alignment had a total length of 1625 bp

(Fig. 4). A comparison between them revealed no p-distance. These five *P. persalinus* sequences clustered closely with another *P. persalinus* (AY835669) having a p-distance of 0.2% and high branch support (Fig. 4; mlBP 92%, BiPP 0.90).

The morphological and molecular identity and the distribution patterns of the third deep-sea isolate belonged to a new ciliate species of the genus *Euplotes* within the family Euplotidae (*E. dominicanus*) which was described in a separate paper (Živaljić et al. 2020).

Survival at high hydrostatic pressures

We checked for the survival of all three ciliate strains in long-term experiments imitating the sedimentation time to the deep sea of about 72 h (e.g., on sedimenting brown algae *Sargassum*, see Baker et al. 2018) establishing final maximum pressures of 200 bar, 350 bar and 430 bar, respectively (Fig. 2). For *E. dominicanus,* only active cells remained after the pressure exposure at all three pressures, no cysts were produced. For *Aristerostoma* sp., active cells only and no cysts were observed after the pressure exposure to 200 bar and cysts only were observed after exposure at 350 and 430 bar (Fig. 5b). For *P. persalinus,* only active cells were recorded after the pressure exposure at 200 and 350 bar, and cysts only were registered after exposure at 430 bar (Fig. 5c).

The abundances of *Euplotes dominicanus* recorded after exposure of 200 bar were similar to that exposed to 1 bar [Fig. 5a; ANOVA, F (1, 10) = 1.226, p = 0.294]. However, abundance after pressure treatments at 350 bar was lower and at 430 bar significantly lower in comparison to control treatments [Fig. 5a; 350 bar, ANOVA, F (1, 10) = 8.555, p = 0.015; 430 bar, ANOVA, F (1, 10) = 17.423, p = 0.002]. The two other ciliates, *Aristerostoma* sp. and *Pseudocohnilembus persalinus,* showed a significant decrease of abundances after the release from each established pressure in comparison to control treatments [Fig. 5b; *Aristerostoma* sp., 200 bar, ANOVA, F (1, 10) = 47.456, p < 0.001; 350 bar, ANOVA, F (1, 10) = 304.318, p < 0.001; 430 bar, ANOVA, F (1, 10) = 214.136, p < 0.001; Fig. 5c, *P. persalinus*; 200 bar, ANOVA, F (1, 10) = 5.262, p = 0.045; 350 bar, ANOVA, F (1, 10) = 64.692, p < 0.001; 430 bar, ANOVA, F (1, 10) = 30.600, p < 0.001]. However, several individuals of all species survived even the highest established pressure.

To check for the viability of cysts, the cuvettes with organisms were left for additional 144 h after releasing the pressure at atmospheric pressure, room temperature and in darkness. This treatment stimulated the hatching of cysts produced by *Aristerostoma* and *Pseudocohnilembus*

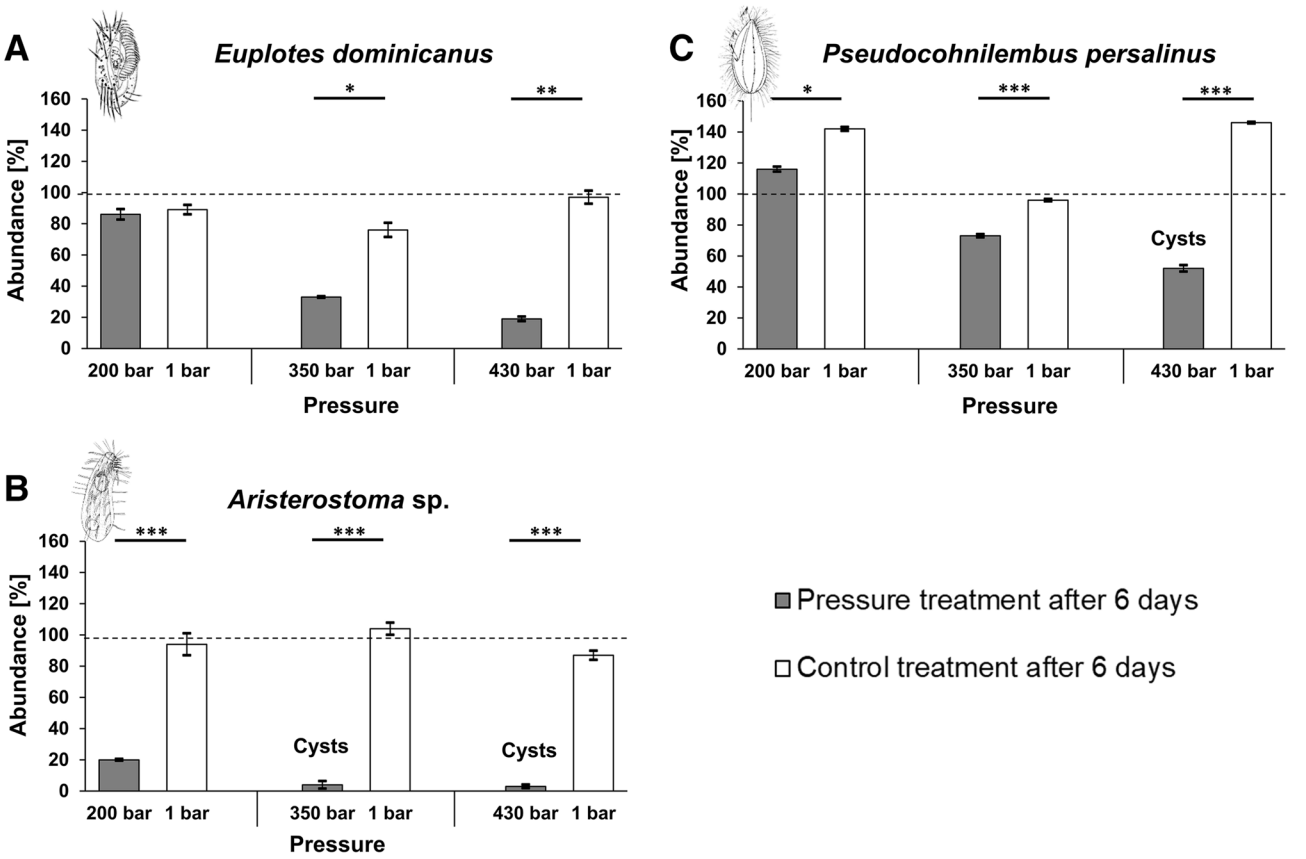

Fig. 5 **a**–**c** Mean abundance (%) of *Euplotes dominicanus* (**a**), *Aristerostoma* sp., (**b**) and *Pseudocohnilembus persalinus* (**c**) strains after 6 days exposed to different pressures (200 bar, 350 bar and 430 bar) and control treatment (1 bar) relative to the start abundances (set to 100%, dashed line). Vertical bars represent 95% confidence intervals. Above the columns, it is indicated when only cysts were observed after pressure exposure. One-way ANOVAs with Tukey's HSD test as post-hoc analysis were conducted and significant differences between pressure and control treatments for each ciliate strain are indicated by '*' (p < 0.05), '**' (p < 0.01), '***' (p < 0.001). Temp. = 4 °C, n = 6 (per species and treatment)

indicating that those cysts recorded after exposure to high pressure were viable and populations could recover. The individuals of *Euplotes* which remained after exposure were viable and dividing specimens were observed.

Behaviour at different hydrostatic pressures

For all three deep-sea ciliates, we analysed the effect of high pressure on their activity in comparison with the data obtained in control treatments (Fig. 6a–c). The number of active cells decreased with increasing pressure for all three ciliate species. At the maximum applied pressure of 500 bar, 50% of the *Euplotes* cells were still active. More than half of the *P. persalinus* individuals (57%) were active at 300 bar, but no specimen survived a pressure of 350 bar (Fig. 6c). *Aristerostoma* sp. had 33% of active cells at a pressure of 400 bar, but no cells survived 450 bar (Fig. 6b). Concerning all control treatments, all ciliates were alive at the end of the experiment. We analysed the changes of the behaviour of all ciliate species when exposed to increasing pressure for the range of pressure the respective species survived (Fig. 6a–c). There was a clear tendency for all three species for a reduction of time spent at short and long lasting elements with increasing pressure. At the highest pressure survived by the three species, other elements like flickering, rotation and only cilia movement characterised their behaviour.

Short lasting elements

Side-stepping reaction (ssr) was mostly recorded for *Euplotes dominicanus*. This element was recorded at a pressure of up to 150 bar when the time spent for this element significantly decreased to 0.4% (Fig. 7a, Kruskal-Wallis test, $H_9 = 33.236$, $p = 0.024$). Individuals of *Aristerostoma* sp. performed this element only at 150 bar (Fig. 8a) and *P. persalinus* individuals at up to 200 bar (Fig. 8d). The behavioural changes in the control treatments in the course of the experimental time were not significant.

At 100 bar, *Euplotes* individuals performed 2% of the total observation time a rough trajectory change (rtc), while the time decreased to 1% at 250 bar (Fig. 7a). For *Aristerostoma* sp. individuals, the time used to perform this element significantly decreased from 19% at 50 bar to 1% at 350 bar (Fig. 8a, Kruskal–Wallis test, $H_7 = 33.133$, $p = 0.041$). At 50 bar, 9% of the total time was used to perform the rtc element by *P. persalinus* and it significantly decreased to 2% at 300 bar (Fig. 8d, Kruskal–Wallis test, $H_5 = 16.669$, $p = 0.039$). In control treatments, the rtc element was very

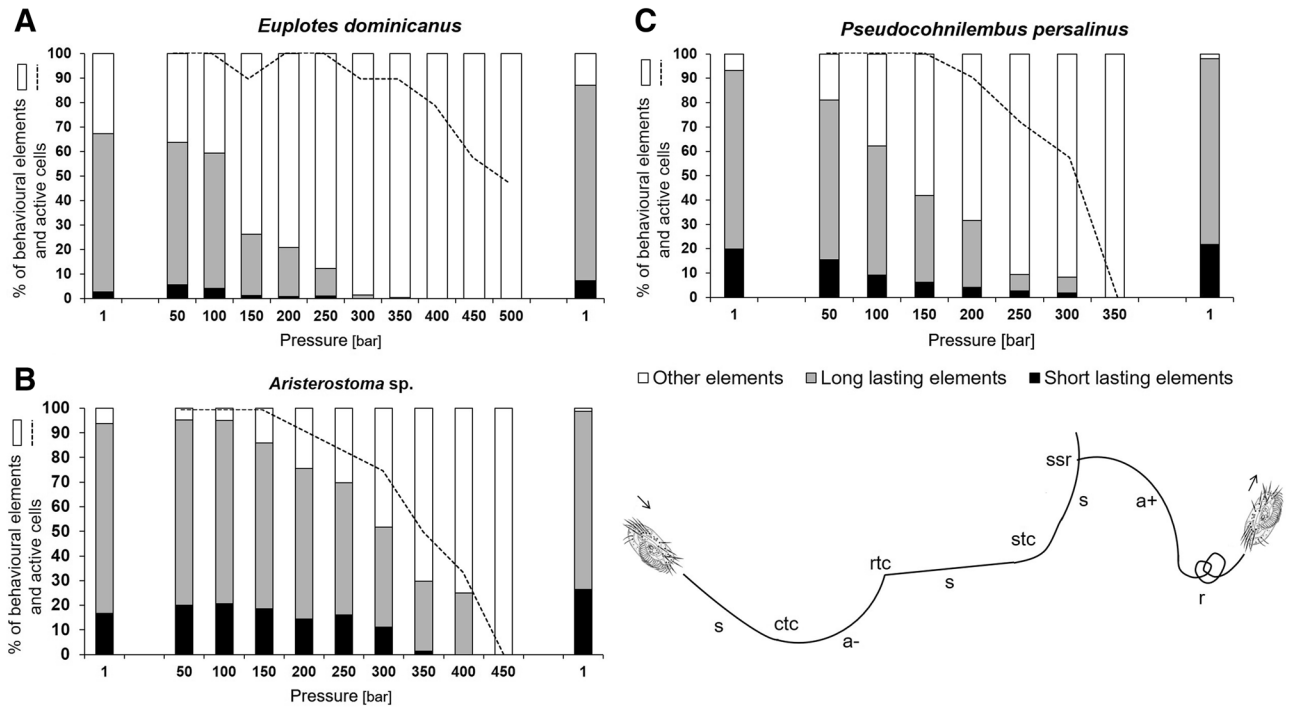

Fig. 6 **a**–**c** The percentage of behavioural elements and active cells recorded for total observation time for three deep-sea ciliates *Euplotes dominicanus* (**a**), *Aristerostoma* sp. (**b**), and *Pseudocohnilembus persalinus* (**c**) during pressure and control treatments (1 bar) (Temp. = 4 °C, $n = 10$–12). The behavioural elements are indicated as columns and active cells as dashed lines. The schematic drawing below the legend shows the short lasting elements (continuous trajectory change, ctc; smooth trajectory change, stc; rough trajectory change, rtc; side-stepping reaction, ssr) and the long lasting elements (linear segment, s; rightward arc, a+; leftward arc, a−). The rotation (r) is an example for other elements

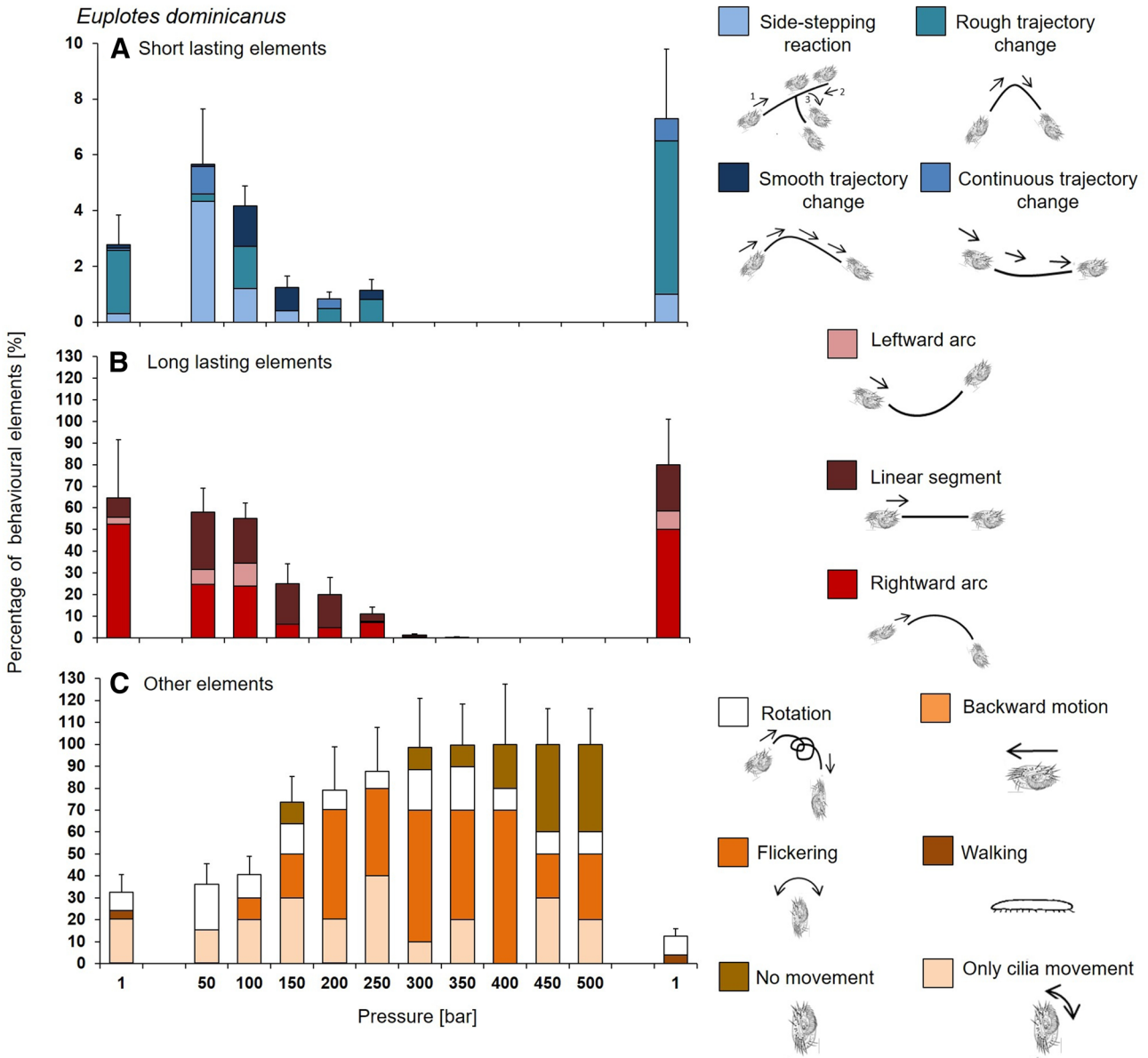

Fig. 7 **a**–**c** The percentage contribution of different types of short (**a**), long lasting (**b**) and other (**c**) behavioural elements performed during total observation time for *Euplotes dominicanus* in pressure and control (1 bar) treatments. The first and the last columns in all three graphs represent the behaviour at the beginning and at the end of the control which was observed in parallel to the pressure treatments. For a better understanding, the schematic drawings are included for all behavioural elements in the legend. Vertical bars represent ± SD (Temp. = 4 °C, $n = 10$)

frequently recorded (Figs. 7, 8a, d). The time to perform this element significantly decreased from 2 to 6% for *E. dominicanus* (Fig. 7a, Wilcoxon rank sum test, $W = 23.5$, $p = 0.044$) and from 16 to 23% for *Aristerostoma* sp. (Fig. 8a, Wilcoxon rank sum test, $W = 36$, $p = 0.040$).

Euplotes individuals performed smooth trajectory change (stc) at up to 250 bar and the highest percentage of the total time was 2% at 100 bar (Fig. 7a). For individuals of *Aristerostoma*, the total time decreased to 0.5% at 150 bar (Fig. 8a). The observation time of stc decreased from 5% at 50 bar to 1% at 250 bar for *Pseudocohnilembus* individuals (Fig. 8d). In control treatments, *Euplotes* individuals performed this element very rarely in comparison to *Aristerostoma* and *Pseudocohnilembus* individuals (Figs. 7, 8a, d).

In pressure and control treatments, a continuous trajectory change (ctc) was recorded only for *E. dominicanus* and *P. persalinus*. For *Euplotes* individuals, this element was performed at 50 and 200 bar (Fig. 7a); and with a higher percentage of the total time at 50 bar. *P. persalinus* displayed

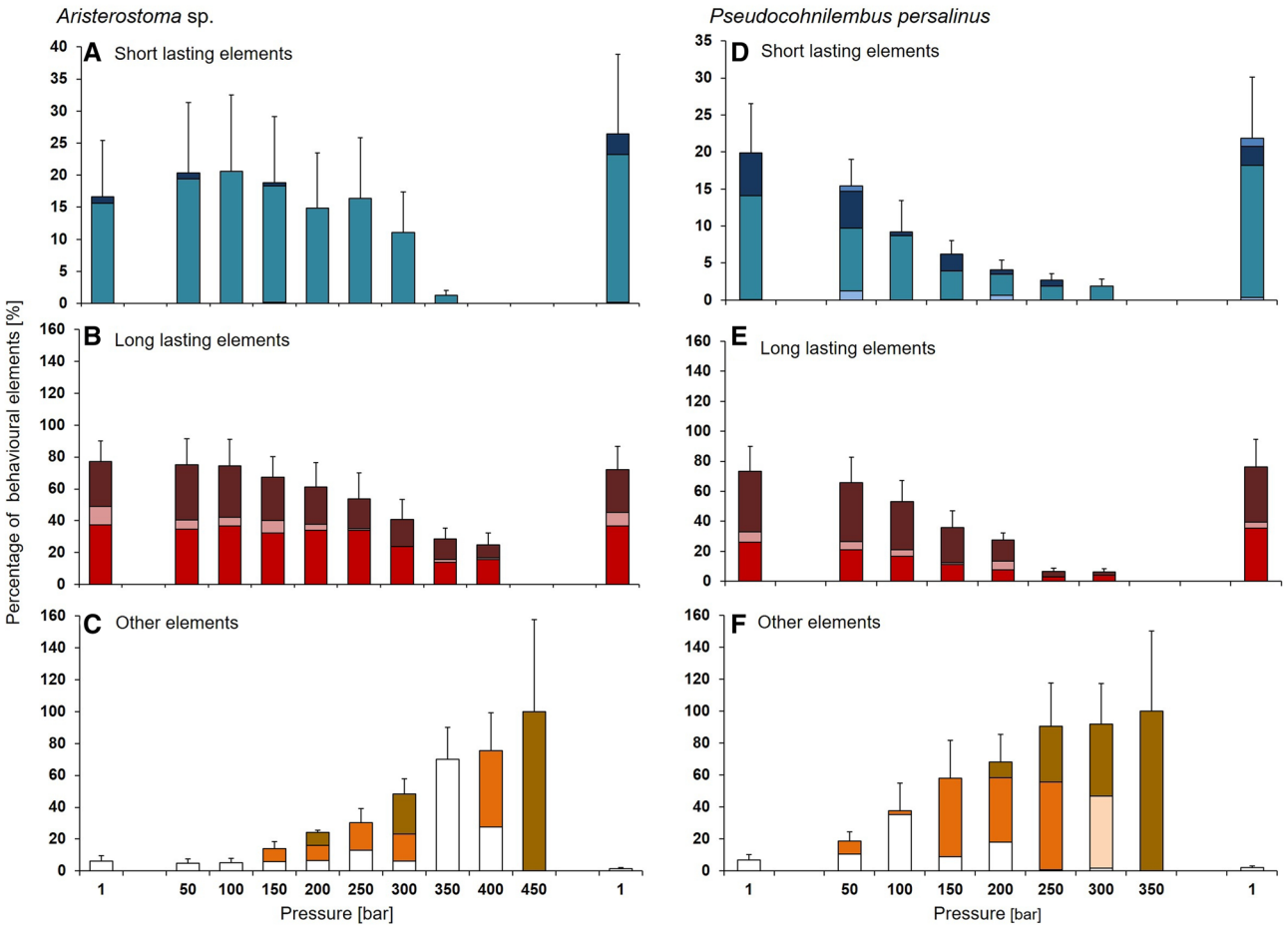

Fig. 8 **a**–**f** The percentage contribution of different types of short (**a**, **d**), long lasting (**b**, **e**) and other (**c**, **f**) behavioural elements performed during total observation time for *Aristerostoma* sp. (**a**–**c**; $n=12$) and *Pseudocohnilembus persalinus* (**d**–**f**; $n=10$) in pressure and control (1 bar) treatments. The first and the last columns in all three graphs represent the behaviour at the beginning and at the end of the control which was observed in parallel to the pressure treatments. The detailed legend is shown in Fig. 7. Vertical bars represent ± SD (Temp. = 4 °C)

ctc element only at 50 bar (Fig. 8d). In control treatments, the ctc element was not frequently recorded for both ciliates.

Long lasting elements

For *Euplotes dominicanus*, rightward arc (a+) was recorded up to 300 bar. The time used to perform this element significantly decreased from 25% at 50 bar to 1% at 300 bar (Fig. 7b, Kruskal–Wallis test, $H_9=37.997$, $p=0.010$). A significant decrease of the time showing this element was also recorded for *Aristerostoma* sp. decreasing from 35% at 50 bar to 16% at 400 bar (Fig. 8b, Kruskal–Wallis test, $H_7=22.604$, $p=0.021$). For *P. persalinus*, the time used to perform this element decreased from 21% at 50 bar to 4% at 300 bar (Fig. 8e). In control treatments, rightward arc was observed for all three ciliates, but most frequently for *Euplotes* individuals.

The leftward arc (a-) element was not often performed by all three ciliates. For *Euplotes* individuals, the highest percentage of the total time spent to perform this element was 11% at 100 bar (Fig. 7b). For *Aristerostoma* sp., the highest percentage of time was 8% at 150 bar (Fig. 8b). For *Pseudocohnilembus* individuals, this element was recorded at 50 and 200 bar with 6% of the total observation time (Fig. 8e). In control treatments, the leftward arc element was not very frequently recorded.

For all three ciliates, the time used to perform linear segment (s) movement significantly decreased with increasing pressure. Recorded decrease was from 26% at 50 bar to 0.8% at 300 bar for *E. dominicanus* (Fig. 7b, Kruskal–Wallis test, $H_9=35.477$, $p=0.019$), from 34% at 50 bar to 8% at 400 bar for *Aristerostoma* sp. (Fig. 8b, Kruskal–Wallis test, $H_7=25.215$, $p=0.003$) and from 39% at 50 bar to 2% at 300 bar for *P. persalinus* (Fig. 8e, Kruskal–Wallis test, $H_5=23.144$, $p=0.002$). In control treatments, this element

was observed for all three ciliates, but less frequently for *Euplotes* individuals.

Other elements of movement

All three ciliates performed rotation (r) during pressure treatments. For *Euplotes* individuals, the time used to perform this element decreased to 10% at 500 bar (Fig. 7c). For *Aristerostoma* sp., the total time of 70% was recorded at 350 bar and it decreased to 28% at 400 bar (Fig. 8c). Significant decreases from 11% at 50 bar to 1% at 250 bar were recorded for *P. persalinus* (Fig. 8f, Kruskal–Wallis test, $H_5 = 24.970$, $p = 0.047$). In control treatments, all three ciliates performed this element very frequently. The time to perform this element significantly decreased from 7 to 2% only for *P. persalinus* (Fig. 8e, Wilcoxon rank sum test, $W = 76$, $p = 0.049$).

The time used to perform flickering (f) elements increased up to 70% at 400 bar and decreased to 30% at 500 bar for *E. dominicanus* (Fig. 7c). The time to perform this element increased up to 48% at 400 bar for *Aristerostoma* sp. (Fig. 8c) and up to 55% at 250 bar for *P. persalinus* (Fig. 8f). For all three ciliates, this element was not recorded during control treatments.

In pressure treatments, the only cilia movement (ocm) element was recorded only for *E. dominicanus* and *P. persalinus*. For *Euplotes* individuals, the time spent to perform this element increased up to 40% at 250 bar and decreased to 20% at 500 bar (Fig. 7c). *Pseudocohnilembus* individuals performed this element only at 300 bar and it was recorded for 45% of the total observation time (Fig. 8f). Only *Euplotes* individuals performed this element in control treatments and it was recorded for 20% of the total observation time (Fig. 8c).

The increase of no movement (nm) element was frequently observed from 150 bar up to maximum pressures for all three ciliates. The time for no movement increased for *Euplotes* individuals from 10% at 150 bar to 40% at 500 bar (Fig. 7c). At maximum pressure, individuals of *Aristerostoma* sp. and *P. persalinus* performed this element for 100% of the observation time (Fig. 8c, f). In control treatments, this element was not recorded for all three ciliates.

Backward motion (bm) was performed only by *Euplotes* individuals (Fig. 7c). This element was observed at up to 200 bar and the time used to perform this element slightly decreased to 0.2%. In control treatments, this ciliate performed a bm element with no significant changes in the course of the experimental time.

Walking (w) element was specific only for *Euplotes* individuals because they posses bundles of cilia (cirri) with which the cell walks on solid surfaces. In control treatment, it was recorded for 4% of the total observation time (Fig. 7c). During all pressure steps, *Euplotes* individuals did not perfome this element.

Discussion

All three deep-sea ciliates in this study survived exposure to high hydrostatic pressures. Individuals of *Euplotes dominicanus* stayed active at a pressure of up to 500 bar and individuals of *Aristerostoma* sp. up to 400 bar. For both ciliates, the pressure at which they survived resembles the pressure present at their isolation depth. In contrast, *Pseudocohnilembus persalinus* survived only up to 300 bar, which corresponds to a depth of about 3000 m which is lower than the depth of its isolation (4000 m). The experiments were hampered from the necessity to rear the different deep-sea isolates at atmospheric pressure until experiments could be carried out. Ciliate cultures were stored at atmospheric pressure from 4 to 6 months prior to the pressure experiments. Thus, ciliates might have been already accommodated to atmospheric pressure conditions and/or there was a selection against specimen adapted to higher hydrostatic pressures (Schoenle et al. 2017). However, our deep-sea isolates showed much better accommodation to higher pressures (> 200 bar) in comparison to isolates of ciliates from surface waters such as *Aspidisca* sp. which survived only up to 150 bar and where cells were destroyed after release from the pressure (data not shown). All three deep-sea isolates displayed normal behaviour at least up to 200 bar. To our knowledge, this was the first direct observation of the behaviour of ciliates from deep-sea environments under high pressure. Pasulka et al. (2019) could recently document grazing activity of a deep-sea ciliate community collected from a hydrothermal vent at 2000 m depth. After releasing ciliates from pressure during sampling, they found ciliates consuming bacteria after re-exposure of the sample to 200 bar.

There is a lack of behavioural studies of ciliates species in general. Up to now, the behaviour of only a few ciliate species belonging to the Spirotrichea, Heterotrichea, Litostomatea and Oligohymenophorea was analysed at normal atmospheric pressure (e.g., Ricci et al 1988, 1995; Ricci 1990; Leonildi et al. 1998). In the present studies, the typical behaviour was described using the two different types of elements, the long and the short lasting elements. The ciliates generally display long lasting elements for their spatial distribution by forming tracks which are combined with different reactions for changing the direction of the movement, the so-called short lasting elements (Leonildi et al. 1998). In our study, all known short lasting elements (continuous trajectory change, smooth trajectory change, rough trajectory change, side-stepping reaction) were recorded for *Euplotes* individuals in pressure treatments up to 200 bar as well as in the control treatments. In comparison with our deep-sea

Euplotes strain, *Euplotes crassus* isolated from surface waters perform continuous trajectory change and leftward arc movements more frequently (Ricci 1990). We could also record similar behavioural elements for *Aristerostoma* and *Pseudocohnilembus*. Most frequently, they performed a rough and smooth trajectory change, elements which were recorded in control and pressure treatments. Similar to *E. dominicanus*, these two ciliates frequently performed rightward arc and linear segment in both treatments. For *Aristerostoma*, no related ciliate species has been investigated regarding its detailed behaviour for comparison yet. *Aristerostoma marinum* can rotate fast around its own longitudinal axis while free-swimming (Dunthorn et al. 2009). Individuals of *Aristerostoma* sp., used in this study, were mainly creeping on the substrate and their behaviour could be described by already known short and long lasting behavioural elements. Among oligohymenophoreans, to which *Pseudocohnilembus* is belonging, two *Tetrahymena* species have been investigated with a similar behaviour (Ricci et al. 1995).

In addition to short and long lasting elements, we introduced the third set of the elements, so-called "other elements". One of those elements, "rotation" was frequently recorded and appeared to be similar to the "maximum rotation reaction" shown for *Euplotes* species (Ricci et al. 1998). We observed additional other elements such as "flickering" and "only cilia movement" during pressure and control treatments, which have not been reported in literature data so far. *Aristerostoma* sp. and *P. persalinus* showed more similar behaviour between each other in comparison to *E. dominicanus*. This may be due to a more similar body geometry of *Aristerostoma* sp. and *P. persalinus.* On the other hand, Bohatová and Vďačný (2018) argued that the behaviour of two phylogenetically distant ciliates with a similar body geometry should be more reflected by evolution rather than cell geometry. However, it has been found that the behaviour might be difficult to describe with general features, even for closely related species (Leonildi et al. 1998). In our experiments, we observed variability in behaviour between individuals of the same species as indicated by the high standard deviations. Ricci (1990) explained these variations by "individuality" of each cell.

On the one hand, we aimed to study the principal behaviour of deep-sea ciliates, and on the other hand, experiments intended to see whether the hydrostatic pressure influences behavioural elements. In our experiments, all three ciliates showed significant changes in their behaviour when exposed to high hydrostatic pressure. The so-called "other elements" dominated at higher pressures. For *E. dominicanus,* short and long lasting elements were not performed above 350 bar which we interpret as a stress response, the elements "flickering" and "no movement" prevailed at 500 bar. For *Aristerostoma* sp. and *P. persalinus*, "only cilia movement" and "rotation" became more prevalent at higher pressures. Frequently, performance of these elements might be caused by the short accommodation time between each pressure increase applied in our pressure experiments. Due to the necessity to compare individuals of a similar physiological stage and due to methodological constraints, behavioural studies had to be carried out within a short time frame which certainly increased the stress for the cells. At least until 200 bar, however, behaviour was still normal. In our experiments, the pressure increase was in the range as that faced by organisms attached to sinking macrophytes (e.g., *Sargassum*, sinking speed about 1000 m/day, see Baker et al. 2018) but it was approx. 10-times faster than pressure increases faced by organisms associated to sedimenting marine snow. Under the latter conditions, organisms might be exposed to a pressure increase of about 10 bar per day which might be easier to tolerate for ciliates. This can also be derived from our survival experiments which lasted several days and where ciliates easily recovered from high hydrostatic pressures.

Several factors can explain the possible changes of the behaviour of ciliates: depolarization of the membrane potential upon contact with specific obstacles or substances, adhesion to the substrate, cell size, individual variation, phase of reproduction and starvation (Ricci 1990). To rule these different factors out, control and pressure incubations were run in parallel with similarly conditioned (on average) individuals and the observed behavioural changes in experimental vessels should have been caused by the stress exerted on the organisms by the increasing hydrostatic pressure.

Beside the changes in behaviour, we observed certain changes in the morphology of all three ciliates. Some of the active cells became more spherical at higher pressures and the normal cell shape was detected after the pressure release. According to Kitching (1954), the protoplasm spreads back to the ciliate pellicle after pressure release, usually within a few minutes, and over a period of many hours, the wrinkled and expanded pellicle slowly reorganises to its normal shape and size. Individuals of *Aristerostoma* sp. and *P. persalinus* built cysts at 430 bar in survival experiments. Cysts production might play an important role for the survival in the deep sea (Atkins et al. 1998). In our experiments, ciliates were able to hatch from the cysts and retrieve their activity upon returning to lower pressures.

The pressure and temperature were described to affect the functioning of biological membranes (Pond et al. 2014). 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). The organisms increase the proportion of unsaturated fatty acids in the membrane phospholipids as the response to both increasing pressure

and/or decreasing temperature (DeLong and Yayanos 1985). However, these mechanisms still need to be investigated for ciliates.

Survival ability under deep-sea conditions was also recorded for different deep-sea and surface isolated heterotrophic flagellates. At higher pressures, better survival (higher growth rate) was observed for deep-sea isolates of *Rhynchomonas nasuta* and *Caecitellus parvulus* than their surface counterparts (Atkins et al. 1998). According to Morgan-Smith et al. (2013), some isolates of *Cafeteria roenbergensis* and *Neobodo designis* from surface waters were able to survive after exposure to 500 bar at 2° C and even positive growth rates were recorded under these conditions. Although they had a high mortality rate initially, in all cases a small portion of the population remained and was able to reproduce once favourable temperature and pressure conditions returned. Turley et al. (1988) found a barophilic (better growth at high pressure) bodonid flagellate isolated from 4500 m depth, which grew only at 450 bar and 2 °C, indicating an adaptation to deep-sea conditions. Also, a *Cercomonas*-like species isolated from the deep sea only grew at pressures of ≥ 300 bar (Turley and Carstens 1991). These data and the data regarding the successful survival and normal behaviour of deep-sea ciliates under deep-sea conditions within this study might point to potentially active protistan deep-sea communities (Živaljić et al. 2018; Gooday et al. 2020 in revision).

In conclusion, our experiments revealed that all three ciliate strains used in this study survived changes in hydrostatic pressure and therefore should be considered as being barotolerant. To our knowledge, barophily—a higher growth rate at high hydrostatic pressure compared to growth rates at low hydrostatic pressure—was not yet recorded for ciliates. One of the reasons for this might be the difficulty to maintain the original pressure for a large volume of sample for long periods and to monitor the behaviour of ciliates at the same time. In our long-term survival experiments, we could show that all three ciliates isolated from the deep sea were able to survive the pressure exposure up to 430 bar, and were able to recover their activity after returning back to atmospheric pressure. The exposure to pressure had a significant impact on the behaviour of all three ciliates; however, the typical behavioural elements were observed at least up to a pressure of 200 bar, which corresponds to 2000 m depth. These findings indicate that all three ciliates might be active in the deep sea. Ciliates are not only important components of oceanic surface waters (e.g., Worden et al. 2015) but should play also an important though up to now underestimated role in deep-sea microbial food webs (Gooday et al. 2020 in revision).

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Author contributions AS, MH, AS, HA and SŽ were involved in the sampling and cultivation of the ciliates. SŽ conducted the pressure and the survival experiments and performed all statistical analyses. AS, FN and SŽ performed the phylogenetic analysis. SŽ and HA wrote the manuscript, and HA supervised the studies. All authors reviewed the manuscript.

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Data availability All data generated during and/or analysed during this study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of organisms were followed.

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Conclusive Summary and Perspectives

The overall results of this thesis indicate that protists can survive and be active at high hydrostatic pressures. The barotolerance has been shown for several heterotrophic flagellates and ciliates isolated from the deep sea and surface waters. Also, a better adaptation to high pressure had been observed for deep-sea isolates in comparison to isolates from surface waters. The first ciliate isolated from the deep sea (>4000 m) was successfully cultivated and described as a new species belonging to the genus *Euplotes*. For the first time, the behaviour of deep-sea ciliates and heterotrophic flagellates was directly observed under high hydrostatic pressures. For ciliates, the normal behaviour was recorded up to 200 bar, which corresponded to a depth of 2000 m. Summarizing all of this data, ciliates and heterotrophic flagellates might be an active part of deep-sea microbial communities and might play an important role in deep-sea microbial food webs.

We summarized the available knowledge on pressure tolerance of deep-sea and surface heterotrophic flagellates (HF; e.g., Turley and Carstens, 1991; Atkins et al., 1998) and added a significant number of additional experiments on strains belonging to a very wide range of different taxonomic groups surviving hydrostatic pressure up to 670 bar (Chapter 1). Many of these different species survived drastic changes in hydrostatic pressure, indicating their barotolerance. 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). 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. Surface isolates of several strains belonging to bicosoecids, chrysomonads, kinetoplastids, and choanoflagellates 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). 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). A clear barophilic behaviour – 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. We suggested that survival (and sometimes growth) of HF recorded at high hydrostatic pressures, which were equal or higher than pressures present at the depth of their depth of isolation, might indicate that some isolates of HF have really originated from vital deep-sea populations and were not contaminants from surface waters during sampling.

The high hydrostatic pressure effect on behavioural motion of three newly described euglenid strains (*Keelungia nitschei* HFCC166, *Petalomonas acorensis* HFCC220, *Ploeotia costaversata* HFCC1508) was analysed (Chapter 2). To our knowledge, *K. nitschei* is the first euglenid which had been cultivated from the deep sea (5000 m). *Petalomonas acorensis* and *Ploeotia costaversata* were isolated from surface waters. This was the first time that the behaviour of heterotrophic flagellates could be directly observed at high hydrostatic pressures. However, there are several pressure systems allowing direct microscopic observation which have been used to study pressure effects on different organisms and cells types (e.g. ciliates *Discophrya piriformis*, *B. undulans* and *P. caudatum*, *Spirogyra* algae, bacteria *Escherichia coli*, HeLa cells (human cancer cell line), the planktonic crustacean *Bosmina longirostris*, eggs of the sea urchin *Arbacia punctulata*; see Marsland, 1950; Kitching, 1954; Salmon and Ellis, 1975; Koyama et al., 2001; Frey et al., 2006; Bao et al., 2010; Nishiyama and Kojima, 2012). Experiments under deep-sea conditions (low temperature and high pressure) showed that the deep-sea strain *K. nitschei* was the only strain still moving at high hydrostatic pressures up to 500 bar at 4°C and the velocity and displacement of *K. nitschei* under pressure at 4°C was similar to the movement of species kept at atmospheric pressure which served as a control. Surface strains, *P. acorensis* and *P. costaversata*, did not move at high hydrostatic pressures. This indicates the possible activity of *K. nitschei* in the deep-sea environment.

From deep-sea sediment samples we could recover and cultivate a ciliate belonging to the *Euplotes* genus. Based on morphology and molecular data, we described this ciliate as a new species *Euplotes dominicanus* (Chapter 3). The morphological comparison of *E. dominicanus* with seven already described species pointed out on certain similarities and differences regarding their body size, cell shape and in number of adoral membranelles, caudal cirri, dorsolateral kineties and dikinetids in mid-dorsolateral row. However, a quite high morphological similarity was recorded with *E. parabalteatus* and *E. dominicanus*, since many of their basic characteristics are overlapping. Relying only on the morphological description without a molecular analysis, it might be impossible to discriminate between these two species. Based on the phylogenetic analyses of 18S rRNA sequences, *E. dominicanus* is most closely related to *E. curdsi*, with a sequence similarity of 97.6%. Also, both species are able to handle the lower salinities. Apart from marine environments, the presence of *E. curdsi* was also reported from brackish waters. *Euplotes dominicanus* was able to survive in medium with low salinity (5 PSU) for several days. However, the long-term effect of the reduction of the salinity on the survival of this species was not studied. A common characteristic of all *Euplotes* species is their isolation from surface water habitats. Up to our knowledge, *E. dominicanus* is the only species belonging to this genus which has been isolated from the deep sea. We could confirm that sequences being 100% identical to the V9 region of the 18S rRNA of *E. dominicanus* were present in six out of 12 deep-sea basins in the Atlantic and in the Pacific Ocean. A 100% identity regarding the V9 region does not mean that sequences belong to exactly the same species, however, our study might indicate that *Euplotes* species at least very similar to *E. dominicanus* are not seldom and might be a component of deep-sea microbial communities. In contrast, we did not find sequences being 100% identical to the V9 region of the 18S rRNA of *E. dominicanus* in the database of surface-water samples from the Tara Ocean project. Also, we compared the similarity of the V9 region of *E. dominicanus* sequence with all other *Euplotes* sequences available in GenBank. Our results show that *E. dominicanus* has a unique and specific V9 region, only found in this species of all currently available sequences from species within the genus *Euplotes*. In our pressure experiments, individuals of *E. dominicanus* were able to survive exposure to atmospheric pressure as well as to high hydrostatic pressures up to 500 bar. Neither in these pressure

experiments nor in cultures grown at atmospheric pressure, we could observe any formation of cysts. This is in contrast to observations on *Pseudocohnilembus persalinus* which showed a stimulated cyst production when exposed to high hydrostatic pressure (Schoenle et al., 2017). Kitching (1957) investigated the effects of pressure on behaviour and survival of an unidentified *Euplotes* species isolated from freshwater. He showed that moderate pressures (69-207 bar) caused the increase of the locomotion activity of ciliates and high pressure (>600 bar) depresses ciliary movement. While some individuals failed to recover on release from a pressure of 689 bar, others instantly resume swimming after a few seconds returning to their normal behaviour. These results clearly support our observation on the potential ability of *Euplotes* species to survive exposure to high hydrostatic pressure and therefore indicate their possible activity in deep-sea ecosystems.

Besides the survival, the behaviour on the high hydrostatic pressures of the newly described *Euplotes* species and two additional deep-sea ciliates, *Aristerostoma* sp. and *Pseudocohnilembus persalinus,* were analysed in more detail (Chapter 4). Prior to behavioural experiments, the survival ability on high pressure was checked for *Aristerostoma* sp. and *P. persalinus.* Individuals of *Aristerostoma* sp. stayed active up to a pressure of 400 bar and this pressure resembles the pressure present at their isolation depth. *P. persalinus* survived only up to 300 bar, which corresponds to a depth of about 3000 m which is lower than the depth of its isolation (4000 m). Before conducting the pressure experiments, our ciliate cultures were stored at atmospheric pressure from four to six months. Thus, it might have been that they already adapted to atmospheric pressure conditions and/or there was a selection against specimen adapted to higher hydrostatic pressures (Schoenle et al., 2017). Our behavioural experiments showed that all three deep-sea isolates displayed normal behaviour at least up to 200 bar. To our knowledge, this was the first direct observation of the behaviour of ciliates from deep-sea environments under high pressure. Up to now, the behaviour of only a few ciliate species belonging to the Spirotrichea, Heterotrichea, Litostomatea and Oligohymenophorea were analysed at normal atmospheric pressure (e.g. Ricci et al., 1988, 1995; Ricci, 1990; Leonildi et al., 1998). The behaviour was described using the two different types of elements, the long and the short

lasting elements. In our study, all known short lasting elements (continuous trajectory change, smooth trajectory change, rough trajectory change, side-stepping reaction) were recorded for *Euplotes* individuals in pressure treatments up to 200 bar and during control treatments. Similar behavioural elements we could also record for *Aristerostoma* and *Pseudocohnilembus*. Most frequently, they performed a rough and smooth trajectory change element which were recorded in control and pressure treatments. Similar to *E. dominicanus*, these two ciliates frequently performed two long lasting elements, rightward arc and linear segment, in both treatments. In addition to short and long lasting elements, we introduced the third set of the elements, so-called "other elements". One of those elements, "rotation", was frequently recorded and appeared to be similar to the "maximum rotation reaction" shown for *Euplotes* species (Ricci et al., 1998). We observed additional other elements such as "flickering" and "only cilia movement" during pressure and control treatments, which were not recorded in literature data so far. For *Aristerostoma*, no related ciliate species has been investigated regarding its detailed behaviour for comparison yet. In comparison with our deep-sea *Euplotes* strain, surface isolated ciliate *Euplotes crassus* perform more frequently continuous trajectory change and leftward arc movements (Ricci, 1990). Among Oligohymenophoreans, to which *Pseudocohnilembus* is belonging, the behaviour of two *Tetrahymena* species have been investigated so far (Ricci et al., 1995). In our experiments, *Aristerostoma* sp. and *P. persalinus* showed more similar behaviour between each other in comparison to *E. dominicanus*. This may be due to a more similar body geometry of *Aristerostoma* sp. and *P. persalinus*. On the other hand, Bohatová and Vďačný (2018) argued that the behaviour of two phylogenetically distant ciliates with a similar body geometry should be more reflected by evolution rather than cell geometry. It seems that the behaviour is difficult to describe with general features, even for closely related species (Leonildi et al., 1998). In our experiments, we observed variability in behaviour between individuals of the same species as indicated by the high standard deviations. Ricci (1990) explained these variations by "individuality" of each cell. Exposure to the high hydrostatic pressures caused significant changes in the normal behaviour of all three ciliates species. The "other elements" dominated at higher pressures. For *E. dominicanus*, short and lasting elements were not performed above 350 bar which we interpret as a stress response, elements "flickering" and "no movement"

prevailed at 500 bar. Also, for *Aristerostoma* sp. and *P. persalinus* "only cilia movement" and "rotation" became more prevalent at higher pressures. Frequently performance of these elements might be caused by the short adaptation time between each pressure increase applied in our pressure experiments. Due to the necessity to compare individuals of a similar physiological stage and due to methodological constraints, behavioural studies had to be carried out within a short time frame which certainly increased the stress for the cells which reflected on their normal behaviour. From the literature it is known that several factors can cause the possible changes of the behaviour of ciliates: depolarization of the membrane potential upon contact with specific obstacles or substances, adhesion to the substrate, cell size, individual variation, phase of reproduction and starvation (Ricci, 1990). To rule these different factors out, control and pressure incubations were run in parallel with similarly conditioned (on average) individuals and the observed behavioural changes in experimental vessels should have been caused by the stress exerted on the organisms by the increasing hydrostatic pressure. Beside the changes in behaviour, we observed certain changes in the morphology of all three ciliates. Some of the active cells became more spherical at higher pressures and the normal cell shape was detected after the pressure release. According to Kitching (1954), the protoplasm spreads back to the ciliate pellicle after pressure release, usually within a few minutes, and over a period of many hours, the wrinkled and expanded pellicle slowly reorganizes to its normal shape and size. Individuals of *Aristerostoma* sp. and *P. persalinus* built cysts at 430 bar in survival experiments and they were able to hatch and retrieve their activity upon returning to lower pressures. For *Euplotes* individuals, cysts formation has not been observed. Cysts production might play an important role for the survival in the deep sea (Atkins et al., 1998). Also, an increase of fluidity of membranes by incorporation of unsaturated fatty acids plays a role in the survival at high hydrostatic pressures at least for the bacteria (DeLong and Yayanos, 1985; Allen et al., 1999). However, the mechanisms, which can be used to prevail the high pressures, still need to be investigated for protists.

In conclusion, the results of this thesis may give new insights on protist activity in extreme habitats such as the deep sea. Also, these results may contribute to the current understanding of

the functioning of deep-sea microbial food webs by showing that two trophic levels, heterotrophic flagellates and ciliates, are potentially active in the deep sea. More investigations of deep-sea sediments to obtain more living cultures of deep-sea protists and more ecological experiments with regard to the reproduction and feeding habits of protists at high hydrostatic pressure still need to be done. This will help to get a more comprehensive knowledge on the role of protists in deep-sea ecosystems.

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Records of Achievement and Subpublications

[1] **Živaljić S., Schoenle A., Nitsche F., Hohlfeld M., Piechocki J., Reif F., Shumo M., Weiss A., Werner J., Witt M., Voss J., Arndt H.,** 2018. 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

Die Konzeption, die statistischen Analysen, ein wichtiger Teil der Literaturarbeit und der wesentliche Teil des Textes wurden von mir durchgeführt bzw. erarbeitet (insgesamt etwa 60%).

[2] **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 acorensis, Ploeotia costaversata*). European Journal of Protistology. 69, 102–116

Die Probenahme und die Kultivierung von Organismus, die Druckversuche und die statistischen Analysen, die Überarbeitung des Manuskriptes in allen Phasen des Publikationsprozesses wurden von mir durchgeführt bzw. erarbeitet (insgesamt etwa 25%).

[3] **Živaljić S., Scherwass A., Schoenle A., Hohlfeld M., Quintela-Alonso P., Nitsche F., Arndt H.,** 2020. A barotolerant ciliate isolated from the abyssal deep sea of the North Atlantic: *Euplotes dominicanus* sp. n. (Ciliophora, Euplotia). European Journal of Protistology. 73, 125664

Die Konzeption, die Probenahme und die Kultivierung von Organismus, die Färbemethoden, die Druckversuche, die statistischen Analysen und die phylogenetische Analysen, ein wichtiger Teil der Literaturarbeit und der wesentliche Teil des Textes wurden von mir durchgeführt bzw. erarbeitet (insgesamt etwa 80%).

[4] **<u>Živaljić, S.</u>, Schoenle, A., Scherwass, A., Hohlfeld, M., Nitsche, F., Arndt, H.,** 2020. Influence of hydrostatic pressure on the behaviour of three ciliate species isolated from the deep-sea floor. Marine Biology. 167, 63

Die Konzeption, die Probenahme und die Kultivierung von Organismus, die Druckversuche, die statistischen Analysen und die phylogenetischen Analysen, ein wichtiger Teil der Literaturarbeit und der wesentliche Teil des Textes wurden von mir durchgeführt bzw. erarbeitet (insgesamt etwa 80%).

Erklärung gemäß § 4 Absatz 1 Punkt 9 der Prüfungsordnung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten 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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Suzana Živaljić