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Benthic flattened cells of the phylogenetically related marine dinoflagellates *Protoceratium reticulatum* and *Ceratocorys mariaovidiorum* (Gonyaulacales): a new type of cyst?[1]

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ABSTRACT

A planktonic-benthic relationship has been described for many dinoflagellate species as part of their ecological strategy to overcome highly variable aquatic environments. Here, the phylogenetically and morphologically related marine dinoflagellates *Protoceratium reticulatum* and *Ceratocorys mariaovidiorum* were studied in relation to an unknown benthic life form. In vivo and fixed samples from cultures were analyzed in detail by light and scanning electron microscopy. In both species, a cell type with a morphology different from that of vegetative cells was observed in cultures grown until stationary phase. This cell type was always benthic, swimming sporadically only when it was disturbed. Its main feature included a strong dorso-ventral compression. These cells originated from vegetative cells whose protoplasm underwent a progressive flattening, resulting in a gradual detachment of the reticulate and thick thecal plates and the formation of very thin non-reticulated new plates with pores. When returned to fresh full strength medium, the cells recovered their spherical

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vegetative-like morphology, including new reticulated thick plates and subsequent cell divisions. The kinetics of flattened cell formation showed that in both species this cell type increased exponentially until the onset of the culture stationary phase and then decreased. The results of this study are discussed in the context of the planktonic-benthic coupling in dinoflagellate life cycles, including those newly appreciated to be well adapted to the benthic environment.

Key index words: benthic, *Ceratocorys mariaovidiorum*, Dinoflagellate, flattened cell, planktonic-benthic coupling, *Protoceratium reticulatum*, thin non-reticulated plates

Abbreviations: BI, Bayesian inference; CCVIEO, culture collection of toxic microalgae at the Spanish Institute of Oceanography in Vigo; ITS rRNA, internal transcribed spacer rRNA; L:D, light:dark; ML, maximum likelihood; NCMA, Provasoli-Guillard National Center for Marine Algae and Microbiota.

INTRODUCTION

Dinoflagellates are highly ecologically diverse eukaryotic organisms and one of the major groups of phytoplankton. Adaptation to a wide variety of environmental conditions and/or to seasonality has made them develop a great diversity of life forms (Hackett et al. 2004, Bravo and Figueroa 2014). In addition to their better known motile vegetative cells, which live in the water column as part of the plankton, ~10% of the all known planktonic marine dinoflagellate species produce benthic resting cysts as part of their life cycle (Dale 1983). Among these dinoflagellate cysts there are enormous differences in the main phenotypic, physiological and resistance properties. The benthic resting stages participate in the planktonic-benthic coupling of phytoplankton and it is known that they play an important role in the ecology of the species, enable its dispersion, and undergo sexual recombination (Anderson and Wall 1978). However, many gaps remain unknown about their origin and functionality. While they remain in the sediment layer when the conditions for vegetative growth are unfavorable, they reinoculate the water column when favorable conditions are restored. These species are therefore constituents of the so-called meroplankton, defined as plankton that spends part of its life in the benthos. They are distinguished from the holoplankton, whose members are planktonic throughout their life cycle (Fogg 1991). Thus, assignment of a species to one or the other classification requires knowledge of its ecology and especially of its life strategy.

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During the last decades, the complexity of dinoflagellate life cycles has been evidenced, which, at least in some species, reflects the adaptive capacities of these organisms with respect to light intensity, turbulence, nutrient availability and other parameters. In addition, diverse functions of cysts in the dinoflagellate life cycle have been recognized, leading to a revised concept of what is considered as a cyst (Bravo and Figueroa 2014). This diversification along with the appearance of cysts differing in their endogenous/exogenous encystment properties, as well as in the factors triggering excystment, demonstrate the intricate life-cycle strategies of dinoflagellates. This flexibility allows the growth and maintenance of cell populations in highly variable environments. In addition to well-studied resting cysts, there is a lack of knowledge about the importance of thin-walled non-dormant cysts in relation to the survival of dinoflagellates. These non-dormant cysts (i.e., without a mandatory dormancy period) were first called "thin-walled" cysts (Fritsch 1935), and later on as ecdysal, pellicle or temporary cysts (Dale 1983, Garcés et al. 2002, Bravo et al. 2010) because of their ecdysal origin, pellicle-layer wall, and absence of dormancy. They were generally described in culture, associated with the vegetative cycle and in short-term or sudden adverse conditions. However, both the sexual and asexual stages of the life cycle can lead to the formation of pellicle cysts (Figueroa et al. 2006). Furthermore, division of cysts or benthic stages of planktonic species seems to be common in some species of dinoflagellates. So, division of non-motile stages or so-called division cysts occurs in a few dinoflagellate species, for example, *Tovellia apiculata* (as *Woloszynskia apiculata* Stosch), *Alexandrium taylori*, *Protoperidinium steidingerae*, *Protoperidinium depressum*, *Kryptoperidinium foliaceum*, *Gymnodinium quadrilobatum* and *Pfiesteria piscicida* (von Stosch 1973, Horiguchi and Pienaar 1994, Garcés 2001, Litaker et al. 2002, Figueroa et al. 2006, Gribble et al. 2009). The flagellated cells shed their flagella and theca, round up, and sink, subsequently forming cysts that undergo division within minutes to hours after encystment. In other species as for example in *Crypthecodinium cohnii*, *Pfiesteria piscicida* and *Alexandrium pseudogonyaulax* swimming cells and division cysts have been described (Bhaud et al. 1991, Zmerli Triki et al. 2015), though division occurs only in the latter forms (Kubai and Ris 1969, Bhaud et al. 1991, Litaker et al. 2002).

Ceratocorys mariaovidiorum has been recently described (Salgado et al. 2018) based on morphological, molecular phylogenetic, and toxin studies of the strains CCMP404 and CCMP1720 previously reported as *Protoceratium reticulatum*. In this study, we investigated the significance of a different life form that occurs during the life cycle of the dinoflagellates *P. reticulatum* and *C. mariaovidiorum*. The results described herein suggest that the ecology of these phylogenetically close species derives from a much more intense planktonic-benthic coupling than previously known, which contributes to the plasticity of the life cycle strategy of both organisms.

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MATERIALS AND METHODS

Culture conditions. The strains of *Protoceratium reticulatum* employed in this study were isolated from the southern Chilean waters, Catalan coast (Mediterranean coast of Spain) and Galician coast (North West of Spain) and kept at the Culture Collection of Harmful Microalgae of the Instituto Español de Oceanografía in Vigo (CCVIEO; http://www.vgohab.es/), and those of *Ceratocorys mariaovidiorum* were obtained from the National Center for Marine Algae and Microbiota (NCMA, ME, USA; Table 1). The cultures were maintained in Erlenmeyer flasks filled with 50 mL of L1 medium (Guillard and Hargraves 1993) without silicate, prepared with Atlantic seawater from off the Ría de Vigo (Spain) and adjusted to a salinity of 32 by the addition of sterile bi-distilled water. The incubation temperatures were 15ºC ± 1ºC for *P. reticulatum* from southern Chile, 19ºC ± 1ºC for *P. reticulatum* from Catalan and Galician coasts, and 24ºC ± 1ºC for *C. mariaovidiorum* from USA waters. The photoperiod cycle was 12:12 h light:dark (L:D) with a photon flux from white fluorescence light of ~100 μmol · m^{-2} · s^{-1}.

Light microscopy and cell measurements. Light microscopy (LM) studies to characterize the morphology and behavior of *Protoceratium reticulatum* and *Ceratocorys mariaovidiorum* flattened cells were carried out with specimens isolated from cultures grown in culture-plate wells of 35mm (Thermo Fisher Scientific, Waltham, MA, USA) incubated in the same conditions mentioned above and using a Zeiss Axiovert 135 inverted microscope (Carl Zeiss, Göttingen, Germany). Digital photos and video recordings of cells were obtained with a Canon EOS 5D Mark II camera (Canon Inc., Tokyo, Japan). From these cultures, live samples were stained with SYBR green (Molecular Probes, Eugene OR, USA) for observation of the nuclei and formalin-fixed samples stained with Calcofluor white (Fluorescent Brightener 28, Sigma; Fritz and Triemer 1985) to characterize the thecal plates using a Leica DMLA microscope (Leica Microsystems, Wetzlar, Germany) equipped with UV epifluorescence and an AxioCam HRc camera (Carl Zeiss, Göttingen, Germany). Additionally, 60 vegetative-like cells of each species, and 25 and 27 flattened cells of *P. reticulatum* and *C. mariaovidiorum*, respectively, were isolated and measured according to their length and width, and depth when it was possible, at 63× magnification using the AxioCam HRc camera. The statistical analyses were performed using IBM SPSS Statistics software v.21. One-way ANOVA or Kruskal–Wallis tests were used to identify significant differences ($P < 0.05$) in morphometric measurements between vegetative and flattened cells. Morphological identification of the species was based on literature descriptions (e.g., Reinecke 1967, Hansen et al. 1996/97, Salgado et al. 2018).

Scanning Electron Microscopy. For scanning electron microscopy (SEM) samples of 3 mL of stationary phase cultures were fixed with glutaraldehyde at a final concentration of 4%. After 24 h at room temperature, the fixed cells were filtered through 5 μm pore size Isopore RTTP polycarbonate filters (Merck Millipore, Billerica, MA, USA), stained with 2% osmium tetroxide for 30 min, rinsed three times with distilled water, and dehydrated in a series of 30, 50, 70, 90, 95 and 100% EtOH (All chemicals for SEM were from Sigma-Aldrich, Darmstadt, Germany). They were then air-dried overnight, coated with gold using a

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K550 X sputter coater (Emitech Ltd., Ashford, Kent, UK), and observed with a Quanta 200 scanning electron microscope (FEI, Hillsboro, OR, USA).

Plate nomenclature. In this study a relaxed Kofoid nomenclature system was used based on homologies of plates of other gonyaulacoid genera. The anterior intercalary plate (1a), which follows the Kofoid system, was considered here as the homologue of the third apical plate (3') of other gonyaulacoid dinoflagellates because it may contact the Po plate in both *Protoceratium reticulatum* and *Ceratocorys mariaovidiorum* (Hansen et al. 1996/97, Salgado et al. 2018). *C. mariaovidiorum* lacks the fourth precingular plate (4"), but the last plate of the series of precingular plates was named here as 6" as in other gonyaulacoids, although *Ceratocorys* has only 5 precingular plates.

DNA extraction, PCR amplification, and sequencing. Exponentially growing strains from southern Chile (PRAY1, PRAY3, PRENM), Catalan (VGO757 and VGO1139), and Galician coasts (VGO904) were harvested by centrifugation (1.5 mL, 13,000 rpm for 2 min). The obtained pellets were washed with sterile bi-distilled water, centrifuged again, and stored until further processing at –20ºC. DNA was extracted using the Chelex procedure described in Salgado et al. (2015). The internal transcribed spacer (ITS1 and ITS2) and 5.8SrRNA gene regions were amplified in a polymerase chain reaction (PCR) using the primer pair EITS2DIR (5'-GTAGGTGAACCTGC(AGC)GAAGA-3'; Guillou et al. 2002) and PERK-ITS-AS (5'-GCTTACTTATATGCTTAAATTCAG-3'; Kotob et al. 1999). The 25 µL amplification reaction mixtures contained 2.5 µL of 10× buffer, 1.25 mM $MgCl_2$, 0.75 U of Taq DNA polymerase (Qiagen), 0.6 mM of each dNTP, 0.2 mM of each primer, and 2–4 µL of the Chelex extracts. The DNA was amplified in an Eppendorf Mastercycler EP5345 under the following conditions: initial denaturation at 94ºC for 2 min, 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 2 min, extension at 72ºC for 3 min, and a final extension cycle at 72ºC for 10 min. A 10 µL aliquot of each PCR was checked by agarose gel electrophoresis (1% TAE, 50 V) and SYBR™ Safe DNA gel staining (Invitrogen, Carlsbad, CA, USA).

The PCR products were purified with ExoSAP–IT (USB, Cleveland, OH, USA), sequenced using the Big Dye Terminator v.3.1 reaction cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), and separated on an AB 3130 sequencer (Applied Biosystems) at the CACTI sequencing facilities (Universidade de Vigo, Spain). The ITS and 5.8SrRNA sequences obtained in this study were deposited in the GenBank database (Acc. Nos. in Fig. 1).

The sequences of the studied strains of *Protoceratium reticulatum* and of *C. mariaovidiorum* and related taxa obtained from Genbank were selected using as reference the phylogenetic analysis by Akselman et al. (2015) and aligned using BioEdit v.7.2.5. A sequence from *Thecadinium kofoidii* (Herdman) Schiller was used as outgroup. The final alignment for the ITS phylogeny consisted of 427 positions. The phylogenetic model was

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selected using MEGA 7 software. Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) with a gamma-shaped parameter (y = 1.84) and proportion of invariable sites (I = 0.10) was selected. The phylogenetic relationships were determined according to the maximum likelihood (ML) method using MEGA 7 and the Bayesian inference method (BI) with a general time-reversible model from MrBayes v.3.2 (Huelsenbeck and Ronquist 2001). The two methods rendered very similar topologies. The phylogenetic tree was represented using the ML results, with bootstrap values from the ML method (n = 1000 replicates) and posterior probabilities from the BI method.

Study of viability of flattened cells. Cultures of *Protoceratium reticulatum* and *Ceratocorys mariaovidiorum* were grown until late exponential phase (~35,000–45,000 cell · mL^{-1} for *P. reticulatum* and ~15,000–25,000 cell · mL^{-1} for *C. mariaovidiorum*) from which flattened cells (13 cells of each species) were manually picked with a glass capillary pipette and transferred into culture-plate wells of 8 mm diameter (Thermo Fisher Scientific). Each of the wells contained 250 µL of L1 medium and were maintained at 15ºC ± 1ºC for Chilean *P. reticulatum*, and 19ºC ± 1ºC for Catalan and Galician *P. reticulatum* and 24ºC ± 1ºC for *C. mariaovidiorum*. The photoperiod cycle was 12:12 h L:D with irradiance about 100 µmol photons · m^{-2} · s^{-1}. The behavior and viability of the flattened cells was checked daily for one month. Cultures were considered viable when cells divided, at least, for four generations.

Kinetics of vegetative and flattened cells. The kinetics of flattened cell formation were studied in *Protoceratium reticulatum* strain PRENM and *Ceratocorys mariaovidiorum* strain CCMP1720. Exponentially growing cells of each strain (4,000–6,000 cell · mL^{-1}) were inoculated to a final concentration of 2,000 cell · mL^{-1} in 2 mL of L1 medium in 18 culture wells of 18mm diameter (Thermo Fisher Scientific). Culture conditions were the same as

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described above for culture maintenance. Every 3 or 4 d, flattened cells were counted in 2 wells of each strain and, later on, cultures stained with Lugol's solution and counted for total cells at 40× magnification using a Sedgwick–Rafter chamber and an inverted microscope. Flattened cells and cells with the typical appearance of vegetative cells were counted separately until the total cell counts decreased; observation period was 32 d.

RESULTS

Phylogenetic and morphological characterization of Protoceratium reticulatum *and* Ceratocorys mariaovidiorum *strains.* ML analysis of the region of the ITS/5.8SrRNA showed that the sequences of all Chilean and Spanish strains obtained in the present study were identical to most other sequences of *Protoceratium reticulatum* from isolates throughout the world (Fig. 1). The ML tree revealed that the isolates from this study were part of a monophyletic clade which separated from the clade of *Ceratocorys mariaovidiorum* composed by strains CCMP1720 and CCMP404 from USA.

The morphological characterization was based on the study of the most abundant cells in the cultures, which were motile flagellated cells. Cell morphology and tabulation of the thecal plates confirmed that all of the studied strains of *Protoceratium reticulatum* (Fig. 2, a–d) agreed with the description given by Reinecke (1967; as *Gonyaulax grindleyi*) and Hansen et al. (1996/97), and those strains of *C. mariaovidiorum* (Fig. 3, a–d) with the description given by Salgado et al. (2018). The two species presented very similar morphological features, among which were the thick reticulated plates with one pore within each reticule (Figs. 2b and 3b), the displacement of the descendent cingulum by one width, with no overhang, and a narrow and excavated sulcus and cingulum (Figs. 2b and 3b). Other characteristics that showed in both species were the variability between the contact of the plates Po and 3', the same number and disposition of sulcal plates, and a large ventral pore on the anterior right margin of the first apical plate (1'), although in *P. reticulatum* this plate was less narrow than that of *Ceratocorys mariaovidiorum* (Figs. 2b and 3b). There were also morphological differences between both species. In the case of *P. reticulatum*, cells had a subsphaeroidal body (Fig. 2a), a shorter epitheca than hypotheca (Fig. 2b), and without horns or spines. While *C. mariaovidiorum* cells were globular in shape (Fig. 3a) with an epitheca almost as long as the hypotheca (Fig. 3b), and some cells had small spines in the second antapical plate (2""). But the principal differences between both species were: 1) the number of precingular plates, with six in *P. reticulatum* and five in *C. mariaovidiorum* (Figs. 2c and 3c) and 2), the clear contact between 1' and Sa in *P. reticulatum* (Fig. 2b), while the contact of these plates in *C. mariaovidiorum* was slight (Fig. 3b) or absent, generating that plates 6" and 1" may be in contact. Thus, the plate formula of *P. reticulatum* was Po, 4', 0a, 6", 6c, ~7s, 5"', 0p, 2"", and that of *C. mariaovidiorum* Po, 4', 0a, 5", 6c, ~7s, 5"', 0p, 2"".

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Characterization of flattened morphotypes. A cell type with a morphology different than that of vegetative cells was observed in all isolates of *Protoceratium reticulatum* (Fig. 2, e–r) and *Ceratocorys mariaovidiorum* (Fig. 3, e–n). This morphotype presents a strong dorso-ventral compression of the cells, which gave them a peculiar leaf shape that became most evident when the cells swam (Fig. 2, e–g; Video S1 in the Supporting Information). In both species, this type of cell formed from a spherical motile cell with the typical morphology of a vegetative cell. The flattening process occurred in all strains and in both species in the same way, and consisted of a progressive flattening of the cell that became impressively flat (Figs. 2g and 3e). The thecal surface of the flattened cells initially resembled that of the vegetative cells with reticulated plates which, due to the strong compression, become detached over time (Fig. 2, e–h; Video S2 in the Supporting Information). Consequently, cells with different degrees of flattening (compare Fig. 2g, i and j), and more or less loosened plates were observed in the cultures. Cells that had already lost their original reticulated plates, exhibited very thin, non-reticulated new plates (Figs. 2k and 3f; Video S3 in the Supporting Information). They were mostly motionless, with their flattened surfaces attached to the bottom edges of the culture flasks. Although they were usually motionless they possessed flagella and when disturbed they initiated swimming, always close to the bottom.

Morphology of Protoceratium reticulatum *flattened cells*. Flattened cells of *P. reticulatum* were characterized by a strong dorso-ventral compression with a mean (± standard deviation) height/depth ratio of 0.40±0.15 µm (n = 5) [mean (±SD) height and depth of 42.7±0.4 µm and 17.3±5.7 µm, respectively, n = 5]. The mean (±SD) length and width of the cells were 45.6±5.6 µm (range 35.2–57.7 µm, n = 25) and 37.3±4.8 µm (range 29.6–47.7 µm, n = 25), respectively. The flattened cells were significantly (Kruskal–Wallis test: $P <$ 0.001) longer and wider than vegetative cells (Fig. 4). Most cells showed a single transverse and a single longitudinal flagellum, but a few flattened cells with two trailing flagella were also observed (image not available). Most of the flattened cells showed numerous gold-colored chloroplasts with more or less star-shape arrangement (Fig. 2, m and n) and other cytoplasm features similar to those of vegetative cells. Although cells with many granules, lighter chloroplasts concentrated in the center of the cell, and contraction of the protoplasm with a more apparent cell wall, were also observed (Fig. 2o). Nuclei were flattened and located in the posterior part of the cell (Fig. 2, m, o and p). Flattened cells presented two types of theca: 1) the old reticulated thecal plates from the original vegetative cell which still remained after flattening (Fig. 2e) or 2) very thin, non-reticulated new plates with pores (Fig. 2, k, l, q and r), formed after releasing the old vegetative cell plates (Fig. 2h). This latter type of theca had very elongated thecal plates in comparison with the plates of the vegetative cells (compare Fig. 2, q and r with 2, b and c). In the epitheca the long plates were 2''–4'' and in the hypotheca 2'''–4''' and 2'''' (Fig. 2, q and r). In spite of all the differences, flattened cells always maintained the tabulation of the plates of vegetative cells.

Morphology of Ceratocorys mariaovidiorum *flattened cells.* Vegetative cells of *C. mariaovidiorum* experienced a cell compression that gave rise to cells strongly flattened (Fig. 3, e–n) with a mean (±SD) height/depth ratio of 0.40±0.02 µm (n = 5) [mean (±SD) height and depth of 44.1±5.3 µm and 17.9±2.8 µm, respectively, n = 5]. The flattened cells were

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significantly ($F_{1,85} = 24.57$, $P < 0.001$, one-way ANOVA) longer than the vegetative ones (Fig. 4) [mean (±SD) length and width of the cells was 46.1±5.4 µm (range 36.2–56.5 µm, $n = 27$) and 40.4±4.0 µm (range 32.3–48.4 µm, $n = 27$), respectively]. They had a transverse flagellum and a longitudinal flagellum, many transparent granules with appearance of reserve material, and one or two large orange bodies located under the nucleus (Fig. 3g). The nucleus was elongated and positioned in the posterior part parallel to the transversal axis (Fig. 3g). They presented golden-orange chloroplasts that radiated from the center of the cell (Fig. 3, g–j). Once the flattened cells lost the old vegetative cell's plates, their new theca had non-reticulated plates with pores and depressions at each reticule (Fig. 3, f, j and k). Under the optical microscope the thecal plates were apparently more robust than those of *Protoceratium reticulatum* (compare Figs. 2l and 3j). The flattened shape allowed meticulous observation of thecal tabulation (Fig. 3, l–p). *Ceratocorys mariaovidiorum* flattened cells had the same plate formula and plate features as vegetative cells: 1) in the epitheca, Po plate was oval with a λ-shaped pore (Fig. 3m), 2) the 1' plate was much longer than wide (Fig. 3, i and m), 3) the absence of the 4" plate in the series of precingular plates was evident (Fig. 3, m and n), 4) the contact between 1' and Sa plates was slight (Fig. 3, l and m) or absent, which allowed plate 6" to touch 1" (Fig. 3o), 5) six cingular plates (Fig. 3, m and n) and seven sulcal plates were clearly identified (Fig. 3p), 6) in the hypotheca the 2"" antapical plate showed small spines, which formed from the vertex of each reticule (Fig. 3, f, i, l, and m).

Viability of flattened cells. An experiment to observe the viability of single flattened cells showed that 2 d after being transferred ($n = 26$) into culture-plate wells containing fresh full strength medium, their vegetative-like appearance, including reticulated plates, was restored either directly or following the release of their theca (Fig. 2s). This change from flat to vegetative-like cell reached up to 61% in *Protoceratium reticulatum* and 77% in *Ceratocorys mariaovidiorum.* The remaining cells were degraded. The resulting cells divided within the two subsequent days forming two vegetative-like cells (Fig. 2t) and giving, all of them, viable normal cultures.

Kinetics of vegetative and flattened cells. The study of the kinetics of flattened cell formation in full strength medium showed that total and flattened cells increased exponentially up to days 18 and 21, respectively, in *Protoceratium reticulatum*, and on day 25 in *Ceratocorys mariaovidiorum* (Fig. 5). In both species, empty thecae were observed in the bottom of the cultures from early stationary phase (Fig. 2s). Flattened cells represented up to 0.22% (98 cell · mL^{-1}) and 0.18% (50 cell · mL^{-1}) of the total cells of *P. reticulatum* and *C. mariaovidiorum* cultures, respectively (Fig. 5). Total cells reached a maximum of 46,650 cell · mL^{-1} (day 18) in *P. reticulatum* and 27,580 cell · mL^{-1} (day 25) in *C. mariaovidiorum* (Fig. 5).

DISCUSSION

The flattened cells and the process by which motile vegetative cells turned into distinctive benthic forms in cultures of the phylogenetically related dinoflagellates *Protoceratium reticulatum* and *Ceratocorys mariaovidiorum* are described. The flattening

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process in both species include: cell compression, detachment of the reticulated thecal plates of the vegetative cell, and the formation of new thin plates without reticulation but with pores and the typical tabulation pattern of the species. The strong flattening of these cells and the absence of reticulation allowed the characterization of some key features of thecal plates which are usually difficult to see in vegetative cells. For example, the sulcal area in the flattened cells of both species was wider and flattened allowing the observation of the pattern of sulcal plates in detail (Figs. 2, q and r and 3, l–o).

Flattened cells occurred in cultures subjected to adequate growth conditions in which the cells were physiologically well acclimated. The kinetics of their formation were proportionally related to vegetative growth following a similar progression to the culture growth curve. The appearance of these cells, although in low amounts, was regular and repetitive; it was also reversible when cells were returned to fresh full strength medium. As the cells appeared through all the growth phases, being completely healthy and without signs of stress in the cultures, flattening is highly unlikely to have been a culture artifact. Further, the possibility of a contaminant specimen was rejected. This is supported by the fact that they appeared distinctively in the two phylogenetically close species keeping the thecal tabulation from vegetative cells. In addition, it appears as consistent trait of both species because flattened cells were observed in strains from different geographical regions. In the case of *P. reticulatum*, from regions as distant as southern Chile, Mediterranean Sea and Atlantic coast of Spain. Rather, it can best be thought as a life cycle stage that, although as yet unknown in the natural environment and requiring further laboratory and field studies, seems to be related to benthic environments. In fact, some features displayed in different degrees in flattened cells suggest them to be cysts, so: their benthic and motionless behavior, the star-shape of chloroplasts concentrating in the center of the cells, the reserve granules and reduced cytoplasm as well as the ecdysis and formation of new vegetative cells after transferring them to nutrient replete medium. Nevertheless, having flagella that allow them to move is one distinctive characteristic that distinguishes them from typical cysts. As far as we know, the process by which a vegetative cell flattens is reported for the first time in dinoflagellates. And, although it is not possible at this stage of knowledge to know their significance in the life cycle of *P. reticulatum* and *C. mariaovidiorum*, it deserves a debate about whether these forms could be considered as cysts. It is evident that the progress of knowledge on the life strategy of dinoflagellates in the last two decades is showing that the benthic life forms in planktonic dinoflagellates are much more diverse than previously thought (Kremp 2013, Bravo and Figueroa 2014).

There are different types of benthic life forms in dinoflagellate life cycles which show that, in shallow coastal environments, morphological and physiological changes would equip certain species with an alternative benthic life-form and thus a significant adaptive advantage through evolution. The most well known benthic stage of dinoflagellates is the so-called resting cyst which is characterized by resistant properties—its thick and resistant wall as well as a more or less long dormancy period—that allow them to survive to long unfavorable periods. However, the flattened cells from the present study seem not to be related to these cysts. Not only because of having flagella but also for the spontaneous ecdysis they underwent in culture. This behavior fits better with the excystment of short-term pellicle or thecate cysts, also called temporary and ecdysal cysts (Bravo and Figueroa 2014). Moreover,

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the resting cyst of *P. reticulatum* is well known to be a thick-walled cyst with slender processes (Matsuoka et al. 1997, Marret and Zonneveld 2003, Salgado et al. 2017) and, though the resting cyst of the phylogenetically close *C. mariaovidiorum* is still unknown, it would also have at least the characteristics of the known resting cysts, that is, no flagella and a thick and resistant wall. In addition, the kinetics of flattened cell formation showed a distinctive pattern different to that of the resting cysts reported in the literature. Resting cyst production in dinoflagellates has been related both to nutrient limitation and to optimal conditions of vegetative growth (Binder and Anderson 1987, Montresor and Marino 1996, Zohary et al. 1998, Olli and Anderson 2002), and in cultures they are commonly formed in nutrient deficient medium and associated with stationary phase (Turpin et al. 1978, Anderson et al. 1985). Our study of kinetics of flattened cells showed that these cells were steadily formed from the beginning of the growth curve and followed its progress. This suggests a different kinetics than the most commonly one reported in resting cysts (associated with nutrient limitation) and might be more related to short-term pellicle cysts of, for example, *Alexandrium taylori* or *Fragilidium* sp. (Owen and Norris 1985, Garcés et al. 1998), which we have observed to encyst throughout all the cultures phases. Yet, unlike those of the first species, we have not seen division in flattened cells.

As knowledge about the ecology of phytoplankton increases, more species whose life history is based on a stronger planktonic-benthic coupling are being recognized (Marcus and Boero 1998). Meroplankton, in which the benthic phase was presumed to be limited to a dormant resting stage that remains in the seabed until the return of favorable conditions, was contrasted with permanently planktonic holoplankton species. However, with increasing understanding of the life strategies of dinoflagellates, the picture has become more complicated and now it must include a closer, more integrated relationship between plankton and benthos (Boero et al. 1996). Thus, between holoplankton and strictly benthic dinoflagellates there is probably a complex network of adaptive strategies and life cycles tailored to the ecological variety of the multiple habitats in which dinoflagellates live, giving rise to numerous planktonic-benthic relationships. Planktonic species whose life form includes benthic division cysts, a type of thin-walled pellicle cyst that undergoes consecutive divisions, are indicative of benthic-type behaviors. For example, the dinoflagellate species that inhabit tide pools are characterized by a rapid planktonic-benthic coupling mediated by a benthic non-motile stage that sinks to the sea bottom for a few hours during low tide and a motile stage that swims in the water column during high tide (Hoppenrath et al. 2014). This strategy allows the avoidance of temporarily adverse conditions (risk of drying at low tide), minimizes advective cell losses and thus ensures short-term survival (Kita et al. 1985, Basterretxea et al. 2005).

There are examples of a strong planktonic-benthic coupling in dinoflagellates that, in some cases, have been proved to be essential in their bloom dynamics. This occurs in planktonic dinoflagellate species in which both vegetative motile cells and division cysts can undergo division, and even species considered planktonic in which the benthic phase is the unique stage that undergoes division. For example, the formation of intensive blooms of *A. taylori* in the Mediterranean is based on a rapid interchange between planktonic and benthic stages. The strategy is the avoidance of advective cell losses, with cells forming division

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cysts that are deposited on the seabed during the night, followed by a return to the planktonic phase after a few hours (Garcés et al. 1999). Moreover, a benthic behavior is still more intense in those species in which the benthic stage is the unique dividing phase. This has been described, for example, in *Crypthecodinium cohnii*, *Pfiesteria piscicida*, *Alexandrium pseudogonyaulax*, *and Gymnodinium quadrilobatum* (Bhaud et al. 1991, Horiguchi and Pienaar 1994, Montresor 1995, Parrow and Burkholder 2004, Zmerli Triki et al. 2015). These thin-walled division cysts of dinoflagellates are considered to be true cysts because they derives from the flagellated mobile planktonic phase (Garcés 2001). During cyst formation, cells lose their flagella and theca and sink to the seabed as non flagellated, motionless forms similar to the resting cysts of dinoflagellates. However, a cyst is a quiescent phase characterized by a greatly reduced metabolic rate and the absence of division (Von Dassow and Montresor 2011). According to that definition, those division cysts should instead be considered as a benthic population that divides to finally form motile cells.

Yet, there was no evidence of division in flattened cells in our study. There are morphological and behavioral differences between the flattened cell type of *P. reticulatum* and *C. mariaovidiorum* in comparison with the pellicle cysts aforementioned. The most obvious ones are the presence of flagella and theca as well as their strongly flattened shape. Rather, the characteristics of the *P. reticulatum* and *C. mariaovidiorum* flattened cells resemble those of many benthic dinoflagellates, for example *Prorocentrum* and *Amphidinium* species in which flattened (sometimes strongly) shapes are very common (Hoppenrath et al. 2013, 2014). This feature may facilitate movement in interstitial habitats as well as substrate attachment (Hoppenrath et al. 2014). Indeed, flattened resting cysts have been reported for *Alexandrium minutum* and *A. taylori* (Bravo et al. 2006). The increase in the amount of surface contact with the substrate by a flat shape is consistent with prior attachment of the cells to the bottom by means of mucus. This morphology is also beneficial because it increases the nutrient uptake efficiency in benthic oligotrophic environments by increasing the surface/volume ratio (Fraga et al. 2012). Although the alternative benthic form of *P. reticulatum* and *C. mariaovidiorum* described in this study showed a low proportion of formation, it suggests an additional adaptation that probably contributes to survival of the species. It must be taken into account that no optimization of flattened cell formation was performed in the present study and the way how they are developed in nature has to be investigated. Whatever the circumstances may be, low encystment rates have already been assumed to play an important role on the bloom dynamics of some dinoflagellates (Garcés et al. 2004). Those authors show that massive proliferations of *A. minutum* in the Mediterranean are formed without requiring high encystment percentages (<1%).

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FIGURE LEGENDS

Fig. 1. Phylogenetic relationships among *Protoceratium reticulatum* and *Ceratocorys mariaovidiorum* strains from this study and related taxa based on ITS/5.8SrRNA sequences. The phylogenetic tree was constructed using the ML method. Numbers at the branches indicate the percentage of bootstrap support (n = 1000) and the posterior probabilities based on BI method. Bootstrap values <60% and probabilities <0.6 are denoted by hyphens. Names in bold represent isolates sequenced in this study.

Fig. 2. Light microscopy images of vegetative and flattened cells of *Protoceratium reticulatum*. (a–d, s and t) Vegetative-like cells. (e–r) Flattened cells. (a) Cell in ventral view (Nomarski microscopy). (b–d) Cells stained with Calcofluor in ventral (b), apical (c), and antapical (d) views. (e) Living cell with detached reticulated thecal plates. (f and g) The same cell as in (e) but viewed from different positions. (h) Cell showing detachment of reticulated thecal plates. (i and j) Living cells with different degrees of flattening and with completely detached reticulated plates (n indicates nucleus). (k) Ventral and (l) dorsal view of the same cell as in (i) with very thin non-reticulated plates. (m) Cell with star-shape chloroplasts (n indicates nucleus). (n) Chloroplasts of the same cell as in (m) viewed with UV microscopy. (o) Cell with many granules, chloroplasts, and an apparent cell wall (n indicates nucleus). (p) Cell showing the nucleus stained with SYBR Green. (q) Calcofluor stained cell showing thecal tabulation in ventral view. (r) The same cell as in (q) in dorsal view from inside the cell (mirror view). (s) Living cell recently formed from a flattened cell exhibiting the new reticulated theca (arrow) after releasing the non-reticulated one (arrowhead). (t) Vegetative-like cells resulting from the division of the cell (s). Scale bars: 10 µm. Vp: ventral pore.

Fig. 3. Light microscopy and SEM images of vegetative and flattened cells of *Ceratocorys mariaovidiorum*. (a–d) Vegetative cells. (e–p) Flattened cells. (a) Cell in ventral view. (b–d) Cells stained with Calcofluor in ventral (b), apical (c), and antapical (d) views. (e) Cell in lateral view showing the strong flattening. (f) SEM image of a cell with a non-reticulated wall but with pores in the plates and small spines in the 2"" plate (arrows). (g) Cell with granules and elongated nucleus (asterisk). (h) The same cell as in (g) showing chloroplasts (chl), its nucleus (asterisk), and orange bodies (arrowhead). (i and j) Cell viewed in different focal planes showing the new thin theca non-reticulated with small spines (arrow) in the 2"" (i), and with depressions (j) at each reticule. (k) SEM image of the same 5"' plate as in (f) showing in detail pores (Por) and depressions (Dep). (l) Cell in ventral view showing thecal tabulation with non-reticulated plates, small spines in the 2"" (arrows), and a slight contact between 1' and Sa (arrowhead). (m) Thecal tabulation of a cell in ventral view (arrowhead indicates a slight contact between 1' and Sa). (n) Thecal tabulation of the same cell as in (m) in dorsal view from inside the cell (mirror view). (o) Contact between 6" and 1" plates (arrow) which avoid contact between 1' and Sa. (p) Detail of sulcal plates. Scale bars: 10 µm, except (f): 20 µm. Vp: ventral pore.

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Fig. 4. Box plots of vegetative and flattened cell sizes of *Protoceratium reticulatum* and *Ceratocorys mariaovidiorum.*

Fig. 5. Kinetics of flattened cell formation and growth of *Protoceratium reticulatum* and *Ceratocorys mariaovidiorum.*

Video S1. *Protoceratium reticulatum* flattened cell (strain PRAY10). Swimming cell showing a leaf shape. Magnification 10×.
Video S2. *Protoceratium reticulatum* flattened cell (strain PRENM). Swimming cell showing strong dorso-ventral compression and the detachment of their reticulated thecal plates. Magnification 40×.
Video S3. *Protoceratium reticulatum* flattened cell (strain PRAY10). Swimming cell with very thin non-reticulated plates. Magnification 40×.
Table 1. Information on isolates used in this study. Identification of isolates, origin, year of isolation, culture origin, and isolator.

Isolate name	Origin	Year of isolation	Culture origin	Location	Isolator
PRAY1-B11	Queulat Sound, Aysén region, Chile	2010	Wild resting cyst	-44º29'28"; -72º36'12"	Present work
PRAY3	Queulat Sound, Aysén region, Chile	2010	Wild resting cyst	-44º29'28"; -72º36'12"	Present work
PRAY10	Queulat Sound, Aysén region, Chile	2010	Wild resting cyst	-44º29'28"; -72º36'12"	Present work
PRENM	Englefield Island, Magallanes región, Chile	2013	Vegetative cell	-53º04'28"; -71º49'38"	Pizarro, G.
VGO904	Ría de Pontevedra (Bueu), Galicia, Spain	2006	Vegetative cell	42º21'; 8º46'	Escalera, L.
VGO757	Els Alfacs (Ebro Delta), Catalonia, Spain	2003	Wild resting cyst	40°37'18"; 0º41'11"	Bravo, I.
VGO1139	El Fangar (Ebro Delta), Catalonia, Spain	2005	Wild resting cyst	40°46'34"; 0°45'27"	Bravo, I.
CCMP404	Salton Sea, CA, USA	1982	Vegetative cell	33º22'; 116°0'	Dodson, A.
CCMP1720	Biscayne Bay, Miami, FL, USA	1994	Vegetative cell	25º48'; 80º19'	Hargraves, P.

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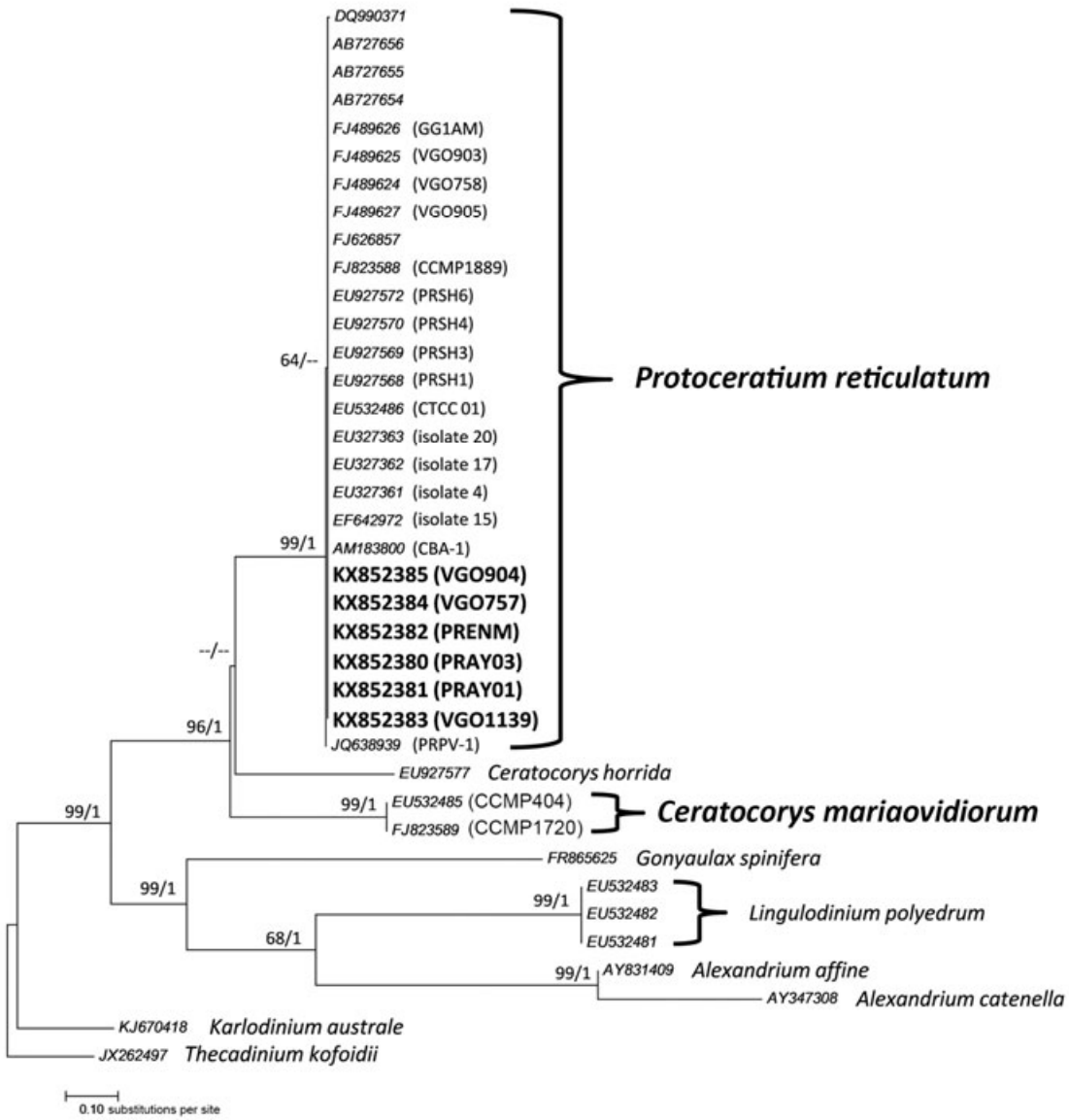

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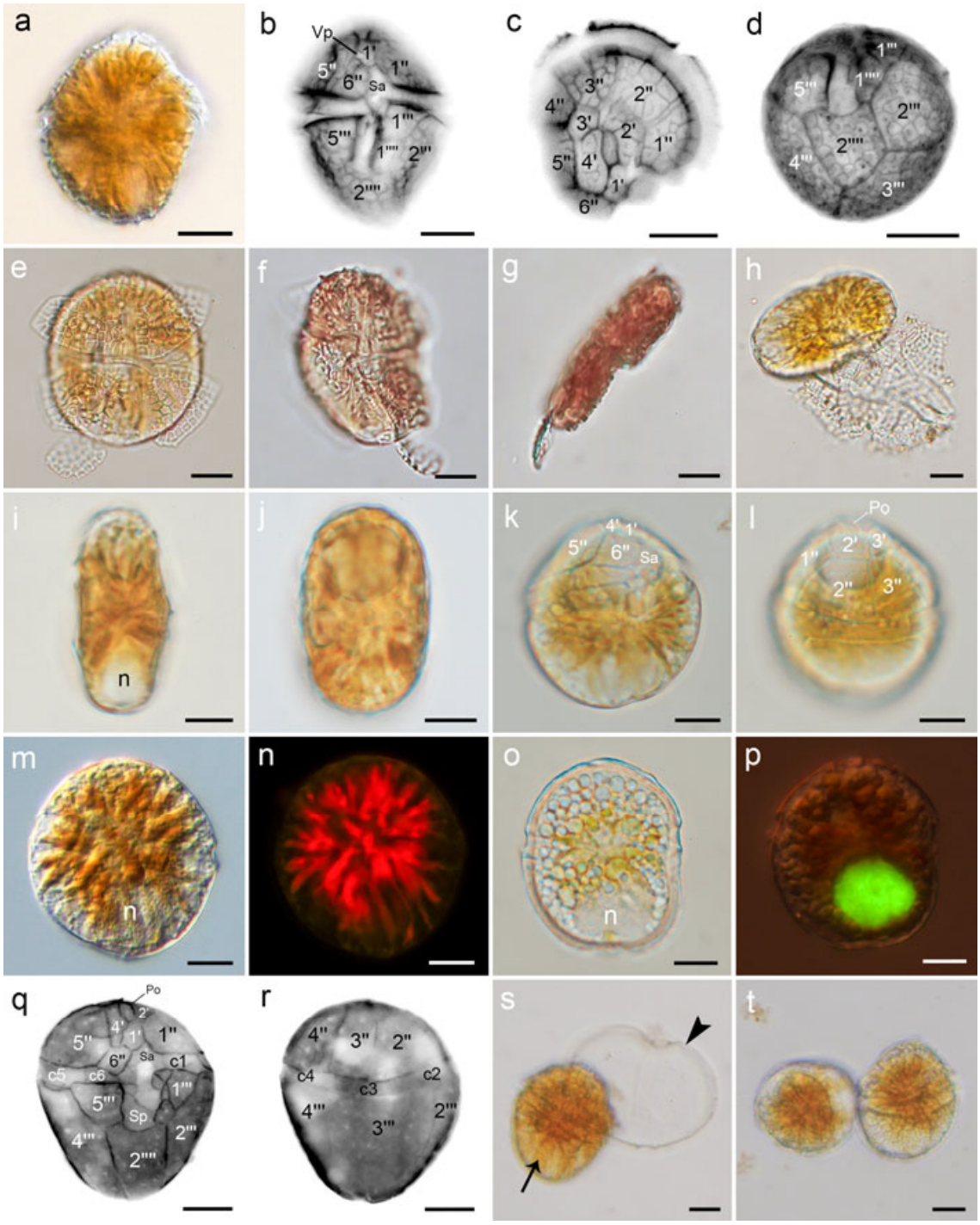

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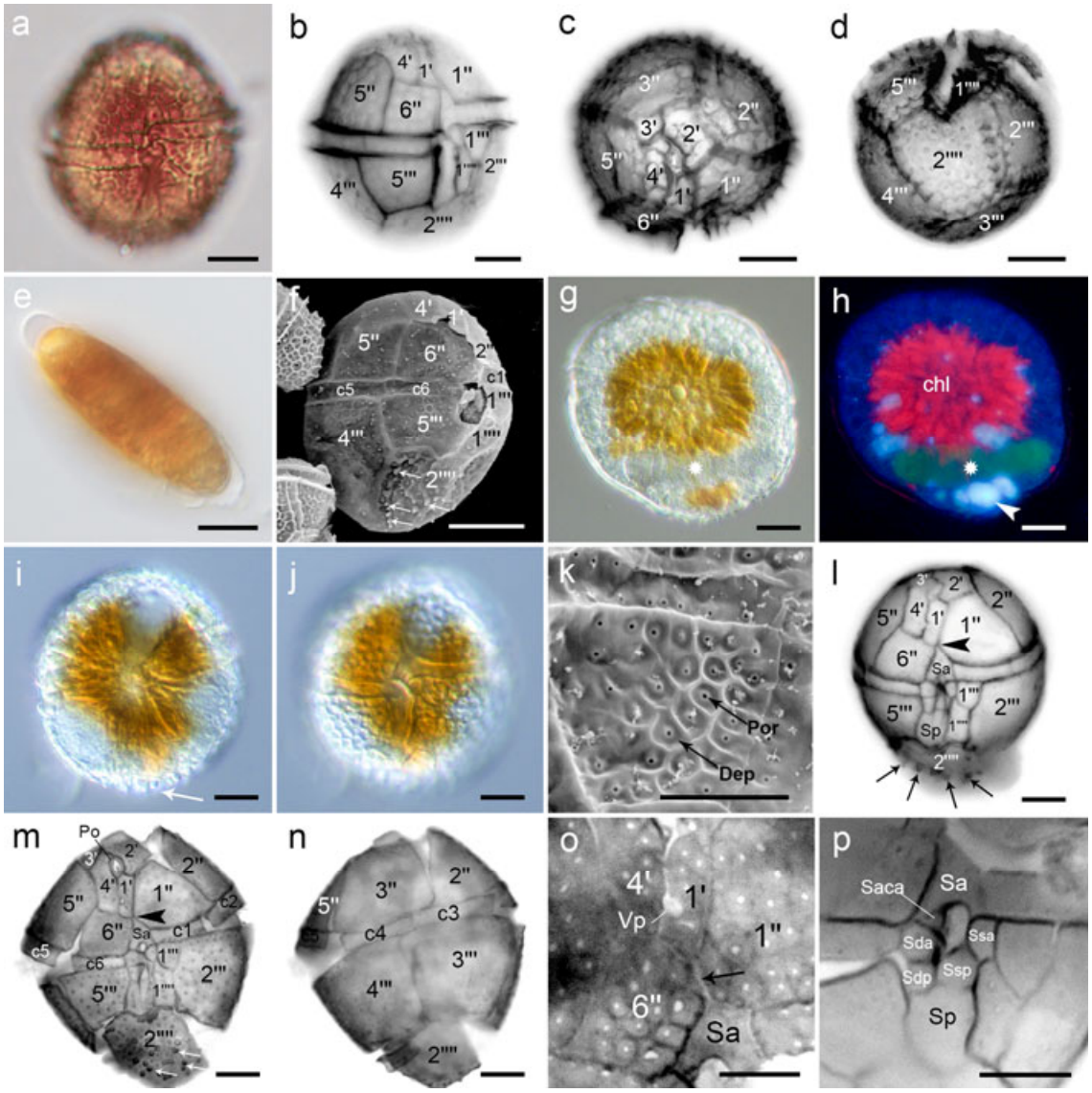

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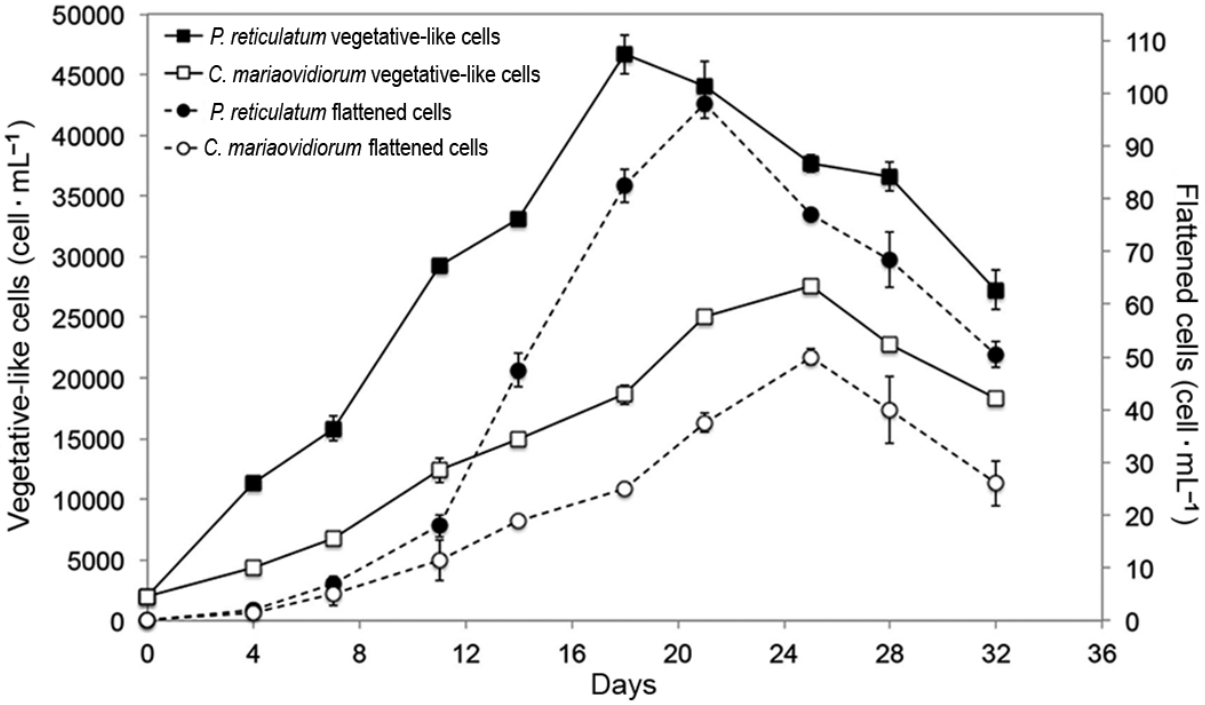

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