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Hydrurus foetidus (Chromista, Chrysophyceae): A large freshwater chromophyte alga in laboratory culture

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SUMMARY

The psychrophilic freshwater alga *Hydrurus foetidus* (Villars) Trevisan has previously resisted laboratory culturing in liquid media. We investigated the use of microbiological techniques adapted to specific requirement for low temperature and high turbulence of the alga. We found that successful culturing required cleaning steps, where pieces of the alga were dissected out and plated on agar under cold conditions. From here, unialgal colonies could be isolated and inoculated into liquid media under appropriate conditions. Turbulence, created by a laboratory shaker, is critical. Sufficient light (30–100 μmol m^{-2} s^{-1}) and low temperature (here 2–3°C) are necessary. This rather common but highly under-investigated freshwater alga has potential for basic and applied research when available as laboratory cultures.

Key words: algal culture, freshwater alga, *Hydrurus*, periglacial, psychrophile.

INTRODUCTION

The large (up to 10–30 cm), branched, freshwater golden alga *Hydrurus foetidus* (Villars) Trevisan is distributed worldwide (Wehr & Sheath 2003), but restricted to cold streams, particularly in alpine and periglacial environments (French 2007). In climates with defined seasons, it is mainly found in the spring during snowmelt, when the light conditions are improving. However, the alga may also be found throughout the summer and autumn in streams with low water temperatures; for example, in rivers that drain glaciated landscapes (Ward 1994; Rott *et al*. 2006a) or in the cold springs of the Alps (Cantonati *et al*. 2006). *Hydrurus* has previously resisted laboratory culturing in liquid media (e.g. Rostafinski 1882a; Esser 2000) and is not available from any culture collection. Pioneers like Klebs (1896) and Geitler (1927) kept *Hydrurus* alive for some time under natural or laboratory conditions, but never succeeded in establishing laboratory cultures in liquid media. It is extremely sensitive to temperature changes, transport and careless laboratory conditions, which may lead to cellular changes, sometimes sporulation, bacterial attacks, deterioration and lysis. In order to fix samples for electron microscopy, scientists brought their fixatives to the river banks to perform the initial processing (Vesk *et al*. 1984; Hoffman *et al*. 1986). Very few studies have been done on gene sequences from *Hydrurus* – only three sequences from natural material are available via the National Centre for Biotechnology Information (NCBI) GenBank database – one (5S) from Japan (Lim *et al*. 1986) and two (18S and 28S rRNA) from Norway. Today, cultures of unicellular (e.g. *Ochromonas, Mallomonas*) and colonial (e.g. *Dinobryon, Synura*) golden algae (the classes Chrysophyceae and Synurophyceae) are maintained to some extent in culture collections and laboratories; but golden algae may be more demanding and require closer attention than members of many other algal classes. Among the more critical studies of golden algae under exacting laboratory conditions are those investigating their physiological requirements (e.g. Hutner *et al*. 1953; Klaveness & Guillard 1975; Lehman 1976; Maberly *et al*. 2009).

The Kingdom Chromista, in which the golden algae are affiliated, also includes the large brown algae (the class Phaeophyceae), which provide shelter and food to fauna of the near-shore marine hydrosphere. Large brown algae contain protective carotenoids and life-supporting unsaturated lipids. *Hydrurus* is the only large freshwater representative of the Kingdom Chromista; it has a wide inland distribution (cf. Wehr & Sheath 2003) and represents an interesting candidate as a source of nutrients comparable to its marine counterparts. *Hydrurus* is a valuable food source for fungi and protists (Bursa 1934; Rott *et al*. 2006a,b) and for larvae and adult insects in cold streams (Ward 1994; Milner *et al*. 2001, 2009)

A comprehensive database on the distribution and locations of *Hydrurus* in Norway is available to us

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(Lindstrøm & Klaveness, manuscript in preparation) for primary information on the environmental requirements of *Hydrurus* in Norway. A large amount of environmental information from locations where *Hydrurus* grows in abundance is available from the international literature (e.g. Ström 1926; Geitler 1927; Bursa 1934; Wehr & Sheath 2003; Rott *et al*. 2006b). This kind of information is helpful for culture initiation. But reliable clonal cultures are necessary to sort out important and critical factors for growth and reproduction, and to test environmental factors individually and in combination. For culturing, we note that a large alga like *Hydrurus* with its pronounced mucopolysaccharide sheath might have a limited capacity for gas exchange in water, unless turbulence can keep every branch of the thallus exposed. Furthermore, very cold water has a large carrying capacity for dissolved gases, and may also provide a continuous supply of nutrients under natural conditions. In the laboratory, the agar plating technique, pioneered in the case of *Hydrurus* by Geitler (1927, p. 796 and footnote), provides direct access to air as well as nutrients. The early works of the scientists Klebs (1893, 1896), Geitler (1927) and Bursa (1934) therefore provided inspiration to continue the work towards achieving consistent *Hydrurus* growth in liquid media. The major purpose of this work was therefore to develop a method for growing reproducible cultures of *H. foetidus* in liquid medium. Our results, materials, and cooperation are offered to improve the knowledge of this widespread and interesting organism.

MATERIALS AND METHODS

Easy access to the Alpine Research Center at Finse (www.finse.uio.no located at 60°36′ N, 7°30′ E) in southern Norway, and to the torrent river that drains lake Finsevatn (map at Fig. 1) close to the center, provided us with fresh material throughout the spring months. The research center has a cold incubator (Sanyo MIR-153 with a door window) with external light sources and a timer to supply a 14:10 h LD (light : dark) cycle. The prominent spring populations of *Hydrurus* in this river were first identified by Ström (1926) as *H. foetidus*. It is still present in abundance seasonally, and we fully confirmed Ström's identification based on the most precise and detailed morphological descriptions available: Rostafinski (1882b), Klebs (1893), Pascher (1913), Geitler (1927), Bursa (1934), Mack (1953), Hovasse and Joyon (1960), Fukushima (1962), Joyon (1963), Canter-Lund and Lund (1995), Nicholls and Wujek (2003). The taxonomic history of this genus is complicated, and the morphological separations between species proposed earlier are uncertain due to a pronounced phenotypic plasticity. For more information, we suggest the overviews by Rostafinski (1882a) and Silva (1950). Until investigations have been done by modern methods on material and cultures from different areas, we follow the recommendations by recognized authorities, from Pascher (1913) to Kristiansen and Preisig (2001), treating this genus as monotypic with *H. foetidus* as the only species.

For sequencing and for the culturing efforts reported here, material was secured from the river on 7 March 2007. The river was still close to a minimum of winter flow, and the water was flowing with a depth of 2–15 cm at the site of collection. Snowmelt is still minimal at this location in the beginning of March, so the river temperature was close to that of the ice-covered lake near the surface (1–2°C). The gross morphology of the thalli collected were typically arbuscular, 3–7 cm in length, with central axes and branches upon which the minor branchlets were organized. The entire thallus is covered by a characteristic polysaccharide sheath. Information on the partial 18S and 28S rRNA gene sequences from this material are now available at the NCBI GenBank at accession numbers FM955256 and FM955257. Unialgal cultures from this material are offered to culture collections, to generate interest and faciliate comparison with local strains.

The methods chosen for isolation and culturing of *Hydrurus* are modifications of standard classical microbiological techniques; glasswares were used in most cases, and when plastics were necessary we avoided direct contact with the biological material (cf. Blankley 1973; McDonald *et al*. 2008). We exposed tiny fractions of the alga directly to moist air within Petri dishes filled with an agar substrate that contained dissolved nutrients (Fig. 2). The medium was Guillard & Lorenzen's WC (Guillard & Lorenzen 1972) made with glass-distilled water, without organic buffer, without added silicate, and no further pH-adjustments (WC simplified; here = SWC, Table 1). The liquid medium was autoclaved as 70-mL batches in 125-mL Bellco flasks with stainless steel caps (Fig. 2). This medium could be solidified with 1.25% Difco Bacto Agar, this should be a three-vessel procedure to avoid harmful interference between components (cf. Allen 1968) and loss of nutrient availability by precipitation. First, 1 mL of the diPotassium-hydrogenphosphate stock solution (= stock solution 5 in Table 1) was diluted to 10 mL in a glass culture tube with a stainless steel cap; then, 490 mL of glass-distilled water was placed in a 1-L (or larger) culture flask with stainless steel cap and the remaining six stock solutions (Table 1) were added in appropriate amounts to achieve a final volume of 1 L of medium; finally, 500 mL of glass-distilled water was mixed with 12.5 g of agar in another flask. All three vessels were autoclaved at 121°C for 15 min; then the three solutions were mixed while hot and poured into sterile plastic Petri dishes. After cooling, the condensed water on the

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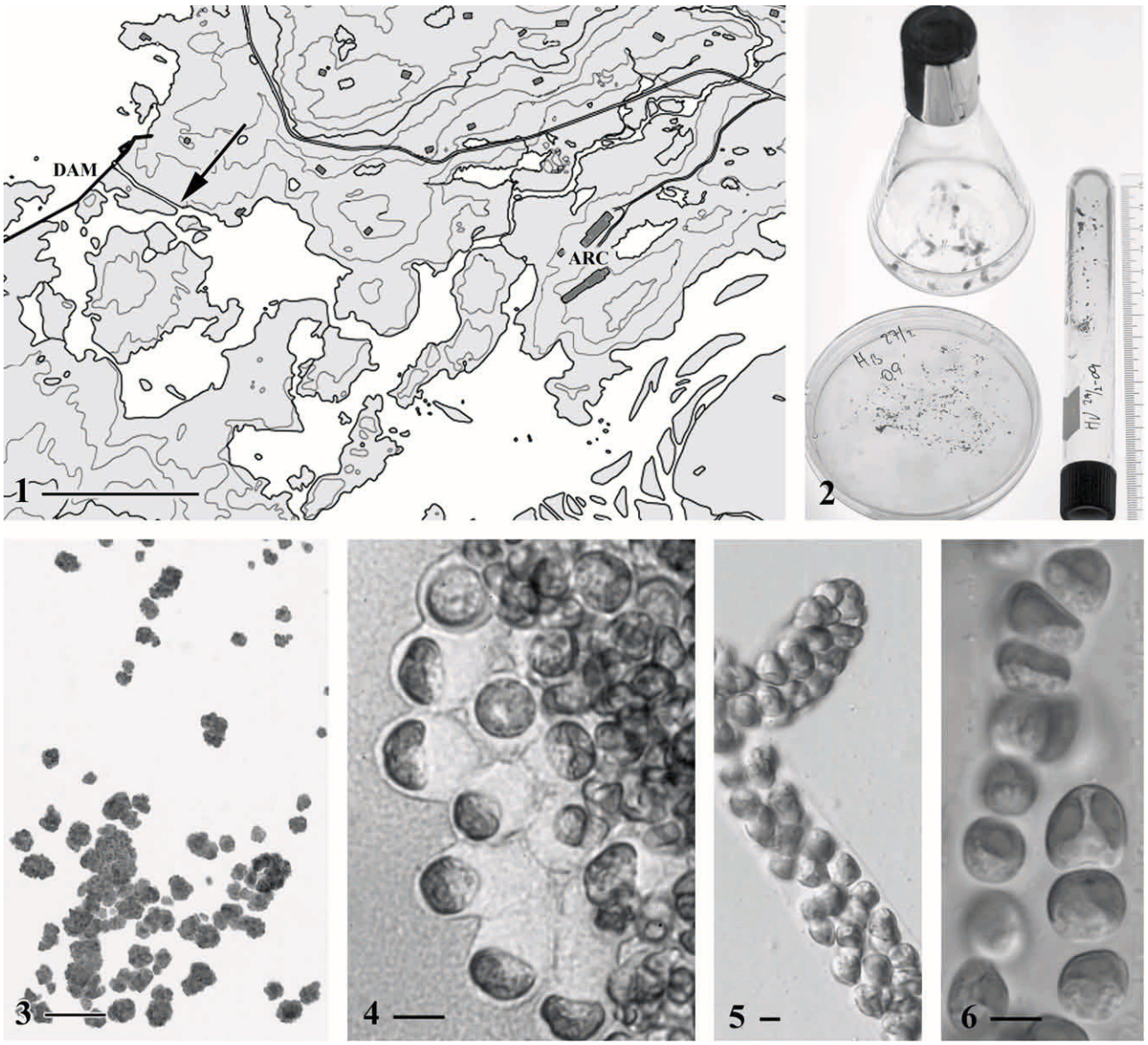

Figs 1–6. 1. Map of area and location from where *Hydrurus foetidus* was collected. The major watercourses and ponds are white, landscape with topography are indicated in grey. Equidistance of terrestrial height curves are 5 m. Cottages and buildings are indicated as dark grey structures, while the dirt road is shown by a double line. The Alpine Research Center, indicated by ARC, is at the center to the right side of the map. Scale bar = 200 m. Lake Finsevatn (in the upper left corner) is dammed up to a water level of 1215 m above sea level, by a concrete structure (indicated by black solid line, at DAM in figure). The river does not freeze during winter and is accessible at the spot indicated by the large arrow – with some effort and technical gear. 2. Culturing vessels for growing *H. foetidus.* (lower left) a 9-cm Petri dish with agar medium; (lower right) a 15-cm screwcapped culture tube with an agar slant; and (top) a 175-mL Bellco bottle with 50 mL of medium. 3. Macrophoto of colonies on an agar plate, grown for 9 weeks. Scale bar = 2 mm. 4. Fringe of a colony on an agar plate, photographed through a microscope (40× objective without a coverglass). Spreading out on the agar plate appeared to be difficult; colonies remained quite compact. Scale bar = 10 μm. 5. Multicellular branch of a thallus in liquid culture. Scale bar = 10 μm. 6. Larger magnification of the distal part of thallus grown in liquid culture. Note a broadly conical apical cell (middle right) initiating a lateral branch, but still within the common polysaccharide sheath. The surface of the polysaccharide sheath at this stage of isolation still appears rugulose due to epiphytic bacteria. Scale bar = 10 μm.

lids was carefully drained, and the plates were stacked and stored upside-down in polyethylene bags at the ambient temperature within the culture facility. The liquid medium attained a pH within the range of 7.1–7.4 upon equilibration with air; the addition of agar did not change this substantially.

A glass Pasteur pipette was melted closed and curved at the tip with a spiritus flame. Tiny pieces of

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Table 1. List of stock solutions for liquid medium SWC (=Guillard and Lorenzen's WC-medium simplified).†

No. of stock solution	Components	Amount of the component dissolved in distilled water to make a 100 mL stock solution
1	$CaCl_2 \cdot 2H_2O$	3.68 g
2	$MgSO_4 \cdot 7H_2O$	3.70 g
3	$NaHCO_3$	1.26 g
4	$NaNO_3$	8.50 g
5	K_2HPO_4	871 mg
6	Na_2EDTA	436 mg
	$FeCl_3 \cdot 6H_2O$	315 mg
	$CuSO_4 \cdot 5H_2O$	1.0 mg
	$ZnSO_4 \cdot 7H_2O$	2.2 mg
	$CoCl_2 \cdot 6H_2O$	1.0 mg
	$MnCl_2 \cdot 4H_2O$	18 mg
	$Na_2MoO_4 \cdot 2H_2O$	0.6 mg
	H_3BO_3	100 mg
7	Thiamin·HCl	10 mg
	Biotin	50 µg
	Vitamin B_{12}	50 µg

†To make a 1 L medium, add 1 mL of each stock solution. Stock solutions 1–6 can be stored for some time in refrigerator. Stock solution 7 should be split in small batches and kept frozen.

fresh material from the river were carefully macerated and then streaked with this fine tool in a criss-cross pattern on the agar plates. The inoculated plates were stored in zip-locked polyethylene bags and maintained upside-down in the cold incubator at 3°C, with approximately 30 ± 15 µmol m^{-2} s^{-1} (depending on the distance from the light source) of fluorescent light, provided on a 14 : 10 h LD cycle. The inoculated agar plates were later moved to Oslo inside cold thermos bottles, and then placed in a cold room maintained at 2.6°C with 14 : 10 h LD cycles and 30–100 µmol m^{-2} s^{-1} of soft white fluorescent light during the L phase. Light was measured at the positions of the culture vessels in the directions of the light sources, with a recently calibrated, cosinuscorrected Li-Cor Quantum sensor, mounted on a LI-1000 DataLogger. Temperatures in the culture chamber and room were logged with calibrated Onset TidBit v1 and v2 dataloggers.

Observations, photography and measurements of cell sizes were done with a Nikon Diaphot microscope equipped with an eyepiece reticle and a Nikon D1 digital camera. Both the eyepiece reticle and the photographic records were carefully calibrated by a stage micrometer (Zeiss Oberkochen, Germany), at all magnifications used.

Two methods were tested for growing *Hydrurus* in liquid medium. The simplest was the inoculation of single colonies from agar plates into flasks (Fig. 2). The flasks were then mounted on a shaker (simple linear movement, 20 mm amplitude at 40 rpm). The other method was inoculation into 40 × 300 mm tapered glass tubes that had an air inlet at the bottom; this method still requires more work for optimization. Here, we report the most important experiences during the work that resulted in successful flask culturing of *Hydrurus*.

RESULTS

Periodic inspection of the agar plates at the Alpine Research Center revealed that the survival of algal fragments was quite successful, with visible growth along the edges and a substantial volume increase (Figs 3,4). There was, however, a heavy growth of bacteria on the plate surface. To eliminate the bacteria, *Hydrurus* colonies were dissected out, shaken, rinsed in the medium, and restreaked/reisolated; this reduced the bacterial problem and removed all foreign algal and cyanobacterial epiphytes. We found that this 'clean' *Hydrurus* could be kept alive on an agar surface for at least one year without transfer when the Petri dishes were stored in sealed polyethylene bags under moderate light in cold room conditions. Agar slants in screwcapped tubes (Fig. 2) were also useful for cold transport and mailing samples.

When clean colonies from one agar plate, grown from the macerated tissue of one single branch tip of material from the river, were picked and inoculated into flasks with medium, a certain degree of morphological plasticity was expressed. In some bottles, aggregations of cells proliferated as typically colored, slimy layers on the glassware and loosely branched filaments in the liquid. In other flasks, star-shaped or branched colonies developed from the agar plate colony. These consisted of fast-growing, weakly colored central axes (cf. Bursa 1934: 'Gallertzylinder' p. 77, 'Zentralzylinder' p. 115) that developed branches of typical structure and color (Figs 5,6). A complete plant of *Hydrurus* consisted of three types of cells: (i) central axis cells that were apparently able to produce firm polysaccharide for the central axis structure (cf. Tab. II in Rostafinski (1882b), or figs 13–26 in Graham & Wilcox 2000); and: (ii) more or less isodiametric cells that formed branches emanating from the axial structures (as in Figs 5,6); these developed within soft to fluid polysaccharide coats (cf. also fig. 297 on p.161 in Canter-Lund & Lund 1995) – and 3) the meristematic top cell of each branch.

To conserve both types of 'tissue' – the central axis and the branches within soft polysaccharide coats – it was initially considered important to propagate pieces consisting of both types. However, we noticed that colonies transferred from agar plates very soon after the initial isolation led significantly to the formation of firmer central axes. Under the conditions described

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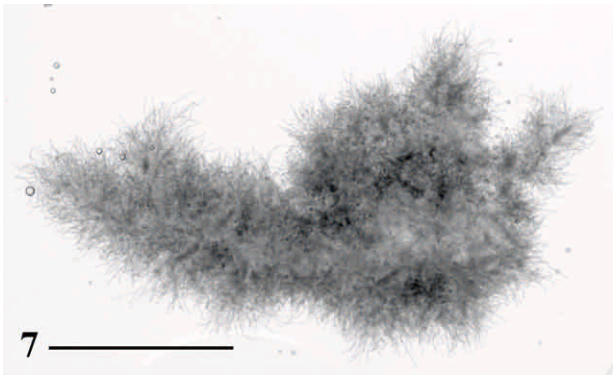

Fig. 7. Recently excised branch of a larger (>30 mm), free-floating *H. foetidus* colony. The colony was grown in a flask on a shaker, this branch was excised and transferred into new medium and grown for one week before this picture was taken. It displays the typical shape of a free-floating colony grown through several transfers (as explained) in liquid cultures on a shaker with non-directional turbulence at 2.6°C and a photosynthetically active radiation quantum flux of up to 100 μmol m^{-2} s^{-1}. Scale bar = 5 mm.

here, the continued transfer of excised pieces from liquid cultures to new liquid cultures every 3 weeks (or when a thallus in a flask reached approximately 30 mm in size), favored the growth of branch tissue. These colonies showed gradual rearrangements of the central axes into loosely organized, but still discrete structures. This organization of the thallus then remained stable under the type of turbulence we applied here. The size of the different cells in a thallus from culture (as shown in Fig. 7) was measured under the microscope and are given in Table 2.

Algal cultures with good growth rates often outgrow the bacteria during exponential growth phases, probably because the leachates of secondary metabolites are kept under control. The foul smell characteristic of *H. foetidus* was not apparent in these cultures, despite the presence of bacteria. As long as the algae were growing rapidly, only few bacteria were observed along the edges of the polysaccharide sheaths or in the medium. However, if the growth of the alga slowed, within a short period of time (days or weeks) a decomposition process commenced. Then, the characteristic smell of *H. foetidus* became palpable.

DISCUSSION

We succeeded in establishing and propagating *H. foetidus* in liquid cultures by using simple, well-established techniques, ensuring that only clean glassware (no plastics) was in direct contact with the alga, and maintaining some degree of skill and patience. The pioneer connoisseurs of the art of protist and algal culturing (e.g. Ogata, Geitler, Lwoff, Droop, Provasoli, Guillard) never renounced cleanliness and quality – obligate requirements for success – and attention and patience gave results of interest.

Our simplification of the medium described by Guillard and Lorenzen (1972) avoided adding silicate; this may be rational for media intended for isolation purposes, given that glassware is involved in their preparation and storage. Studies of silicate requirements in cultures indicated early that silicate leaching from glasswares, from silicate contamination of chemicals, and from the use of glass stills could supply silicate compounds (e.g. Werner 1977). A pH adjustment was not required for the SWC medium, when time was allowed after autoclaving for equilibration with air at the ambient temperature. Chlorinity was kept low by avoiding the addition of HCl, and other potentially inhibiting ions for golden algae (e.g. potassium; cf. Lehman 1976) were well controlled by moderate concentrations. The buffering capacity was provided by the bicarbonate system; therefore, this required attention during the cleaning and rinsing of glassware and preparation of medium. Continuous equilibration of the bicarbonate system was controlled by applying turbulence during algal growth. The careful handling of bottles (e.g. placing the bottles on melting ice in polystyrene containers when outside the growth facilities) may have prevented sporulation during periods without turbulence; moreover, secondary establishment was also avoided in the splash zones on the glass walls of the bottles. The shaker speed was chosen by trial and error and had to be reduced to avoid spilling medium. Thus, the degree of turbulence was not optimized for algae growth. This compromise may have influenced the developmental morphology of the pieces inoculated into our bottles. Bursa (1934, p. 77) concluded from field studies that the water flow rate ('Strömung') had an influence on the development of the central axis; low flow rates appeared to have led to poor development. The turbulence in our cultures was non-directional with respect to the free-floating plants. However, in the river, there is a substantial directional drag on the sessile thallus; under those conditions, growth may require a different central axis. The quality of the central axis may be regulated by turbulence, laminar flow rate, and shear stress; this should be investigated in future studies under rigorous experimental conditions.

The cell sizes (particularly the categories 2., 3., and combined in category 5., in Table 2) agree well with the size ranges reported by other authors from wild material (e.g. 8–16 × 12–22 μm by Mack 1953; 7–20 μm by Joyon 1963; 5–15 μm by Vesk *et al*. 1984) – but there are no separate measurements of the different cell categories in the literature, as reported here. It has been shown that cells 'aus dem Innern des Lagers' (=central axis cells?) may attain extreme shapes and

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Table 2. Cell sizes of thallus in liquid medium (as Fig. 7), under the present culture conditions

Cell category	Cell shape	*n*	Apical-antapical (AA) distance and range (R), µm	Lateral diameter (LL) and range (R), µm
1. Apical cell of branches	Half spheroid to conical with flat or angular base	10	AA = 12.0 ± 2.3 R = 9–16	LL = 14.8 ± 2.5 R = 12–19
2. Intercalary cell of dense branches	Angular or compressed	10	AA = 11.1 ± 1.7 R = 9–13	LL = 13.0 ± 2.3 R = 10–18
3. Free cells of loose branches	Sphere to spheroid	10	AA = 13.5 ± 1.7 R = 12–17.5	LL = 12.6 ± 1.1 R = 11.5–14
4. Central axis cells	Ellipsoid to ovoid	10	AA = 25.2 ± 4.7 R = 20–32	LL = 10.2 ± 1.2 R = 8–12
5. Branch cells (2. + 3. above) combined		20	AA = 12.3 ± 2.1 R = 9–17.5	LL = 12.8 ± 2.1 R = 10–18

The first 10 cells (*n*) observed of each cell category was measured. Apical end of cells were towards the top of thallus or branches (= direction of growth), close to where the chloroplast is located (cf. Fig. 6). Lateral diameter was taken as maximum diameter 90° on the apical-antapical distance. Data given are means ± standard deviations. R indicates ranges encountered among the cells of each category.

AA-elongation (e.g. Berthold 1878, cf. also Mack 1953).

Answers to questions and speculations that have arisen in the literature regarding the morphological plasticity or nature of the highly polymorphous species *H. foetidus,* will require more careful observations (e.g. observations and biometry on different cell types, cf. Table 2 here), well-designed experiments in the laboratory on cultivated strains like this one (numbered G 070301), and new isolates from other locations. New isolates may provide new sequences that reveal genetic diversity behind the apparent morphological plasticity of *H. foetidus*. Bacteria-free cultures are obligatory for the study of physiological processes and certain nutrient requirements (e.g. Mainx 1929; Hutner *et al*. 1953; Klaveness & Guillard 1975; Vieira and Klaveness 1986; Danger *et al*. 2007). When phagotrophic protists or potential mixotrophs, like golden alga, are involved (e.g. Hardin 1942; 1944; Bird & Kalff 1987), knowledge of the bacterial flora is essential (cf. Jennings 1908: '*To make the conditions of existence the same, it is not sufficient to attend merely to the basic fluid; the bacteria must also be the same*'). In our current state of knowledge, it is a primary challenge to obtain bacteria-free cultures of *Hydrurus* for different kinds of physiological studies, *without* use of antibiotics that could be mutagenetic or harmful to organelles. Because *Hydrurus* is a large, freshwater, inland alga that is phylogenetically affiliated with marine seaweeds, it may be of nutritional and biotechnological interest in addition to serving as a model system for the brown branch of plants (see Baweja *et al*. 2009 for challenges).

We invite academic laboratories to share our agar slants with 'Palmellen' in order to take part in improving culturing conditions and to further study this strain (G 070301). This study may also inspire the investigation of new local strains of *H. foetidus*.

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