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

Suzana Živaljić[1] · Alexandra Schoenle[1] · Anja Scherwass[1] · Manon Hohlfeld[1] · Frank Nitsche[1] · Hartmut Arndt[1]

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).

✉ Hartmut Arndt
  hartmut.arndt@uni-koeln.de

1 Institute for Zoology, General Ecology, University of Cologne, Zuelpicher Str. 47b, 50674 Cologne, Germany

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
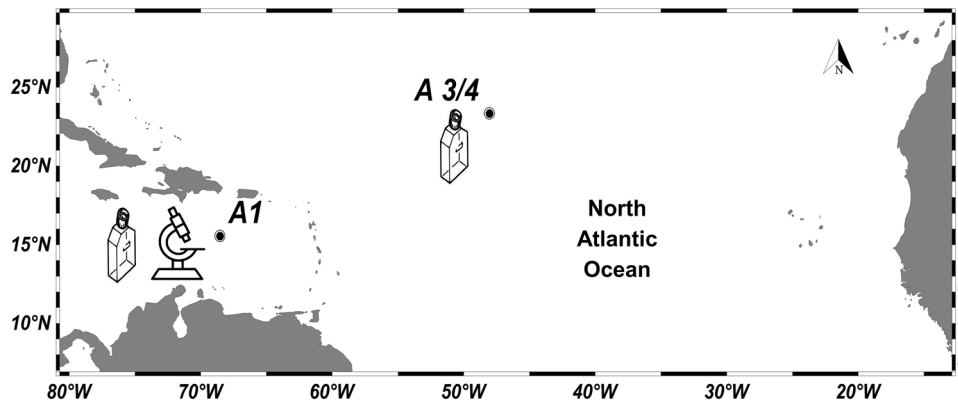
specimens were further cultivated in 50-mL tissue-culture flasks (Sarstedt, Nümbrecht, Germany) filled with 30 mL of autoclaved 35 PSU Schmaltz-Pratt medium (a litre contained 28.15 g NaCl, 0.67 g KCl, 5.51 g $MgCl_2 \times 6H_2O$, 6.92 g $MgSO_4 \times 7H_2O$, 1.45 g $CaCl_2 \times 2H_2O$, 0.10 g KNO_3, 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$_2$O), 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$_2$O) 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$_2$O, 25 μL of Mastermix (VWR Red Taq DNA Polymerase Master Mix by VWR Chemicals, USA), 10 μL ddH$_2$O 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 $GTR + I + \Gamma$ 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 $20 \times LD$ 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 $p < 0.001$.
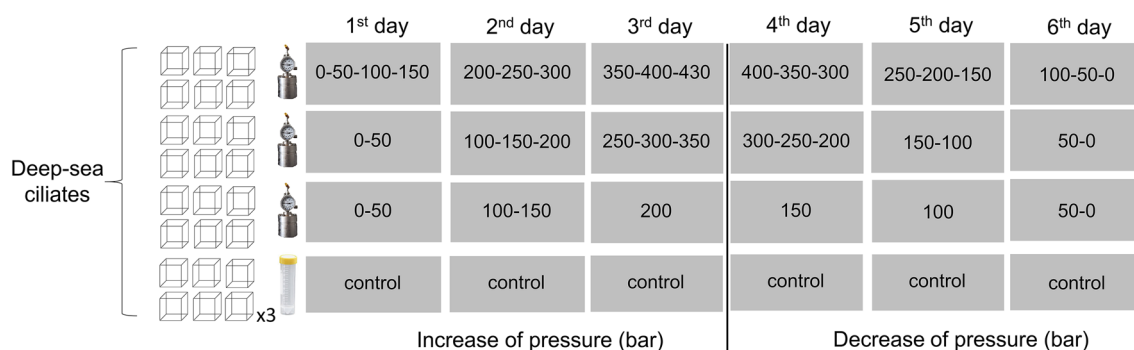

Deep-sea ciliates	1st day	2nd day	3rd day	4th day	5th day	6th day
	0-50-100-150	200-250-300	350-400-430	400-350-300	250-200-150	100-50-0
	0-50	100-150-200	250-300-350	300-250-200	150-100	50-0
	0-50	100-150	200	150	100	50-0
	control	control	control	control	control	control

Increase of pressure (bar) — Decrease of pressure (bar)

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 $20 \times$ 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
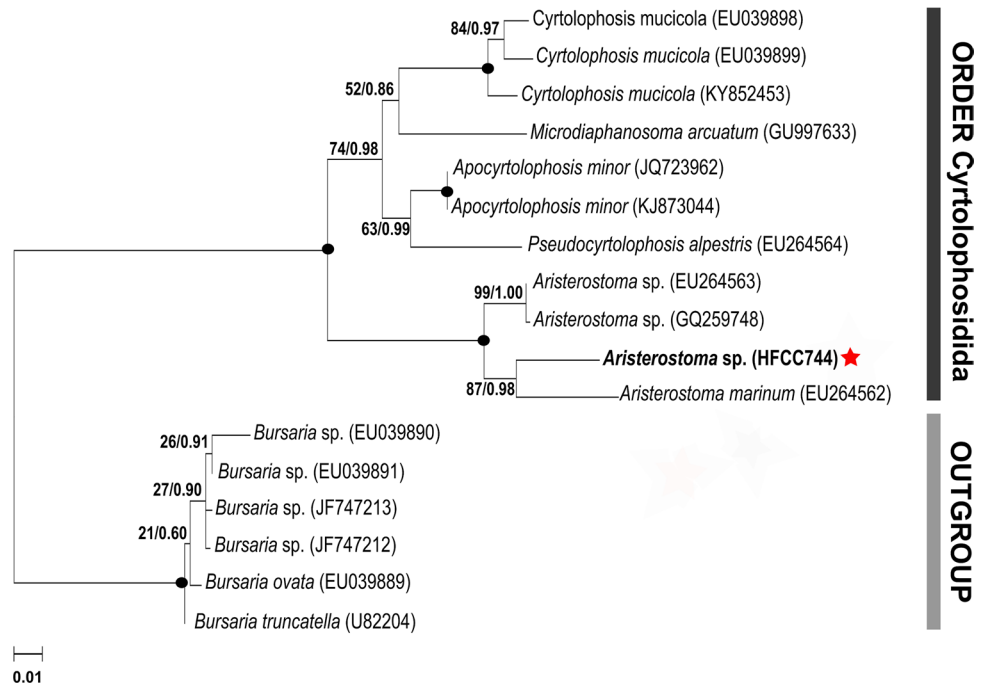
Fig. 3 Maximum likelihood (ml) phylogenetic tree of small subunit (SSU) rDNA of the order Cyrtolophosidida. New sequence is in bold marked with red star. Numerical support values are given at the respective nodes as: maximum likelihood (ml) bootstrap percentages (RaxML, 1000 replicates)/ Bayesian posterior 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
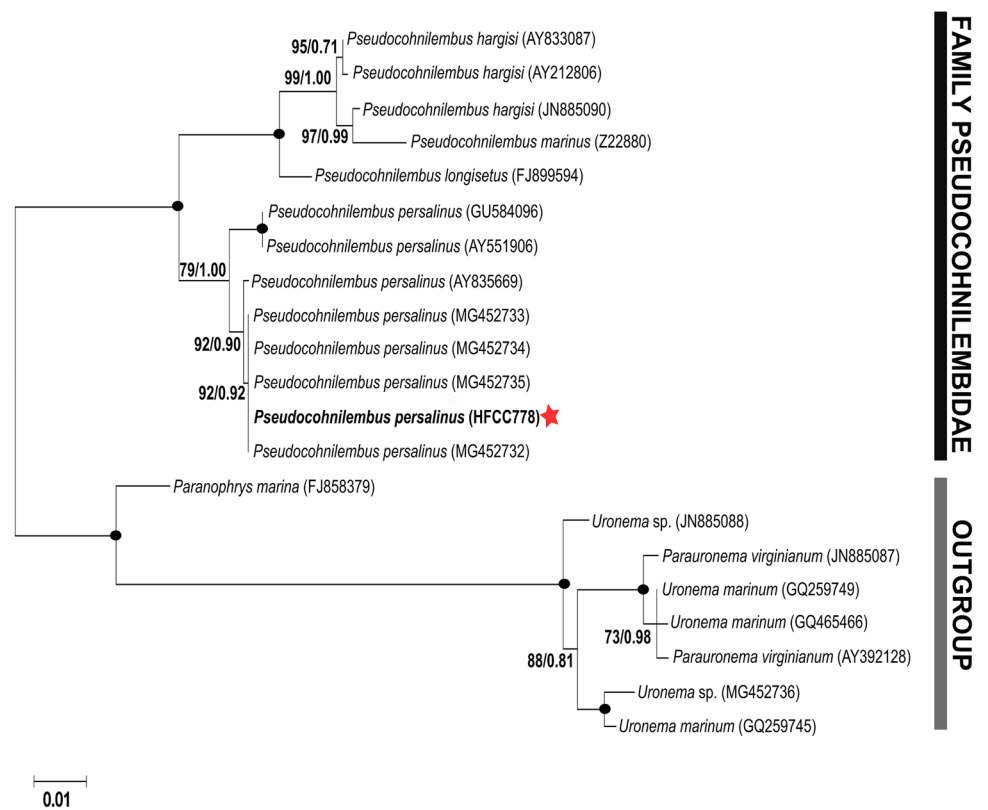
(Fig. 4). A comparison between them revealed no p-distance. These five *P. persalinus* sequences clustered closely with another *P. persalinus* (AY835669) having a p-distance of 0.2% and high branch support (Fig. 4; mlBP 92%, BiPP 0.90).

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*
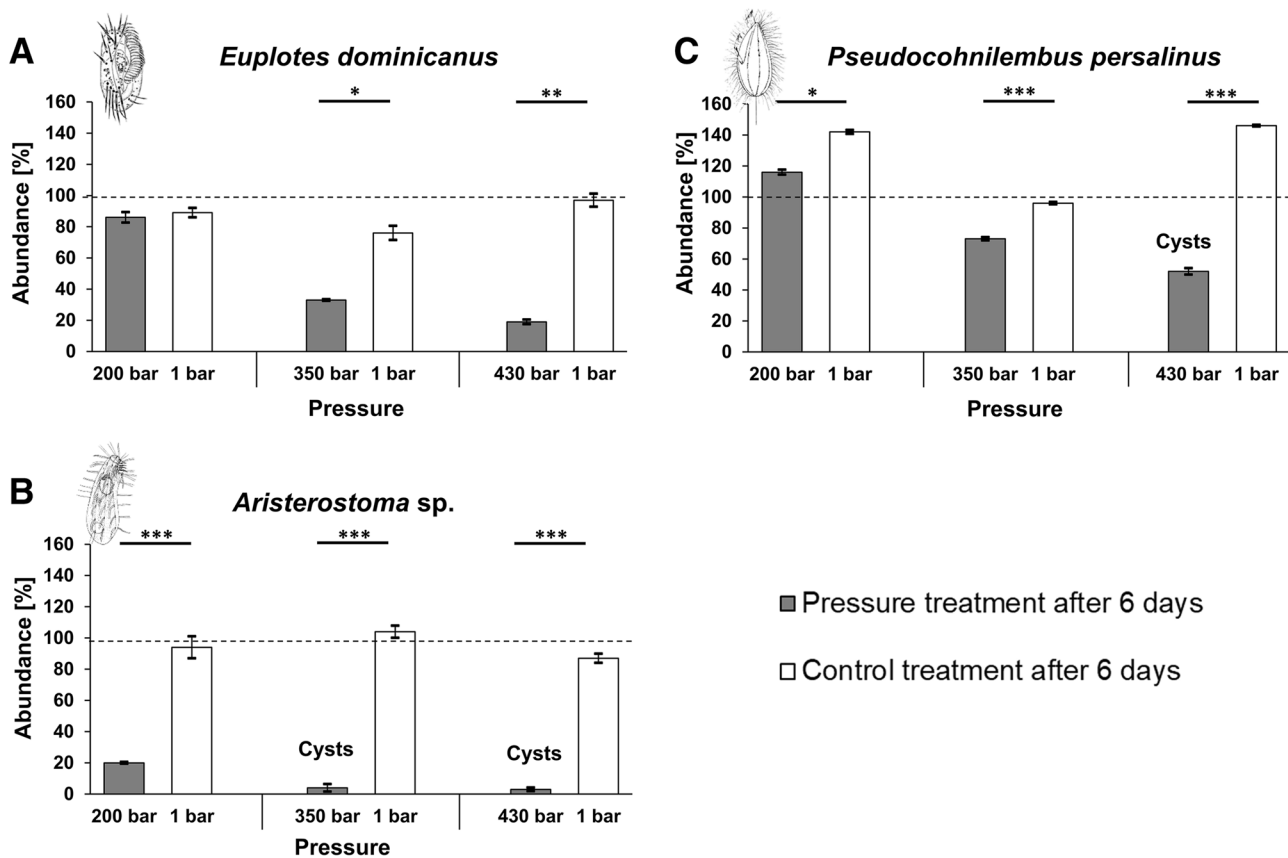

Fig. 5 **a–c** Mean abundance (%) of *Euplotes dominicanus* (**a**), *Aristerostoma* sp., (**b**) and *Pseudocohnilembus persalinus* (**c**) strains after 6 days exposed to different pressures (200 bar, 350 bar and 430 bar) and control treatment (1 bar) relative to the start abundances (set to 100%, 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
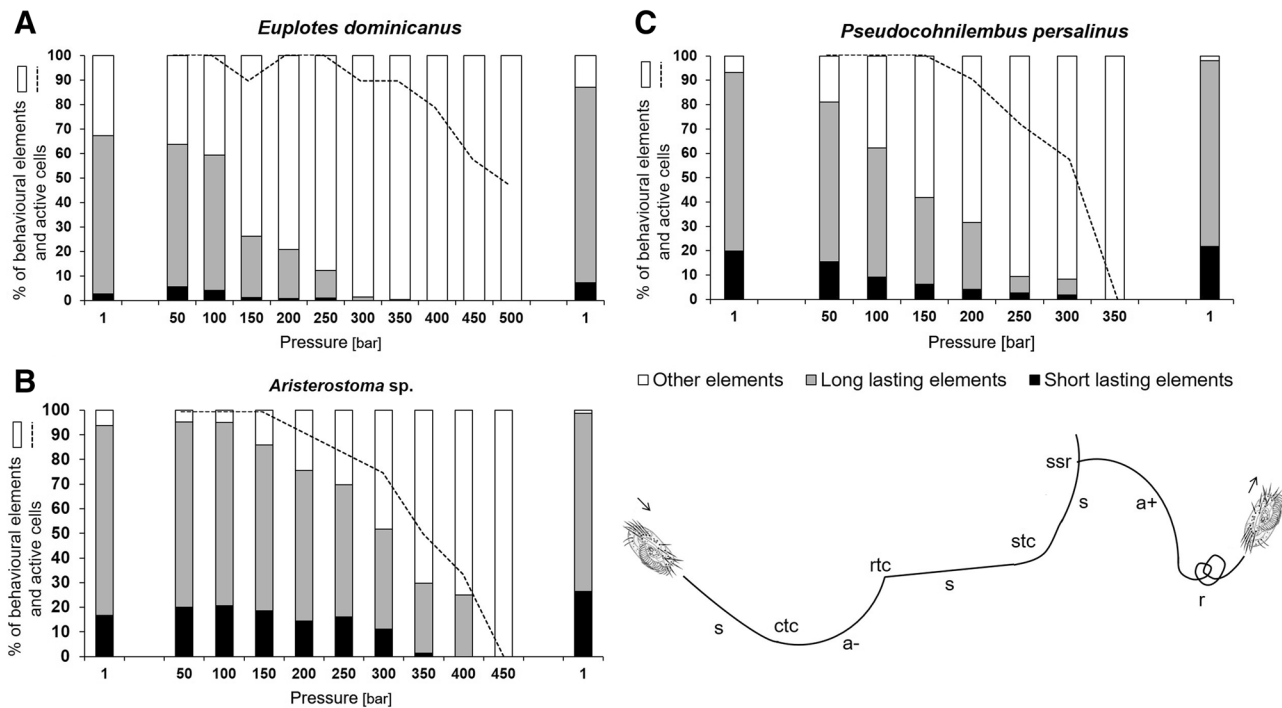

Fig. 6 a–c The percentage of behavioural elements and active cells recorded for total observation time for three deep-sea ciliates *Euplotes dominicanus* (**a**), *Aristerostoma* sp. (**b**), and *Pseudocohnilembus persalinus* (**c**) during pressure and control treatments (1 bar) (Temp. = 4 °C, $n = 10$–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
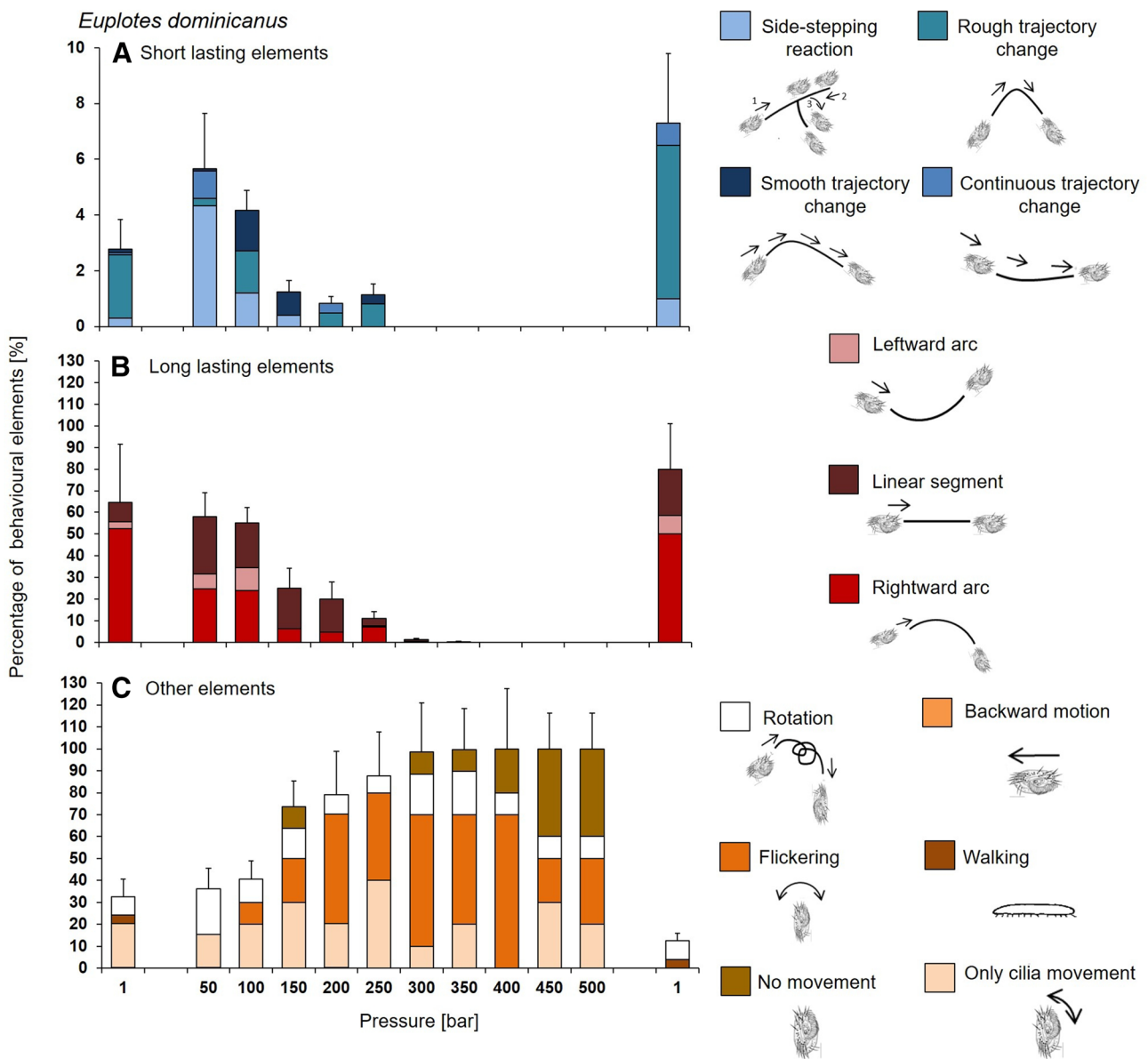
Fig. 7 **a–c** The percentage contribution of different types of short (**a**), long lasting (**b**) and other (**c**) behavioural elements performed during total observation time for *Euplotes dominicanus* in pressure and control (1 bar) treatments. The first and the last columns in all three graphs 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
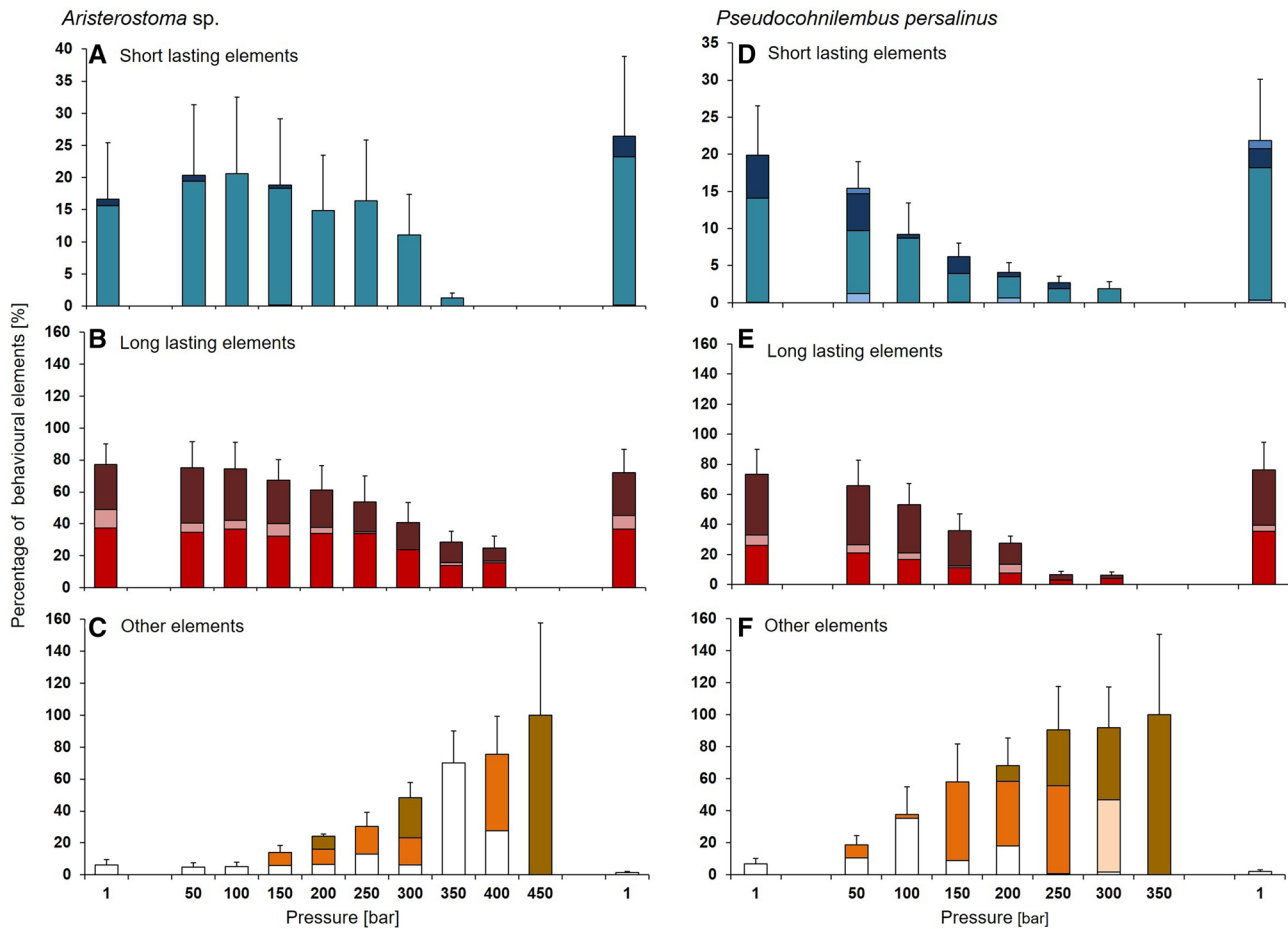
Fig. 8 a–f The percentage contribution of different types of short (**a**, **d**), long lasting (**b**, **e**) and other (**c**, **f**) behavioural elements performed during total observation time for *Aristerostoma* sp. (**a–c**; *n* = 12) and *Pseudocohnilembus persalinus* (**d–f**; *n* = 10) in pressure 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 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).

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.

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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