Permanent ectoplasmic structures in deep-sea *Cibicides/oides* taxa – long-term observations at in situ pressure

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Abstract. Deep-sea *Cibicidoides pachyderma* (forma *mundulus*) and related *Cibicidoides* spp. were cultured at in situ pressure for 1-2 days, or 6 weeks to 3 months. During that period, fluorescence analyses following BCECF-AM (2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester) or Calcein (Bis[N,N-bis(carboxymethyl)aminomethyl]-fluorescein) labelling, revealed a persisting cytoplasmic sheet or envelope surrounding the *Cibicidoides* tests. Thus, the *Cibicidoides* shell can be considered rather as an internal than an external cell structure. A couple of days to a week after being transferred into high-pressure aquaria and adjusted to a pressure of 115 bar, the foraminifera changed from a mobile to a more or less sessile living mode. During this quasi sessile way of life, a series of comparably thick static ectoplasmic structures developed that were not resorbed or remodelled but, except for occasional further growth, remained unchanged throughout the experiments. Three different types of these 'permanent structures' were observed: A) Ectoplasmic 'roots' were common in adult *C. pachyderma, C. lobatulus* and *C. wuellerstorfi* specimens. In our experiments single ectoplasmic 'roots' grew to maximum 700 times the individuals shell diameter and were presumably used to anchor the specimen in an environment with strong currents. B) Ectoplasmic 'trees' describe rigid ectoplasmic structures directed into the aquarium's water body and were used by the foraminifera to climb up and down these ectoplasmic structures. Ectoplasmic 'trees' were so far only observed in *C. pachyderma* and enabled the 'tree'-forming foraminifera to elevate itself above ground. C) Ectoplasmic 'twigs' were used

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to guide and hold the more delicate pseudopodial network when distributed into prevailing currents, and were, in our experiments, also only developed in *C. pachyderma* specimens. Relocation of a specimen usually required to tear apart and leave behind the rigid ectoplasmic structures, eventually also the envelope surrounding the test. Apparently, these rigid structures could not be resorbed or reused.

1 Introduction

Our knowledge on form and functioning of ectoplasmic extensions in benthic foraminifera is based on laboratory observations of a few shallow-water species under atmospheric pressure. In 1835 Felix Dujardin published a series of short papers where he not only noticed that the investigated animals were no micro-cephalopods but also that these animals interacted with filaments, which he called rhizopoda, with the environment why he proposed the name Rhizopoda for the group (Dujardin, 1835a, b, c, d). Subsequent studies describe complex networks of branching and anastomosing pseudopodia that are rapidly and alternately extended and withdrawn into the surrounding environment (Bowser and Travis, 2002; Hedley, 1964; Lee and Anderson, 1991; Lee, 1985; Schultze, 1854). The almost continuously remodelling pseudopodia are used for motility, attachment, food collection, the formation of cysts, growth and certain aspects of reproduction (Goldstein, 1999; Heinz, 2005; Travis, 2002; Tyszka et al., 2019).

Numerous cytoplasmic particles give the pseudopodia a granular appearance when viewed under the light microscope (Goldstein, 1999; Hedley, 1964; Schultze, 1854). The main components of granule are mitochondria, (secretory, excretory, and storage) vesicles or vacuoles, and occasionally symbionts (Bowser and Travis, 2002; Goldstein, 1999; Hedley, 1964; Lee, 1985). Independently of whether pseudopodia modify their shape or are in a stationary state, they display constant bidirectional streaming (Bowser and Travis, 2002; Rinaldi, 1964). Coupled to this cytoplasmic streaming, particles are transported bidirectional along the extracellular surfaces of pseudopodia (Bowser, 1985, 1984a). Foraminifera use this extracellular conveyor belt to collect particles for agglutination or nutrition (Bowser and Travis, 2002).

The majority of foraminifera of the genus *Cibicides* (e.g. *C. refulgens, C. antarcticus*) and a significant proportion of *Cibicidoides* species (e.g. *C. lobatulus, C. wuellerstorfi,* and *C. pachyderma* with the morphotypes *C. pachyderma, C. kullenbergi* and *C. mundulus*, see (Schweizer, 2009) for the genetic versus morphological classification) are epibenthic

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(Jorissen et al., 1995; Linke and Lutze, 1993; Lutze, 1989; Nyholm, 1962) although Rose Bengal-stained specimens are occasionally found at 1-4 cm sediment depth (e.g. (Hunt and Corliss, 1993; Wollenburg and Mackensen, 1998b). However, an affinity of *Cibicides/-oides* species to settle in places exposed to currents has been inferred from the preferential colonization of elevated structures exposed to currents or on filter feeding invertebrates (e.g. (Alexander, 1987; Linke and Lutze, 1993; Schönfeld, 2002). Although facultative grazing on phytodetritus and bacteria on the sediment is proposed for some species such as *C. antarctica* (Alexander and DeLaca, 1987), the majority of *Cibicides/-oides* species are assumed to be passive suspension feeders (Lipps, 1983) trapping phytodetritus by deployment of a pseudopodial network in the prevailing current.

Main target of this study was *C. pachyderma*, of which we continuously observed 57 specimens under *in situ* pressure, temperature, and current activity conditions over a time span of 3 months. Daily observations allowed us to shed light on the development of temporary and lasting ectoplasmic extensions in *C. pachyderma*, one of the most important species for palaeo-reconstructions of the deep sea.

To determine if the observed ectoplasmic structures are unique to *C. pachyderma* or common to the related genera *Cibicides* and *Cibicidoides*, 40 *C. lobatulus* and 3 *C. wuellerstorfi* specimens were cultured at corresponding conditions and visually inspected daily to weekly for a time period of 6 weeks. To prove that shells were covered by living cytoplasm, in addition, fluorescence studies on the ectoplasmic envelope of *C. lobatulus* were carried out for 1-3 days.

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2 Methods and Material

Central to this study are more or less daily observations on permanent ectoplasmic structures in 57 *C. pachyderma* specimens that were cultured for 3 months during the 'experiment (1)' of 2017. In 2018, we complimented this data set by daily to weekly observations on permanent ectoplasmic structures in 40 *C. lobatulus* and 3 *C. wuellerstorfi* specimens cultured for 6 weeks using the same set-up and experimental design as for *C. pachyderma* (Tab. 1). In 2019 fluorescence studies on the ectoplasmic envelope of *C. lobatulus* were carried out for 1-3 days.

hat gelöscht: (Wollenburg et al., 2018).

High-pressure culturing with small aquaria, like we have used during these experiments, require to keep a stock of foraminifera at atmospheric pressure for some weeks or months in advance. The decision in favour of *Cibicidoides pachyderma* and *C. lobatulus* species was made as both species live from the shelf to water depths >1000 m and can, thus, be cultured at

atmospheric conditions until they are used in high-pressure experiments. Although it has been shown that barophil *C. wuellerstorfi* is able to survive depressurisation for weeks and can reproduce when subsequently been cultured at *in situ* pressure (Wollenburg et al., 2015), so far there is no proof that the cell functioning is not altered under such conditions.

During the RV Polarstern expedition PS101 in 2016, pebbles from surface sediments were collected with a multicorer (MUC) at 79°27.09′N, 7°30.93′E, 856 m water depth and used as stock for the *Cibicidoides pachyderma* experiment (Wollenburg et al., 2018). During the RV G.O. Sars expedition GS2018108 (Juli -August 2018) pebbles with attached living *C. lobatulus* and *C. wuellerstorfi* specimens were collected at 900 m water depth on the Norwegian continental slope (68° 00' N, 15° 00' E). Pebbles of both expeditions were transferred in large lid-covered petri dishes and used as stock cultures for all observations (see Wollenburg et al., 2018 for handling of the stock cultures). From these stock pebbles, specimens with strong cytoplasm staining were detached with a cactus-spine under a stereomicroscope, temporarily stored in small (ø 3 cm) seawater-filled petri dishes in the cold laboratory, and then transferred into the high-pressure aquaria.

Species	***C. pachyderma***	***C. lobatulus***	***C. wuellerstorfi***
Specimen number	57	40	3
Pressure (bar)	115 ± 1	115 ± 1	115 ± 1
pH	8	8	8
O_2 (mmol/L)	340–396	340–396	340–396
Tp (°C)	2.5 ± 0.2	2.5 ± 0.2	2.5 ± 0.2
Pumping rate (mL/min)	0.3 (1st month)	0.3 (week 1-3)	0.3 (week 1-3)
	0.6 (month 2-3)	0.6 (week 4-6)	0.6 (week 4-6)
Feeding (Chlorella/Spirulina)	0.005 mg weekly	0.005 mg weekly	0.005 mg weekly
Sediment	partly*	yes	yes
Observations	daily	irregular	irregular

Table 1. Basic parameters of the culture experiments. Oxygen and pH values were measured with a combined O_2 and pH measuring device (WTW Multi 3620 IDS) and respective O_2 (WTW FDO®925) and pH (SenTix®980) sensors, three times per week. Fine-grained siliceous oxide (1–5 µm) was used as artificial sediment in one out of four aquaria in the *C. pachyderma* (*), and in all aquaria of the *C. lobatulus/C. wuellerstorfi* culture experiments.

High-pressure culturing observations on *C. pachyderma* were performed from February to May 2017 (Wollenburg et al., 2018), observations on *C. lobatulus* and *C. wuellerstorfi* from August to October 2018, and confocal microscope investigations from October to December 2020.

For this study, a total of 200 L sterile-filtered (0.2 µm mesh) North Sea water was adjusted to a salinity of ~35, by addition of 1 g Hobby Marine sea salt per L and psu-offset, and to a pH of 8.0 under atmospheric pressure. The normal culture seawater (160 L) was tagged with Calcein (4,5-Bis((N,N-bis(carboxymethy)amino)methyl)fluorescein) (200 mg/L) to allow for identification of newly precipitated calcite (Wollenburg et al., 2018). To observe ectoplasmic structures under fluorescence light (excitation wavelength of 470 nm, emission wavelength >490 nm) required to rinse the aquaria with unlabelled seawater from the remaining sterile-filtered batch of 40 L. This was done every 2–3 weeks for two days. Tagged and non-tagged seawater was stored in multiple 10-L Schott glass bottles with Bola-connections in a cold room and refrigerator running at 2.5°C. A high-pressure pump (ProStar218 Agilent Technologies) was used to supply a continuous one-way isobaric and isocratic seawater flow through the serially arranged aquaria running at an experimental pressure of 115 bar. Weekly, with a second high-pressure pump, 0.005 mg of dried *Chlorella* and *Spirulina* algae dispersed in seawater were pumped in each individual aquarium containing foraminifera (Wollenburg et al., 2018).

Cibicidoides specimens and the development of momentary and durable ectoplasmic extensions were observed under a Zeiss Axio Zoom V16 microscope and pictures were taken with an Axiocam 506 colour camera.

In 2019, 1 to 3 day-lasting high-pressure (100 bar) fluorescence measurements with *C. lobatulus* were performed. For these investigations, *C. lobatulus* specimens from the 2018 stock were transferred in a ~10 mL aquarium with windows on both sides and installed in a portable cooling table running at 1.5°C. A volume of 0.6 mL/min of non-labelled culturing water was directed through the high-pressure aquarium. For examination, a Confocal- Leica TCS SP5 II equipped with a HCX PL Fluotar

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objective (10x/0.30) and an argon laser (λex = 488 nm) was used. Fluorescence emission was measured at 494 - 504 nm. The assessment and evaluation of the images were done with the software LAS AF Lite (Leica Camera AG). A stock solution of BCECF-AM (2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester) in DMSO (1 mg/mL in dimethylsulfoxid) was mixed and stored at -20 °C. Prior to the staining procedure, control observations were made to check for foraminiferal autofluorescence. At used microscope settings there was no autofluorescence of *C. lobatulus* specimens prior to staining. For incubation, the selected specimens were transferred into a petri dish with 2 mL seawater and exposed to 5 µmol/LBCECF-AM. The incubation medium was then gently stirred with a small brush to distribute the dye evenly. The petri dish was covered and stored at 4 °C for 19 hours (incubation time). The properties of BCECF-AM allow to conduct a non-terminal life-dead screening procedure (Bernhard et al., 1995). The nonfluorescent membrane permeable BCECF-AM enters an organism and has to be converted to fluorescent BCECF via intracellular hydrolases, thus, the cell has to be alive to exhibit fluorescence. After incubation, specimens were transferred into the high-pressure aquaria and gradually adjusted to a pressure of 100 bar over a period of 6 hours. The observations were conducted right after the aimed pressure was reached, after 24 hours, and after 48 hours. The settings from the control measurement were used to record the fluorescence activity in the cytoplasm of the *C. lobatulus* specimens. As the *Cibicidoides* test proved to be too thick to be penetrated by the argon laser, only ectoplasmic features could be investigated with the confocal microscope.

3 Results

As the refraction index of foraminiferal cytoplasm approximates that of water, pseudopodia and other cytoplasmic extensions are usually observed with inverted microscopes once they are in contact to or close to the thin glass bottom of the observational dishes (e.g. (Bowser, 2002; Cedhagen and Frimanson, 2002; Röttger, 1982; Travis, 2002). High-pressure culturing requires a thick glass and a certain interior aquarium height, in our case both measuring 4 mm. In these aquaria thin pseudopodia could only be observed occasionally when a specimen positioned itself or the respective ectoplasmic structure close to the aquarium's window. Therefore, our results do not comprise a comprehensive documentation of the fine branched parts of the pseudopodial network but essentially of the thicker ectoplasmic structures.

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153 **3.1 Shell envelope**

154 At all times, all *Cibicidoides* tests were covered by a thin to thick continuous layer of ectoplasm (envelope) making the shell
155 an internal rather than an external cellular structure (Figs. 1-2). The shell envelopes showed numerous granules, and in this
156 respect resembled the appearance of pseudopodia (Fig. 1a-d). Although at an extremely low speed (significantly less than <10
157 µm per 10 min), the envelope-inherent granules gradually changed their position over time. A coherent ectoplasmic structure
158 of the shell envelope is corroborated by BCECF-AM staining / confocal microscope analyses (Fig. 1e1-e2). Extension of
159 pseudopodia from the shell envelope became apparent when algae adhered to these filaments during feeding (Fig. 1c-d),
160 whereas hours to days after feeding a significant portion of the fed algae were found covering parts of the shell envelope. We
161 assume that the shell envelope initiates the formation of the agglutinated cyst that covers *Cibicidoides* tests during shell
162 precipitation/growth or in waters of low pH (De Nooijer et al., 2009; Wollenburg et al., 2018). Similarly, a pure algae-half
163 cyst formed during a period of 6 weeks on the spiral side of an adult *C. lobatulus* (Fig. 2a-b). Figure 2a shows a bright shell
164 envelope covering the umbilical side of the specimen and the algae cyst with ectoplasmic contributions on the spiral side. After
165 6 weeks, the half cyst was shed but still showed parts of what we assume to be ectoplasmic remains (Fig. 2b). Occasionally (n
166 = 2) also abandoned ectoplasmic envelopes were observed, supporting the idea that the cytoplasmic envelope serves as matrix
167 for the cyst formation (Figs. 2c-d).
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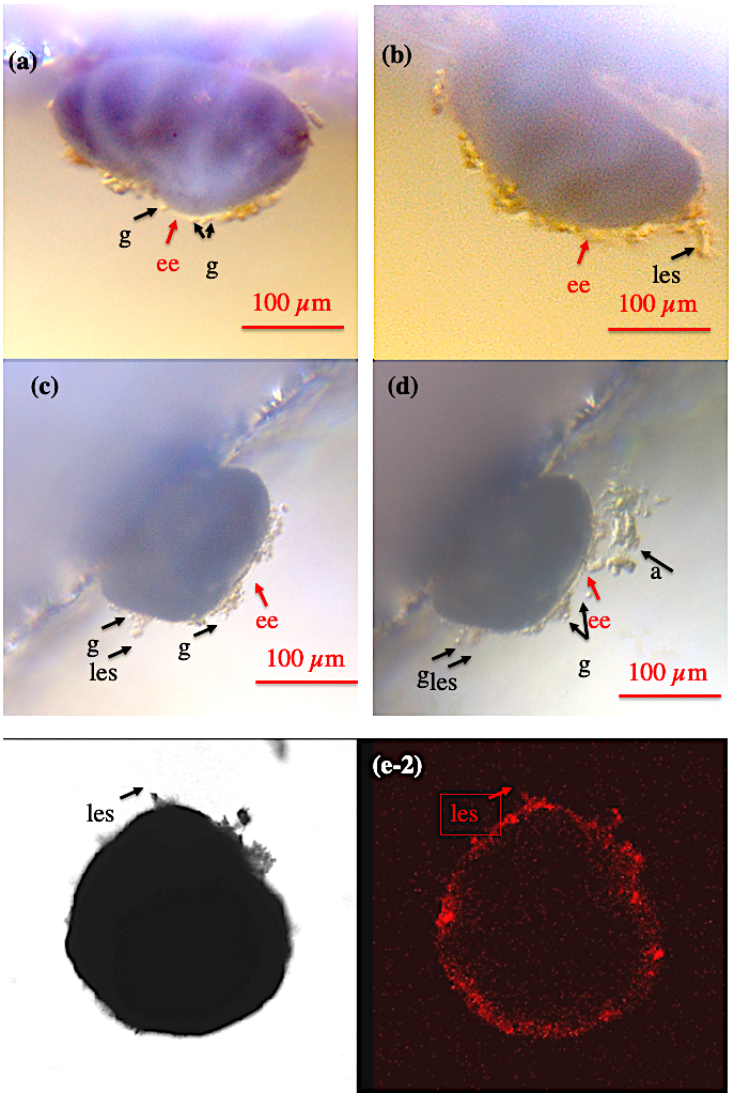
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Figure 1. Shell envelope I. (a-b) Shell envelope (ee) of a *Cibicidoides pachyderma* specimens revealing multiple granule (g) and initial static ectoplasmic structures (les). (c-d) Shell envelope of a *C. pachyderma* specimen 24-hours before (c) and during feeding (d). During feeding multiple mobile granule and attached algae (a) indicate a pseudopodial network presumably originating in the shell envelope. (e-1-e-2) BCECF-AM incubated *C. lobatulus* specimen viewed under normal transmitted light (e-1) and laser excitation exhibiting the BCECF-AM fluorescence (e-2). As *C. lobatulus* specimens possess a thick shell, only the shell envelope, an initial lasting ectoplasmic structure (les), and especially granule reveal bright red fluorescence.

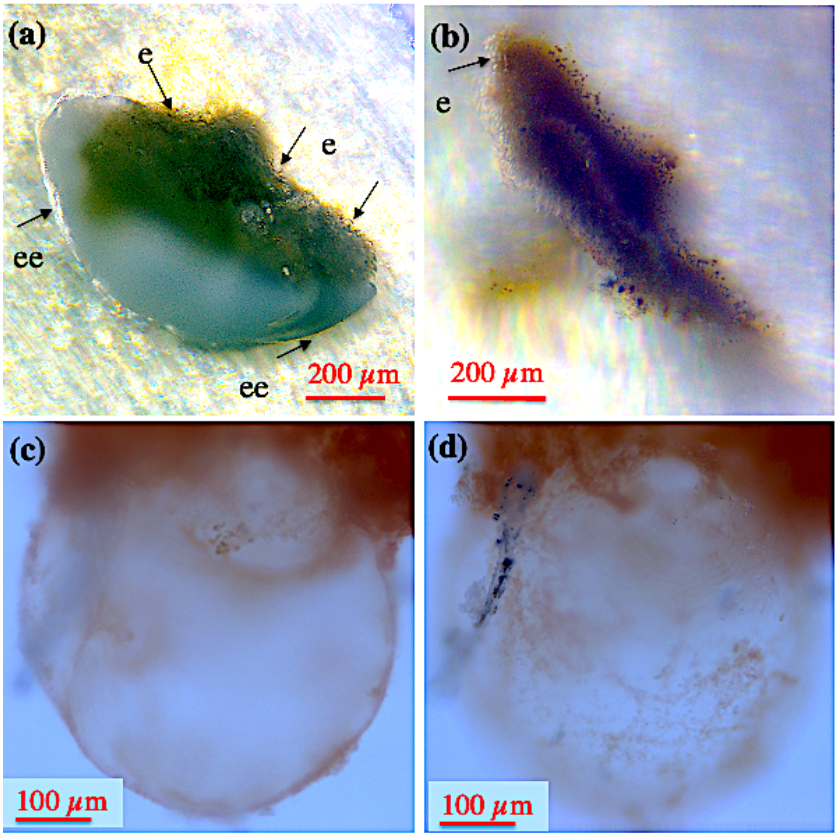

Figure 2. Shell envelope II: (a) Shell envelope apparent on the umbilical side of an adult *C. lobatulus* specimen, whereas an algae half-cyst was formed over a period of six weeks over the spiral side. (b) The half cyst 1-2 days after it has been abandoned (cyst was shed during the weekend). (c-d) Abandoned shell envelope of a *C. pachyderma* specimen retrieved after the termination of the *Cibicidoides pachyderma* experiment. (c) and (d) show the same cyst but different focussing. ee= ectoplasmic envelope, e = remains of ectoplasm.

3.2 Static ectoplasmic structures

Within 24 hours after transfer into the aquaria and adjustment to a pressure of 115 bar, the first type of thick static ectoplasmic structures, ectoplasmic 'roots', appeared in about 50% of juvenile and most adult specimens (Figs. 3-9). In 68 out of 100 specimens ectoplasmic 'roots' were observed. In an unknown proportion of the rest (32 specimens), such structures might have existed but due to the large working distance and/or a less optimal observational position of the specimens in the aquarium not noticed. Juvenile *Cibicidoides* specimens were more mobile than adults (Wollenburg et al., 2018) and likely therefore, the formation of ectoplasmic 'roots' was often delayed. Three days and two weeks after transfer, first ectoplasmic 'twigs' and 'trees', respectively, were formed directing into the water column. All static ectoplasmic structures may have shown continued growth but otherwise changed little over the 3 months of observation. In one case braided ectoplasmic 'roots' even persisted after the termination of the experiment when the two involved specimens were rinsed in deionized water and dried (Fig. 5g). We never observed that these structures were in whole or in part resorbed.

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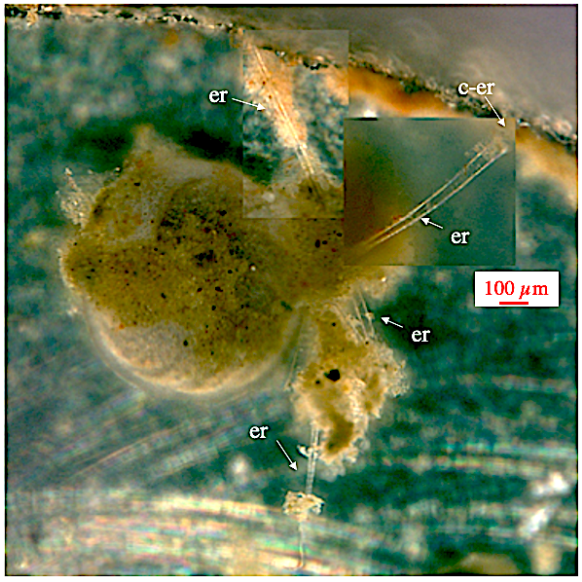
3.2.1 Ectoplasmic 'roots'

The most frequent static ectoplasmic structures were 'root-like', extending along the bottom or adhering to the window of the aquarium (Figs. 3-5). Where the ectoplasmic 'root' came close to the aquarium glass, thereby reducing the distance to the microscope objective, pseudopodia and bidirectional streaming on the outside of the respective ectoplasmic 'root' could be observed (Fig. 4). Ectoplasmic 'roots' were attached to the aquarium glass via thickened endings (Figs. 3-4). The typical ectoplasmic 'root' had a mean thickness of roughly 30 μm and often two 'roots' were twisted to form thicker braid-like structures (Fig. 5). Presumably limited by the dimension of our aquaria, a maximum root length of roughly 5 mm was observed (Figs. 4-5). Over the course of the experiments, the number of ectoplasmic 'roots' increased and some showed ongoing growth (Fig. 4). Figure 5a shows a twisted ectoplasmic 'root' with a total length of 400 μm on the left and a shorter straight 'root' of approx. 100 μm on the right side of *C. pachyderma* specimen 1 (Sp. 1). Both structures had formed in the course of a night. During the following day, Sp. 1 flipped over so that the test periphery was facing the aquarium floor, and moved to the filter ring. There the smaller single ectoplasmic 'root' continued to grow and branch (Fig. 5b-c). Finally, this ectoplasmic 'root' of Sp. 1 combined with the ectoplasmic 'root' of a neighbouring specimen (Sp. 2) and formed a single braid-like ectoplasmic

208 ‘root’ (Fig. 5d). For the remaining 2 months, the two individuals moved along this braided ‘root’ like on rails and positioned
209 themselves sometimes closer to, sometimes further away from each other. Hereby, specimen 2 remained under the filter ring
210 for most of the time.



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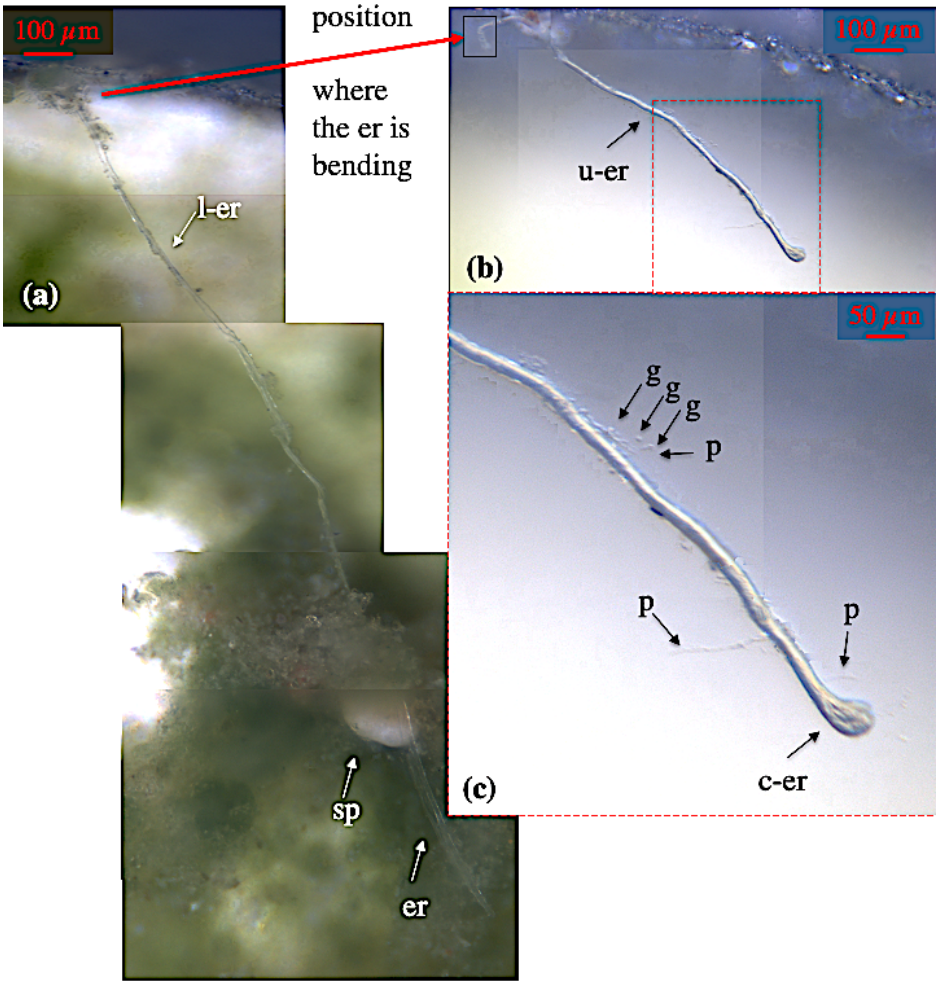
212 **Figure. 3.** Ectoplasmic ‘roots’ of *C. wuellerstorfi*. Starting with the lower ones, ectoplasmic ‘roots’ were developed over a
213 period of 1 week and remained unchanged for the remaining 5 weeks of the experiment. er= ectoplasmic ‘root’, c-er= contact
214 zone of ectoplasmic ‘root’ with the aquarium glass.



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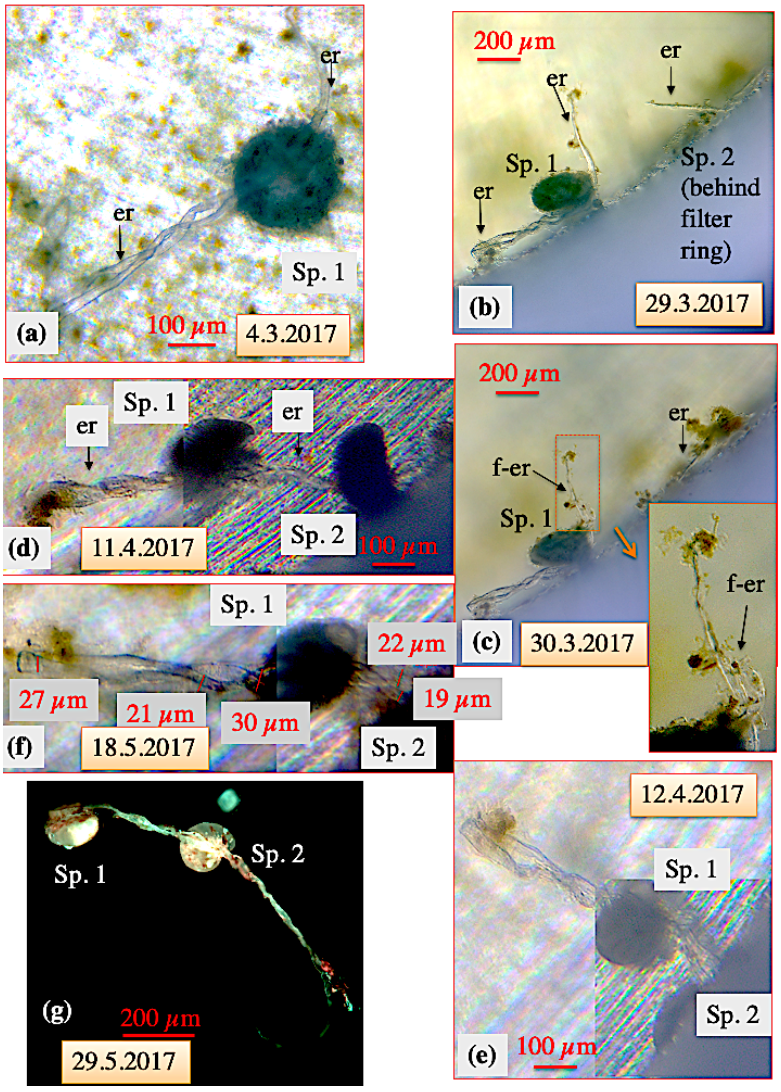
216 **Figure. 4.** Ectoplasmic 'roots' of *C. lobatulus*. (a) *Cibidoides lobatulus* specimen (sp) embedded in algae with two ectoplasmic

217 'roots' (er) extending on the bottom of the aquarium. At one point, the northern ectoplasmic 'root' bends upward at the

aquarium's wall, thus, it is differentiated in a lower (l-er) and an upper (u-er) part. (b) Shows the upper part of the northern ectoplasmic 'root'. (c) Shows the u-er at higher magnification revealing granule (g), pseudopodia (p), and a broad contact zone (c-er) where the ectoplasmic 'root' is attached to the aquarium's window.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19 μm
(f)
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Sp. 2
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Sp. 1
Sp. 2
(e)
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Sp. 1
Sp. 2
(g)
200 μm
29.5.2017

Figure. 5. Ectoplasmic 'roots' of *C. pachyderma* (specimens 1 and 2). (a) Six days after being transferred into the high-pressure aquarium, overnight a twisted ectoplasmic 'root' formed on the left and a short simple 'root' on the right side of the test of specimen 1 (Sp. 1). (b) Thereafter, Sp. 1 moved towards the filter ring, and finally positioned itself close to an ectoplasmic 'root' of specimen 2 (Sp. 2; situated under the filter ring) on March 29. (c) The next day, the right-hand ectoplasmic 'root' of specimen 1 started to fray. (d) Several days later, during a weekend, specimen 2 resurfaced from below the filter ring and its left-hand ectoplasmic 'root' was combined with the frayed right-hand 'root' of specimen 1 to a joined twisted or braided ectoplasmic 'root'. (e) The joined braided ectoplasmic 'root' of specimens 1 and 2 (positioned under the filter ring) on April 12. (f) Thickness measurements of the joined braided ectoplasmic 'root'. (g) Fluorescence picture of the braided ectoplasmic 'root' of Sp. 1 and 2 immediately after termination of the experiment (excitation wavelength 470 nm, emission wavelength 490 nm). The emitted bright greenish Calcein fluorescence of the ectoplasmic 'root' likely indicates recent cytoplasmic activity. er= ectoplasmic 'root', f-er= frayed ectoplasmic 'root'.

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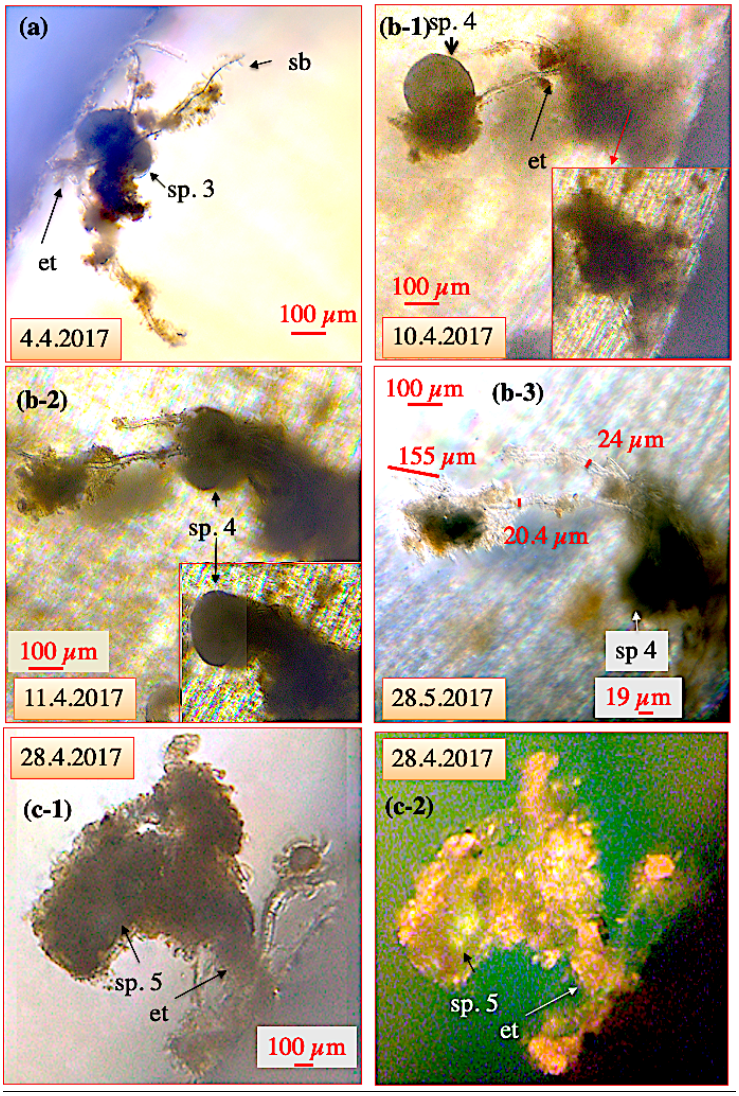
After termination of the experiment, gently washing the specimens over a 30 μm mesh, and drying the residue, both specimens were still attached via the joined braided ectoplasmic 'root' with a final length of at least 5 mm (Fig. 5g).

3.2.2 Ectoplasmic 'trees'

Thick, robust, and permanent ectoplasmic structures, very similar to ectoplasmic 'roots' but extending into the water column, were termed ectoplasmic 'trees'. "Tree"-forming *Cibicidoides pachyderma* specimens could climb up these structures to raise themselves above the bottom. Interestingly, similar structures were not observed in any of the investigated *C. lobatulus* and *C. wuellerstorfi* specimens. Distinct ectoplasmic 'trees' were observed in 6 of the 50 studied *C. pachyderma* specimens, others might have been overlooked as the experimental set-up just allows a vertical view insight the aquarium.

Whereas ectoplasmic 'roots' were eventually formed within 24-hours after transfer into the aquaria, it took about two weeks before the first ectoplasmic 'trees' were formed (Fig. 6). Rather than moving with the foraminifera, as described for the 'roots'of some specimens, ectoplasmic 'trees' were fixed in the aquaria. They reached a maximum height of approx. 2 mm and the foraminifera could climb freely along these tree-like structures (Fig. 6a-c). Regularly spaced short and obviously adhesive side-branches (Fig. 6a), probably with tiny pseudopodia (that are rarely visible in our set-up), collected suspended

algae from the inflow current during feeding. As result ectoplasmic ‘trees’ looked like loosely agglutinated structures, later in the experiment (Figs. 6c-1-2).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28.4.2017
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Figure 6. Ectoplasmic 'trees' of *C. pachyderma*. (a) Ectoplasmic 'tree' of *C. pachyderma* specimen (sp.) 3 with three thick branches originating from a single "stem" fixed to the aquarium wall. *Cibicidoides pachyderma* sp. 3 was positioned approx. 100 µm away from the wall with no contact to the bottom of the aquarium. (b-1-3) Ectoplasmic 'tree' of *C. pachyderma* sp. 4 fixed to the aquarium's bottom and extending at least 2 mm into the water column. (b-1) On April 10, specimen 4 had climbed to the top of the ectoplasmic 'tree'. (b-2) The next day, the specimen had moved to the middle section of the ectoplasmic 'tree'. (b-3) Shows, as an example, specimen 4 at the bottom of the ectoplasmic 'tree' on May 28. Furthermore, thickness measurements on the 'tree' structures are provided. (c-1-2) Ectoplasmic 'tree' of *C. pachyderma* sp. 5. Algae adhering to the adhesive side branches of the ectoplasmic 'tree' obscure the ectoplasmic nature when viewed under normal light (c-1). (c-2) Shows the same ectoplasmic 'tree' under fluorescent light, allowing a better visibility of the 'tree' and the specimen's position. The bright greenish Calcein fluorescence of the cytoplasm illustrates the elevated position of specimen 5 within the accumulated algae.

et= ectoplasmic 'tree', sb= side branches.

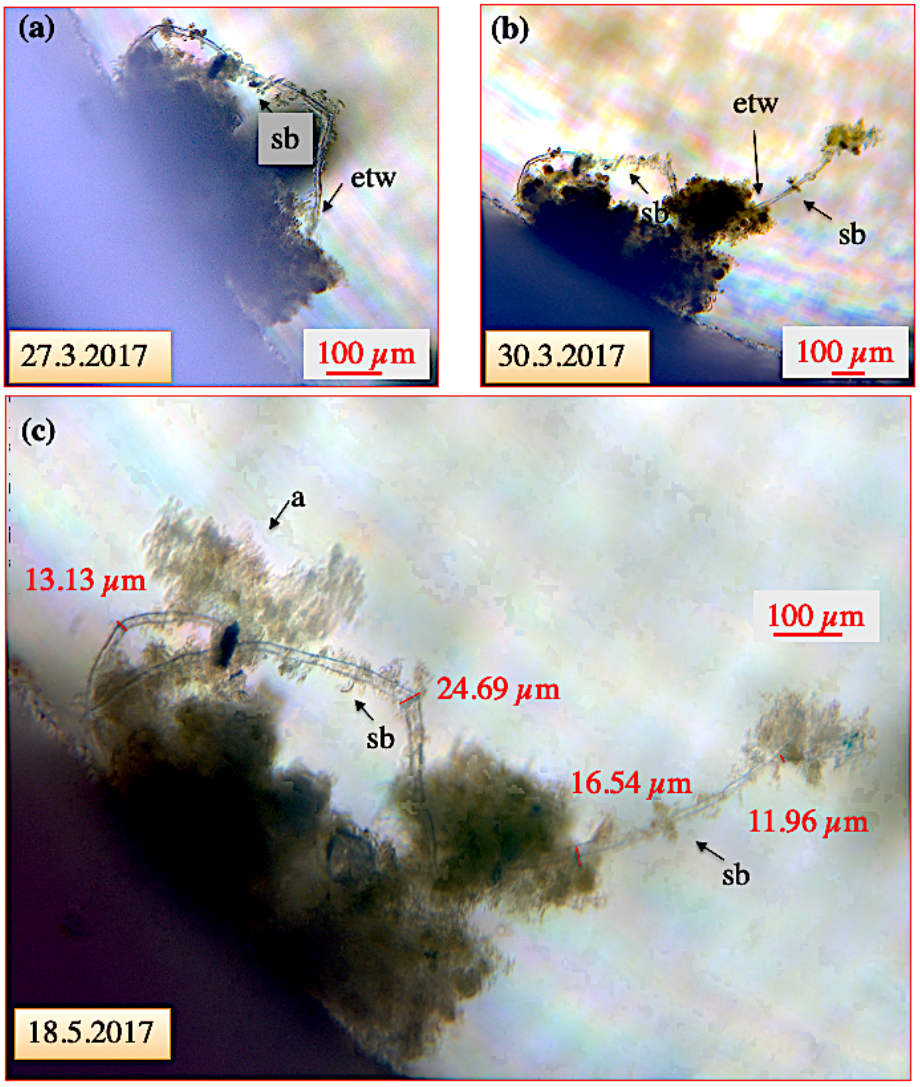
hat gelöscht: Calcein-labelled

3.2.3 Ectoplasmic 'twigs' and pseudopodial network

Thick ectoplasmic structures extending into the water were termed ectoplasmic 'twigs' if the shape and position with respect to the test remained essentially permanent during the experiment (Figs. 7-8). However, ectoplasmic 'twigs' are the least static of the three described ectoplasmic structures and were only observed in *C. pachyderma* specimens so far. Ectoplasmic 'twigs' are directed above the umbilical side into the water column, thus, in our experiments they could only be observed in specimens that had attached themselves on an, in respect to the observation, ideal position on the aquarium's wall. In 16 of the 50 observed *C. pachyderma* specimens ectoplasmic 'twigs' were observed. The first ectoplasmic 'twigs' appeared 3 days after transfer of *C. pachyderma* specimens into the aquaria (Fig. 7a). Additional structures were eventually added over time (Fig. 7a-b), but the original structure was usually not modified (Figs. 7-8). Provided with the same short and obviously adhesive side branches as ectoplasmic 'trees' (Fig. 6), the ectoplasmic 'twigs' probably support a more delicate pseudopodial network (Figs. 7-8). In our experiment, *C. pachyderma* specimens exhibited a strong rheotaxis. In this context it was observed that a specimen had

positioned itself at the hole of the filter ring (where the food entered the aquarium). After this position was occupied the specimen developed a series of crescent-shaped ectoplasmic ‘twigs’ (Fig. 8). From the area in which the ectoplasmic ‘twigs’ were developed, the species directed an anastomosing pseudopodial network into the inflowing water current during feeding (Figs. 8-10). In doing so, the instrumentally visible collection area increased by at least twenty times the specimen’s test size. Hereby, both the pseudopodial network and the respective supportive ectoplasmic ‘twigs’ obviously allowed the animal to collect food from the water current (Figs. 8-10). When we shut down the pumps and, thus, the current activity for some minutes (on May 26, 2017, 25 hours after feeding), the pseudopodial network, visualized by adhering algae, collapsed (Fig. 8b), whereas the ectoplasmic ‘twigs’ kept their original shape (Fig. 8). The shape of the specimen’s ectoplasmic ‘twigs’ was neither affected by the presence or absence of the current nor by the speed of it (~0.1-5 cm/min (Wollenburg et al., 2018)).

For the specimen positioned at the hole in the filter ring, the development and extension of pseudopodia directing into the water current during feeding was immediate (Fig. 10), however, the transport of collected algae towards the shell was extremely slow. Seven hours after feeding, algae were still sticking to the pseudopodia and ectoplasmic ‘twigs’ and no or only low amounts of fresh algae had reached the shell interior (Fig. 10f). Slow food ingestion was also reflected by the extremely slow propagation of anastomoses over time. An anastomosis propagated less than 150 µm within 24 hours (Fig. 10). During and following feeding, the number of granules in the ectoplasmic envelope, the ectoplasmic ‘twigs’, and pseudopodia were significantly increased.


(a)
sb
etw
27.3.2017
100 µm
(b)
etw
sb
sb
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(c)
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13.13 µm
100 µm
24.69 µm
sb
16.54 µm
11.96 µm
sb
18.5.2017

295

Figure. 7. Ectoplasmic 'twigs' of *C. pachyderma* specimen 8. (a) For 3 days, the specimen had gathered algal detritus around its shell envelope and simultaneously developed a loop-like ectoplasmic 'twig' with a total length of ~700 µm from the periphery to the opposite side. (b) Three days later, an ~500 µm-measuring extension directing into the water column was added to the loop-like 'twig'. Both structures persisted for the remaining weeks of the experiment. (c) On May 15, dispersion of algae into the aquarium allowed the specimen to collect additional algae onto the ectoplasmic 'twig'. The algae mass remained in this position and was not ingested during the experiment. etw= ectoplasmic 'twig', a= algae, sb= side branch.

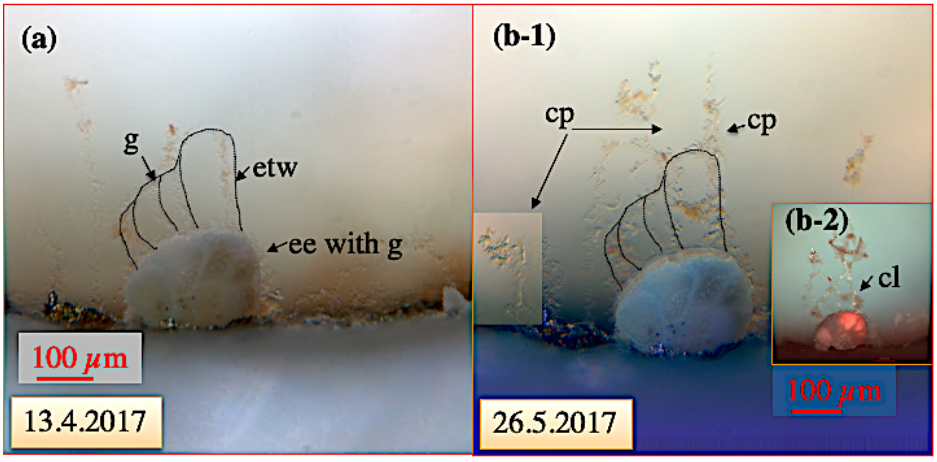

Figure. 8. Crescent-shaped ectoplasmic 'twigs' of *C. pachyderma* specimen 1 positioned at the hole of the sinter ring, i.e. at the inflow of water and algal food into this aquarium. (a) Specimen viewed under normal light when no food was added to the inflow revealing bow-like ectoplasmic 'twigs'. (b) 35 days later, the pumps were stopped[1] to investigate the stability of the ectoplasmic 'twigs' and the pseudopodial network at zero current activity but stable high-pressure conditions. Stable

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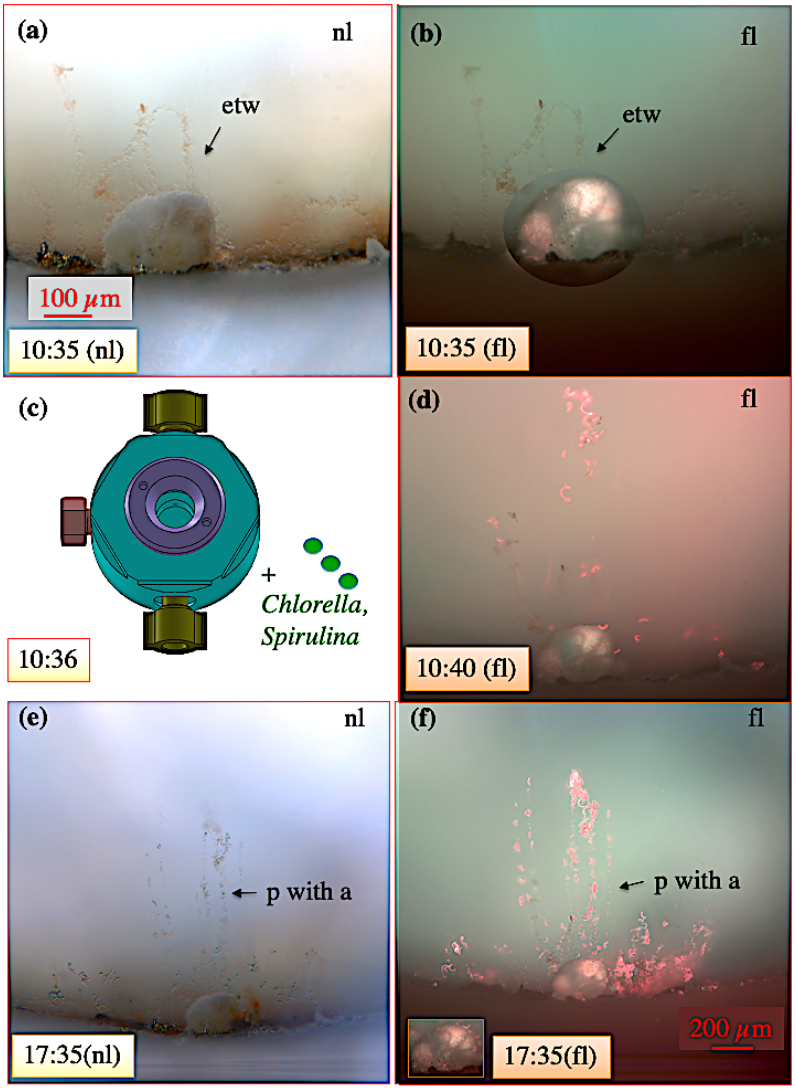
[1] A shut-off valve following downstream the overflow valve prohibited a pressure drop in the high-pressure aquaria when the pumps were shut.

ectoplasmic ‘twigs’ and collapsed pseudopodial (cp) network under normal light (b-1) and fluorescent light (b-2). The red colour of especially older test parts result from ingested *Spirulina* and *Chlorella* algae stored in food vacuoles of the cytoplasm. ee = ectoplasmic envelope, etw= ectoplasmic ‘twig’, g= granule, cl= Calcein-stained cytoplasmic lacuna in the etw and cp.


(a)
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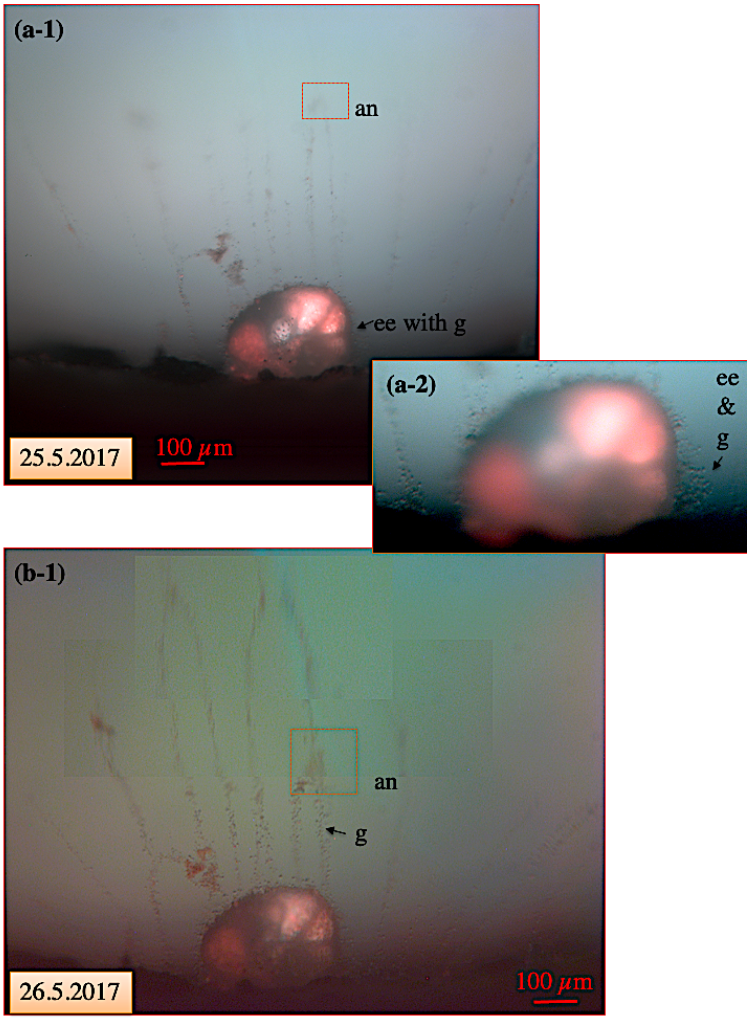
+
Chlorella,
Spirulina
10:36
(d)
fl
10:40 (fl)
(e)
nl
p with a
17:35(nl)
(f)
fl
p with a
200 μm
17:35(fl)

314

Figure. 9. Pseudopodial network of *C. pachyderma* specimen 1 during feeding on April 13 2017.

Specimen 1 before, during, and after feeding with 0.5 µg dried *Spirulina* and *Chlorella* algae. The bright red colour of dispersed algae under fluorescent light provides an excellent tool to document the passage and uptake of algae in the pseudopodia and cytoplasm. (a-b) Specimen 1 prior feeding. (c) Schematic illustration of the aquaria indicating the start of feeding. (d) Specimen 1 during feeding. (e-f) Seven hours after feeding. etw= ectoplasmic 'twig', p= pseudopod, a= algae, nl = normal light, fl = fluorescence light. Numbers state the respective time on April 13.



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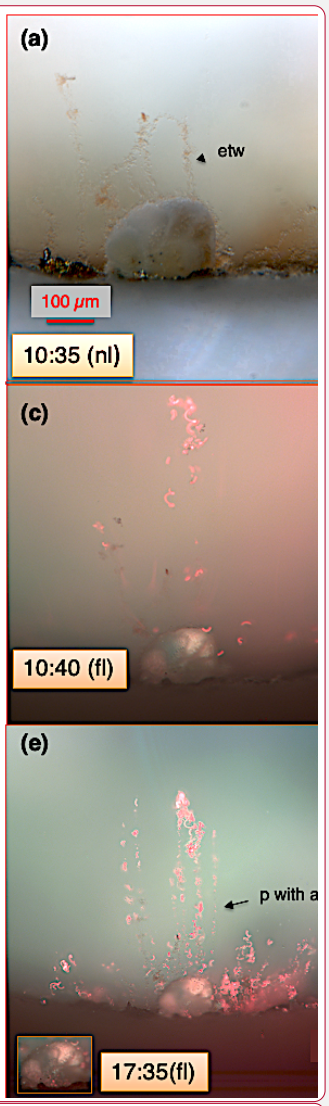

Figure. 10. Pseudopodial network of *C. pachyderma* specimen 1 under fluorescent light on May 25 and 26. Movement of an anastomosis within 24 hours after feeding. (a-1) In course of the experimental running time, a visually increasing amount of algae (intensified red colour of cytoplasm; compare to Fig. 9) had accumulated in the specimen's cytoplasm. A red square indicates the position of a slowly moving anastomosis in the pseudopodial network. (a-2) Shows the test at higher magnification revealing the presence of numerous granules in the ectoplasmic envelope and 'twigs'. (b) 24 hours later, the anastomosis had moved by approximately 150 µm towards the shell. an= anastomosis, ee = ectoplasmic envelope, g= granule.

3.2.4 Torn ectoplasmic remains

When *Cibicidoides* specimens that were virtually sessile for weeks changed position, their static ectoplasmic structures could obviously not be resorbed. These structures were either pulled along by the specimens, as shown for the ectoplasmic 'roots' in Fig. 5, or torn off. Over the duration of the experiment, numerous ectoplasmic 'roots' and 'twigs', or what is supposed to be parts of such structures, were flushed to the aquarium's window (Fig. 11). We had to increase the current speed through the aquaria sporadically to get rid of the torn biomass and clear the view. When we opened the aquaria after termination of the experiments, we found torn ectoplasmic 'roots' with no signs of shrinking or collapsing. Since static ectoplasmic structures can obviously not be resorbed, any relocation is accompanied by material loss for a specimen.

It was also observed that algae (dispersed from the water inflow) adhering to the static ectoplasmic envelope, 'twigs', 'trees', and less marked 'roots', remained almost at the same position throughout the experiment or until the respective structure was torn off (Figs. 6-7).

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hat verschoben (Einfügung) [1]

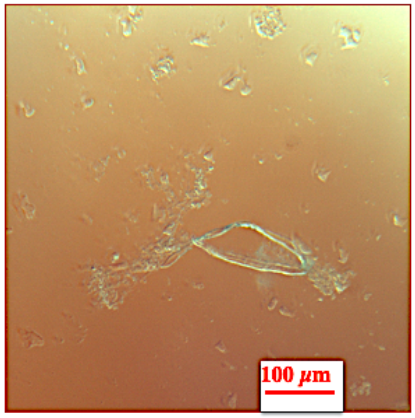

Figure. 11. Torn ectoplasmic 'roots' and 'twigs' at the aquarium window on May 2, 2017.

4 Discussion

4.1 Ectoplasmic envelope

This study describes the shell of *Cibicidoides* spp. as an internal 'sceleton' rather than an external feature. Already in Schultze's work from 1854 (Schultze, 1854) an ectoplasmic sheet can be suspected to cover the illustrated *Elphidium macellum* (as *Polystomella strigilatum*) test plate IV, fig. 1). Cushman (Cushman, 1928) even stated that in many taxa the foraminiferal shell would an internal one but did not elaborate more on which species he had in mind. In studies on foraminiferal calcification processes, in planktonic foraminifera, *Spiroloculina hyalinea*, *Ammonia* sp., and *Amphistegina lessoni* a protective cytoplasmic envelope is described as a structure restricted to times and areas when/where new shell material is precipitated (Angell, 1980; Bé et al., 1979; de Nooijer et al., 2014; Erez, 2003; Tyszka et al., 2019). In our observations, an ectoplasmic envelope covered the tests of the investigated *Cibicidoides* specimens at all times and for shell growth a supplementary surrounding sediment cyst had to develop (Wollenburg et al., 2018). Thus, it is currently unclear whether a permanent ectoplasmic envelope as we have observed it for *Cibicidoides* spp. is developed in only some foraminifera taxa or has simply

hat gelöscht: is the first to describe

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hat gelöscht: However, the observation of

hat gelöscht: or envelope around

hat gelöscht: shells goes back to the early days of foraminiferal observations when it has been described for *Heterostegina depressa* (Röttger, 1973, 1982). The observation of a significantly reduced pH surrounding

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hat gelöscht: . shells during growth (Toyofuku et al., 2017) may point to an envelope also in *Ammonia*. However, so far, no sheet or envelope has been described for this most studied genus. There are also some vague parallels between ectoplasmic envelopes and the Actin-rich lamellipodia that cover the tests of

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hat gelöscht: *lessonii* specimen during chamber formation (Tyszka et al., 2019). Yet, in

hat gelöscht: Thus,

hat gelöscht: is currently unclear whether an ectoplasmic envelope

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been overlooked in others. The ectoplasmic sheet described for *Heterostegina depressa* (Röttger, 1973, 1982) visually resembles the sheets surrounding *Cibicidoides* specimens and as in our experiments had to be during rapid relocation.

hat gelöscht: As described for *Hetereostegina depressa* (Röttger, 1982), also in our experiments the

hat gelöscht: obviously only shed their envelope

4.2 Ectoplasmic extensions – pseudopodial network

Only for a few shallow-water benthic foraminifera, information on ectoplasmic extensions to interact with the environment has been published so far (Bowser and Travis, 2002; Travis et al., 2002). Hereby, the typical ectoplasmic extensions described are pseudopodia characterised by their forceful and rapid extension enabled by actin filaments and extremely dynamic microtubule systems (Bowser et al., 1988; Goleń et al., 2020; Travis and Bowser, 1986; Travis, 2002). Anastomosing, i.e. the fusing of two neighbouring pseudopodia, is abundant and rapidly propagating. Furthermore, a rapid bidirectional transport of both granules and surface-attached particles has been described for the pseudopodia of shallow-water foraminifera. Giving tribute to the granular appearance, the term 'granuloreticulopodia' is widely used for this pseudopodial network and separates it from the globular and lamellar pseudopodia involved in chamber formation (Goleń et al., 2020; Tyszka et al., 2019).

hat gelöscht: (Bowser, 2002; Travis, 2002).

Our study shows that at in situ pressure the pseudopodial network of the examined *Cibicidoides* taxa extends into the water current and exhibits branching and anastomoses, resembling the pseudopodial network of shallow-water foraminifera. However, in the investigated specimens granules, anastomoses, and attached particles moved very slow and could be observed for hours, sometimes even days or weeks with little noticeable movement (Figs. 9-10). In *C. pachyderma* sp. 1 of Figs. 8-10, for example, it took about 6 weeks before a significant ingestion of dispersed algae inside the shell could be noticed (Figs. 9-10).

The rate at which cells can form projections, like pseudopodia, and transport granules and adhering particles is, in part, limited by the rate at which the cell assembles new or reorganises existing actin filaments (Bowser et al., 1988; Goleń et al., 2020; Travis and Bowser, 1986; Travis, 2002; Tyszka et al., 2019). This ATP consuming process is obviously much faster in shallow-water foraminifera than in deep-water *Cibicides/Cibicidoides*-taxa. Presumably due to the large working distance in our high-pressure aquarium set-up fluorescent SiR-actin labelling failed in our confocal studies so far. Therefore, we can just speculate that the ATP demand to form pseudopodia and perform bidirectional streaming increases with hydrostatic pressure and/or at sites of high current activity.

409

410 **4.2 Ectoplasmic extensions –permanent extensions**

411 Besides pseudopodia, this study describes for the first time non-retractable static ectoplasmic structures that, depending on
412 their characteristics, were named ectoplasmic 'roots', 'trees', and 'twigs'. Ectoplasmic 'roots' developed in most specimens
413 and all species investigated. Hereby, minimum 2 mutually opposing ectoplasmic 'roots' developed soon after the start of the
414 experiments. However, over the course of the experiments, the number of ectoplasmic 'roots' increased and most showed
415 ongoing growth. Ectoplasmic 'roots' are long branchless structures extending along the bottom or adhering to the window of
416 the aquarium. Together with pseudopodia emerging from the ectoplasmic 'root', these structures likely act as anchors to
417 stabilize the foraminiferal shell in an area of high current activity. Ectoplasmic 'roots' are likely the 'naked' variant of the
418 agglutinated tubes of *C. lobatulus* described from shallow-water occurrences (Nyholm, 1962). We assume that similar to the
419 sedimentary cyst covering the ectoplasmic envelope (see above), deposition of current-collected sediment particles on top of
420 ectoplasmic 'roots' leads to an increased robustness and protection of these structures.
421 Ectoplasmic 'trees' are thick, robust, and branching structures that, other than 'roots', direct into the water column (Fig. 6).
422 Over the course of weeks in the experiments, ectoplasmic 'trees' were only formed by *C. pachyderma* specimens. Fixed to the
423 aquarium bottom, these protruding structures reached heights of around 2 mm. Ectoplasmic 'trees' likely serve as scaffolding
424 on which the foraminifera can modify or optimise its position with respect to the prevailing current.
425 Ectoplasmic 'twigs' are thick structures extending into the water column whose shape and position with respect to the
426 specimen's test remain largely unchanged. However, they are the least static ones of the three described ectoplasmic structures.
427 Ectoplasmic 'twigs' are perhaps a stabilizing and protective framework that maintains a delicate pseudopodial network when
428 distributed into a current. However, further studies are required to prove our assumptions. In our high-pressure experiments,
429 ectoplasmic 'twigs' were only observed in *C. pachyderma* specimens, yet, recent observations on shallow-water *C. lobatulus*
430 show 'agglutinated' tubes directing into the water column (Fig. 12) that resemble ectoplasmic 'twigs'. In Fig. 12 we see a joint
431 'agglutinated' tube between specimen 1 (juv. *C. lobatulus*) and 2 (adult *C. lobatulus*) with freshly (picture was taken following
432 a feeding experiment) accumulated algae half way. On specimen 2 a second 'agglutinated' tube directs into the water column.
433 From our experience with cyst formation and algae aggregation, we assume that these 'agglutinated' tubes are sediment

hat gelöscht: specimen

covered ectoplasmic ‘twigs’. If *C. lobatulus* just develops ectoplasmic ‘twigs’ at shallow-water/ low-pressure sites, or if they were too thin to be detected with our instrumental set-up in our experiments with this species remains unclear. However, the picture of these freshly fed shallow water *C. lobatulus* specimens supports our assumption that the formation of rigid ectoplasmic ‘twigs’ assists a food-gathering pseudopodial network.

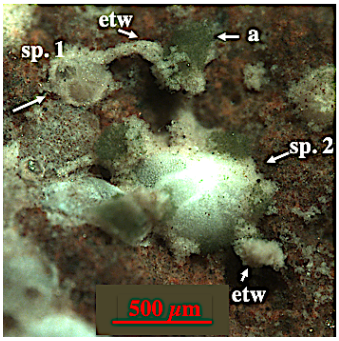

Figure. 12. Epilithic *C. lobatulus* specimen from off Svalbard. A joined ‘agglutinated’ tube, here equated with ectoplasmic ‘twigs’, is developed between specimen 1 and 2. Algae are accumulated half-way the tube. etw= ectoplasmic ‘twig’, a= algae. Picture courtesy of Julia Wukovits (September 2020).

We observed that static ectoplasmic structures did not change in response to current speed and that they could not be resorbed or retracted. It was also observed that algae (dispersed from the water inflow) adhering to the static ectoplasmic envelope, ‘twigs’, ‘trees’, and less marked ‘roots’, remained almost at the same position throughout the experiment or until the respective structure was torn off (Figs. 6-7). This might suggest that, in the absence of sediment particles in the current, the foraminifera try to stabilise lasting ectoplasmic structures by the continuous accumulation of algae (see also below).

In the field, the pseudopodial network of *C. antarcticus* is assumed to be guided by agglutinated tubes extending from the foraminiferal shell into the water column (Alexander and DeLaca, 1987; Hancock et al., 2015). In our experiments the ectoplasmic ‘trees’ and ‘twigs’ accumulated algae over time, but likely would also have accumulated sediments if provided by the inflowing current. Hypothetically, accumulation of sediment particles on ectoplasmic ‘twigs’ and ‘trees’ over longer

hat nach oben verschoben [1]: When we opened the aquaria after termination of the experiments, we found torn ectoplasmic ‘roots’ with no signs of shrinking or collapsing. Since static ectoplasmic structures can obviously not be resorbed, any relocation is accompanied by material loss for a specimen. ¶

hat gelöscht: (Alexander and DeLaca, 1987b; Alexander, 1987; Hancock et al., 2015)

460 periods could result in structures that resemble the agglutinated tubes described for *C. antarcticus* (Alexander and DeLaca,
461 1987) or shallow-water *C. lobatulus* (Fig. 12).
462 The tubes of *C. antarcticus* are made up of silt- and clay-sized minerals, diatom frustules, fine organic detritus, and occasionally
463 sponge spicules. However, although being described as agglutinated structures, the tubes collapsed when the respective
464 foraminifera was taken out of the water (Alexander and DeLaca, 1987). As no analyses on the particle combining cement were
465 made, it is quite possible that the described agglutinated tubes are sediment-covered ectoplasmic structures. In our study
466 provided artificial quartz substrate was not used for agglutination or accumulation on the static ectoplasmic 'roots, 'trees', or
467 'twigs', whereas dispersed algae were collected from the inflowing current and deposited on these structures. As we had no
468 dispersed minerals in the circulating current it can only be assumed that they would also adhere to the lasting ectoplasmic
469 structures described.
470
471 **4.3 Ectoplasmic extensions –biological and evolutionary aspects of permanent extensions and outlook for future**
472 **research**
473
474 Bowser and Travis (2002) speculated that evolutionarily the pseudopodium may have derived from the eukaryotic flagellum
475 because nearly all foraminifera possess flagellated gametes (Goldstein, 1999). Both, flagella and pseudopodia rely on
476 microtubules as a supporting and locomotive framework. Flagella possess an elaborate crosslinking apparatus designed to
477 produce a highly regulated bending form, whereas in shallow-water foraminifera microtubules are constantly transported
478 within the tethered framework of pseudopodia allowing a less rigid but highly flexible motile function. Although, pseudopodia
479 emerged from the static ectoplasmic structures, due to the stiffness of 'roots', 'trees', and 'twigs', they rather resemble flagella
480 than pseudopodia. Yet, future transmission electron analyses or confocal microscope investigations at atmospheric pressure
481 (Goleń et al., 2020; Tyszka et al., 2019) are needed to understand the cellular structure of these lasting ectoplasmic extensions.
482 Application of fluorescent dyes for confocal microscope investigations in high-pressure aquaria is often limited by the large
483 working distance hampering e.g. a noticeable emission from SiR-actin labelling.

hat gelöscht: *antarcticus* (Alexander and DeLaca, 1987b)
hat formatiert: Schriftart: Kursiv
hat gelöscht: (Alexander and Delaca, 1987a).

The static ectoplasmic features described are long-lasting and, thus, presumably energy saving structures of taxa living under significant hydrostatic pressure and current activity. They likely anchor the specimen at low energetic costs in a highly turbulent environment. Furthermore, 'twigs' and 'trees' likely protect a delicate pseudopodial network that, in a habitat with unpredictable food supply has to be immediately developed and extended. However, movement of anastomoses, adhering algae, and bidirectional streaming in the pseudopodial network were extremely slow during our observations suggesting a much slower ingestion time than has been described for shallow-water foraminifera (Bowser, 1984a, 2002; Wollenburg et al., 2018). This may be the reason why, for example, *C. wuellerstorfi* in the Nordic Seas and Arctic Ocean is restricted to times and areas of high food supply but is insensible to sudden primary production/carbon export pulses (Wollenburg and Kuhnt, 2000; Wollenburg et al., 2001; Wollenburg and Mackensen, 1998a).

5. Summary

This is the first report investigating ectoplasmic structures and dynamics in *Cibicidoides* species under *in situ* pressure. In the present study, a protective ectoplasmic envelope completely covered all *Cibicidoides* shells at any time suggesting that the shell is an endo- rather than ectoplasmatic feature.

Our further findings indicate that the life of these deep-sea foraminifera is characterised by energy-saving, long-lasting, static ectoplasmic structures that allow these rheotactic species to position themselves at sites of high current activities. 'Roots' are thick and robust ectoplasmic structures that anchor the specimens on current exposed substrates. They might continue to grow but otherwise could not be reshaped. Ectoplasmic 'trees' are stationary structures that are directed into the water column allowing the foraminifera to climb this structure and thereby elevate itself above ground.

Ectoplasmatic 'twigs' provide a supportive rigid framework from which or around which a delicate food-gathering pseudopodial network emerge.

When the specimen changed their location, the stationary ectoplasmic 'trees' and one or the other ectoplasmic 'root' were torn off. Thus, relocation is associated with a loss of ectoplasm and an additional energy demand required for the formation of new lasting ectoplasmic structures to secure the specimen at its new location. Whereas the deployment of a pseudopodial network

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into an inflowing current with algae is immediate, the propagation of collected algae towards the shell is extremely slow. Perhaps for this reason *Cibicidoides* taxa are poor indicators of primary production pulses.

We assume that the static shape and slow remodelling of 'trees', 'twigs', and 'roots' as well as the slow formation of anastomoses and surface transport arises from an adaptation to a high current activity habitat with unpredictable food fluxes driven by energetic optimization. This assumption as well as the possibility of a different microtubule system in deep-sea pseudopodia have to be addressed in future studies.

Acknowledgments

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