The affiliation of *Hexasterias problematica* and *Halodinium verrucatum* sp. nov. to ciliate cysts based on molecular phylogeny and cyst wall composition

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Abstract :

Species in the genera *Hexasterias* and *Halodinium* have been recorded over the last decades as acritarchs in palynological and/or plankton studies. In paleoenvironmental studies, these resting stages are often interpreted as indicators of freshwater input. The biological affinity of these genera has never been definitely established. Here, a new species, *Halodinium verrucatum* sp. nov., is described and molecular evidence (single specimen SSU and LSU rDNA sequencing) reveals that both this new species and *Hexasterias problematica*, collected from sediment samples in the Skagerrak and Baltic Sea, are resting stages of prorodontid ciliates. Additionally, infrared spectroscopic analysis (micro-FTIR) of *Hexasterias problematica* and *Halodinium* spp. specimens indicates a carbohydrate-based composition of the cyst wall with evidence for nitrogen-containing functional groups. A similar composition is recorded for tintinnid loricae, which further supports the placement of *Hexasterias* and *Halodinium* as ciliate cysts, and the composition is consistent with the heterotrophic nature of ciliates. The morphologically similar species *Radiosperma corbiferum* has a comparable composition, suggesting a similar ciliate affinity and indicating the utility of micro-FTIR in understanding acritarch affinity. *Hexasterias problematica* typically occurs in coastal waters from temperate to arctic regions. *Halodinium verrucatum* sp. nov. is observed in temperate estuarine sediments in the northern hemisphere.

Highlights

► *Halodinium verrucatum* sp. nov. is described, *Hexasterias problematica* is emended. ► LSU and SSU rDNA is obtained for *Halodinium verrucatum* and *Hexasterias problematica*. ► Molecular phylogeny and DAPI-staining demonstrate these are ciliate resting cysts. ► micro-FTIR of resting cysts and tintinid loricae supports this affiliation. ► The distribution and ecology of both palynomorphs is discussed.

Keywords : Acritarch, Ciliate cyst, FTIR, LSU-SSU rDNA, Prostomatida, Taxonomy

Introduction

The informal group of Acritarcha was introduced by Evitt (1963, p. 300) to accommodate organic-walled microfossil remains (i.e., palynomorphs) of apparently unicellular organisms whose biological affinity is unknown and which are resistant to degradation and palynological treatment. Acritarchs consist of a central cavity enclosed by a single- or multilayered wall and are extremely variable in general shape, symmetry, structure, ornamentation and opening of the central cavity (e.g., pore, slit, rupture, pylome). They occur in Proterozoic to modern sediments, and are considered polyphyletic with diverse biological affinities (e.g., Servais 1996). Many palynomorphs originally classified as acritarchs have since been reassigned to other groups, particularly dinoflagellates (e.g., Dale 1977; Colbath and Grenfell 1995), chlorophytes (e.g., Aroui et al. 1999; Kaźmierczak and Kremer 1999; Moczyłowska et al. 2011) and copepod eggs (e.g., McMinin et al. 1992; Van Waveren and Marcus 1993). For fossil species, arguments for these assignments are based on morphological and ultrastructural observations through light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (e.g., Colbath and Grenfell 1995; Willman 2009) and chemical composition through spectroscopic methods (e.g., Marshall et al. 2005; Javaux and Marshall 2006). For species in surface sediments, these are complemented by cytological observations, life history information through incubation experiments and molecular data obtained through methods such as single-cell polymerase chain reaction (PCR) (e.g., Takano and Horiguchi 2005). *Hexasterias problematica* (syn. *Polyasterias problematica*) and species of the genus *Halodinium* are considered to be acritarchs and are often reported in surface sediments. The former species is also known from plankton studies and *Halodinium* has an extensive fossil record in the late Cenozoic. Hypotheses about their biological affinity have varied widely but were never established conclusively.

Ciliates (phylum Ciliophora) are morphologically diverse heterotrophic unicellular eukaryotes (protozoans) that occur in nearly all moist and aquatic environments from soils, freshwater ponds and lakes to estuaries and the open ocean (e.g., Lynn 2008; Foissner et al. 2008).

An important synapomorphy of ciliates is nuclear dualism, in which a micro- and macronucleus can be distinguished (e.g., Lynn 2008). Many of these mainly free-living forms are able to produce cysts as part of their life cycle, often as a response to adverse conditions (Verni and Rosati 2011). These cysts are often more resistant to destruction than any other stage of the life cycle and thus offer higher potential for preservation and fossilization (Dunthorn et al. 2015). The class Spirotrichea is particularly known to produce cysts (Reid and John 1978, 1983). In addition to cysts, the tintinnid ciliates (Class Spirotrichea, Order Tintinnida) are known to have a long fossil record due to their loricae, protective tubular structures that contain the ciliate body throughout the life cycle (Dolan et al. 2013). The chemical composition of ciliate cyst walls is poorly known and limited to a small number of species. Thus far, the major components are characterized as (glyco)proteins and carbohydrates (Wang et al. 2017). Some ciliates are known to cause disease in other organisms (e.g. Lynn 2008), and the management of, for example, sea itch, caused by the parasitic ciliate *Cryptocaryon irritans*, is difficult because of the complexity of the life cycle involving a resting cyst stage (Ma et al. 2017). Clearly, better understanding of ciliate life cycles and resting cyst stages is of interest to workers in a broad range of fields.

In this study, living and empty *Hexasterias problematica* cysts were isolated from the surface sediment of a low salinity site in SW Finland. Also, the new *Halodinium* species, *H. verrucatum* sp. nov. is described from living and empty cysts isolated from surface sediments in a brackish fjord within the Skagerrak, on the west coast of Sweden. Both species are shown, through molecular phylogenetics based on single-cell polymerase chain reaction (PCR) of partial large (LSU) and complete short (SSU) subunit ribosomal DNA (rDNA), to belong to the ciliate order Prorodontida. Staining using the fluorescent 4'-6-diamidino-2-phenylindole (DAPI) was done to confirm this placement. The geochemical composition of the cyst walls was analyzed through micro-Fourier transform infrared (FTIR) spectroscopy and compared with loricae of tintinnid ciliates and with morphologically similar palynomorphs. The species' distributions are assessed

though a literature review and new observations, and the paleoenvironmental application is evaluated.

Material and Methods

Sediment sampling, cyst extraction and microscopic observations

For the study of *Hexasterias problematica*, surface sediment was collected from Sällvik (28 October 2011), a freshwater-influenced low salinity estuary off southwestern Finland with a halocline at 10 m depth and salinities up to 4.6 psu in deeper waters (e.g. Heiskanen and Tallberg 1999), using a Limnos gravity corer (Table 1, Fig. 1). For *Halodinium verrucatum* sp. nov., surface sediment was collected from station Strö 1, south of Havstensund (Skagerrak) using a boxcore (Table 1, Fig. 1). Additional samples from the Skagarrak and Brittany were collected for further observations (Table 1, Fig. 1). All samples were stored in sterile plastic bags at 4°C.

About 0.5–1.0 cm³ of wet sediment was immersed in filtered seawater and after ultrasonication (60 s, As One™ US2R ultrasonic bath), the sediment was rinsed using filtered seawater through a calibrated metallic-mesh Sanpo™ sieve with a 20 µm mesh. From this residue, the cyst fraction was separated using sodium polytungstate (SPT) at a density of 1.3 g cm⁻¹ (Bolch 1997). For microscopic observations and geochemical measurements, residues were prepared using a palynological method similar to that described in Mertens et al. (2009a), involving hydrochloric and hydrofluoric acids and no oxidizing agents.

The LM observations, measurements, and photography were made using a MRc5 camera mounted on a Zeiss Axio Imager A1 (400× and 1000× magnification) at Ghent University. For SEM observations, single cysts were picked from the residue and mounted on a glass slide or filtered on polycarbonate membrane filters (Millipore, Billerica, MA, USA, GTTP Isopore, 0.22 µm pore size), sputter coated with gold, and examined with a Zeiss SIGMA300 Gemini field emission SEM at the Station de Biologie Marine (Ifremer, Concarneau, France). The terminology for describing the morphology of *Hexasterias* and *Halodinium* is clarified in Fig. 2.

Molecular analyses

Four specimens of *Hexasterias problematica* were isolated for single-cell analysis from the Sällvik sediment sample. Two specimens of *Halodinium verrucatum* sp. nov. were isolated for single-cell PCR from the Strö 1 sediment sample of Skagerrak. Cysts were photographed and measured using an Olympus BX51 LM with a Nikon digital sight DS-1L 1 module with 100x oil immersion objectives. Subsequently, the cysts were transferred to an inverted LM, crushed with a fine glass needle, and subsequently transferred into a 200 µL PCR tube containing 3 µL of Milli-Q water. Sequences of partial LSU and SSU rDNA were determined from single cysts. In the first PCR round, the external primers (SR1 and LSU R2; Takano and Horiguchi 2005) were used with PCR mixtures of KOD-Plus-Ver. 2 Kit (Toyobo, Osaka, Japan) under PCR conditions: one initial cycle of denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min and final extension at 72°C for 5 min. In the second PCR, five sets of primers (SR1-SR9, SR4-SR12, SR8-25F1R, SR12cF-25R1 and LSU D1R-LSU R2; Takano and Horiguchi 2005) were used with PCR mixtures of KOD-Plus-Ver. 2 Kit (Toyobo, Osaka, Japan), 0.5 µL of the first round PCR product as the DNA template, and the same PCR conditions except that the extension at 72°C was for 1 min. In the third PCR, five sets of primers (SR-1c-SR5, SR4-SR9, SR8-SR12, LSU D1R-25R1 and LSU D3A-LSU R2; Takano and Horiguchi 2005) were used with PCR mixtures of the TaKaRa EX taq system (Takara Bio Inc., Shiga, Japan), 0.5 µL of the second round PCR products as the DNA template, and the same PCR conditions except that the extension at 72°C was for 30 s. PCR products were sequenced directly using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA).

Sequence alignments and phylogenetic analyses

After an initial analysis containing taxa from all major ciliate groups based on Dunthorn et al. (2014), a final sampling of 26 SSU-rDNA accessions from the Prostomatea, plus two outgroup species (*Furgasonia blochmanni* and *Platyophrya vorax*) was based on Yi et al. (2010). Nucleotides were aligned and ambiguous positions masked with GUIDANCE v1.1 (Penn et al. 2010a,b). The GTR-I-G evolutionary model was the best fit model selected by the Akaike information criterion (AIC) as implemented in jModeltest2 v0.1 (Darriba et al. 2012; Guindon and Gascuel 2003). Maximum Likelihood (ML) analyses were carried out in RAxML-HPC v7.2.5 (Stamatakis et al. 2008), with bipartition support from 1000 multiparametric bootstrap replicates.

DAPI Staining

For DNA-specific fluorescent DAPI staining (Kapusinski, 1995), a specimen of *Hexasterias problematica* was extracted from fresh sediments from the Vilaine Estuary (Brittany). The cyst was slightly crushed by pressing down the coverslip to allow the DAPI to intrude. Observation and imaging was done on a Olympus BX41 microscope with 100× oil immersion objectives (Ifremer, Concarneau).

Geochemical analysis of cyst wall composition

For micro-FTIR geochemical analysis of *Halodinium* spp., palynological residue was used from Skagerrak (Strö 1 station), Brittany (France) and the Irish Sea (see Mertens et al. 2009b and Gurdebeke et al., accepted, for sampling details), and from Brittany (France) and southern France for *Hexasterias problematica* (see Mertens et al. 2009b; Gurdebeke et al., accepted and Mertens et al. 2017, respectively for sampling details). For comparison, additional FTIR spectra were measured on specimens of the acritarch *Radiosperma corbiferum* from Brittany, the Irish Sea (Mertens et al. 2009b; Gurdebeke et al., accepted for sampling details) and the Baltic Sea (see Mertens et al. 2012a for details) and on loricae of the tintinnid ciliates *Parafavella* sp. from the Skagerrak (Strö 1 station, mentioned above) and *Stenosemella* sp. from the German Wadden Sea (see Bogus et al. 2014 for

sampling details). Between palynological processing and the preparation for FTIR, these residues were stored in cool and dark conditions without adding conservation chemicals such as copper sulphate or fungicides.

The palynological residues were treated as described in Gurdebeke et al. (accepted). They were ultrasonicated for 30 s and rinsed three times with organic solvents (methanol and dichloromethane, 1:1 in volume) and MilliQ water over a 20 µm mesh to remove polar and apolar compounds that might have adhered to the outside of the cyst walls. Visually clean individual cysts were manually isolated, placed on an Au-coated mirror and dried. Specimens were analyzed with a Bruker Hyperion 2000 microscope coupled to a Bruker Vertex 80v FTIR spectrometer at the Department of Solid State Sciences of Ghent University. Magnification of the microscope was set at 15× and the aperture at 100×100 µm. The combination of detector (liquid N₂ cooled MCT detector), source (Globar) and beamsplitter (KBr) settings restrict the effective infrared spectral range examined to ~4000–650 cm⁻¹. All presented spectra are recorded in reflection mode (deposits on the Au mirror), at a resolution of 2 cm⁻¹, and averaged over 100 scans.

Data were analyzed with OPUS© spectroscopy software (Bruker 2014). The presented absorbance spectra were obtained after background (direct reflection on the Au mirror) subtraction, atmospheric correction and baseline correction using a rubberband correction method using polynomes. Functional groups were identified based on comparison with Colthup et al. (1990) and published data (e.g., Bogus et al. 2014; Cárdenas et al. 2004).

Results

Species description

Phylum Ciliophora Doflein, 1901

Class Prostomatea Schewiakoff, 1896

Order Prorodontida Corliss, 1974

Genus *Hexasterias* Cleve, 1900 emend.

Synonymy.

- 1900 *Hexasterias problematica* Cl. – Cleve, K. Sven. Vetenskapsakad. Handl. 32: 22 (original description of genus and species).
- 1910 *Polyasterias* g.n. – Meunier, Microplancton, p. 87 (junior objective synonym; unjustified replacement name for *Hexasterias*).

Type species. *Hexasterias problematica* Cleve, 1910

Improved diagnosis. Organic-walled cyst with bilayered wall and subspherical to flattened central body from which coplanar radial processes arise from inner wall, are hollow and not connected proximally or distally. Circular pylome in polar position, parallel to the process plane.

Remarks. Cleve (1900) gave no formal description of the genus *Hexasterias*, which is why a diagnosis is provided here. Meunier (1910), who studied plankton from the Barents and Kara seas, proposed changing the genus name to *Polyasterias*, to emphasize that the number of projections is often not six (instead ranging from four to eight). This is an unjustified replacement and thus *Polyasterias* is an objective junior synonym of *Hexasterias*.

Species classified in *Hexasterias*. *Hexasterias problematica* Cleve, 1900. A second species, *Hexasterias spina-trifida*, was described by Wright (1907, p. 9, Plate 3, Fig. 6), but it is not clear if it has any affinity to *H. problematica*. Likewise for *Polyasterias simplex* of Kufferath (1950).

Hexasterias problematica Cleve, 1900 emend.

Figs. 3a–c; Table 2.

- 1887 ‘Röhrenstatoblast’ – Hensen, Ber. Komm. Wiss. Unters. Meere 5: 67, Plate IV, Fig. 27 (first illustration).
- 1897 ‘Dornige cyste’ – Vanhöffen, Die Fauna und Flora Grönlands: 287, Plate VI, Fig. 3 (record from Egersund Fjord, Norway, with illustration).
- 1900 *Hexasterias problematica* Cl. – Cleve, K. Sven. Vetenskapsakad. Handl. 32: 22 (original description, probably no type material available).

- 1904 ‘*Ovum hispidum problematicum*’ (Clev.) Lohm. – Lohmann, Eier und sogenannte Cysten der Plankton-Expedition: 32, Pl. 5, Figs. 2, 4 (invalid by implication, as the genus was not validly published, see Williams et al. 2017, p. 920).
- 1905 *Xanthidium coronatum* nov. sp. – Pavillard, Dissertation: 60, Pl. 3, Figs. 2–3 (invalid because the name was already occupied: *Xanthidium coronatum* Ehrenberg, 1843, Verbr. und Einfluss des Mikrosk. Lebens in Süd- und Nord-Amerika, Pl. 4, Fig. 26).
- 1907 *Hexasterias problematica* Cleve – Wright, Contrib. Can. Biol. Fish. 1: 8, Pl. 3, Fig. 5 (record from eastern Nova Scotia, with illustration).
- 1910 *Polyasterias problematica* (Cleve) pro parte – Meunier, Microplancton: 87, Pl. 5, Figs. 20–22 (redescription and combination with superfluous replacement name *Polyasterias*).
- 1919 *Polyasterias problematica* Meunier – Meunier, Mém. Mus. r. his. nat. Belg. 8: 40, Pl. 23, Figs. 19-20 (record from the Belgian coast, with illustration).
- 1961 *Polyasterias problematica* (Cleve) Meunier – Bursa, J. Fish. Res. Bd. Canada 18: 76, Fig. 5 (record from the Hudson Bay, with illustration).
- 1962 *Polyasterias problematica* (Cleve) Meunier – Brunel, Phytoplankton de la Baie des Chaleurs: 211, Pl. 66 (record from the Baie des Chaleurs, with illustration).
- 1991 “Dinoquiste A-B” – Martínez-Hernández and Hernández-Campos, Palaeontología Mexicana 57: 60, Pl. 4, Figs. 1-4 (record from the Gulf of California, with illustration).
- 1997 Resting stage type C – Nehring, Bot. Mar. 40: 320, Fig. 36 (record from the German bight of the North Sea, with illustration).
- 1998 *Hexasterias problematica* Cleve – Kunz-Pirrung, Ber. Polarforsch. 281: 98, Pl. 5, Fig. 3 (record from the Laptev Sea, with illustration).
- 1999 *Polyasterias problematica* (Cleve) Meunier – Bérard-Therriault et al., Guide de l’Identification: 323, Pl. 149, Fig. a (record from the Gulf of Saint-Lawrence, with illustration).

2018 *Polyasterias problematica* – Kubiszyn and Svensen, Bot. Mar. 61: Fig. 2 (record from the Balsafjord, Norway, with illustration).

Gene sequence. [to be inserted after acceptance]

Original diagnosis (Cleve 1900, p. 22). “Flat disk, diameter 0.04 mm, with six, at the ends truncate and denticulate empty processes, twice as long as the radius of the disk.”

Improved diagnosis. Cyst circular to subcircular outline in polar view (diameter ~100 µm) and flattened in lateral view. Central cyst wall portion (“central body”; average diameter 35 µm) ornamented with four to nine radially arranged coplanar processes which are about as long as the diameter of the central body (33 µm on average). Processes hollow and empty, attached to the inner wall, show longitudinal striations, are broad at the base, open up distally and terminate in numerous (~10 in intact specimens) denticulations which are sometimes recurved. A central circular pylome without operculum is present when there is no cell content, but inconspicuous elsewhere. Cyst wall transparent and colorless.

Studied material. Surface sediments from the Skagerrak, Baltic Sea and the Vilaine estuary (Brittany).

Description. Table 2 gives detailed morphometrics of *Hexasterias problematica*. The number of processes in most cases is six, but may vary from five to eight. The central body diameter and process length are correlated ($R^2 = 0.85$, Fig. 5). The cysts may be somewhat deformed from the coplanar configuration. In palynological preparations, the processes may be torn off, and the cyst is readily deformed. Cell contents include many small transparent to yellow lipid bodies (Fig. 3a–b). Micronucleus circular (3.7 µm in diameter) and macronucleus more irregular and elongated (10.3×12.7 µm) after DAPI staining (Fig. 6).

Remarks. Meunier (1910) reports specimens with four processes, while Bursa (1961) reports nine. In some environments, e.g., Black Sea (Verleye et al. 2009; Mudie et al. 2010), the processes are typically shorter (~13 µm in Verleye et al. 2009). Previous authors made various assumptions about the affinity of *Hexasterias problematica*. Hensen (1887) proposed it is a bryozoan statoblast, and

Cleve (1900) considered it as an “unicellular alga”, without any further specification. Meunier (1910, 1919) rejected earlier propositions of algal affinity. Erdtman (1954) proposed a phytogenous origin based in cell content. Parke and Dixon (1964, p. 529; 1968, p. 815) assigned *H. problematica* to the Pterospermataceae (prasinophytes); however, in Parke and Dixon (1976), it was deleted from the Pterospermataceae, and they suggested it was possibly a desmid zygote. All of these revisions and changes have led to much confusion to this day, with some authors classifying *H. problematica* as a prasinophyte (e.g., Tappan 1980, p. 818), a tintinnid cyst (e.g., Mudie et al. 2010), and others as *incertae sedis* (e.g., Bérard-Therriault et al. 1999). *Hexasterias problematica* sensu Pospelova et al. (2010, Plate VIII, fig. 7) is a desmid. Cleve (1900) referred to chromatophores with an uncertain colour; here it cannot be assessed to what he was referring to.

Occurrence and stratigraphic range. *Hexasterias problematica* Cleve, 1900 has been reported from both surface sediments and plankton samples. Kubiszyn and Svensen (2018) provided a distribution map for *Hexasterias problematica*, which is reproduced in Fig. 7 and extended with additional references from literature and personal observations. Besides the records presented by Kubiszyn and Svensen (2018), *Hexasterias problematica* is reliably recorded from Newfoundland (Lohmann 1904), Etang de Thau (southern France; Pavillard 1905), Nova Scotia (Wright 1907), Gullmarn Fjord (Erdtman 1954), Gulf of California (Martínez-Hernández and Hernández-Campos 1991), the German Bight of the North Sea (Nerhing 1997), the Beagle Chanel (Tierra del Fuego; Candel et al. 2012, 2013). Additional records that not substantiated by morphological data: the Faroe Islands (Ostenfeld 1903), Elbe River (Volk 1905), the west coast of Scotland (Herdman and Ridell 1911), central Atlantic Ocean (Gran 1912), Kola Gulf (Deriugin 1915), Thyborøn, (Denmark; Hansen-Ostenfeld 1916), British Coastal waters (Parke and Dixon 1964, 1968, 1976), Auray Estuary (Brittany; Paulmier 1972), southern Baltic Sea (Ringer 1973), Romanian coast of the Black Sea (Cărauş 2002), offshore Walvis Bay (Mertens et al. 2009a), the Okhotsk Sea (Medvedeva and Nikulina 2014), Vancouver Island fjords (Gurdebeke et al. 2018). Further observations were

made from the Vilaine Estuary (Brittany), Baltic Sea, German Wadden Sea, Harrington Sound (Bermuda Islands) and the East Chinese Sea (Mertens, Gurdebeke, unpublished data).

The cysts are resistant to chemical degradation, shown by the fact that they withstand palynological treatment using strong (hydrochloric, hydrofluoric) acids (see Material and Methods). As a fossil, *H. problematica* is mostly known from late Holocene sediments, with the oldest occurrence from Kyuquot Sound (BC, Canada), around 6.2 ka BP (P. Gurdebeke, pers. obs.). They were also found in late Holocene sediments from the Laptev Sea (Kunz-Pirrung 1998) and Saguenay Fjord (St-Onge et al. 1999), Santa Barbara Basin (Pospelova et al. 2006), the Aral Sea (Sorrel et al. 2006), Black Sea (Verleye et al., 2009; Shumilovskikh et al. 2013) and Effingham Inlet (Bringué et al. 2016).

Genus *Halodinium* Bujak, 1984

1984 *Halodinium* gen. nov.: Bujak, p. 196 (original description).

Type species. *Halodinium major* Bujak, 1984

Original diagnosis (Bujak, 1984). “Discoidal organic-walled microfossils with circular polar outline and irregular membranous flange. Central pylome present with lid often in place.”

Remarks. The genus *Halodinium* was erected by Bujak (1984), based on specimens isolated from Pleistocene North Pacific sediments. From that assemblage, he described *H. major* and *H. minor*. He considered these to be “Microphytoplankton incertae sedis”. Two other species have since been described: *Halodinium scopaeum* by Head (1993, p. 47) and *Halodinium eiriksonii* by Verhoeven et al. (2014, p. 45). *Halodinium* was suggested to be a rhizopod by de Vernal et al. (1989) and an *incertae sedis* alga by Head (1993). The four species of *Halodinium* have been identified globally in Neogene to modern sediments. The oldest published occurrence is *H. eiriksonii* from the Zanclean (early Pliocene) of Iceland (Verhoeven et al. 2014). In the absence of molecular data and incubation experiments, the systematic positions of *Halodinium* and *Hexasterias* remained inconclusive and they were classified as acritarchs by several authors (e.g., Kunz-Pirrung 1998, p. 98).

Species classified in *Halodinium*. *Halodinium minor* Bujak, *Halodinium major* Bujak, *Halodinium scopaeum* Head and *Halodinium eiriksonii* Verhoeven et al.

Halodinium verrucatum sp. nov.

Figs. 3d–l; Fig 4, a–f; Table 3.

Diagnosis. Bilayered, polar compressed cyst with circular to subcircular (average diameter ~70 µm) outline in polar view. Central body (average diameter ~57 µm) ornamented with low ridges forming irregular reticulum; covered over the polar region with a thin, hyaline outer layer ornamented with discrete, bifurcating sculptural elements appearing as verrucae in LM. Near margin of central body portion, outer layer forms a wrinkled, diaphanous collar-like flange with a varying width. Central pylome with thickened rim and without operculum.

Studied material. Surface sediments from the Vilaine estuary (Brittany), the Skagerrak and the Baltic Sea.

Stratigraphical horizon and type locality. Modern sediments from the Vilaine estuary (47.50°N, 2.39°W, 6 m water depth), Brittany (sample BV5 of Mertens et al., 2009b).

Type material. The holotype specimen, isolated from palynological residue BV5 and mounted on SEM stub 16C05 (Fig. 4, a–e), has been deposited in the collection of the National Botanic Garden of Belgium (NBGB) (accession code BR-4516).

Gene sequence. [to be inserted in proof]

Zoobank registration number of *Halodinium verrucatum*. [to be inserted in proof]

Etymology. The species-group name is derived from the Latin word *verruca* meaning wart, referring to the ornamentation as seen in light microscopy.

Description. Detailed morphometrics are given in Table 3. The holotype has a maximum central body diameter 65.0 µm and a maximum diameter pylome of 12.1 µm; the width of its ambital flange is 11.3 µm. Cyst with a strong polar compression and a circular to subcircular outline in polar view (Fig. 4a–b). Cyst wall is bilayered, with the inner wall forming a central body (Fig. 2).

Inner wall of the discoidal cyst about 1.2 μm thick (N=3) and faintly ornamented with low ridges forming a reticulate pattern with varying mesh size ($< 1 \mu\text{m}$), only visible with high magnification (1000 $\times$) LM and SEM (Fig. 4d). Pylome central to the inner cyst wall, with slightly thickened rim and surrounded by a shallow circular depression of $\sim 2 \mu\text{m}$ (Fig. 4d). The specimen with cell content illustrated in Fig. 3d–f, has a transparent and colorless operculum in place, but the fine structure is obscured by the cell content. Palynological specimens (e.g., Fig. 4) show no trace of an operculum. The hyaline outer layer is much thinner ($\sim 0.2 \mu\text{m}$) than the inner layer and is ornamented with solid, slender sculptural elements with a widened base. With medium magnification (400 $\times$, LM), they often appear as verrucae (Fig. 3i–k) because they are recumbent (Fig. 4e), but higher LM magnifications and SEM reveal them as process-like sculptural elements. The discrete sculptural elements are of equal length (average of 1.7 μm , Table 3) and bifurcate at circa two third of their length (Fig. 4e). SEM reveals an occasional second bifurcation near the top of some sculptural elements (Fig. 4e). The sculptural elements are irregularly distributed and the distance between them averages 1.6 μm (Table 3). The cyst outer wall layer separates from the central body near the periphery and evolves into a folded, translucent outer flange producing a coarse reticulate appearance (Fig. 4b). The delicate outer flange is often observed as torn or bent with an irregular or jagged appearance (Fig. 3h, Fig. 4a). The width of the outer flange observed on well-preserved specimens averages between 9.3 μm and 25.3 μm (Table 3). All observed specimens are pale and are difficult to observe under transmitted LM (Fig. 3). Sometimes, several specimens occur in clusters (Fig. 3g). Cell contents include many small transparent to yellow lipid bodies (Fig. 3d–f). Details on other organelles such as a central vacuole or the nuclear apparatus are lacking. Specimens from different studied populations are identical in all studies features.

Comparison. *Halodinium verrucatum* sp. nov. is distinguished from all other *Halodinium* species by the ornamentation of the outer wall layer (Table 4). The diameter of the central body of *Halodinium major* Bujak, 1984 and *H. minor* Bujak, 1984 are larger (104–116 μm and 46–63 μm , respectively). Furthermore, their outer flange is unornamented. *Halodinium scopaeum* Head, 1993

is much smaller (average diameter central body: 21–24 μm), and possesses a smooth to scabrate ornamentation. *Halodinium eirikssonii* Verhoeven et al., 2014 has a smaller central body (23.9–27.4 μm), a rugulate outer wall layer and a complex ambital flange. Species of the genus *Cyclopsiella* Drugg and Loeblich, 1967 possess a more reduced ambital flange and an eccentric pylome (e.g., Matsuoka and Head 1992). Cysts of *Chattonella* sp., as illustrated by Matsuoka and Iishi (2018), are hemispherical and lack a flange and ornamentation.

Occurrence and stratigraphic range. *Halodinium verrucatum* n. sp. is described here from modern sediments in the type locality (Brittany) and the Baltic Sea, and withstands palynological treatment. This species has been recorded from modern sediments from the Skagerrak, Croatia, and Effingham Inlet (British Columbia) as well (unpublished data; see Fig. 7).

Phylogenetic position of *Hexasterias problematica* and *Halodinium verrucatum* sp. nov. based on LSU rDNA and SSU rDNA

Identical SSU rDNA (1691 bp) and partial LSU rDNA (1230 bp) sequences were obtained from three out of four specimens of *Hexasterias problematica* (Accession numbers: [to be inserted in proof]). For *Halodinium verrucatum* sp. nov., SSU rDNA (1699 bp) was obtained from a single specimen and identical LSU rDNA (1139 bp) from two specimens (Accession numbers: [to be inserted in proof]). The SSU sequences were used for phylogenetic analyses (Fig. 8), as there are more sequences available than for LSU.

In the SSU phylogeny, *Hexasterias problematica* formed a well-supported clade with the planktonic, marine, prostome ciliate *Balanion masanensis* (Fig 7; Kim et al. 2007). This clade showed a sister relationship with another clade that includes *Halodinium verrucatum* sp. nov. and the prostome species *Urotricha* sp., *Plagiocampa* sp. and *Cryptocaryon irritans* Brown (Colorni and Burgess 1997; Foissner et al. 1994, 1999; Ma et al. 2017). Both clades formed a monophyletic clade with 72% bootstrap support (Fig. 8).

Hexasterias and *Halodinium* micro-FTIR analyses

Three specimens of *Halodinium* spp., three specimens of *H. verrucatum* sp. nov. and five specimens of *Hexasterias problematica* were measured (Fig. 9). See Table 5 for absorptions and assignments.

The consistent absorption spectra for the same species from one location (e.g. the *Halodinium verrucatum* sp. nov. spectra from the Strö 6 station) indicates reproducibility of the method. The general consistency of spectra from one species from several locations indicates they truly reflect species composition. The remaining variation (e.g. specimen M4S13) is probably due to differing environmental conditions (cf. Bogus et al. 2014; Mertens et al. 2015; Gurdebeke et al., accepted).

Consistently strong (and often sharp, e.g., M4S28) absorptions occur at $\sim 3280\text{ cm}^{-1}$ (N-H stretch), with a shoulder at $\sim 3400\text{ cm}^{-1}$ (O-H stretch) and a minor peak around 3100 cm^{-1} . Absorptions indicative of aliphatic CH-stretching occur in the $3000\text{--}2800\text{ cm}^{-1}$ region.

The spectral region between 1800 and 1500 cm^{-1} dominates in all measured spectra, with specific maxima at 1620 , 1570 and 1550 cm^{-1} and a distinct shoulder at 1640 cm^{-1} . Nitrogen-containing (amide) group absorptions are evident in the spectra, particularly the amide I and II bands around 1600 cm^{-1} , and minor amide III and V bands around 1312 and 690 cm^{-1} , respectively.

The absorptions in the region between 1200 and 1000 cm^{-1} are indicative of C-O-C asymmetric vibration ($\sim 1160\text{ cm}^{-1}$), glucose ring stretch ($\sim 1110\text{ cm}^{-1}$) and C-O stretching ($\sim 1040\text{ cm}^{-1}$). They are indicative of polysaccharides but lacking diagnostic absorptions of β -linkage around 900 cm^{-1} (Cárdenas et al. 2004; Kačuráková et al. 2002).

There is variability in the composition of *Halodinium* spp. specimens (Fig. 9), in that several of the specimens showed relatively weak absorptions for C-O bonds, and the position of the strongest peak was either 1620 cm^{-1} (*Halodinium* sp. from the Irish Sea and Brittany) or 1570 cm^{-1} (*H. verrucatum* sp. nov. from the Skagerrak). These compositional differences may reflect interspecific or environmental differences. In earlier studies most *Halodinium* specimens from Brittany and the Irish Sea were found to be *H. minor*. The *Hexasterias problematica* spectra also

show variability in the relative strength of the absorptions, specifically the specimen (M4S13) where the absorptions from 1160 to 1040 cm⁻¹ are stronger than in the others.

The absorption spectra recorded for the tintinnid loricae (*Parafavella* sp. and *Stenosemella* sp.) are very similar to *Hexasterias* and *Halodinium*. The two spectra of *Parafavella* sp. specimens from Skagerrak are more or less identical (Fig. 10); the spectrum of *Stenosemella* sp. differs in having more pronounced shoulders at 1640 cm⁻¹ and 1550 cm⁻¹, and there are some differences in the spectral region around 1400 cm⁻¹.

Radiosperma corbiferum cysts (Fig. 10) again show similarities with *Hexasterias* and *Halodinium*, with maximal absorptions at 1620 or 1570 cm⁻¹ and a similar shape in the region around 1400 cm⁻¹. There are some differences, however, for instance there is an additional small absorption around 3440 cm⁻¹ and relatively stronger absorptions in the region 1040–1160 cm⁻¹, especially at 1040 cm⁻¹.

Discussion

Phylogenetic implications and relation to acritarchs

The rDNA sequences extracted from *Hexasterias problematica* and *Halodinium verrucatum* sp. nov. reveal a phylogenetic placement within the ciliate order Prorodontida (Gao et al. 2016). The genealogical structure and monophyly of the Colepidae, Prorodontidae, and Placidae (see Fig. 8) is in agreement with earlier studies (Gao et al. 2016; Lipscomb et al. 2012; Mordret et al. 2016; Zhang et al. 2014). The placement with the ciliates is further supported for *Hexasterias problematica* by the DAPI-staining, which shows a dual nuclear apparatus (Fig. 6), a derived character of the ciliates. This is in line with earlier observations of ciliate nuclei with DAPI staining (e.g., Bellec et al. 2014). Further observations of nuclear dualism should be made with the classic approach using TEM (e.g., Grimes 1973).

In this data set, *Hexasterias problematica* is most closely related to *Balanion masanensis*, described by Kim et al. (2007) from coastal waters off South Korea. *Halodinium verrucatum* sp.

nov. is most closely related to the freshwater *Urotricha* sp. (EU024981) with which it forms a sister clade to *Plagiocampa* sp. and the fish parasite *Cryptocaryon irritans*, both marine species (Wright and Colorni 2002; Zhang et al. 2014). The clade formed by these has been reported previously (Zhang et al. 2014), but the nature of the relationship with the Class Plagiopylea remains unclear (Gao et al. 2016).

Within the free-living Ciliophora, the formation of resting cysts is common (see reviews in Gutiérrez et al. 2001 and Verni and Rosati 2011), but detailed information on morphology and physiology is only known for a very limited number of species (Verni and Rosati 2011). Most well-described palynologically preservable cysts are assigned to the class Spirotrichea (Reid and John 1978, 1983). For the class Prostomatea, cysts have been described for *Prorodon* (Hiller and Bardele 1988) and *Cryptocaryon irritans* (Ma et al. 2017).

It is hypothesized that the palynomorphs *Hexasterias problematica* and *Halodinium verrucatum* sp. nov. represent cryptobiotic cyst stages of prorodontid ciliates. The classification of the Order Prorodontida is based on characteristics not present in cyst stages, such as the nature of the cytostome, kinetids and the brosse (Lynn 2008), and life cycle stages of a single species can be significantly heteromorphic (Hiller and Bardele 1988). Thus far, there are no morphological observations of the ciliates that supposedly produce the cysts *Hexasterias problematica* and *Halodinium verrucatum* sp. nov., and consequently the placement of these species within the order Prorodontida cannot be evaluated in light of diagnostic characteristics of their planktonic stages. Incubation experiments, which have been unsuccessful to date, or increased molecular taxon sampling could provide further insights. This situation stresses the importance of including cyst morphology in future ciliate species descriptions.

The average size of *Halodinium verrucatum* and *Hexasterias problematicum* is within the range of other known ciliate cysts (see Table II in Verni and Rosati 2011). The disk-shaped morphology, however, is unusual for ciliate cysts, which are typically globular or flask-shaped. This suggests that the morphological disparity within ciliate cysts might be larger than expected. The

double cyst wall texture of *Halodinium verrucatum* sp. nov. agrees with the observation that ciliate cyst walls in general differentiate between a thick inner and a thinner outer cyst wall (Matsusaka et al. 1989; Verni and Rosati 2011). TEM would be needed to evaluate the ultrastructure of the inner cyst wall, which in many cases is multi-layered in ciliate cysts (Verni and Rosati 2011).

It is expected that morphologically similar, two-layered marine acritarchs with a pylome, such as the other *Halodinium* species, *Radiosperma corbiferum*, and *Cyclopsiella* (e.g. Matsuoka and Head 1992) may also be affiliated with the ciliates. An affinity with the ciliates has also been tentatively proposed for other acritarchs with pylomes, such as species of *Palaeostomocystis* (Roncaglia 2004; Warny et al. 2009). It should be noted, however, that other organic microfossils with a circular opening exist (e.g., testate amoeba, desmids, cysts of the raphidophycean *Chattonella*), but these differ in structure, composition and autecology. For many extinct acritarch species with a pylome structure, described from older sediments, a ciliate affinity may be considered, e.g. the Proterozoic *Appendisphaera* (Moczyłowska et al. 2011) or the Ordovician *Velatasphaera* (e.g., Miller and Williams 1988). However, because of the impossibility of obtaining molecular data for these species, combined observations of detailed morphology, wall structure, chemical composition and paleoecology would be needed to support this affiliation (Aroui et al. 1999; Marshall et al. 2005).

Distribution and ecology of *Hexasterias problematica* and *Halodinium verrucatum* sp. nov.

Specimens of *Hexasterias problematica* are typically recovered from plankton and surface sediments in estuarine and coastal areas in temperate zones of the northern and southern hemisphere (Fig. 7). Occurrences range from fresh to fully marine waters. Mudie (1992) linked the abundances in surface sediments with river/meltwater input. Indeed, sediment trap studies in British Columbia found a positive relationship between *H. problematica* abundance and freshwater input (Pospelova et al. 2010; Price and Pospelova 2011). Consequently, *H. problematica* has been used as an indicator for freshwater input in paleoecological studies (e.g., Bringué et al. 2016). In all, it seems

that *H. problematica* is a euryhaline species, occurring from fresh to marine environments, but with maximal abundances in the case of lowered salinities (brackish water) due to freshwater discharge. The short (average of 16.2 μm , $n = 4$ in Verleye et al. 2009) processes noted for specimens from the Black Sea (e.g., Verleye et al. 2009; Mudie et al. 2010) suggest a possible covariance of process length with environmental variables such as salinity. Similar relationships were noted for dinoflagellate cyst species *Lingulodinium machaerophorum* (Mertens et al. 2009b; Verleye et al. 2009) and *Operculodinium centrocarpum* (Mertens et al. 2011) and allow application of these microfossils as a proxy for paleoenvironmental conditions (e.g., Mertens et al. 2012b; Bringué et al. 2016). The relation should be explored quantitatively for *Hexasterias problematica*.

For *Halodinium*, many authors do not distinguish species, preventing definite statements about the distributions of individual species. The sparse occurrence data of *Halodinium verrucatum* sp. nov. suggests it occurs in coastal estuarine environments with brackish marine salinities (Aure et al. 1993; Arapov et al. 2017). A similar ecology is often assigned to “*Halodinium* sp.” in palynological studies (e.g. Matthiessen 1995) and to *Halodinium minor* in the sediment trap study of Price and Pospelova (2011).

In the light of their phylogenetic position (Fig. 8), both *Hexasterias* and *Halodinium* are grouped in a clade with mainly marine species, except for *Urotricha* sp., that is most closely related to *Halodinium verrucatum* sp. nov. and was sequenced from lacustrine plankton (Auinger et al. 2008). This large genus includes mostly freshwater but also marine species (e.g., Foissner et al. 1999; Wilbert and Song 2008).

The irregular flange rim and the monospecific clusters of specimens of *Halodinium verrucatum* sp. nov. (Fig. 3g) are not unlike the morphology described by Matsuoka and Head (1992) for *Cyclopsiella*, suggesting an attached, perhaps encrusting mode of life.

Geochemical composition

The measured spectra bear some similarity to a carbohydrate-based macromolecule described from heterotrophic dinoflagellates (Bogus et al. 2014; Mertens et al. 2015), e.g., the abundant sugar group component, specifically in *Halodinium* spp. from Brittany and the Irish Sea, and the amide functional groups. There are significant differences, however, suggesting that the composition here is not dinosporin. In particular, strong C-O bonds and evidence for a β -glycosidic linkage are lacking. The spectra also differ from chitin (Cárdenas et al. 2004), which was the cyst composition found by e.g. Foissner et al. (2005) and Verni and Rosati (2011) using a Van Wesselingh test. This evidence supports the rejection by Agatha and Simon (2012) for a chitinous composition of ciliate loricae and cysts.

The spectra of the loricae of the tintinnids *Parafavella* sp. and *Stenosemella* sp. indicate a comparable composition and corroborate the results of Agatha and Simon (2012), who evaluated tintinnid loricae using a multitude of methods, and suggested a nitrogen-rich proteinaceous composition. These authors also found, however, that polysaccharides are never the main component of tintinnid loricae.

The compositional similarity of *Halodinium verrucatum* and *Hexasterias problematica* with the loricae supports their placement in the ciliates revealed by the genetic data. The scarce reports on ciliate cyst wall chemistry (Izquierdo et al. 2000; Wang et al. 2017) report a composition of mainly carbohydrates and (glyco)proteins. Wang et al. (2017) found, using electrophoresis combined with ionization mass spectrometry, that the protein composition within a single species is very diverse (42 proteins), a resolution that cannot be achieved by single specimen micro-FTIR.

The chemical composition of both *Hexasterias* and *Halodinium* indicates that they are formed by heterotrophic organisms, evidenced by amide groups in the wall chemistry (see Table 5), a result also found for cysts of heterotrophic dinoflagellates (Bogus et al. 2014). This is also in accordance with their placement with the ciliates, which are nearly all heterotrophic (Lynn 2008). This illustrates that cyst wall chemistry is a more reliable indicator for trophic preference of the

producing organism than are cyst color and autofluorescence, the approaches often applied with dinoflagellate cysts (Brenner and Biebow 2001; Bogus et al. 2014).

Finally, the acritarch *Radiosperma corbiferum* shows a similar composition, which would further support a ciliate affinity of acritarchs with a similar morphology, although genetic data is needed to confirm this. These results complement earlier studies applying spectroscopical techniques to fossil acritarchs to test hypotheses about their affinity (e.g., Arouri et al. 2000; Dhamelincourt et al. 2010).

5. Conclusions

Two palynomorphs, *Hexasterias problematica*, and a new species of the genus *Halodinium*, *H. verrucatum* sp. nov., were retrieved from surface sediments from Brittany, the Skagerrak and the Baltic Sea. Phylogenetic analysis using rDNA (LSU and SSU) revealed that these species belong to the ciliates (phylum Ciliophora), more specifically within the order Prorodontida. The ciliate nature is supported by the presence of a micro- and macronucleus. Both species represent cryptobiotic cyst stages of hitherto undescribed ciliate species.

This placement is supported by the cyst wall chemistry, which, evaluated using micro-FTIR, is found similar to that of loricae of the tintinnid ciliates *Stenosemella* sp. and *Parafavella* sp. The cyst wall is carbohydrate-based and contains nitrogen-containing functional groups. The composition is in accordance with the heterotrophic nature of the ciliates.

Hexasterias problematica and species of *Halodinium* have a global distribution, mainly in temperate coastal waters, and are often encountered in palynological studies. The affinity with the ciliates provides a better understanding of their ecological significance as an indicator for coastal conditions and freshwater influence.

Finally, the results of this study highlight the potential utility of micro-FTIR in understanding acritarch affinity, especially for fossil taxa for which molecular data are unavailable. As a test case, an acritarch with a similar morphology, here represented by *Radiosperma*

corbiferum, was found to have a similar composition and possibly has a similar affinity, which should be confirmed by molecular data.

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Fig. 1. Map showing the five sampling sites (red dots) of surface sediments in this study. 1. Vilaine estuary, Brittany; 2. stations ‘Strö 1’ and Strö 6’, South of Havstenstund (Skagerrak, Sweden); 3. Lysekil, station ‘Lyse 3’ (Skagerrak, Sweden); 4. Galterö (Skagerrak, Sweden); 5. Sällvik, Pojo Bay (Baltic Sea, Finland).

Fig. 2a–e. Schematic drawing of *Hexasterias problematica* (a, b) and *Halodinium verrucatum* sp. nov. (c–e), depicting the morphological features discussed in text.

Fig. 3a-l. *Hexasterias problematica* (a-c) and *Halodinium verrucatum* sp. nov. (d-l), in LM (a-b, d-l) and SEM (c). **a-b:** cysts with cytoplasm from the Baltic Sea (Sällvik); **c:** empty cyst from the Skagerrak (Lyse 3); **d-f:** cysts with cytoplasm from the Skagerrak (Stro 1); **g-l:** empty cysts from the Skagerrak (Lyse 3), showing a cluster (g) and a specimen in polar view, obscured by amorphous organic matter.

Fig. 4a-f. *Halodinium verrucatum* sp. nov., holotype from the Vilaine estuary (a-e) and specimen from Kaštela Bay, Croatia (f), in SEM. **a**: oral view; **b**: oblique oral view; **c**: detail of wall rupture and sculptural elements; **d**: detail of pylome, with arrows indicating the texture of the inner wall (left) and the thickened rim of the pylome (right); **e**: detail of sculptural elements. Scale bar is 20 µm unless otherwise indicated.

Fig. 5. Correlation of central body diameter and process length of *Hexasterias problematica*.

Fig. 6. DAPI-stained cell of *Hexasterias problematica* from the Vilaine Estuary (Brittany), showing micro (MI)- and macronucleus (MA).

Fig. 7. Map showing the distribution of *Hexasterias problematica* (red and small brown circles) and *Halodinium verrucatum* sp. nov. (yellow squares). The occurrences of *Hexasterias problematica* are based on Kubiszyn & Svensen (2018), supplemented with additional published and personal observations (see section Occurrence and stratigraphic range in Species descriptions). The small brown circles represent occurrence data from OBIS, as in Kubiszyn & Svensen (2018).

Fig. 8. Phylogenetic placement of *Hexasterias problematica* and *Halodinium verrucatum* sp. nov. based on SSU rDNA sequences, with indication of GenBank accession numbers, existing classification (e.g., Gao et al. 2016) and ecological preferences (e.g., Lynn 2008).

Fig. 9. Normalized micro-FTIR absorbance spectra of *Halodinium* spp. (Irish Sea and Brittany), *Halodinium verrucatum* sp. nov. (Strö 6) and *Hexasterias problematica*, with indication of the major absorptions (cf. Table 5). Specimen numbers (MxSy) refer to unique measurement codes.

Fig. 10. Normalized micro-FTIR absorbance spectra of ciliate loricae (German Wadden Sea and Skagerrak) and *Radiosperma corbiferum* (Brittany, Irish and Baltic Seas), with indication of the major absorptions (cf. Table 5). Specimen numbers (MxSy) refer to unique measurement codes.

Table 1. Region, site location, latitude, longitude and water depth of samples from Brittany, the Skagerrak and the Baltic Sea used in this paper.

Region	Sampling site	Latitude (°N)	Longitude (°E)	Water depth (m)
Brittany	Vilaine Estuary	47.5	2.39	6
Baltic Sea	Sällvik (Pojo bay, Finland)	60.02	23.48	34.5
Kattegatt	Galterö	57.11	11.81	40.5
Skagerrak	“Strö 1”, South of Havstensund (Sweden)	58.73	11.17	31
Skagerrak	“Strö 6”, west of Strömstad (Sweden)	58.85	10.76	100
Skagerrak	“Lyse 3”	58.34	11.36	29

Table 2. Morphometric data for *Hexasterias problematica*, based on material from Brittany and the Skagerrak.

	Arithmetic mean	Median	SD	CV	Min	Max	N
Total diameter (µm)	102.2	99.1	14.9	14.58	82.8	130.2	12
Pylome diameter (µm)	22.4	22.1	1.8	8.04	18.9	25.5	9
Central body diameter (µm)	35.2	34.5	3.9	11.08	29.5	42.4	15
Process length (µm)	33.4	31.6	5.5	16.47	26.6	43.9	12
Process width (µm)	2.7	2.7	0.8	28.41	2	3.6	9
Number of processes	6.5	6	0.9	13.85	5	8	12
Number of denticulations	9.7	10	0.5	5.15	9	10	6

Table 3. Morphometric data for *Halodinium verrucatum* sp. nov. from Brittany and the Skagerrak

	Arithmetic mean	Median	SD	CV	Min	Max	N
Total diameter (µm)	70.2	67	8.5	12.1	60.4	87.5	21
Pylome diameter (µm)	14.2	13.7	2	14.1	10.5	18.5	24
Central body diameter (µm)	56.5	53.7	8.1	14.3	47	74	24
Flange width (µm)	13.4	12.4	3.9	29.1	9.3	25.3	15
Length of sculptural elements (µm)	1.7	1.7	0.2	9.4	1.6	2.1	10
Width of sculptural elements (µm)	0.13	0.12	0.02	15.4	0.1	0.18	15
Width of sculptural element basis (µm)	0.39	0.38	0.03	7.7	0.36	0.45	8
Distance between sculptural elements (µm)	1.6	1.6	0.3	16	1.3	2.3	21

Table 4. Comparison of diagnostic features of the species of *Halodinium*.

Species	<i>Halodinium major</i>	<i>Halodinium minor</i>	<i>Halodinium scopaeum</i>	<i>Halodinium eiriksonii</i>	<i>Halodinium verrucatum</i> sp. nov.
Body diameter	104 - 116 µm	46 - 63 µm	21 - 24 µm	23.9 - 27.4 µm	47.0 (56.5) 74.0 µm
Pylome diameter	24 - 25 µm	11 - 22 µm	5.5 - 8.0 µm	4.6 - 8.8 µm	10.5 (14.2) 18.5 µm
Max. flange width	9 - 27 µm	4.5 - 9 µm	3 - 5.5 µm	1.6 - 6.8 µm	9.3 (13.4) 25.3 µm
General shape	Discoidal with circular polar outline and irregular membranous flange.	Discoidal with circular polar outline and irregular membranous flange.	Central body has a strong polar compression and circular to subcircular outline in polar view.	Small, polar compressed, with circular to subcircular outline.	polar compressed cyst with a circular to subcircular outline in polar view.
Wall	-	-	Bilayered, pale yellow-brown. Periphragm much thinner than endophragm, to which it is appressed except at ambitus.	Bilayered, outer wall is appressed to the inner wall over the greater part of the cyst body.	Bilayered,
Nature of the wall and ornamentation	The wall of the main body has a granulate or occasionally rugulate surface	The wall of the main body has a smooth surface	Smooth or faintly scabrate. Periphragm visible as small wrinkles over the surface of the endocyst.	Scabrate to smooth outer wall has a rugulate surface consisting of occasionally anastomosing wrinkle-like ridges.	Central body ornamented with low ridges forming an irregular reticulum, and is covered over the polar region with a thin, hyaline outer layer ornamented with discrete, bifurcating sculptural elements.
Pylome	Central pylome with well-defined ridge on, or close to edge of pylome. Operculum often in place; circular with a smooth edge that is usually thickened, although the thickening may occur a few micrometers away from the pylome on the main body.	Central pylome present, with operculum often in place. The central pylome is circular with a smooth edge that is occasionally thickened.	Pylome located centrally and circular or subcircular, with an entire and usually unthickened margin.	Circular without operculum.	A central pylome with a thickened rim; operculum not observed.
Ambital flange	The surrounding membrane extends around the main body and is thin, pale and often broken or torn	The surrounding membrane extends around the main body and is thin, pale and often broken or torn.	Diaphanous flange of approximately constant width; its outer margin usually ragged but may be partially entire on some specimens.	At the periphery of the cyst, the outer wall forms a collar-like ambital flange, consisting of irregular septate or foldlike structures.	The outer layer forms near the margin of the central body a wrinkled, diaphanous collar-like outer flange with a varying width.
Reference	Bujak (1984)	Bujak (1984)	Head (1993)	Verhoeven et al. (2013)	This paper

Table 5. FTIR assignments of functional groups present in *Halodinium* spp. and *Hexasterias problematica* (absorptions in bold are shown on Figs. 9 and 10).

Wavenumber (cm ⁻¹)	Assignment*	Comments
~3400	vOH	shoulder
3280	vNH	
3100	vNH	
2970	vCH3	
2950	vCH2	
2860	vCH3	
1640	vC=O	
1620	vC=O	Amide I
1580, 1550	vCN+ δNH	Amide II
1420	δCH ₂	
1370	δCH + δC-CH ₃	
1312	vCN+ δNH	Amide III
1255	δNH	
1160	vC-O-C	Ring
1110	vC-O	
1040	vC-O	
930	γCH ₃	
860, 770	ρCH ₂	
690	γNH	Amide V

* v means bond stretching, δ means deformation, γ means wagging and ρ means rocking of a bond within a molecule.