

Asymmetric cell division and its role in cell fate determination in the green alga *Tetraselmis indica*

Mani Arora^{1,2}, Arga Chandrashekar Anil^{1*}, Karl Burgess³, Jane Delany² and Ehsan Mesbahi⁴

¹CSIR-National Institute of Oceanography, CSIR, Dona Paula, Goa- 403004, India

²School of Marine Science and Technology, Newcastle University, Newcastle Upon Tyne NE1 7RU, UK

³Glasgow Polyomics, University of Glasgow, Glasgow, G12 8QQ, UK

⁴Faculty of Science, Agriculture and Engineering, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK

* Corresponding author: Email: acanil@nio.org; Tel.: +918322450404; Fax: +918322450615

Abstract

The class Prasinophyceae (early diverging Chlorophyta), consisting of simple unicellular picoeukaryotes occupies a critical position at the base of the green algal tree of life, and are viewed as the cell form most similar to the first green alga, the ‘ancestral green flagellate’ (Lewis and McCourt 2004; Leliaert *et al.* 2012). Relatively large-celled unicellular eukaryotic phytoflagellates (such as *Tetraselmis* and *Scherffelia*), traditionally been placed in Prasinophyceae but now considered as members of Chlorodendrophyceae (core Chlorophyta), have retained some primitive characteristics of Prasinophytes, but are as evolved as any other green alga or land plant. These organisms share several ultrastructural features with the other core Chlorophytes (Trebouxiophyceae, Ulvophyceae and Chlorophyceae). However, the role of Chlorodendrophycean algae as the evolutionary link between cellular individuality and cellular cooperation has been largely unstudied. Here, we show that clonal populations of a unicellular Chlorophyte, *Tetraselmis indica*, consist of morphologically and ultrastructurally variant cells which arise through asymmetric cell division. These cells also differ in their physiological properties. The structural and physiological differences in the clonal cell population correlate to a certain extent with the longevity and function of cells.

Keywords: *Tetraselmis*; unicellular Chlorophyte; asymmetric cell division; morphological distinction; ultrastructural variations; physiological differences

1. Introduction

Individual cells of Prasinophytes are the basic living entities of the green plastid lineage Viridiplantae. Viridiplantae consists of the phyla Chlorophyta- the 'green algae', Streptophyta- the true land plants and several green algal lines which were previously considered part of the Charophyceae. Prasinophyte algal abundances have been in a long-term decline since the Triassic period (Falkowski *et al.* 2004). Relatively large-celled unicellular eukaryotes, traditionally considered as prasinophytes (such as *Tetraselmis*) but now considered as a member of the core Chlorophytes (Chlorodendrophyceae), can still be found in the oceans. These unicellular flagellates have retained some primitive characteristics of Prasinophytes but are as evolved as any other green alga or land plants. They share several ultrastructural features with the core Chlorophyta (Trebouxiophyceae, Ulvophyceae and Chlorophyceae), including closed mitosis and a phycoplast (Mattox and Stewart 1984, Melkonian 1990, Symand Pienaar 1993). A phylogenetic relationship has been confirmed via molecular data (Fawley *et al.* 2000; Guillou *et al.* 2004; Marin 2012), and both Prasinophyceae and Chlorodendrophyceae are considered as early diverging Chlorophytes. Sexual reproduction is unknown in Chlorodendrophyceae.

Asymmetric cell division generates two cells with different fates and plays an important role in producing diverse cell types and for maintaining stem cell niches in animals, plants and multicellular algae (Abrash and Bergmann 2009; Horvitz and Herskowitz 1992; Knoblich 2008; Scheres and Benfey 1999; De Smet and Beeckman 2011). However, the understanding of asymmetric cell division in the simplest eukaryotic unicellular green algal forms is fragmentary. Three representative examples of asymmetric cell division in unicellular Chlorophytes are: the first report is in *Platymonas impellucida* (now known as *Tetraselmis impellucida*) from Puerto Rico (McLachlan and Parke 1967); the second is in *Prasinoderma singularis* from the south east Pacific Ocean (Jouenne *et al.* 2010); and the third is in *Tetraselmis indica* from the salt pans of Goa, India (Arora *et al.* 2013). Interestingly, two out of the three reports of asymmetric division in Chlorophytes are in *Tetraselmis*. Asymmetric cell division, and the resulting cellular heterogeneity, undoubtedly has functional consequences in *Tetraselmis*, and hence the interrogation of a single cell is important for gaining a complete understanding of variability and the role that it plays.

2. Materials and Methods

The material for this study (*Tetraselmis indica*) was collected from a water pool in salt pans from Panaji, Goa, India. A sample of dark green water between the salt lumps was collected and diluted fivefold with autoclaved sea water. Unialgal clonal cultures of the organism were established by

micropipetting from the enriched crude culture. Clonal cultures were established for a single strain by picking cells and then serially diluting to obtain a single cell. Single-celled progeny were then allowed to grow under culture conditions (Arora *et al.* 2013). These cultures are maintained at the National Institute of Oceanography, India, in F/2 media (Guillard and Ryther 1962) without silicates, at 25°C, photon flux density 80 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and a 16:8 h L: D cycle. The culture is deposited with National Facility for Marine Cyanobacteria, Govt of India, Bharathidasan University, Tiruchirappalli, India (accession number BDU GD001).

Tetraselmis population was studied using a combination of epifluorescence microscopy, confocal laser scanning microscopy (CSLM), and phase contrast microscopy. Individual cells were analysed for their fluorescence signals using CLSM. Nuclear division in an unequally dividing cell was studied using CLSM after staining with Hoechst 33342.

The *Tetraselmis* sp. isolated from salt-pan blooms are subjected to very high temperatures. Hence we assessed the effect of temperature on cells in order to determine how unequal daughter cells respond to temperature stress. Temperature stress was instigated by incubation of cells at 37 °C for 6 hrs. Early onset of cell death was assayed using Alexa fluor conjugated Annexin V to determine the binding of Annexin V to externalized phosphatidyl serine. The cells were harvested by centrifugation and dissolved in a mixture of 5 μl of Alexa fluor labelled annexin (Molecular probes, invitrogen) and Annexin binding buffer containing 10 mM HEPES- NaOH (pH 7.8), 140 mM NaCl, and 2.5 mM CaCl_2 . Cells stained with Alexa fluor labelled Annexin were visualised in 488 nm wavelength using Olympus microscope BX 51 and Image Pro cell imaging software. Apoptotic cells were distinguished from nonapoptotic cells based on their yellow green fluorescence.

Light and confocal microscopy

Live cells were observed using an Olympus BX 51 microscope equipped with Image-Pro software and an Olympus confocal microscope (CLSM).

Transmission electron microscopy

For electron microscopy (EM), cells were primarily fixed by rinsing several times in 2% glutaraldehyde (TAAB Lab. Equip., Aldermaston, Berks) in sea water containing 0.1 M cacodylate buffer (pH 7.0). The cells were post-fixed overnight in 1% cold osmium tetroxide (Agar Scientific, Stansted, Essex) in 0.1 M cacodylate buffer, rinsed in buffer for 10 min and then dehydrated in an acetone series (30 min each in 25, 50 and 75% acetone followed by 100% acetone for 1 h at room temperature). Following dehydration, cells were impregnated using an epoxy resin kit (TAAB Lab. Equip., Aldermaston, Berks) for 1 h each with 25, 50 and 75% resin (in acetone), followed by 100%

resin for 1 h, with rotation overnight. The embedding medium was then replaced with fresh 100% resin at room temperature and the cells transferred 5 h later to an embedding dish for polymerisation at 60 °C overnight.

Sections were cut with a diamond knife mounted on a RMC MT-XL ultramicrotome. The sections were stretched with chloroform to eliminate compression and mounted on pioloform (Agar Scientific, Stansted) filmed copper grids. Sections were stained for 20 min in 2% aqueous uranyl acetate (Leica UK Ltd, Milton Keynes) and lead citrate (Leica UK Ltd, Milton Keynes). The grids were examined using a Philips CM 100 Compustage (FEI) transmission electron microscope and digital images were collected using an AMT CCD camera (Deben) at the Electron Microscopy Research Services facility, Newcastle University.

3. Results and discussion

Asymmetric cell division gives rise to phenotypic heterogeneity in T.indica

Asymmetric division and differential cell specification could be a direct effect of cell size, nuclear control or nuclear cytoplasmic interactions (Kirk *et al.* 1993). Asymmetric divisions in *T. indica* set apart large and small sister cells or one large and two small sister cells (figure 1-4 and Supplementary figure S1-S3). Ultrastructural analysis revealed that the smaller division products (daughter cells) were enclosed by two cell walls (figure 5-8), and in addition, characteristic structures similar to phagosomes (Kielian and Cohn 1980; Swanson *et al.* 1998; Matile and Wiekmen 1967; Spormann *et al.* 1992; Li and Kane 2009) were observed in the vacuoles or lysosomes of the smaller division products (figure 5-11). The cell specification blue print may be directly dependent on cell size, but nuclear control or nuclear cytoplasmic interactions can also lead to cell specification (Kirk *et al.* 1993). Nuclear staining at the very start of cell division suggested an unequal partitioning of the nucleoplasm (Supplementary figure S4 & S5).

To gain insight into the pigment composition at a single cell level, absorption spectra for specific daughter cells were obtained by scanning the cells over a range of wavelengths, from 570 nm to 700 nm using an Olympus confocal microscope (CLSM). Absorption peaks for the daughter cells were obtained to reveal pigment differences. A single zone of absorption at 680 nm, demonstrating the presence of chlorophyll A, was obtained for the small (S) cells, whereas the larger daughter cells (L) gave two peaks, a major peak at 570 nm, indicating the presence of carotenoids, and another small peak at 680 nm (Supplementary figure S6 & 7). Carotenoids are mainly localized in the eyespot and, in addition to serving as a collector of quanta for photosynthesis, these pigments are also known to provide a reserve for nitrogen and are classed as protective pigments.

The benefit of cellular heterogeneity: differential resistance to environmental and chemical stressors

Genetically homogenous individual cells within clonal cultures of the unicellular eukaryotic phytoflagellate *Tetraselmis* exhibit considerable phenotypic heterogeneity and diversity. It was observed that under temperature stress the smaller daughter cells, S, exhibited programmed cell death (figure 12-15); whereas the larger division products, L were resistant to this stresses (figure 12-15). L cells had the ability to respond to temperature stress more efficiently (figure 12).

Both types of cells were kept under observation to study their cell division patterns. S cells divided equally into two daughter cells. L cells exhibited two patterns of asymmetric division: Pattern 1. One cell gives rise to two daughter cells, one small and one large (figure 1, 2 & 4). Pattern 2. One cell gives rise to three daughter cells, two small and one large (Fig. 3 and Supplementary figure 8). In any case when these L cells divided, one daughter cell usually retained the features of the original cell, a feature similar to that exhibited by animal stem cells. The smaller daughter cell gradually lost its contents through lysis as the culture was placed under unfavourable environmental conditions, particularly temperature stress (Supplementary figure S9-11). The inherent resistance of L cells to stress may be a contributing factor to the reappearance of populations following unfavourable conditions. These cells can also rapidly effluxed the toxic fluorescent stain Hoechst 33342, thereby providing an explanation to their resistance to chemicals, as some active transporters appear to be present in these cells (Supplementary figure S12, 13).

Heterogeneous stress resistance is widely documented in bacteria, yeast, and mammalian stem cells, whereas in unicellular phytoplankton populations there are seldom any reports of this behaviour. 'Survivor cells' within microbial populations (bacteria and yeast) and the benefit of variant subpopulations, which have the potential to better withstand perturbations and to exploit new niches, have been demonstrated to have a significant effect (Booth 2002; Sumner and Avery 2002). However, the presence and role of such 'survivor cells' in unicellular phytoplanktonic populations is poorly understood. It is also important to note that these cells in *Tetraselmis* are different from the dormant stages of phytoplankton, such as cysts, spores, auxospores, etc., since these cells are the result of unequal cell division.

Difference in size between the products of an unequal division is probably accompanied by some invisible qualitative difference that is the actual cause of their divergent development (Horvitz and Herskowitz 1992). Mechanisms that drive asymmetric cell division in animals might also work in algae, but this still needs to be explored.

These observations highlight the significant role of cell division patterns in determining population dynamics, which in this alga appear to be regulated by equilibrium between different cell types. The point of equilibrium appears to be controlled by various environmental factors such as temperature. Many cells lose their capacity to divide as they mature or divide only rarely, whilst other cells are capable of rapid cell division. Hence the structural and physiological differences in the clonal cell population correlate to a certain extent with the longevity and function of cells.

4. Conclusions

Our investigation demonstrates that unicellular phytoplankton exhibit cellular heterogeneity and suggests that variant subpopulations work in close cooperation for the persistence of the whole population. This study has also changed our view of evolutionary transition in cell individuality within the green algal lineage, which has not previously been studied in unicellular members. Given the involvement of various cells, it is likely that small subsets of a population, such as the L cells, regulate the survival and may influence the capacity of a population to maintain its existence.

We provide experimental evidence that integrated division products of a single cell are ultrastructurally different and differentially sense stress signals. Whether the ultrastructural features such as the vacuolar globular structures in S cells are responsible for cell degradation as observed in yeast, remains unknown. Much additional work is required to characterise the composition of the vacuoles and the intracellular trafficking more precisely by molecular criteria, before integrating these findings into a structural and functional framework.

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Figure Legends

Figs 1-4. Asymmetric cell division in *T. indica*. **1.** A live *T. indica* cell soon after cell division, with the two unequal division products still enclosed in the parental theca. **2.** Unequal cells following their release from theca. **3, 4.** Asymmetric cell division giving rise to unequal cells. Scale bars: 10 μm

Figs 5-11. Ultrastructural TEM comparison of unequally divided products soon after cell division. **5.** The smaller division product is surrounded by two wall layers (indicated with arrows), whereas the larger division product has a single wall layer. It is known that *Tetraselmis* produces cyst that consists of several layers of wall under adverse conditions. The two wall layers in Fig. 5 & 7 seem to be the step towards cyst stage. **6.** Magnification of Fig. 5; **7.** Presence of specific globular structures in the vacuoles (V) of the smaller daughter cell (SDC), whereas no such structures can be observed in the larger daughter cell (LDC). **8.** Magnified view of Fig. 7. **9-10.** Magnified view of Fig. 7 SDC showing the globular structures (P) present in the vacuoles (V) of smaller cells; notably, these structures are similar in appearance to yeast phagosomes. **11.** Magnified view of Fig. 7 SDC showing cytoplasmic strands (CS) extend into the vacuoles and connect to the globular structures. Scale bars: Figs 5-8, 2 μm ; Fig. 9, 100 nm; Fig. 10, 300 nm; Fig. 11, 100 nm.

Figs 12-17. Unequal division products demonstrate differential resistance to temperature stress **12-15.** Fluorescence and corresponding light micrographs of annexin stained cells: the smaller division product is undergoing programmed cell death following temperature stress resulting in a green stain signal, whereas the larger product is red due to chlorophyll autofluorescence. **16, 17.** Fluorescence and light micrographs of cells with the annexin staining without temperature stress. Scale bars: 10 μm .

Supplementary Figure Legends:

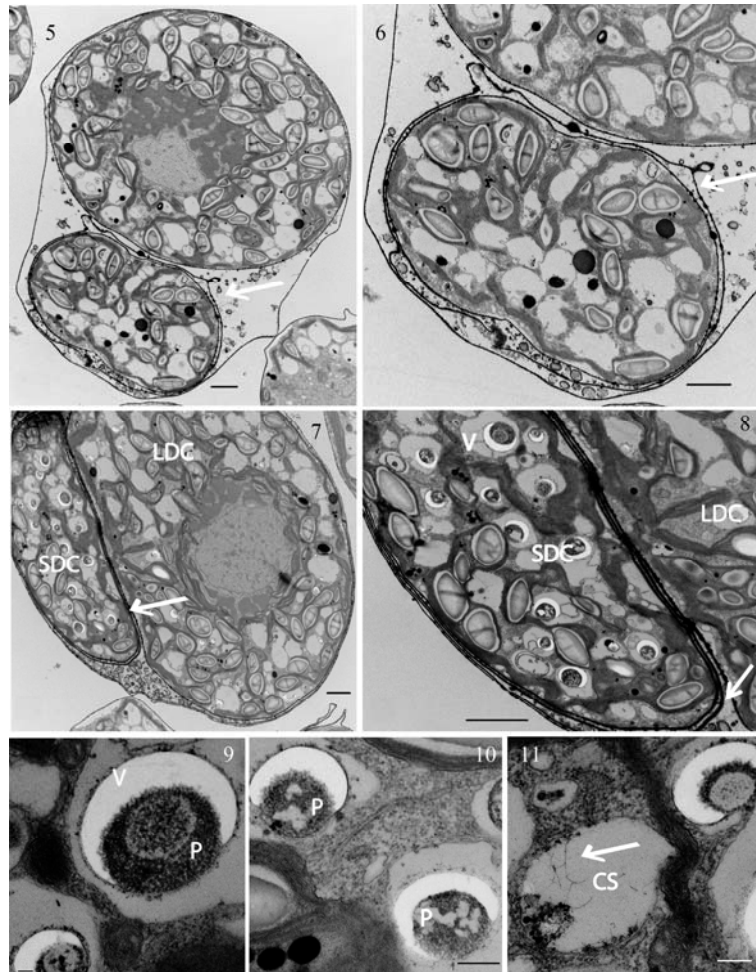
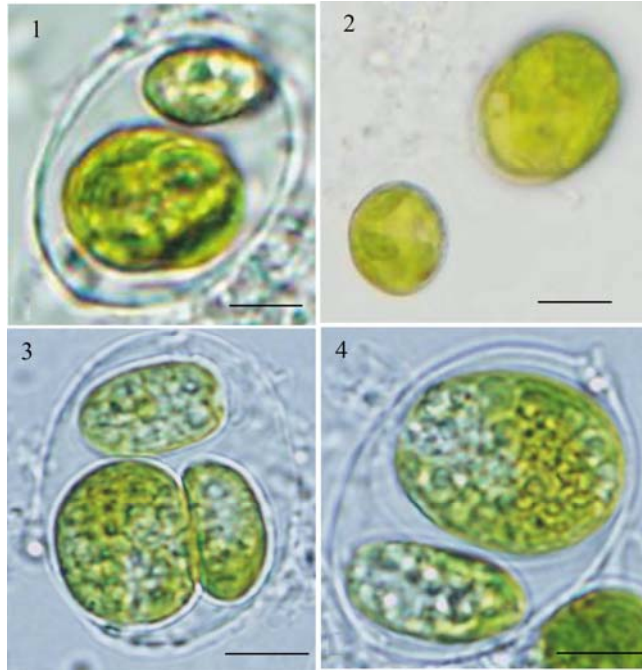
Figs 1-3. Light micrograph of an asymmetrically dividing *T. indica* cell at various time intervals. Progress over a 12 hr period, showing that the larger component does not undergo further division. **1.** 0 hr, cell division at an early stage. **2.** 5 hrs, lower half of cell undergoing further division. **3.** 12 hrs, differentiated phenotypes in terms of cell size. Scale bars: 10 μm

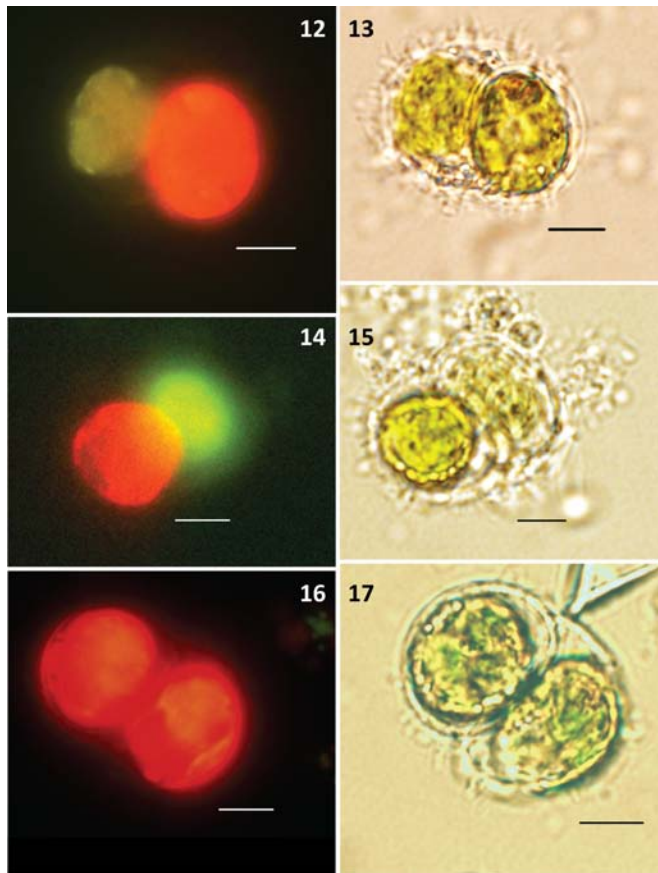
Figs 4-5. **4.** DIC image of a cell at an early stage of division **5.** Confocal laser scanning micrograph of a cell passing through the X-Z plane at an early stage of cell division demonstrating the unequal partitioning of nuclear material; comparatively more nuclear material is going to a predestined region. Scale bars: Fig. 4, 22.71 μm ; Fig. 5, 11 μm .

Fig. 6- 7. Absorption peaks of individual cells showing the variation in absorption maxima and hence pigment differences of different types of daughter cells. **6.** L cells always demonstrate a major peak in the range of accessory pigments, whereas **7.** S cells yield only a single peak in the range of chlorophyll *a*.

Fig 8-11. **8.** Control **11.** Unequal division products demonstrating differential resistance to temperature stress. The smaller daughter cell gradually lost its contents through lysis under unfavourable environmental conditions. Scale bars: 10 μm

Figs 12, 13. Fluorescence and Light micrograph showing Hoechst staining and efflux of the stain by the bigger division product indicating that certain cells are resistant to its toxicity and can efflux or exclude the stain; however, whether this efflux is due to active transporters is not yet understood. Scale bars: 10 μm





Supplementary Figure Legends:

