

# The cell cycle method for phytoplankton growth studies

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**Knowledge of *in situ* species-specific growth rates of marine phytoplankton is a crucial information for understanding the structure and function of phytoplankton communities and populations.**

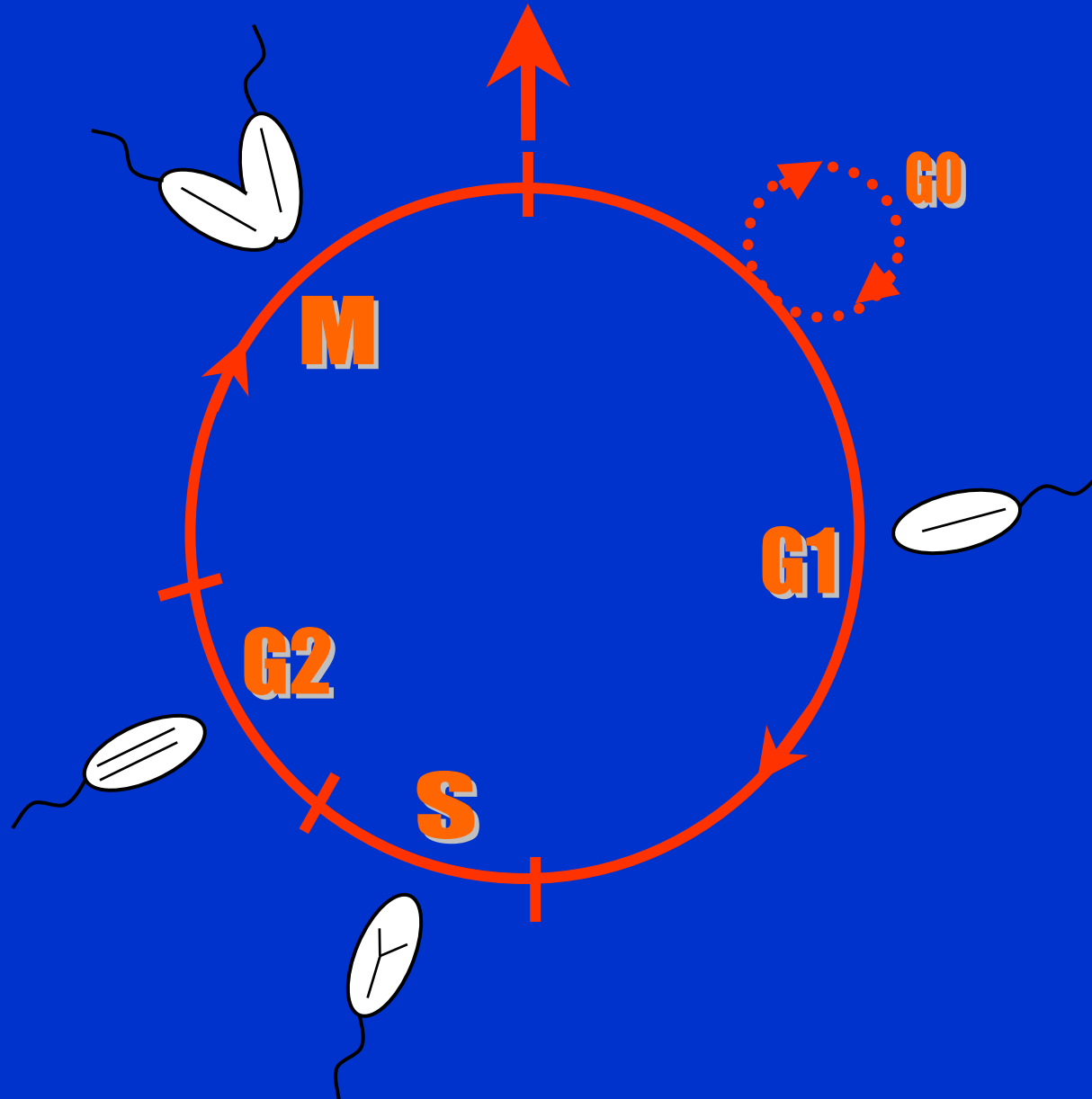
**Such information is relatively limited due to the difficulty in measuring rates**



**Cell cycle approach for growth rates estimation**

**In a unicellular organism, cell population growth is the direct result of the completion of a cell division cycle**

# Eukaryotic cell cycle



# Cell cycle approach for growth rates estimation

**Concept of the method:**

estimation of population growth rates by monitoring how fast a cell population passes through a certain phase of the cell cycle

Advantages: free from bottle effects

not affected by cell loss due to death or grazing

**Need for a 'TERMINAL EVENT'**

segment of the cycle which begins at any point but terminates at the end of mitosis

$T_x$  = time required to travel through the terminal event

$f_x$  = daily averaged fraction of a population in a terminal event

## MARKERS

- **Mitotic Index** (*McDuff and Chisolm, 1982; Vaultot, 1992*)
- **Diel DNA synthesis cycle** (*Carpenter and Chang, 1988*)
- **Cell cycle proteins** (*Lin, Chang and Carpenter, 1995*)

## Frequency of dividing cells method (McDuff and Chisolm, 1982; Vaultot, 1992)

Use of a Mitotic Index :

count double-nucleated cells (second half of M) and estimate  $f_x$  (fraction of cells in terminal event)

But difficult to assess  $T_x$  (duration of the event) since M is relatively short → requires frequent sampling and not easily applicable *in situ*

$$\mu = \frac{1}{nT_d} \sum_{i=1}^n \ln(1 + f_i) \quad (1) \quad \mu = \text{daily mean growth rate (d}^{-1}\text{)}$$

## DNA quantification method (Carpenter and Chang, 1988)

Based on diel DNA synthesis cycle

Terminal event longer than M phase → need a different technique to identify cells in G1, S and G2 phases.

$$\mu = \frac{1}{n(T_s + T_{G2M})} \sum_{i=1}^n \ln(1 + f_{S_i} + f_{G2M_i}) \quad (2)$$

**Measure cellular DNA content by using a DNA-specific fluorochrome and quantitative microscopy or flow cytometry**

**Cells fixed in chilled MeOH (-20°C)**



**Stained with DAPI (at least 300 cells)**



**Quantitative epifluorescence microscopy (quantify intensity of staining)**

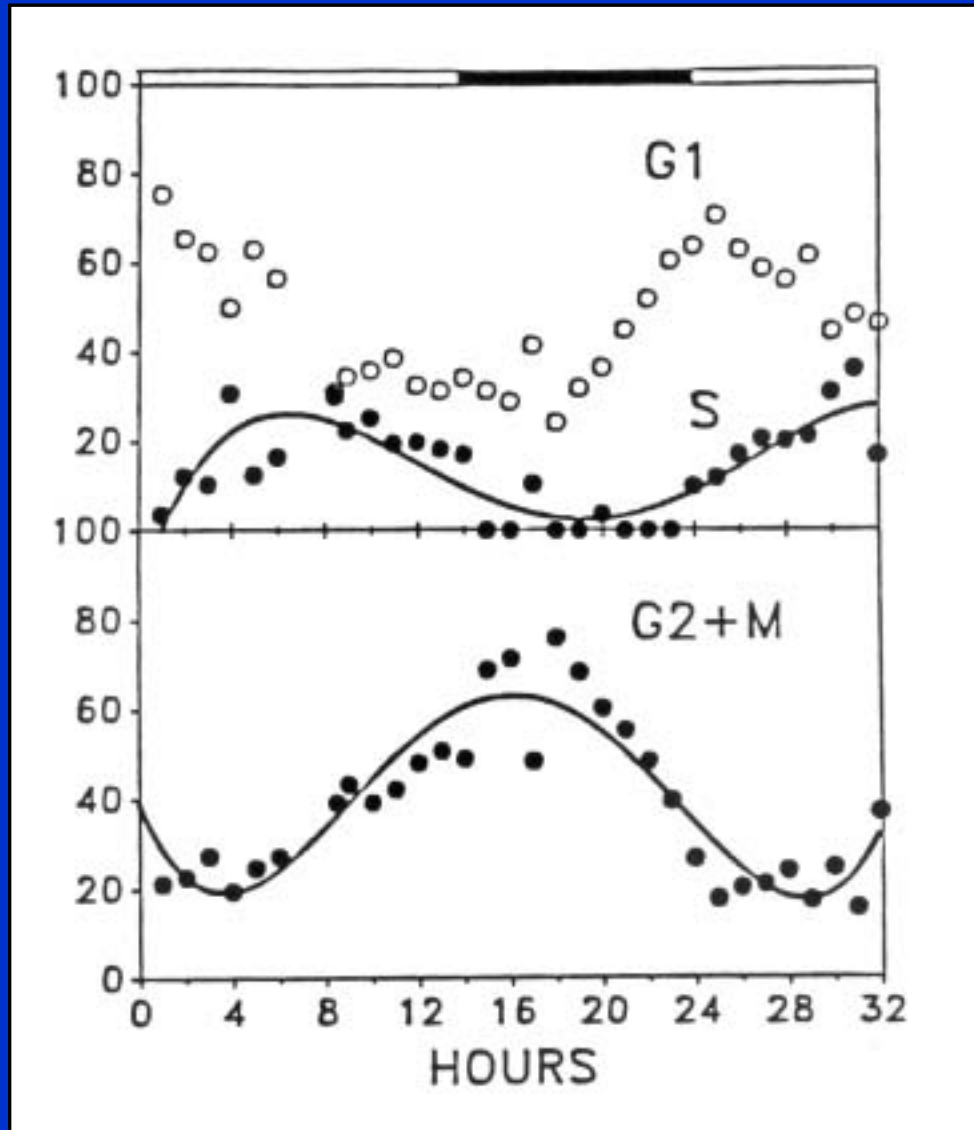


**DNA histogram and curve fitting to estimate n. of cells in each curve**

**Has been successfully applied to dinoflagellates (cfr. Chang and Carpenter 1991, 1994)  
and oceanic picoplankton (Vaulot *et al.*, 1995),  
but requires a sophisticated computer-interphased microscope or flow cytometer  
and a data deconvolution program**

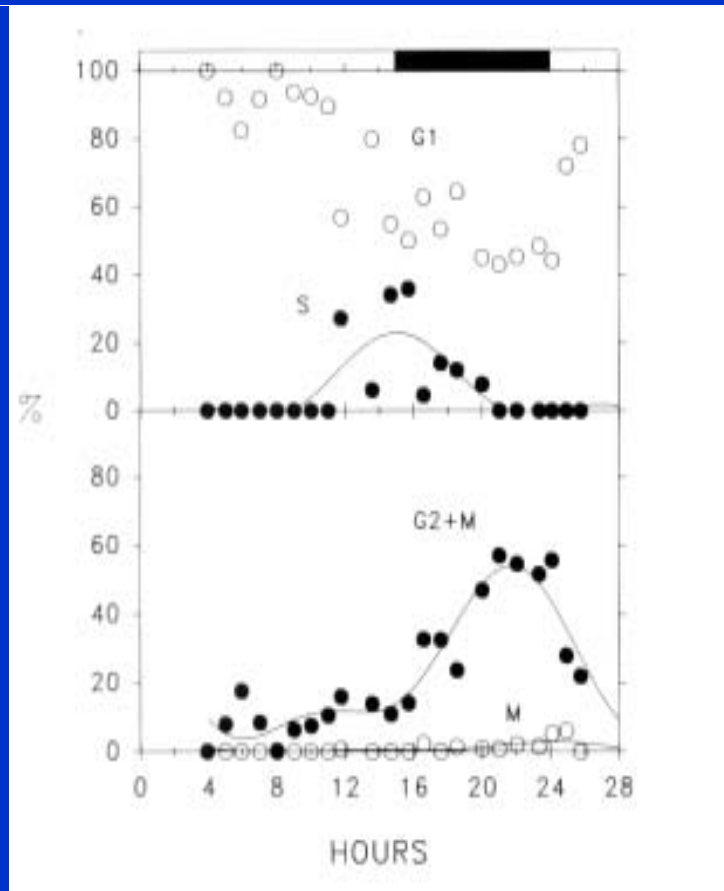
## Diel synthesis cycle:

DNA quantification and model verification with the dinoflagellate *Heterocapsa triquetra*

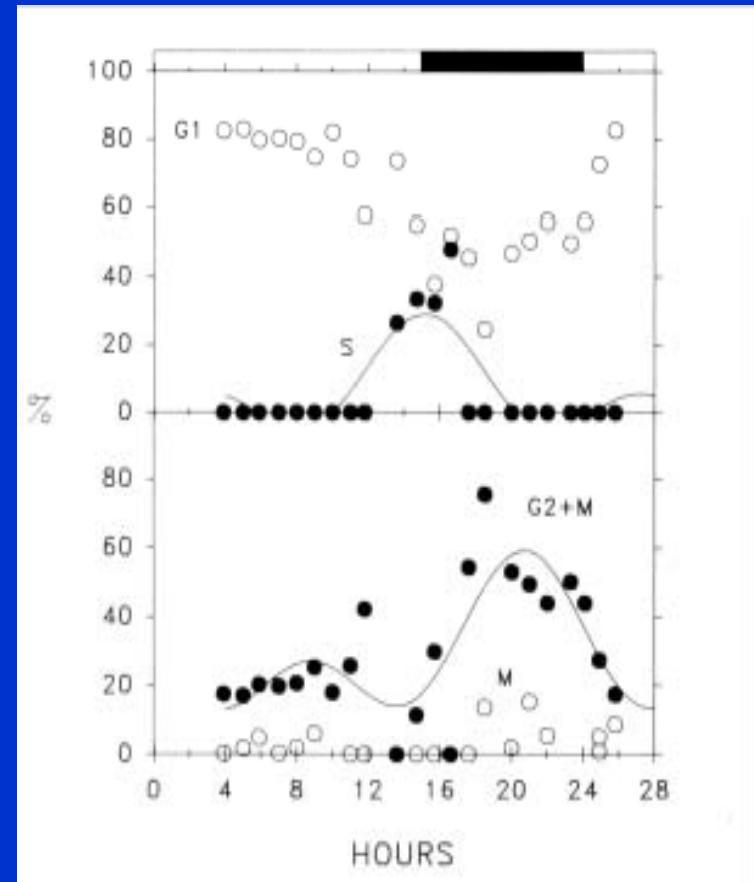


(Chang and Carpenter, 1988)

## Diel synthesis cycle: Application to natural populations

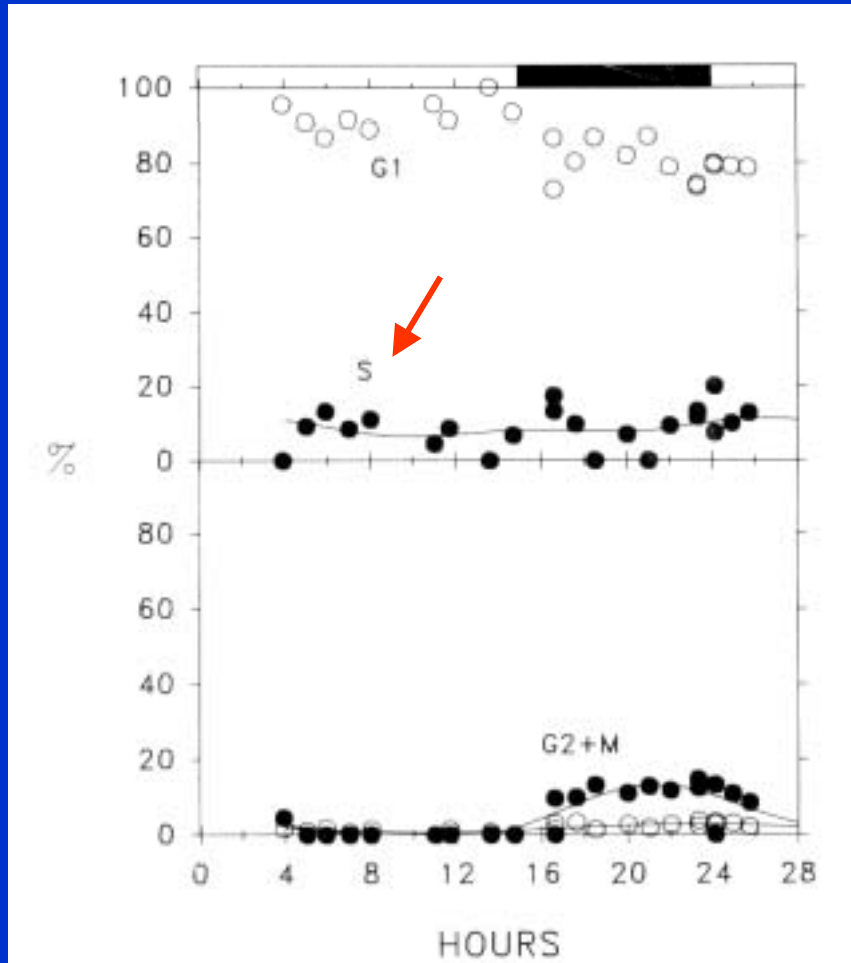


*Prorocentrum triestinum*, Long Island Sound



*Dinophysis acuminata*, Long Island Sound

## Diel synthesis cycle: Application to natural populations (continue)



*Leptocylindrus danicus*, Long Island Sound

*“Difficulties will be inevitable when peaks cannot be reliably located with the curve fitting techniques”*

## Cell cycle proteins method (Lin, Chang and Carpenter, 1995)

- Cell cycle-related proteins can be used to identify different cell cycle phases, resolving the uncertainty of phase fraction estimation.
- Method simpler in equipment requirement and less time consuming: only regular epifluorescence microscope and an immunostaining technique needed.

$$\mu = \frac{1}{nT_d} \sum_{i=1}^n \ln(1 + f_i)$$

### Requisites of a cell cycle protein candidate:

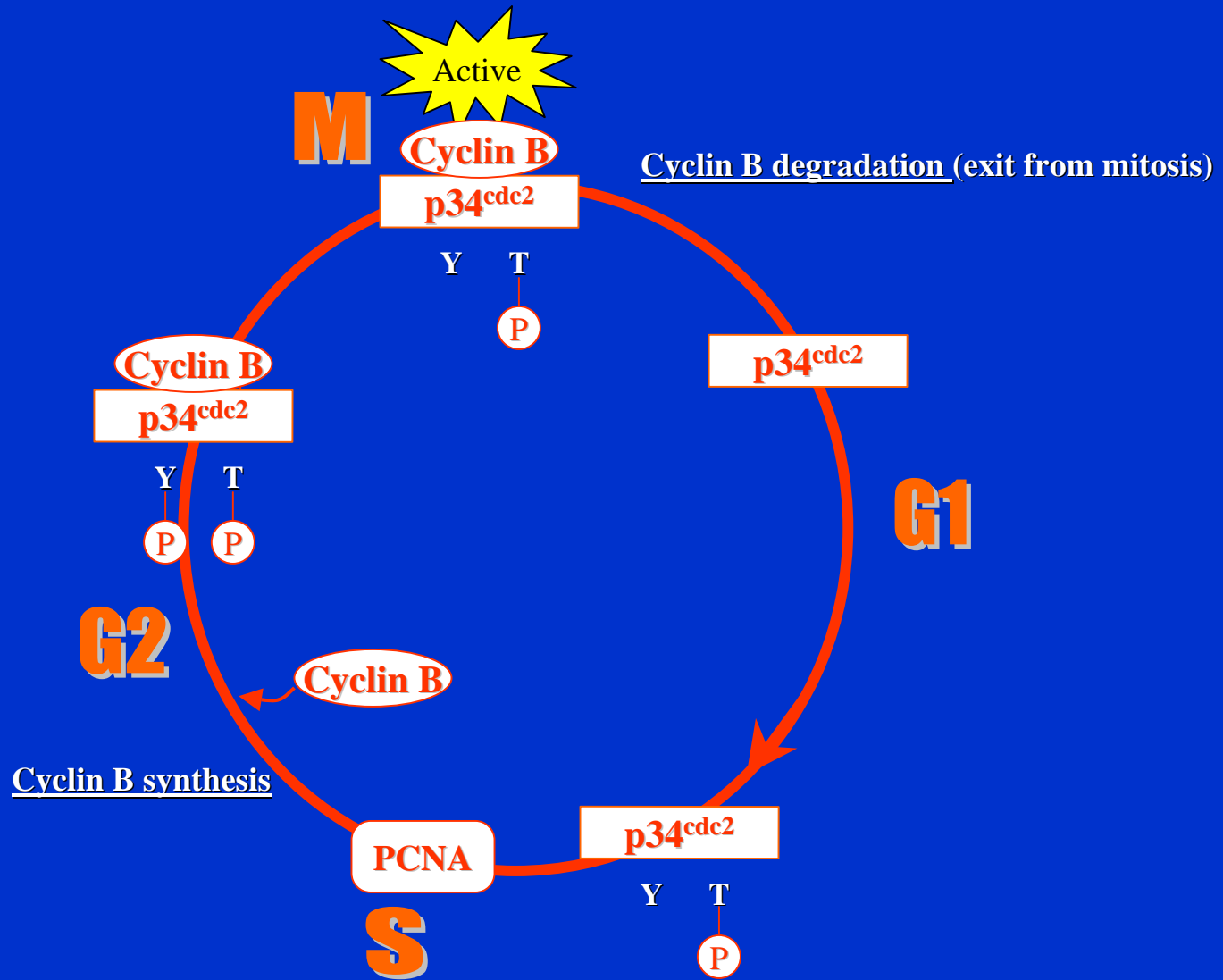
- ✓ Widely present in different organisms
- ✓ Growth stage-related and cell cycle phase-dependent
- ✓ (Ideally) aminoacid sequence highly conserved

# Regulation of the cell cycle

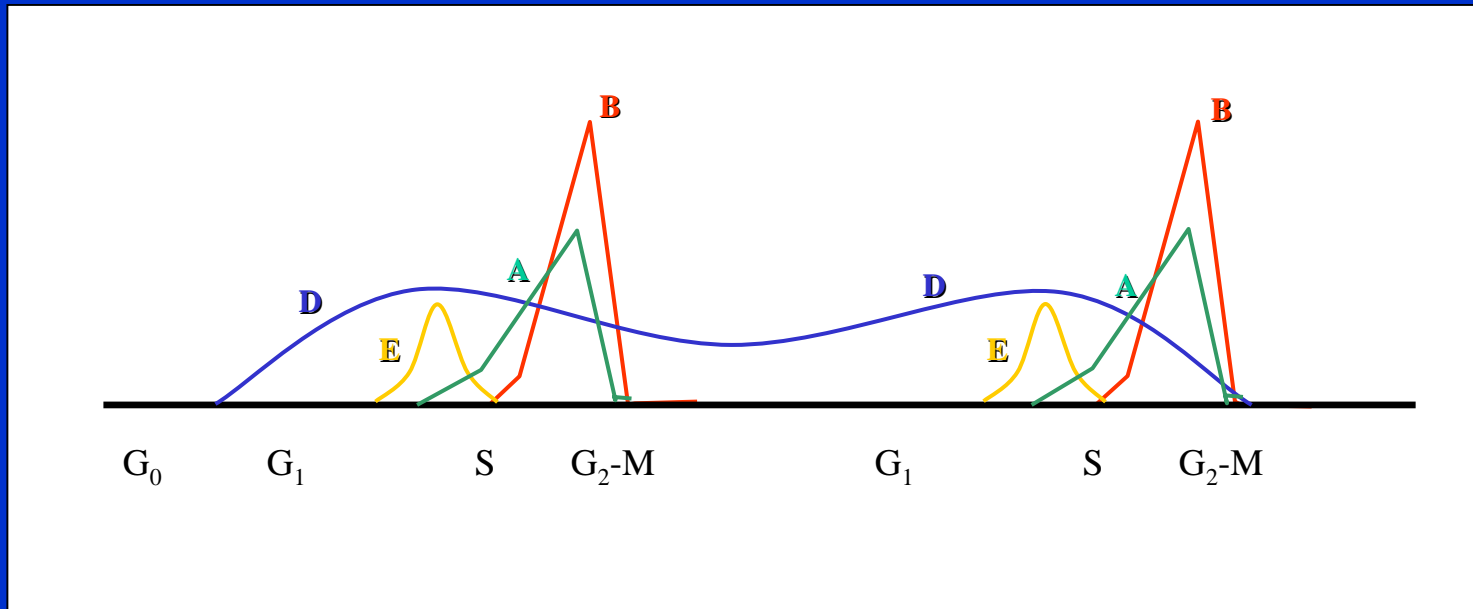
- **Cyclins**
  - Mitotic cyclins (**cyclin B**)
  - S-phase cyclins (**cyclin A**)
  - G1 cyclins (cyclin C, D, E and F)
- **Cyclin-dependent kinases or CDKs** (i.e. **p34<sup>cdc2</sup>**)
- **CDKs inhibitory proteins**
- **Proliferating Cell Nuclear Antigen or PCNA** (S-phase)

- Detected in phytoplankton

Y: tyrosine residue  
T: threonine residue



# Fluctuation of cyclins during the cell cycle



(Redrawn from C.J. Sherr, *Science*, 1996)

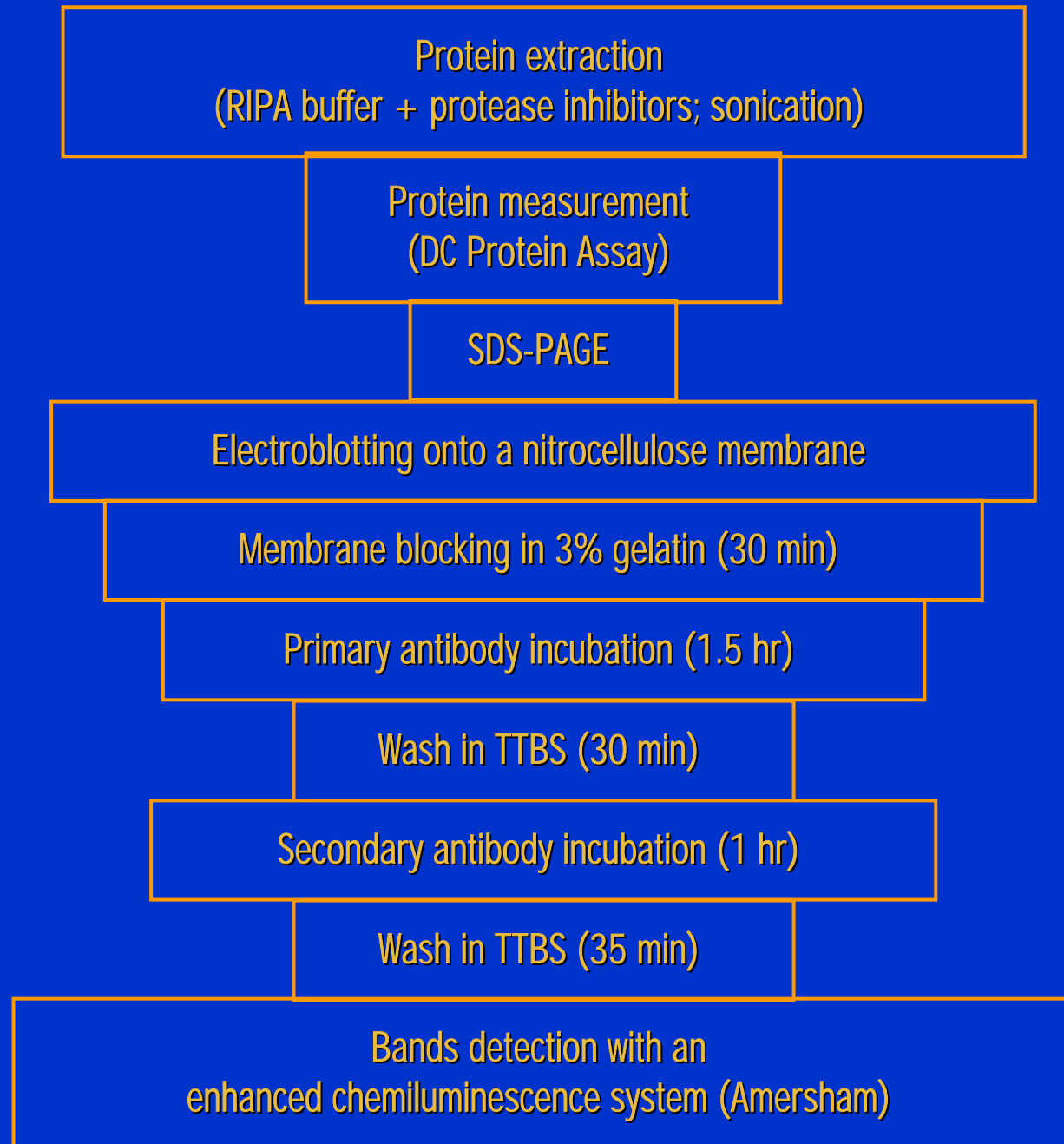
## Cell cycle-related proteins detected in phytoplankton

Protein homolog	Species	References
p34cdc2	<i>Chlamydomonas reinhardtii</i> (FW) <i>Cryptocodinium cohnii</i> <i>Gambierdiscus toxicus</i> <i>Dunaliella tertiolecta</i> <i>Prorocentrum minimum</i> <i>Alexandrium tamarense</i> <i>Aureococcus anophagefferens</i>	John <i>et al.</i> , 1989 Rodriguez <i>et al.</i> , 1993 Van Dolah <i>et al.</i> , 1995 Lin <i>et al.</i> , 1996 Magaletti <i>et al.</i> , unpublished Magaletti <i>et al.</i> , unpublished Magaletti, 1998
PCNA	<i>Thalassiosira weissflogii</i> <i>Skeletonema costatum</i> <i>Heterocapsa triquetra</i> <i>Ethmodiscus rex</i> <i>Tetraselmis chui</i> * <i>Isochrysis galbana</i> * <i>Dunaliella tertiolecta</i> * <i>Prorocentrum minimum</i> <i>Aureococcus anophagefferens</i> <i>Pleurochrysis carterae</i> *	Lin <i>et al.</i> , 1994 Lin <i>et al.</i> , 1994 Lin, 1995 Lin and Carpenter, 1995 Cheng <i>et al.</i> , 1997 Lin and Carpenter, 1998 Lin and Carpenter, 1998 Magaletti <i>et al.</i> , unpublished Magaletti, 1998 Lin and Corstjens, 2002
Cyclin B	<i>Prorocentrum minimum</i> <i>Dunaliella tertiolecta</i> <i>Aureococcus anophagefferens</i> **	Lin, 1995 Lin <i>et al.</i> , 1996 Magaletti, 1998; Lin <i>et al.</i> , 2000
Cyclin A	<i>Dunaliella tertiolecta</i> <i>Prorocentrum minimum</i> <i>Aureococcus anophagefferens</i>	Lin <i>et al.</i> , unpublished Magaletti <i>et al.</i> , unpublished Magaletti, 1998

\*Gene identification and preliminary characterization.

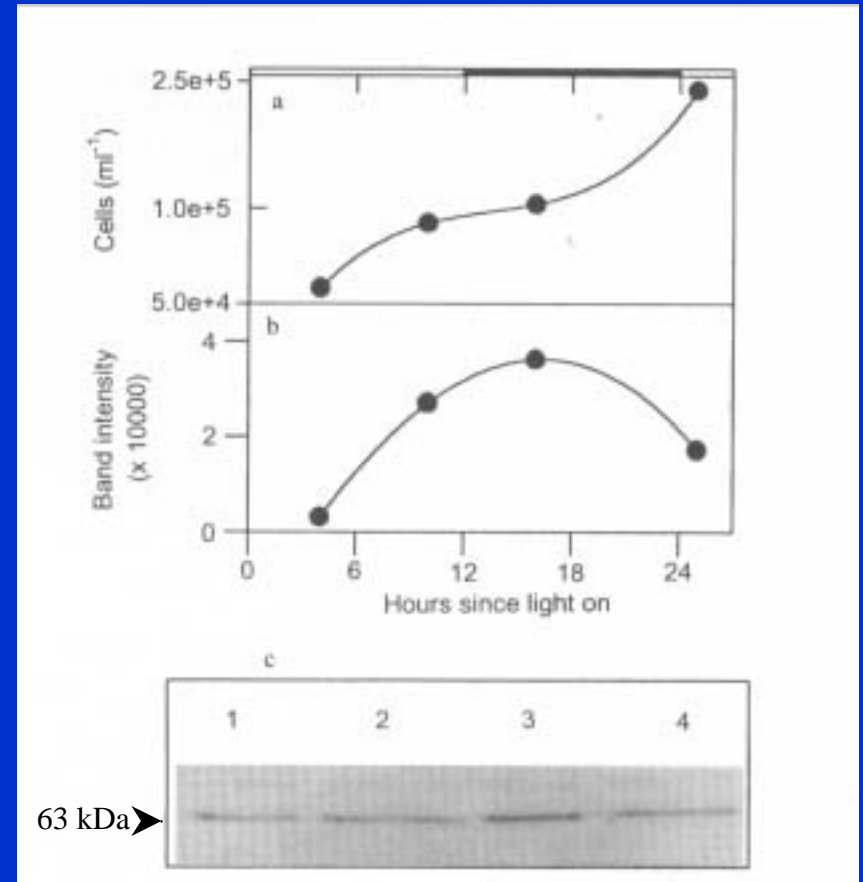
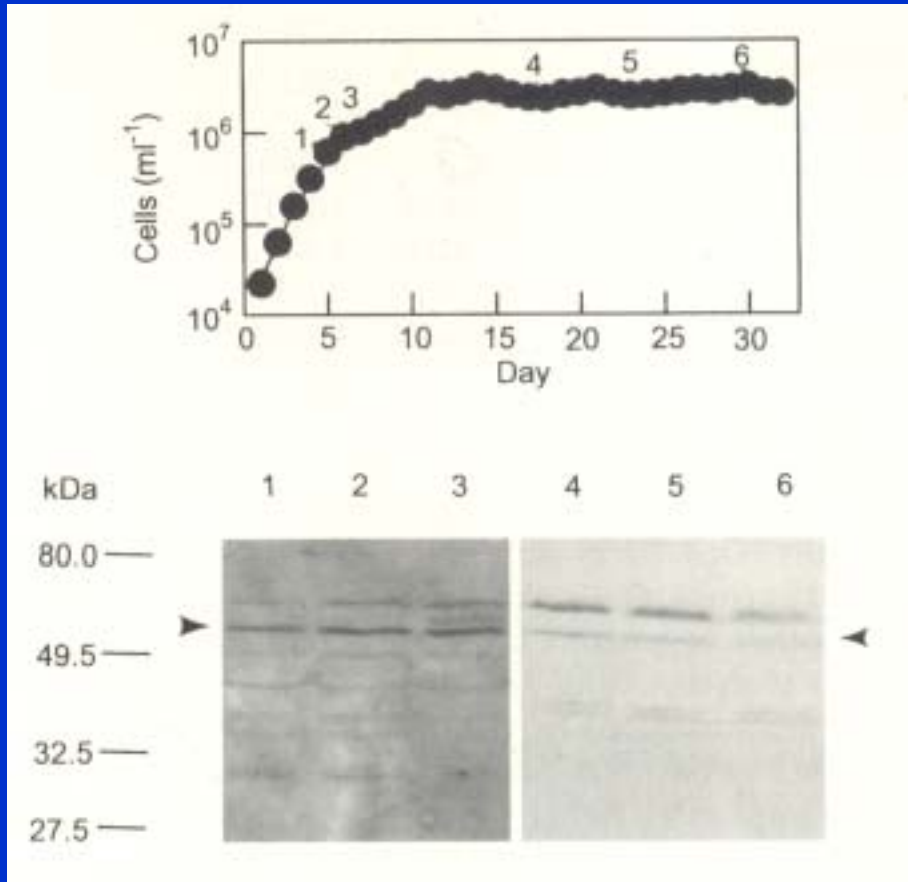
\*\*Gene fragment identification, antibody development.

# Western blotting



# Cyclin B-like protein in different cell cycle phases and growth stages

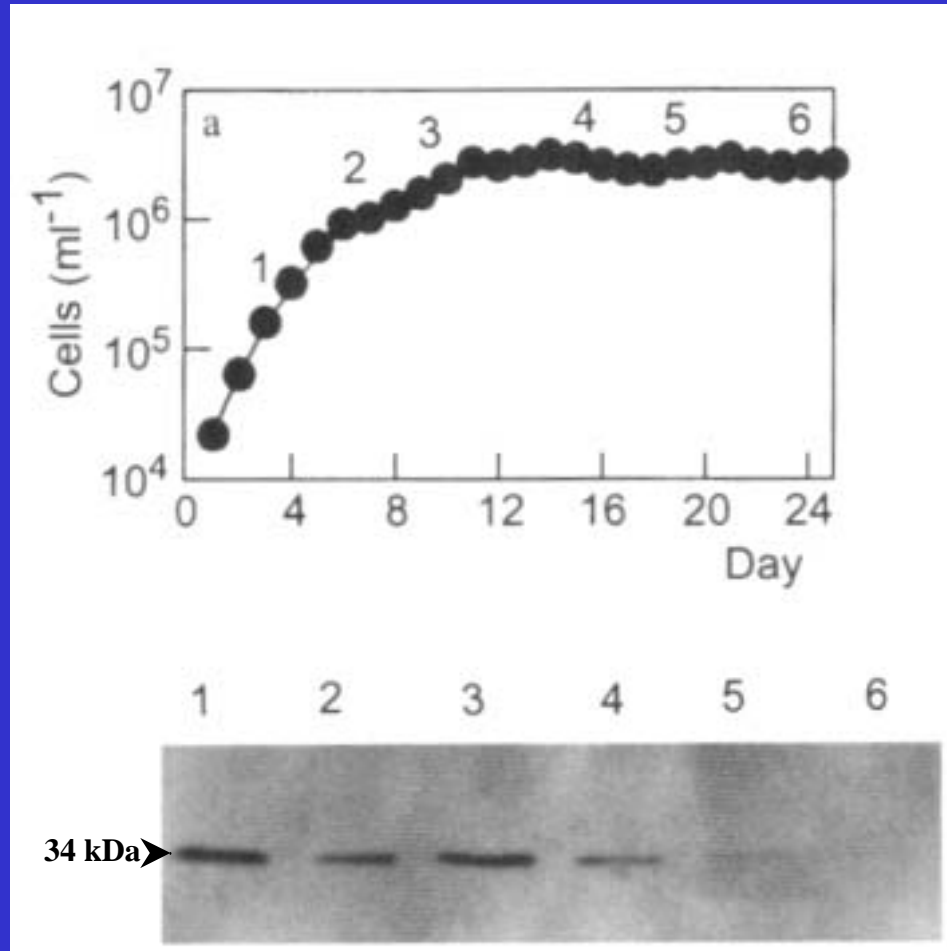
*Dunaliella tertiolecta*



(Lin, Carpenter and Chang., 1996)

# p34cdc2-like protein in different growth phases

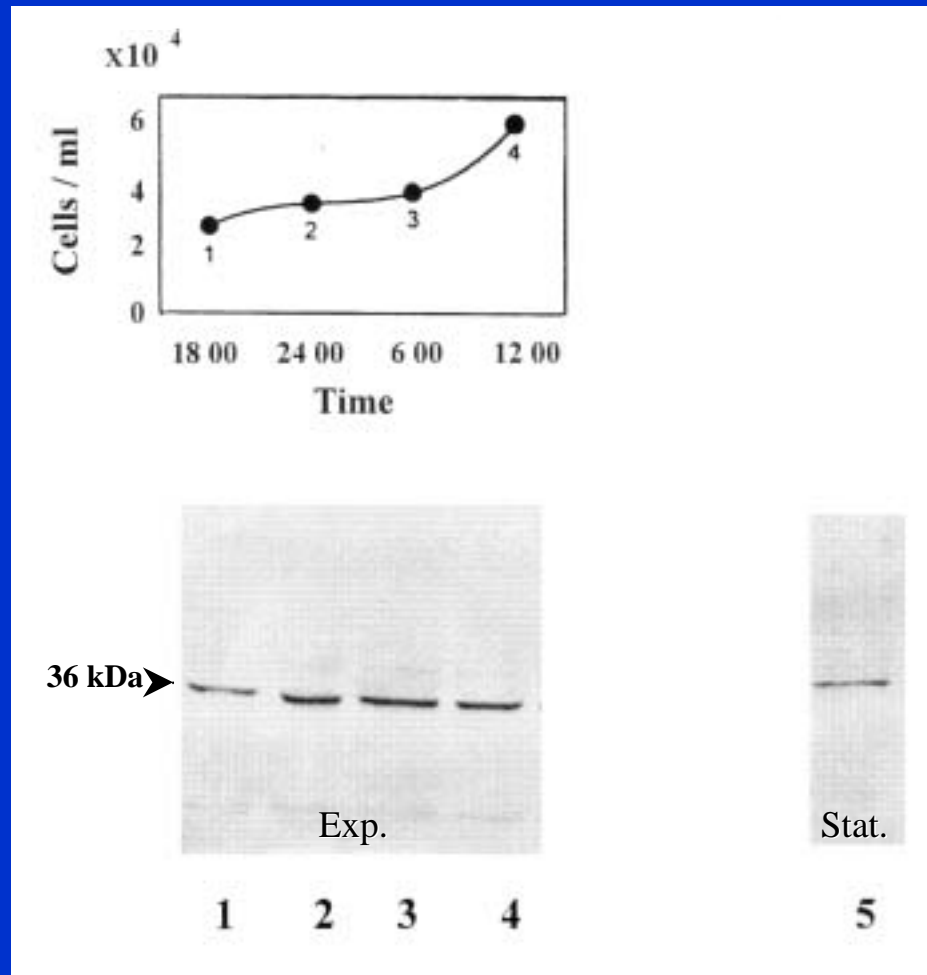
*Dunaliella tertiolecta*



(Lin, Carpenter and Chang, 1996)

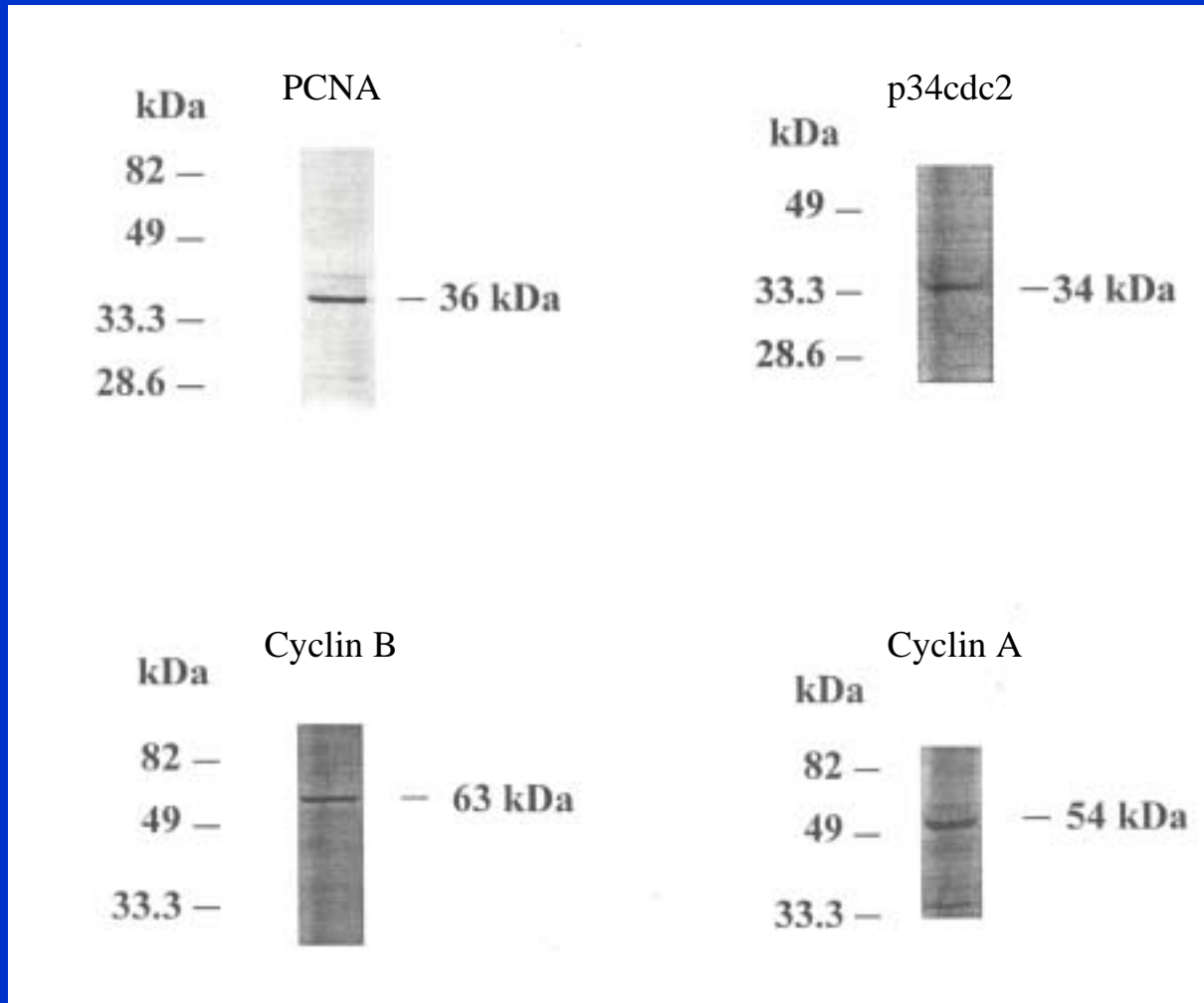
# PCNA-like protein in different cell cycle phases and growth stages

## *Prorocentrum minimum*



(Magaletti et al., unpublished)

# Detection of cell cycle-related proteins in *Aureococcus anophagefferens*



(Magaletti, 1998)

# Immunostaining protocol

Sample fixation (2% PFA f.c., 6 hrs, 4°C)

Sample storage (MeOH, -20°C)

Filtration (0.6 mm Millipore filter, 1 atm)

Transfer cells from filters onto slides

Permeabilization (DMSO 0.5%, 15 min, 4°C)

(Incubation in normal goat serum, 30 min, RT)

Primary antibody incubation (4 hrs, 4°C)

Wash in PBS (3x2 min)

Secondary antibody incubation (FITC or AMCA, 1 hr, RT)

Wash in PBS (3x2 min)

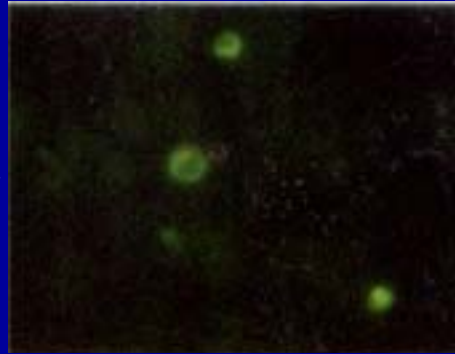
(Counter-stain with DAPI, 10 min, 4°C)

Rinse

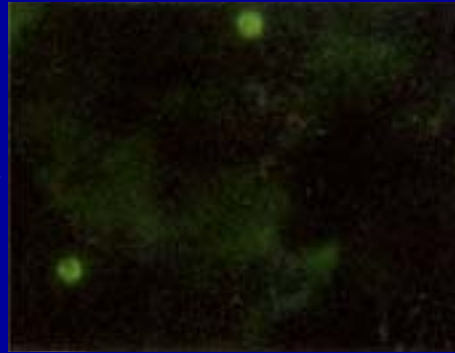
Mount (Gel Mount)

Storage in MeOH, -20°C

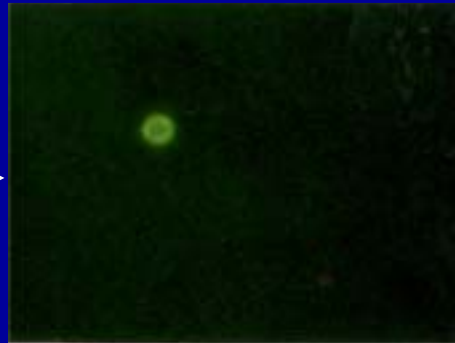
2 weeks →



5 weeks →



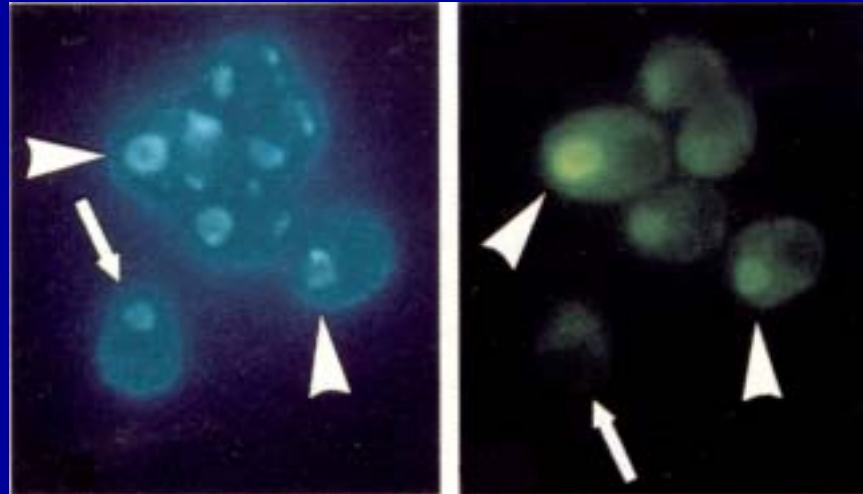
2 months →



*Aureococcus anophagefferens*

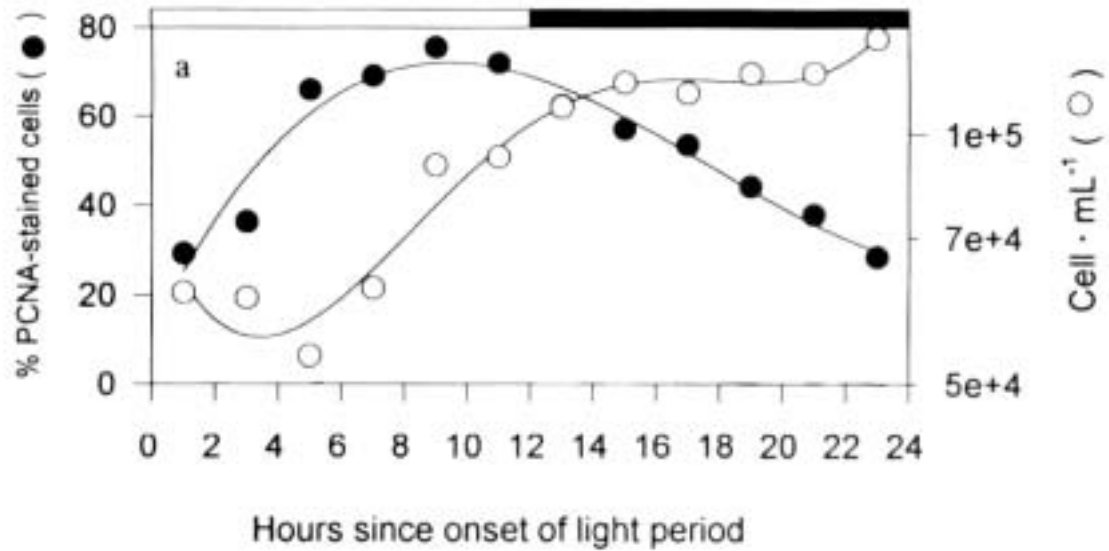
Immunostaining with polyclonal  
anti-*Isochrysis* Rubisco antibody

## PCNA immunostaining of *Dunaliella tertiolecta*



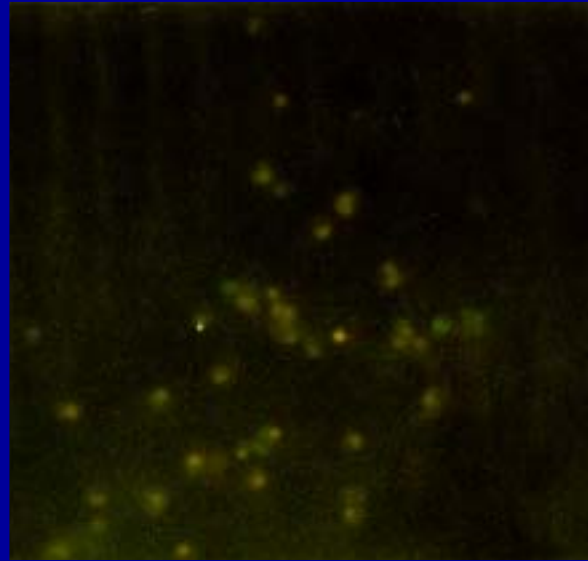
DAPI

Anti-rat PCNA monoclonal ab



*Aureococcus anophagefferens*

Anti-rat PCNA  
monoclonal antibody



Anti-BT cell surface  
polyclonal antibody  
(by D.M. Anderson)



# Molecular cloning and antiserum development of Cyclin box in the brown tide alga *Aureococcus anophagefferens*

(Magaletti, 1998; Lin, Magaletti & Carpenter, 2000)

RNA extraction

cDNA synthesis (RT)

RT-PCR

Agarose gel DNA electrophoresis

Subcloning

Bacteria transformation

Screening of the transformed bacteria

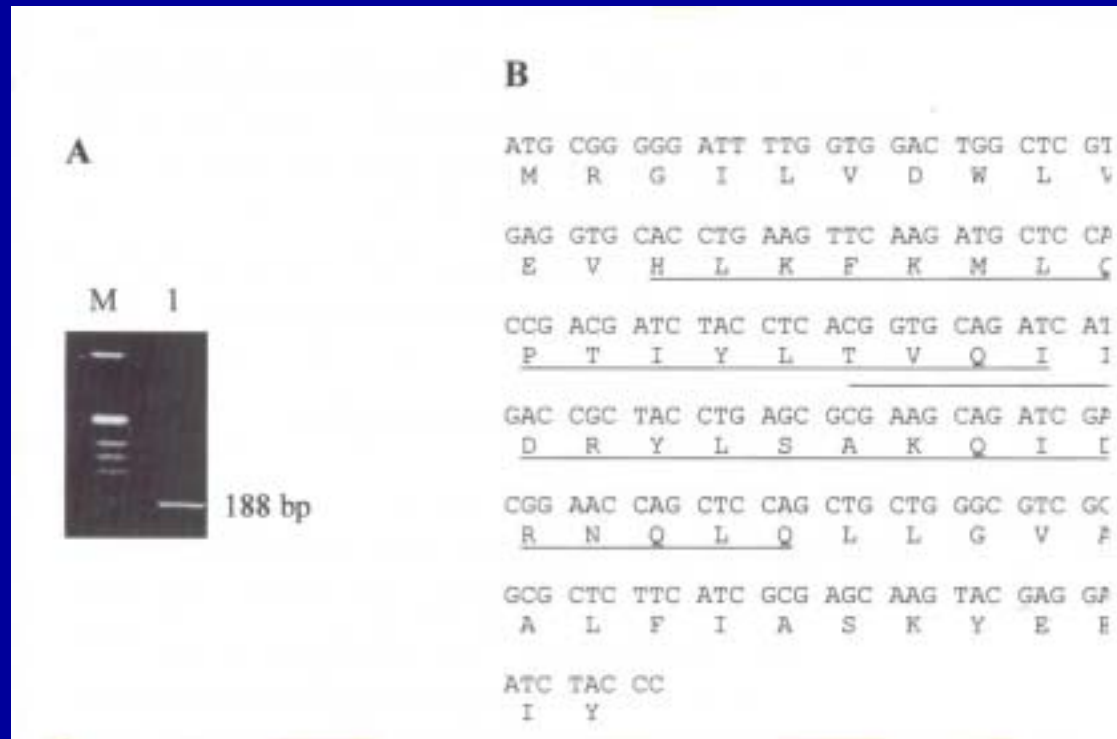
Plasmid purification

Sequencing

Synthesis of polypeptide encoded by gene sequence

Rabbit immunization and antiserum collection (Biosynthesis)

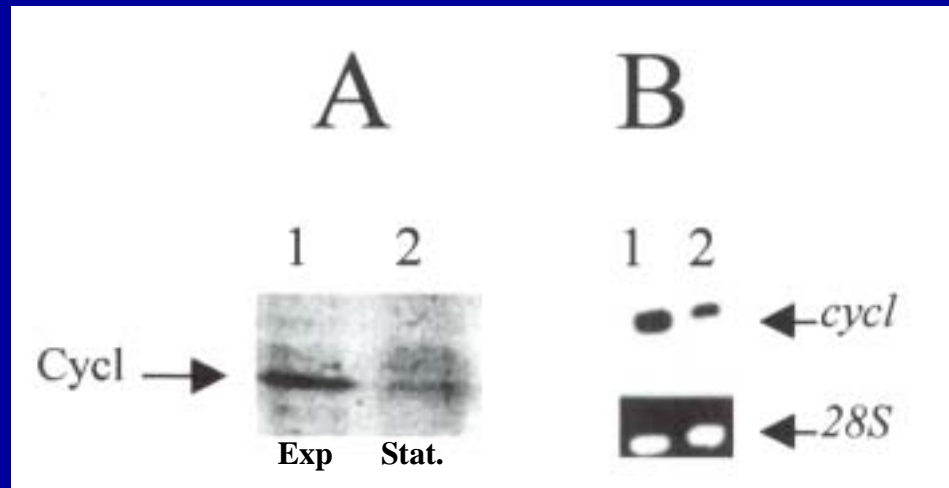
# Cloning of Cyclin box in *Aureococcus anophagefferens*



A: Agarose gel electrophoresis (1%)  
of PCR product stained with EB

B: Nucleotide and deduced aminoacid sequence

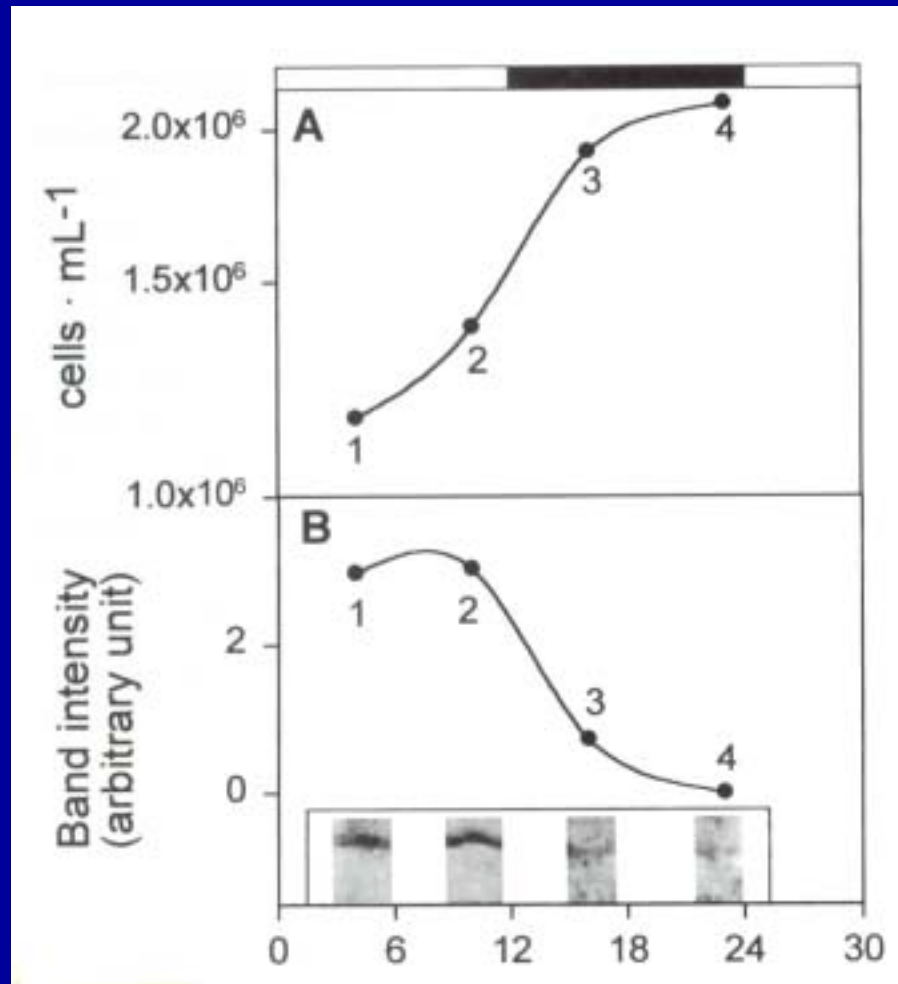
## Variation in the expression of *Btcycl1* gene with growth stage



**A:** Western blot with the newly developed anti-*Btcycl1* antibody

**B:** RT-PCR Southern hybridization of *Btcycl1* and RT-PCR of 28S rRNA

# Variation in abundance of the cyclin B-like protein with the cell cycle



# Micrograph of immunofluorescence and DAPI staining

Anti-BtCYCL1  
polyclonal antibody



DAPI



- The determination of *in situ* growth rates is one of the key elements in studying bloom dynamics, and is a particularly relevant information for toxic/nuisance species
- The use of cell cycle-related proteins as markers for the cell cycle method is a very promising approach
- More work needs to be done, at the single species level, in order to
  - detect the cell cycle protein of interest
  - identify the genes involved
  - perform field work with antibodies or gene probes