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Control mechanisms of cell proliferation in marine dinoflagellates

Mecanismos de control de proliferación celular en dinoflagelados marinos

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ABSTRACT

The effects of animal growth factors (platelet derived growth factor, PDGF), animal hormones (Hydrocortisone), artificial mitogenes (phorbol ester monoacetate 12 phorbol, TPA), lectins (Concanavalin A, Phaseolous limensis PHA), phytohormones (zeatine, abscisic acid) and okadaic acid on cell yield and acclimated maximal growth rates were analyzed in axenic clonal cultures of the dinoflagellates: *Prorocentrum lima*, *Prorocentrum minimum*, *Gyrodinium impudicum*, *Alexandrium minutum* and *Gymnodinium catenatum*. All the mitogenes analyzed were able to increase significantly the growth rates in the five species tested except with the okadaic acid which showed a mitogenic effect only with *P. lima*, increasing up to tree times its growth rate. However, the presence of okadaic acid did not affect the growth on the other species analyzed. These results showed that dinoflagellates respond to growth factors in a similar way to mammalian cells. This is thought to be due to the existence of a universal mechanism controlling the cell cycle in all eukaryotic cells. Due to the fact that growth factors act in a paracrine manner on the neighboring cells, cell density plays an important role on dinoflagellate growth. This could be because the likelihood of a telemediator encountering its target cell is function of cell density. The tentative idea that eukaryotes are able to control their cell proliferation themselves suggests eukaryotic phytoplankton could regulate the growth rates of multiespecies aggregates process in plankton growth.

Key words: mitogenes, cell division cycle, growth factors, *Prorocentrum lima*, *Prorocentrum minimum*, *Gyrodinium impudicum*, *Alexandrium minutum*, *Gymnodinium catenatum*.

RESUMEN

Se analizaron los efectos de factores de crecimiento animales (factor de crecimiento derivado de plaquetas, PDGF), hormonas animales (hidrocortisol), mitógenos artificiales (monoacetato 12 de forbol, TPA), lectinas (concanavalina A, *Phaseolus limensis*, PHA), fitohormonas (zeatina, ácido abcísico) y ácido okadaico sobre las tasas de crecimiento aclimatadas en cultivos axénicos de cinco especies diferentes de dinoflagelados, *Prorocentrum lima*, *Prorocentrum minimum*, *Gyrodinium impudicum*, *Alexandrium minutum* y *Gymnodinium catenatum*. En general, todos ellos fueron capaces de incrementar las tasas de división en todas las especies analizadas. La única excepción fue el ácido okadaico que no modificó significativamente la tasa de crecimiento en ninguna especie analizada excepto en el caso de *P. lima* que se triplicó. Estos resultados indican que los dinoflagelados responden a la presencia de factores de crecimiento en el medio de forma semejante a como lo hacen las células de mamíferos, confirmado la existencia de un mecanismo universal que controla el ciclo celular en los organismos eucariotas. Debido a que los factores de crecimiento actúan de una forma paracrina sobre las células vecinas, la densidad celular juega un papel importante en el crecimiento de los dinoflagelados, probablemente debido a un efecto telemediador.

Palabras clave: mitógenos, ciclo de división celular, factores de crecimiento, *Prorocentrum lima*, *Prorocentrum minimum*, *Gyrodinium impudicum*, *Alexandrium minutum*, *Gymnodinium catenatum*.

INTRODUCTION

It is generally assumed that unicellular phytoplanktonic organisms proliferate and are limited only by the rate at which nutrients can be taken up from the medium and converted into cell materials. From this point of view, dinoflagellate blooms are thought to be controlled by abiotic variables (i.e. water column stratification, wind, tides and currents, light, temperature and other hydrographic features) and by nutritional factors (i.e. eutrophication, pollution, micro and macro-nutrients).

Other mechanisms regulating the rate of mitosis, such as biological controls, could however, play an important role in the autocontrol of algal proliferation. In this regard, circadian and annual rhythms, endogenous control of mitosis, and biosynthetic rates could be controlling factors in determining the growth of bloom-forming or toxic dinoflagellates (Fraga & Prego 1989, Margalef 1989, Taylor 1990).

With respect to biological control of mitosis, the dependence on growth factors and mitogenes, contact inhibition, anchorage dependence and cells genetically programmed to die are among the main mechanisms which influence the multiplication of higher eukaryotic cells (Alberts *et al.* 1989). They depend upon growth rates, genes and gene products that respond to growth factors (Cantley *et al.* 1991). Although these have been interpreted as adaptations for regulating cellular proliferation in multicellular organisms, several of the mechanisms have recently been detected in unicellular algae. The animal growth factor PDGF as well as the phorbol ester monoacetate 12 phorbol increased the rate of mitosis of several marine phytoplanktonic organisms (Costas *et al.* 1993b). Furthermore, these mitogenes were also able to germinate resting cysts of dinoflagellates and some dinoflagellates showed contact growth inhibition (Costas *et al.* 1993c).

Moreover, some evidence suggests that dinoflagellates are also able to secrete telemediators, which act paracrinely on the closets cells. In this regard, studies on rhythmicity show that dinoflagellates are able to communicate intercellularly by telemediators (Cornelissen *et al.* 1986). Dinoflagellates are apparently also able to inhibit diatom growth by means of telemediators (Arzul & Gentien 1990). These facts suggest that telemediators could be a biological control mechanism on dinoflagellate survival and proliferation. Thus, cell density could play an important role in dinoflagellate growth, because the probability of a telemediator encountering target cell is a function of cell density (Alberts *et al.* 1989). In animal and plant cells, these telemediators are growth factors and hormones.

As an approach to understanding the role played by biological control of mitosis in dinoflagellates growth, the mitogenic effect of animal growth factors, hormones, phorbol esters, lectins, phytohormones and okadaic acid on the proliferation of five different species were studied. We also analyzed the effects of cell density on the proliferation and survival of *P. lima.*

MATERIALS AND METHODS

CULTURES: Five different dinoflagellate species were analyzed, *P. lima* (clone Pl8V), *P. minimum* (clon Pmin), *G. impudicum* (clon Gg7P), *A. minutum* (clon Am1V) and *G. catenatum* (clon Gc9V). The cultures were grown under 12:12 h LD cycle at 50 µmol photons m^{-2} s^{-1} and a temperature of 20±1 °C in 250 Erlenmeyer flasks with 100 ml of f/2 medium (Sigma). The culture was maintained in exponential growth by trasferring an inoculum of $10^4 \pm 10^2$ cells to fresh medium once every 2 weeks.

The cultures were treated for 2 h with 150 mg l^{-1} penicillin, 100 mg l^{-1} streptomycin and 205 µg l^{-1} amphotericine (Sigma) to obtain axenic cultures. Before the experiments took place, cultures were tested for the presence of bacteria by adding acridine orange and examination in the epifluorescence microscope (Costas 1990). The inhibitory effects of the antibiotics on growth rates were avoided performing the treatment 30 days before the experiment took place.

EFFECT OF MITOGENES: To analyze the effect of mitogenes, three replicates of each culture in exponential growth phase (mid log) were grown in multiwell dishes with 2 ml in each of the following media:

1. Mitogene supplemented media (f/2 medium with 10 ng ml^{-1}) of:

-Platelet derived growth factor (PDGF, Sigma).
-12 phorbol monoacetate (TPA, Sigma)
-Hydrocortisone (Sigma).
-Lectin from *Phaseolus limensis* (PHA, Sigma).
-Lectin from *Canavalia ensiformis* (ConA, Sigma).
-The phytohormone Zeatine (Sigma).
-The phytohormone Abscisic acid (Sigma).
-Okadaic acid.

2. Unsupplemented controls.

The effects of all the mitogenes were analyzed by measuring the cellular density in cells ml^{-1}. Cells were counted just after inoculation and subsequent counts were performed at days 2, 3 and 7, 15 and 20 by direct observation of the multiwell dishes in an inverted Zeiss Axiovert 35M microscope. All cultures were subcultured on day 7, inoculated into fresh medium at the same density as the initial cultures and the experiments were repeated. The number of samples in each replicate was estimated by using the progressive mean procedure with a confidence limit of ±1% (Williams 1977).

Acclimated maximal growth rates were also determined in mid-exponential growth phase as doublings day^{-1} (dd^{-1}=(1/Ln2)(Ln(Nt/No)/t) where Nt=cells ml^{-1} at time t, No=cells ml^{-1} at time 0, and t=number of days between times t and 0. To avoid the presence of traces of previous media and to achieve complete acclimatization, strains were grown for 15 days in each experimental conditions before the experiments took place. The position of the cultures in the incubator was changed randomly twice daily. More details are give in Costas (1990).

EFFECTS OF CELL DENSITY: to evaluate the importance of cell density on cell proliferation of *P. lima*, groups of 1, 10 and 100 cells were isolated, using a Zeiss Eppendorf micromanipulator, from exponentially growing cultures and placed in well microdishes plated, each microdish contained 0.25 ml of culture medium. The cells were maintained at 20±1 °C and 12:12 h LD photocycles; 48 h later all the cells in each microdish were counted in a Zeiss Axiovert 35M invertoscope.

To detect the effect of cell density on the survival rate of cells, groups of 1, 2, 4 and 8 cells were isolated using a Zeiss-Eppendorf micromanipulator-microinjector from exponentially growing cultures and placed in well microdishes, each containing 0.25 ml of culture medium. The cells were maintained at 20±1 °C and 12:12 h LD photocycles and, 48 h later, checked the invertoscope to detect dead cells using the yellow acridine exclusion procedure (Costas 1990). Each of the initially isolated cells had two possibilities, to be dead or divided. The dead rates of these cells were estimated in each group of 1, 2, 4 or 8 cells as the ratio of the number of initial dead cells/total number of initial cells. The survival rates were (1- death rates).

In both experiments, just after placing the isolated cells in the well microdish plates, all the cells were microscopically checked to remove the cells killed by the manipulation effects. The methods for the isolation, manipulation and counting low numbers of cells were previously described in more detail by Costas & López-Rodas (1990).

Both experiments were performed in three different culture media, in an attempt to detect the effect of growth factors and telemediators on proliferation and survival:

i) f/2 (Sigma; ii) f/2 supplemented with 25% of CEM (conditioned exponential medium) f/2 medium which contained about 500 exponentially growing cells ml^{-1}, which were removed by filtration (0.25 µm pore); iii) f/2 with 10 ng ml^{-1} TPA (Sigma)

The methods for using growth factor supplementation and conditioned media in unicellular algal are described in more detail in Costas *et al.* (1993a).

RESULTS AND DISCUSSION

The acclimated maximal growth rates of the different species with and without mitogene supplement are summarized in Fig. 1. The data for *P. lima* are represented in Fig. 1a, four of the seven mitogenes analyzed, PDGF, TPA, the lectin PHA and the okadaic acid significantly increased the growth rates with respect to unsupplemented controls ($p<0.01$ Mann-Whitney U test). The animal hormone hydrocortisone and the phytohormone abscisic acid slightly increase the cell growth, whereas, the lectin ConA and the phytohormone zeatine decreased the growth rates ($p<0.01$ Mann-Whitney U test).

The growth rates obtained when *P. minimum* was tested are represented in Fig. 1b. In this species, all the mitogenes analyzed increased the growth rates significantly ($p<0.01$). The highest growth rate was induced by TPA (from 0.19 div/day to 0.92), PDGF and the lectins (ConA and PHA) only were able to increase up to three times the growth rates. The lowest rates were achieved when hydrocortisone, okadaic acid and the two phytohormones were added to the media, although the growth rates were doubled in the supplemented media.

Figure 1c shows the data for *G. impudicum*. The results were very similar for this particular species, almost all the mitogenes increased significatively the growth rates ($p<0.01$) except for the okadaic acid. As always, the TPA exhibited the highest mitogenic activity followed closely by the cells incubated with the lectins PHA and ConA (the growth rates were increased from 0.12 div/day to 1.01, 0.96 and 0.94 respectively). PDGF and hydroxicortisone well as the phytohormones were the mitogenes with lower reaction, they were able to multiply by two the controls growth rates.

With *G. catenatum* the results were slightly different (Fig. 1d). In this case, the most effective mitogenes were both, lectins with growth rates up to ten times higher than the control ones (from 0.15 to 1.3 for ConA and 1.5 for PHA). The other mitogenes analyzed reached very similar growth rates among them and significantly higher than the controls ($p<0.01$). There was no statistical differences between the unsupplemented controls and the cells incubated with okadaic acid for this dinoflagellate ($p>0.01$).

Finally, the growth rates of *A. minutum* has been represented in Fig. 1e. TPA was again the most potent mitogene increasing the growth rate more than ten times followed by the two lectins. PDGF and the phytohormones zeatine and abscisic acid had similar results around 0.4 div/day in comparison with the 0.13 div/day obtained for the controls. There were no statistical differences between the unsupplemented controls and the cells incubated with hydrocortisone and okadaic acid for this dinoflagellate ($p>0.01$).

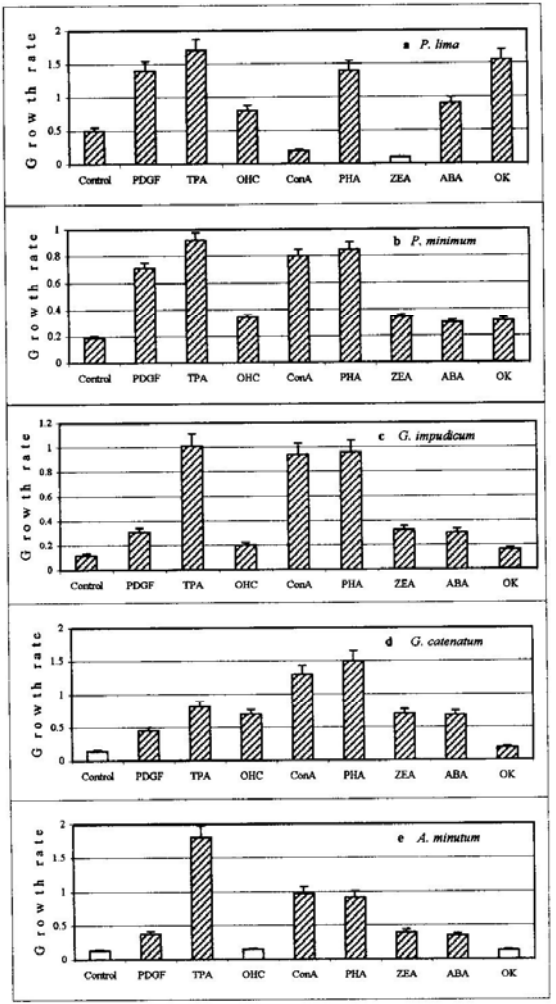

Figure 1. Acclimated growth rates (div/day) of *P. lima* in mitogene supplemented media and unsupplemented control. PDGF= Platelet Derivated Growth Factor 10 ng ml^{-1}, TPA= (12- Phorbol) Monoacetate 10 ng ml^{-1}, OHC=Hydrocortisone 10 ng ml^{-1}, ConA= *Canavalia ensiformis* 10 ng ml^{-1}, PHA= *Phaseolus limensis* 10 ng ml^{-1}, ZEA= Zeatine 10 ng ml^{-1}, ABA= Abscisic Acid 10 ng ml^{-1}, OK= Okadaic Acid 10 ng ml^{-1}. Bars represent ±1 SD for each treatment.

Figura 1. Tasas de crecimiento (div/día) aclimatadas en medios suplementados con factores de crecimiento y controles. PDGF= Factor de Crecimiento Derivado de Plaquetas 10 ng ml^{-1}, TPA= Monoacetato 12 de forbol 10 ng ml^{-1}, OHC= Hidroxicortisona 10 ng ml^{-1}, ConA= *Canavalia ensiformis* A 10 ng ml^{-1}, PHA= *Phaseolus limensis* 10 ng ml^{-1}, ZEA= Zeatina 10 ng ml^{-1}, ABA= Acido Abcísico 10 ng ml^{-1}, OK= Acido Okadaico 10 ng ml^{-1}. Las barras representan la desviación estándar.

The increase in acclimated maximal growth rates in growth factor-supplemented media is thought to be due to the specific mitogenic effect of these growth factors and not due to nutrient deficiency in the unsupplemented culture medium. Previous work has rejected the possible indirect estimulation of microalgal growth by mitogenes, which are not directly related to mitogenic activity (i.e. acting as organic nutrients, chelators or trace metals, etc) (Costas *et al.* 1993a, b) and, furthermore, only 10 ng ml^{-1} of each mitogene as added.

Molecular mechanisms which allow mitogenes to increase mitosis have been widely discussed in pervious papers (Costas *et al.* 1993a,b). Briefly, PDGF is a specific mitogen encoded by the c-sis oncogene, which acts in the phosphatidyl inositol pathway to induce DNA synthesis and cell division (Cascales 1989, Cantley *et al.* 1991). In the phosphatidyl inositol pathway, diacylglycerol (DAG) is one of the most important second messengers, which activates protein kinase C. TPA is an important tumor promoter, analog of DAG, which penetrate inside the cell and directly acts on protein kinase C increasing the amount of calcium phospholipid-dependent protein kinase associated with the plasma membrane (Kraft & Wayne 1983). This fact could explain the similarity between growth rates obtained with PDGF and TPA, assuming that they act on the same pathway.

Lectins are also able to activate or inhibit cell division cycle genes. Some lectins which specifically conjugate with membrane proteoglycans activate second messenger pathways, inducing mitosis just as with growth factors (Slifkin & Doyle 1990). The mitogenic effect of *Phaseolous limensis* on the proliferation of *P. lima* could, therefore, be due to the presence of membrane receptors for lectins on the surface of the dinoflagellate. Then, the lectin could act as an analog of the corresponding growth factor by activating the inositol-phospholipid pathway and, subsequently, activating cells proliferation.

However, some other lectins act in a citotoxic way on the control of the cell division cycle in mammalian cells (Hart 1980, Damjanov 1987, Slifkin & Doyle 1990). This kind of citotoxic effects have been widely described, due to the fact that a lot of substances have an antagonic effect to that growth factors or, because they are in an excessive high concentration (Matsunami *et al.* 1990, Reuse *et al.* 1990, Traxler *et al.* 1991). This sort of substances inhibit the tyr-kinase activity of their receptors and produce a decrease in the cell growth. In our case, the lectin from *Canavalia ensiformis* exhibits a high degree of citotoxicity with respect *P. lima* proliferation, so this effect could be produced by an antagonism to the corresponding growth factor, then inhibiting the tyr-kinase activity of the membrane receptor. The presence of lectins in many marine organisms and its abundance in macrophyta, suggest that lectins could play a significant, positive or negative, role in the occurrence and proliferation of dino-flagellates, specially in epiphytic species as *P. lima*.

In the same regard, okadaic acid is a potent mitogene on *P. lima* cells. Although the molecular effects of okadaic acid on eukaryotic cells are well known, paradoxically its biological function is believed to be a metabolite of only some dinoflagellates. It had recently been proved that okadaic acid is a potent inhibitor of the phosphatases 1 and 2A, possibly enhancing calcium influx through voltage dependent calcium channels (Novelli *et al.* 1993). Therefore, if okadaic acid is an inhibitor of these phosphatases, then okadaic acid is an indirect activator of mitosis (Cyert & Kirschener 1988, Murray 1993). Previous papers suggest the phosphatidyl inositol

pathway is the main biochemical trigger to control cell proliferation in dinoflagellates (Costas *et al.* 1993a,b). In interphase extracts, okadaic acid treatment can drive cdc 25 into its fully phosphorylated and active form, providing a simple premature entry into mitosis (Murray 1993).

Specificity is a current property of mitogenes and growth factors which only acts on their target cells (Cantley *et al.* 1991). In this respect, *P. lima* (a benthic dinoflagellate) has mechanisms to control its cell proliferation (i.e. contact inhibition of growth) that other planktonic dinoflagellates lack.

In a similar way, phytohormones regulate the division cycle in plant cells as growth factors do in animal cells. The molecular mechanism of phytohormones seems to be similar to that of growth factors, increasing the transcription of several cell cycle-dependent genes and modifying the cell division cycle, inhibiting (as zeatine does) or stimulating it (as abscisic acid does) (Peaud-Leonel 1977).

Table 1 summarizes the total cell of *P. lima* number 48 h after that groups of 1, 10 or 100 exponentially growing cells had been placed in three different culture media. Apparently cell density has an influence on cell proliferation. So in f/2 medium without any mitogene or conditioned, initial groups of 100 cells proliferate statistically significantly more than initial groups of 10 or 1 cell (tested by Kruskal-Wallis H test, $p<0.05$).

Table 1. Total cell numbers of *P. lima* 48 h after groups of 1, 10 or 100 exponentially growing cells had been placed in three different culture media. Results are corrected to compensate for the initial differences in cell numbers by multiplication by 100, x10 and x1, the 1, 10 and 100 cell groups respectively. Significance of Kruskal-Wallis H test are summarized in rows and columns. n= number of replicates, m± sd= mean ± standard deviation.

Tabla 1. Número total de células de *P. lima* después de 48 h de haber sido distribuidas en grupos de 1, 10 o 100 células en tres medios cultivo diferentes. Los resultados han sido corregidos para compensar las diferencias iniciales en el número de células multiplicando por 100, 10 y 1 respectivamente. n= número de replicados, m±sd= media aritmética ±desviación estándar

	No=1 cells (x100 Nt)		No=10 cells (x10Nt)		No=100 cells (x1Nt)	
	n	m ± sd	n	m ± sd	n	m ± sd
f/2	25	124±42	12	128±15	12	151±11 **
f/2+25% CEM	27	167±47	12	174±25	12	173±13 *
f/2+TPA	35	157±50	12	167±24	12	161±14 *
		***		***		***

* = No significative differences ($p>0.05$).
** = Significative differences ($p<0.05$).
*** = Significative differences ($p<0.01$).

Furthermore, cell of *P. lima* density has also influence on cell survival. Table 2 summarizes the survival rate per cell (S) 48 h after groups of 1, 2, 4 or 8 exponentially growing cells had been placed in three different culture media. In f/2 medium, a cell from an 8 cells microdish has a survival rate of 38 % but an isolated cell only has a 22 %.

It has been suggested that telemediators, which act as paracrine growth factors, could be implied in control of cell proliferation of marine dinoflagellates (Costas *et al.* 1993a). In this respect, the addition of conditioned exponential media (CEM) increased the cell proliferation with respect to f/2 without any supplementation

($p<0.05$, Mann-Whitney U test). Also the CEM addition statistically increased the survival rate with respect to f/2 controls. Furthermore, conditioned exponential media (CEM) suppresses the effects of cell density on proliferation and survival. In fact, when *P. lima* cells were grown in CEM, the growth rates from isolated cells were similar to growth rates reached by 10 or 100 cell inocula. A similar feature also occurred with the survival rates (Table 2).

Table 2. Survival rates per cell 48 h after groups of 1, 2, 4, or 8 exponentially growing cells has been placed in three different culture media. n= number of replicates, s = survival rates.

Tabla 2. Tasas de supervivencia por célula después de 48 h de haber sido distribuidas en grupos de 1, 2, 4 o 8 células en tres medios de cultivo diferentes. N= número de replicados, s= tasa de supervivencia.

	1 cell		2 cells		4 cells		8 cells	
	n	s	n	s	n	s	n	s
f/2	31	0.22±0.05	18	0.26±0.08	13	0.27±0.05	11	0.38±0.08
f/2+25% CEM	33	0.59±0.06	16	0.58±0.1	11	0.63±0.12	11	0.61±0.13
f/2+TPA	31	0.35±0.09	15	0.37±0.07	11	0.41±0.9	11	0.43±0.07

Diffusible substances seem to play an important role as a mechanism to intercellular communication in dinoflagellates (Cornelissen *et al.* 1986). Apparently dinoflagellates control their cell division cycle just like the highest eukaryotic cells do, and several observations show that dinoflagellates are able to secrete diffusible growth factors that act paracrinely on neighboring cells inducing cell division (Costas *et al.* 1993a,b).

Little is known of the origin of growth factors in marine ecosystems. As in other eukaryotic cells, dinoflagellates may obtain growth factors mainly via endogenous production. Okadaic acid is produced by some dinoflagellates, the autocrine action of these growth factors would explain how dinoflagellates can proliferate in artificial media without growth factors, while their paracrine action would indicate that a minimal cell density may be required for rapid proliferation. Growth factors may also originate through bacterial activity. Recently, the production and secretion of diacylglycerol, an activator of protein kinase C for cell division, has been reported in bacteria (Morotomi *et al.* 1990). Bacteria are frequently able to grow on the dinoflagellate wall, and as endocytobionts in the cytoplasm or nucleus (Costas & Lopez-Rodas, 1990). Although many questions about the role played by growth factors are still unanswered, research on the biological mechanisms controlling phytoplankton proliferation can be an important complement to traditional studies on environmental factors. Perhaps cell density plays an important role on dinoflagellate abundance and occurrence, and perhaps minimal cell density is needed to guarantee survival and proliferation. If true, the tentative idea that eukaryotes control cell proliferation themselves but prokaryotes do not imply that eukaryotes i.e. "ordinary phytoplankton", are able to regulate the rates of all the cellular processes, in the way that predators regulate their prey (Wyatt, personal communication).

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