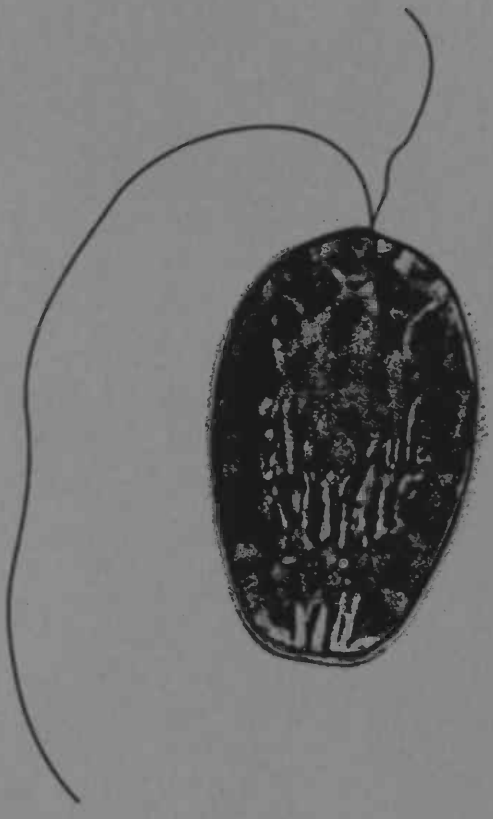
The Relation between Toxin Production of *Fibrocapsa japonica* (Raphidophyceae) and available Nitrate and Phosphate



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Under supervision of Engel G. Vrieling and
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Acknowledgments

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Contents

Abstract

Changing nutrient conditions in the North Sea, have raised the question whether the species composition of the algae may be influenced in favor of potentially toxic species over non-toxic species. An understanding of the environmental factors influencing growth and toxin production is necessary before predictions on the occurrence of toxic algal blooms can be made and warnings of potential damage by can be issued. The nutrient physiology of the potentially harmful raphidophyte species *Fibrocapsa japonica* Toriumi et Takano was studied in relation to its production of toxins. Cultures were grown under different ratios of nitrate and phosphate. Cell numbers were determined and the concentration of phosphate and nitrate in the medium was measured. Growth was found to be faster under low nitrate concentrations and lower under phosphate limitation. The differences were however not significant except at the lowest phosphate concentration, where growth was significantly lower. Cultures grown in a low nitrate or phosphate medium had a shorter growth phase than control cultures (10-11 days in stead of 16 or more days). A possible effect of bacteria on growth was observed when comparing growth between trials. Toxin samples were analyzed by High Performance Liquid Chromatography (HPLC). The total toxin yield was highest when cultures declined and under limitation of phosphate. A shift was seen in the toxin profile during the culture period. Most measured components diminished towards the end of the culture period, but the first eluting component increased. A total of 8-9 components that are possibly toxic were identified, 3 or 4 of them new. Toxicity tests confirming their toxicity have not been performed yet.

Introduction

Harmful Algal Blooms: Causes and Consequences

The occurrence of unusually high concentrations of marine phytoplankton in the water column is called a "bloom". Algal blooms which cause nuisance or damage, such as foam on beaches or fish mortality, are called Harmful Algal Blooms (HAB's). For this phenomenon the term "Red Tides" was often used in the past. A true red tide was already described in the bible and sums up possible effects of a harmful algal bloom quite nicely: "all the waters that were in the river were turned to blood. The fish that was in the river died, the water stank, and the Egyptians could not drink of the water of the Nile" (Exodus 7, verse 20 to 21). The term "Red Tides" was until recently used for most noxious algal blooms irrespective of the color. Because of the general nature of the term its use is now abandoned (Anderson 1998).

The damage by HAB's can be caused by either 1) high biomass or foam which may result in anoxia, 2) by clogging or damaging the gills of fish, or 3) toxins (Hallegraeff 1993). Toxins from algal blooms may find their way through the food chain by bioaccumulation to top predators such as birds, whales, seals and even humans. Filter feeding shellfish bio-accumulate poisons and transmit these to human consumers causing shellfish poisoning (Shumway and Cembella 1993).

A discussion has been taking place if there has been an increase in the occurrence of HAB's in the last decades. An increase in the number of recorded harmful algal blooms has been suggested by Hallegraeff (1993).The increase has even been termed a "global epidemic" (Smayda 1990). The increase in observed HAB's is however also attributed to an increase in "observer effort". The discussion is short- cutted by Anderson (1998) pointing out that whether there is a global increase or not, the problem is evident.

Human- induced factors which may have a positive influence on the occurrence of HAB's are eutrophication, disruption of the nutrient balance and new dispersal means. Eutrophication causes an increased availability of nutrients, enabling higher biomass of phytoplankton (Hallegraeff 1993, Anderson 1994). Ballast water and the import and export of shellfish seed provide a new pathway for the dispersal of harmful algae (Hallegraeff and Bolch 1992, Scholin *et al.*1995). Control measures to reduce eutrophication in the North Sea have led to a reduction of phosphate loads from the river Rhine. Nitrate loads, however, are much more expensive to reduce. Diminishing phosphate levels and fairly constant nitrate levels (1976 - 1990 period) have lead to an increase in the nitrate: phosphate ratio (Rijkswaterstaat 1992). This has raised the question whether a higher nitrate : phosphate ratio than the Redfield - ratio may favor toxic over non-toxic species in competition (Peperzak 1994, Riegman *et al.* 1996).

The Raphidophyceae

Until recently the taxon Raphidophyceae was rare in the North Sea and was not observed during monitoring before 1991 (Vrieling *et al.*1995). The Raphidophyceae are a monophyletic group (100 % Bootstrap value, Potter *et al.* 1997) with nine genera, both freshwater and marine (van den Hoek *et al.* 1995). Harmful Raphidophyte species are *Chattonella marina* (Subrahmanyan) Hara et Chihara, *Chattonella antiqua* (Hada) Ono,

Fibrocapsa japonica Toriumi et Takano and *Heterosigma akashiwo* (Hada) Hada ex Sournia. These species have caused HAB's in the coastal areas of Japan resulting in a major damage in commercial aquaculture (Endo *et al.* 1992, Onoue *et al.* 1990, Khan *et al.* 1995, 1996a, b, 1997). *F. japonica* caused major damage by killing large numbers of cage-reared yellowtails (*Seriola quiqueradiata*) in Japan (Okaichi 1972). Raphidophyte species are in general undesirable food for copepods (Uye and Takamatsu 1990) which may contribute to bloom formation. A recent fish mortality, reported in the Dutch press, in the North Sea and the Skagerrak along the Danish coast was linked to *Chattonella* spp.(press release in appendix). This suggested that a Raphidophyte species may cause considerable damage under the right environmental circumstances inducing bloom formation. The recent occurrence of blooms of Raphidophyte algae in combination with changing nutrient ratio's in the North Sea requires research to understand nutrient eco-physiology of this taxon.

The presented study has been focused on *Fibrocapsa japonica*, one of the Raphidophyte species observed in the North Sea. *F. japonica* has a global distribution and now occurs along the west European coasts of France, Belgium (Billard 1992), Holland and Germany (Vrieling *et al.*1995).

Fibrocapsa japonica*; Appearance and Ultrastructure*

Cells of *F. Japonica* are highly motile and possess two unequal flagella. In culture, strong migratory movements can be observed when removed from under the light source. The cells are yellowish brown to golden brown. Their shape is slightly flattened, oval to obovate. The cells are able to eject extended threads of biopolymers originating from mucocysts (White arrows Fig. 1a, Ultrastructure Fig. 2b) up to 300 μm long outside the cell body (Hara and Chihara, 1985).The cell membrane is not rigid due to the absence of a skeletal structure. For this reason cell morphology is severely altered in plankton samples fixed with lugol or formalin (Billard, 1992). The cells are, however, readily visible by light microscopy (Fig. 1) as they are 25-40 μm long and 15-20 μm broad (van den Hoek *et al.* 1995). Ultrastructurally the most distinguishing characteristics (Hara and Chihara 1985) are the presence of mucocysts with thread like inclusions in the posterior part of the cell and the organization of the chloroplasts (Fig. 2). The chloroplasts appear to form a network when viewed under the light microscope, but are in fact separated by cytoplasmic layers (Hara and Chihara 1985).

Optimal culturing conditions according to Khan *et al.* (1996a) are temperatures of 15-25 °C (tolerance range 10-30), a salinity between 25-35 PSU (tolerance range 15-40 PSU), a light intensity between 60 and 140 $\mu E/m^2/s$ (tolerance range 20 - 200 μmol quanta/m^2/s) and

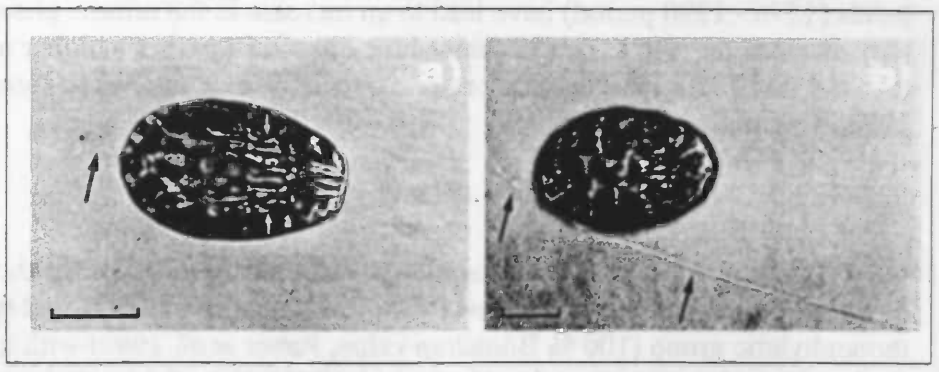

Figure 1: *Appearance of* Fibrocapsa japonica *under a light microscope a)live, b) fixed. The white arrows mark the position of the mucocysts, black arrows indicate the position of the flagella. Bar length is 15 μm. (from Vrieling* et al. *1995)*

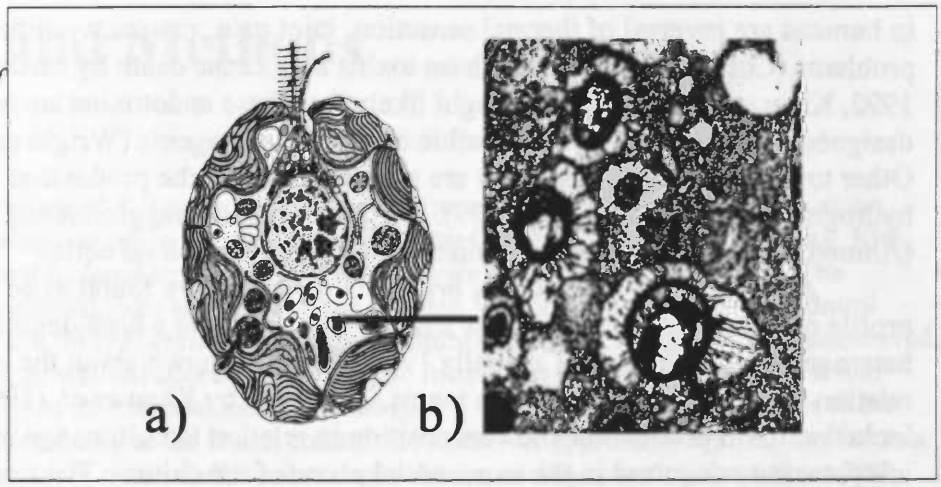

Figure 2: *Ultrastructure of* F. Japonica *(a)(Hara et Chihara 1985), EM photo of a mucocyst (b)(Vrieling unpubl.)*

pH values between 7.5 and 8.5 (tolerance range 6.5-8.5). Optimal phosphorous and nitrogen concentrations are 0.03-0.3 and 0.3 mg/l respectively (Ono, 1988). Under optimal conditions cell proliferation was found to be up to 0.7 divisions a day. The maximum cell number found in culture was $5.6 \cdot 10^4$ cells/ml.

Toxin Production

Algal toxins are most probably secondary metabolites whose function is not yet clear (Wright and Cembella 1998). Hypotheses concerning possible functions include xenobiotics, chemical defense inhibitors, co-factors, trace metal ligands, and hormones (Wright and Cembella 1998). *F. japonica* has been found to produce five analogues to the toxins produced by *Gymnodinium breve* Davis (formerly *Ptychodiscus brevis* Steidinger) by Khan *et al.* (1996b) using Thin Layer Chromatography (TLC) followed by High Performance Liquid Chromatography (HPLC). These "brevetoxins" are known to induce Neurotoxic Shellfish Poisoning (NSP, Collins 1978) by activating voltage sensitive sodium channels in neuroblastoma cells (Yasumoto and Murata 1993). So far 8 different brevetoxins have been identified based on two basic polyether skeletons (Fig. 3). These substances are known to be lipophilic and highly unstable (Collins 1978, Wright and Cembella 1998). Symptoms of NSP

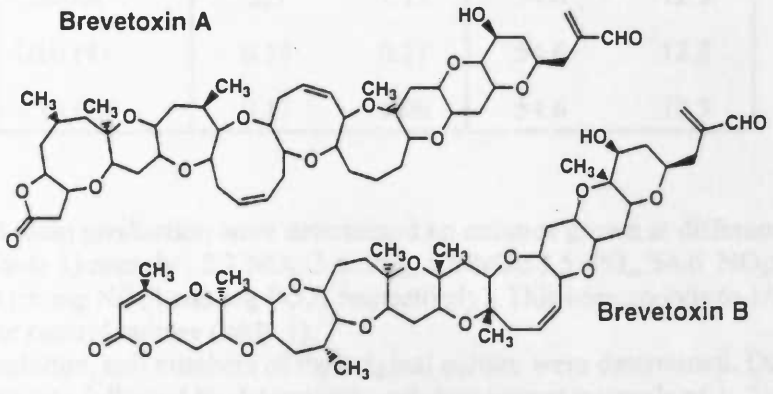

Figure 3: *Structures of the brevetoxin skeletons (Wright and Cembella 1998)*

in humans are reversal of thermal sensation, joint pain, nausea, vomiting, and cardiovascular problems (Collins 1978). In fish these toxins may cause death by cardiac disorder (Endo *et al.* 1992, Khan *et al.* 1996). It is thought likely that these endotoxins are not specifically designed as bacteriostatic, allelopathic or ichtyotoxic agents (Wright and Cembella 1998). Other toxic effects of *F. Japonica* are accounted for by the production of superoxide and hydrogen peroxide (Oda *et al.* 1997). In *Chattonella* hemagglutinating substances were found (Ahmed *et al.* 1995) which can cause agglomeration of blood cells.

Although in *Gymnodinium breve* toxin yields were found to be fairly constant, the profile of the different components shift in time and have a high degree of interclonal heterogeneity (Wright and Cembella 1998). Little is known about the nutrient physiology in relation to the production of these toxins. The study by Khan *et al.* (1996b) mentioned above looked at toxin production and composition in relation to culture age in *F. Japonica*. Highest ichtyotoxicity occurred in the exponential phase of the culture. The toxin composition varied; one of the brevetoxin-like components was found to be higher at the stationary phase, while three other components had their maximum yields at the height of the exponential phase.

Factors named for possible influence on toxin production include salinity, light, and nutrient conditions (Anderson *et al.* 1998). Nutrient concentrations are known to induce toxin production in the toxic diatom species of the genus *Pseudo-nitzschia* (Bates *et al.* 1998) and some dinoflagellates (Anderson *et al.* 1998, Cembella 1998). Although these toxins contain nitrogen and are therefore dependent on the relative availability of this element, other elements (Si in *Pseudo-nitzschia*) are also triggers. In *Gymnodinium breve* toxin synthesis was found to decrease linearly with DNA and polysaccharide synthesis. Therefore, stress is thought to be a stimulating factor for the production of brevetoxins (Wright and Cembella 1998).

To examine the influence of nutrient stress on the toxin production for *F. japonica*, the relation between growth under different nitrate and phosphate concentrations and toxin production was examined in the project described here. The toxin composition and yield was studied by HPLC analysis and related to culture age and culturing conditions. The aim was to create comparable profiles of the different toxic components for different clones and under different nitrate and phosphate concentrations.

Materials and Methods

Cultures

A multiclonal culture of *F. japonica* was received from the group of Prof. F. Colijn at the Forschungs- und Technologiezentrum Westküste (FTZ) of the Christian Albrechts University, Kiel, Germany. This culture and 6 clonal strains derived from it were used for the experiments. The original culture was established from an isolate of the German Wadden Sea near Büsum. Control cultures were grown in 200 ml F/2- enriched seawater medium (Guillard 1975) made with autoclaved North-Atlantic seawater; Si was not added to the medium. Incubation took place in 250 ml serum flasks. A standard salinity of 25 Practical Salinity Units (PSU) was used, which corresponds to the observed salinity in the proximity of the Dutch coast. Irradiation was approximately 70 $\mu Em^{-2}s^{-1}$ in a 16 hours light and 8 hours dark cycle. Cell numbers were determined by counting three times 200 cells or 4 vertical rows and one horizontal row of cells in a Sedgwick-Rafter chamber, whichever came first. Before counting the cells of a subsample of 1400 μl were immobilised with 100 μl 20 mM $NiCl_2$ (Hara and Chihara 1985, Watanabe *et al.*1987) and 5 μl Lugol. $NiCl_2$ also inhibits mucocyst excretion, avoiding aggregation of the cells. Division rates (K in divisions per day) were calculated using the following formula (Guillard 1973, Khan *et al.* 1996a):

$$K = \frac{\ln X_2 - \ln X_1}{\ln 2(t_2 - t_1)}$$

Differences in cell numbers between cultures were tested using the Students t-test for comparing two means at $\propto = 0.05$ (Zar 1984). Differences between the means of the division rates of the nutrient conditions were tested using single factor ANOVA and the Tukey-Kramer test.

Table 1: *Nutrient conditions*

Condition	[Phosphate]		[Nitrate]	
	in mg PO_4/l	in mg P/l	in mg NO_3/l	in mg N/l
1/20 NO	3.5	1.13	2.7	0.62
1/10 NO	3.5	1.13	5.5	1.23
Control	3.5	1.13	54.6	12.3
1/10 PO	0.35	0.11	54.6	12.3
1/20 PO	0.17	0.06	54.6	12.3

Growth and toxin production were determined on cultures grown at different Nitrate : Phosphate ratios (Table 1) namely : 2.7 NO_3: 3.5 PO_4, 5.5 NO_3: 3.5 PO_4, 54.6 NO_3: 0.35 PO_4 and 54.6 NO_3: 0.17 PO_4(in mg NO_3/l and mg PO_4/l respectively). This corresponds to $1/10^{th}$ and $1/20^{th}$ concentrations of the control culture (table 1).

Before inoculation, cell numbers of the original culture were determined. During a period of 10 to 21 days growth was followed by determining cell numbers at intervals of 1, 2 or 3 days. In the same time schedule samples were taken for measuring the concentrations of toxin and nutrients. All

samples were kept at -20 °C until further analysis. The experiments were performed in sequential duplicates ("trials") to test for reproducibility. Phosphate and nitrate conditions were applied alongside separate controls. This resulted in four sequential batch culture experiments; first 2 trials for phosphate conditions followed by 2 trials for nitrate conditions. Samples of 10.0 ml culture medium (containing at least 10^4 cells) were taken after careful homogenisation. After centrifuging for 15 minutes at 3300 RPM during 15 minutes 9.5 ml of supernatant was removed and stored at -20 °C for nutrient measurement. The cells were stored at -20 °C along with the remaining supernatant for toxin analysis.

Measurement of Nutrients

Concentrations of nitrate and phosphate were measured using a segmented flow analyser (Skalar Analytical Inc.). Nitrate was determined by first using the cadmium reduction method to reduce nitrate to nitrite followed by complexation with ∝-naphthylenediamine dihydrochloride; the intensity of the absorption of the complex was measured in a spectrophotometer at 540 nm. Phosphate was measured by formation of a blue phosphomolybdate-complex and measured at 880 nm. The detection limits were 0.25 gNl^{-1} for nitrate and 0.01 gPl^{-1} for phosphate measurement with standard errors of 0.025 for both analyses. Further details can be found in the Skalar Auto- Analyser Handbook.

Toxin Analysis

The High Performance Liquid Chromatography measurements were done at the Terramare Institution, Wilhelmshaven (Germany) under supervision of Dr. G. Liebezeit. The extraction method will be published in detail by Liebezeit *et al.* (in prep.). Shortly, the samples were thawed in the dark, centrifuged for 2 minutes at 5000 RPM at 4 °C, and the supernatant decanted before extraction. Ice-cold methanol (200μl) was added and the cell pellet was extracted for 4 minutes in an ultrasonic bath at approximately 0 °C. Cell debris was removed by centrifugation for 1 minute at 3200 RPM and the extract was pipetted into a 200μl microvial and directly placed in an autosampler.

The used HPLC system consisted of a degassing unit, a gradient pump and an autoinjector (all Thermo Separation Products) . A Diode Array Detector (DAD) (Merck -Hitachi) and a fluorescence detector (Thermo Separation Products) were used in line (Fig. 5). The DAD measured absorbency between 190 and 500 nm. The fluorescence detector (excitation at 215 nm) measured emission at 290 nm. The applied C-18 column was a 256 x 4 mm Spherisorb ODS2, 5 µm (Bischoff). All analyses were carried out at room temperature using a gradient elution of eluent A (20% water, 80 %

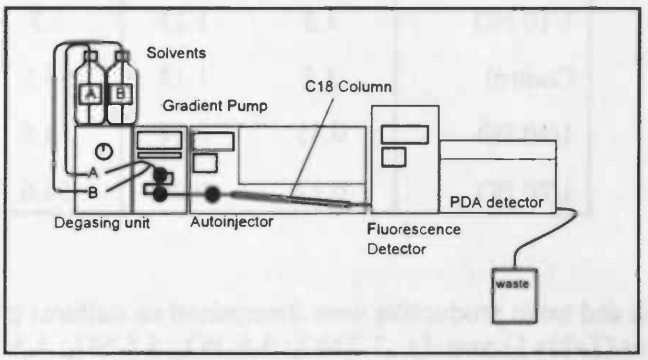

Figure 5: *The HPLC system used*

methanol) and eluent B (100 % acetonitrile). The gradient employed was 65 % A and 35 % B (t = 0 min) to 45 % A and 55 % B (t = 10 min) to 100 % B (t = 15 - 22 min). The solvents methanol and acetonitrile were bought from Fluka.

Fluorescent peaks were recovered from the HPLC and the fluorescence spectra recorded on an SFM 25 fluorimeter (Kontron Instruments) with 5 nm slit width.

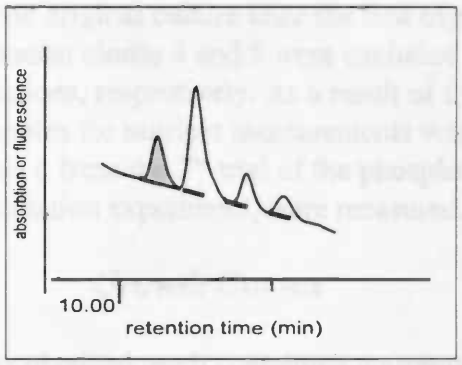

Figure 6: *Baseline and peak area calculation*

As brevetoxin-like substances are known to absorb at 215 nm (Khan et al. 1996b, Liebezeit pers. com.) and many other substances also absorb at this wavelength, standards were used to identify peaks. As pigments tend to absorb at 215 nm but also between 400-450 nm, standards of chlorophyll-*a*, fucoxantin, zeaxantin and ≥ -carotene were analyzed for analysis of the sample peaks. Diadinoxanthin was identified by comparison to a spectral library at Terramare.

To identify other non- toxin peaks the fluorescently active amino acids phenylalanine and tyrosine (obtained from Sigma), the short chained acids acetic acid and oxalic acid, and the sugars glucose and fructose (obtained from Merck)were analysed using the same method. Sample chromatograms were compared with the above mentioned standards and with oxidised brevetoxin-2. All chemicals and solvents were of the highest available purity.

Peak areas were calculated using Merck software after manually adjusting the baseline to fit the gradient slope (Fig. 6). Areas of the different peaks identified as possible toxins were monitored in relation to culture age. Relations were tested between toxin profiles, total toxin production and division rates of the differently grown cultures.

Results

Due to poor growth of the original culture after the first experiment (first phosphate limitation trial). For the same reason clones 4 and 5 were excluded from the first and second experiments under nitrate limitations, respectively. As a result of the extensive experimental design, a limited number of samples for nutrient measurements was selected. A selection of samples from the clones 1, 3 and 6 from the 2nd trial of the phosphate limitation experiment and the 1st trial of the nitrate limitation experiment, were measured.

Growth Curves

A total of 23 graphs was obtained, each containing a growth curve of a control and

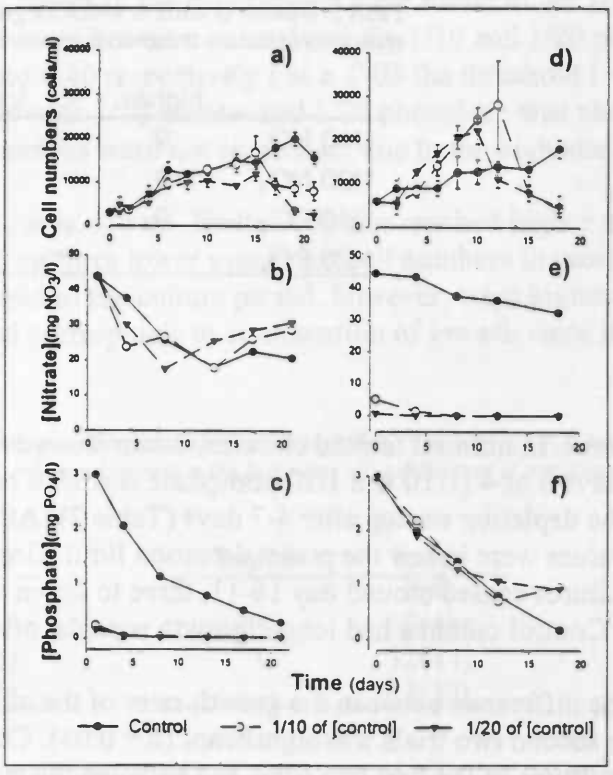

Figure 6: *Growth curves, [nitrate] and [phosphate] for the phosphate (a,b,c) and nitrate (d,e,f) limited cultures*

1/10 and 1/20 limited cultures. Observations on the second nitrate limitation experiment were done for only ten days. The other experiments were sampled and counted for 14 (1st phosphate), 18 (2nd phosphate) and 21 (1st nitrate) days. Here a set of representative growth and nutrient curves for both nitrate and phosphate limited cultures has been presented (Fig. 6); all curves are included in the appendix. As can be seen by examination of the error bars, standard deviations were considerable (40 % of the mean in the 1st phophate trial, all other trials 20 % of the mean). Variability among clones and replicates was high (20 to 50 % of the mean)

Nutrient curves were obtained for the 2nd phosphate and 1st nitrate experiments. In the corresponding growth curves a lag-phase of about 2 days could be observed (as in figure 6). After the lag phase, the control cultures grew steadily until reaching the stationary phase around day 16 (Table 2). At the end of the growth period, cell numbers decreased sharply in

Table 2: *Growth period and nutrient depletion for the different [phosphate] and [nitrate](1st trial phosphate and 2nd trial nitrate were excluded due to incomplete observations)*

	Growth periods (days)	Depletion after (days)
Control	16	>16
1/10 PO_4	10	3-4
1/20 PO_4	11	3-4
1/10 NO_3	11	4-7
1/20 NO_3	9	3-4

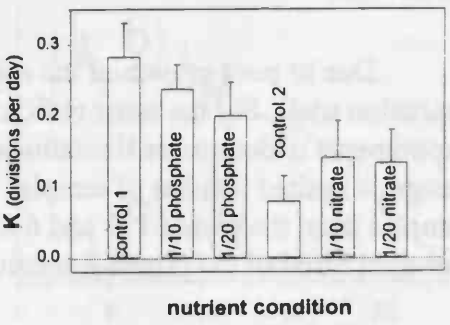

Figure 7: *Mean division rates and standard-deviations under different nutrient conditions*

Table 3: *Number of cases in which the growth rate of the limited culture is higher than control*

	higher	lower
1/10 NO_3	9	1
1/20 NO_3	9	1
1/10 PO_4	9	4
1/20 PO_4	4	9

most cultures. In nutrient limited cultures, diminishing of nitrate and phosphate was observed between days 3 or 4 (1/10 and 1/20 phosphate and 1/20 nitrate), while for the 1/10 nitrate cultures the depletion started after 4-7 days (Table 2). After these periods the determined nutrient values were below the preset detection limit. Growth in nitrate- and phosphate-limited cultures ceased around day 10-11, three to seven days after the observed depletion of nutrients. Control cultures had longer growth periods, often even longer than the observation period.

The difference between the growth rates of the control cultures in the first two trials and in the second two trials was significant ($\alpha = 0.05$). Comparisons were therefore made between cultures of the first two trials and between the cultures of the second two trials. The cultures grown at low nitrate concentrations had higher growth rates than the corresponding control in all cases, except one (table 3). The exception was seen in the second nitrate experiment, in this case the control culture had higher growth rates than the low nitrate cultures.The difference in mean growth rates of the nitrate limited cultures were however not significantly different from control cultures.

Low phosphate concentrations had a less distinct effect on division rate (Table 3). The difference between growth rates of the 1/10 limited culture and the 1/20 limited cultures was positive in 6 out of ten cases for nitrate, and 8 out of 13 for phosphate. The mean division rate of the 1/20 phosphate culture, however, was significantly lower than the control culture (Tukey-Kramer test $\alpha = 0.05$).

Highest division rates for the growth period were found to be up to 0.4 divisions day^{-1} (mean = 0.24 standard deviation 0.10). Highest observed growth rates between sampling days

were between 0.4 and 1.7 with a mean of 0.5 (standard deviation = 0.26) divisions day^{-1}. The pooled variance of the individual division rates could not be calculated due to the low degrees of freedom (2), differences between growth rates could therefore not be tested statistically.

Table 4: *Highest observed cell numbers in culture period*

	highest cell numbers	std. dev.
Control	33900	4506
1/10 nitrate	37217	9864
1/20 nitrate	24697	8460
1/10 phosphate	21052	3876
1/20 phosphate	12812	1185

The highest observed cell numbers in the experiments were found in the 1/10 nitrate limited culture (Table 4). The differences between control and the 1/10 and 1/20 phosphate-cultures were significant (t= 3.05 and 6.40 respectively (at α 0.05 the threshold t = 2.92). The difference between the cultures with 1/10 nitrate- and 1/20 phosphate was also significant (t= 3.47). All other differences were not significant due to the high standard deviations.

In comparison to control cultures, nitrate- limited cultures reached higher maximum cell numbers and phosphate limited cultures lower maximum cell numbers in most cases (table 5a). The cell numbers at the end of the culture period, however, were highest in control cultures in most cases (table 5b) and corresponds to continuation of growth since nutrients were still available (Fig. 6).

Table 5: *Highest cell numbers (a) and cell numbers at the end of the growth period (b), numbers are # of cases in which the limited culture is higher or lower than control, between brackets is the # of cases were difference is significant*

a)

	higher	lower
1/10 NO_3	7(1)	3
1/20 NO_3	6(3)	4
1/10 PO_4		13(5)
1/20 PO_4	1	12(5)

b)

higher	lower
3	7(4)
2(1)	8(4)
1	12(11)
	13(10)

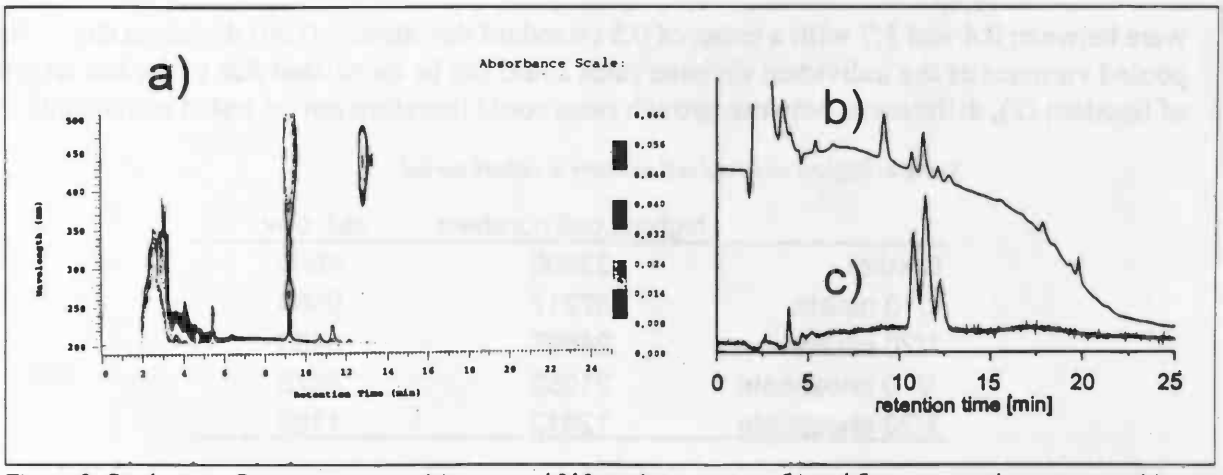

Figure 8: *Diode Array Detector spectrum (a), extracted 215nm chromatogram (b) and fluorescence chromatogram (c)*

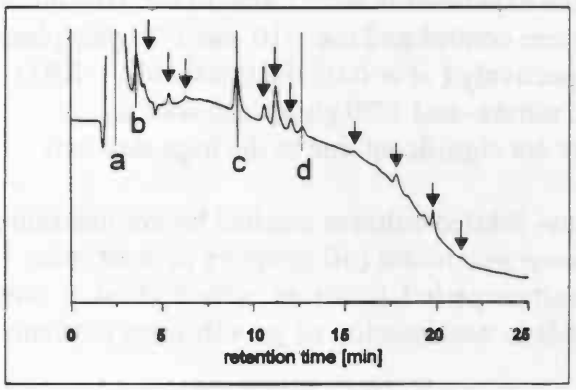

Figure 9: *215nm chromatogram, peak identification, peaks of interest are marked with an arrow. a) injection peak + tyrosine, phenylalanine, glucose and fructose, b) methanol, oxalic acid, acetic acid, brevetoxin 2, c) Fucoxanthin, c) Diadinoxanthin*

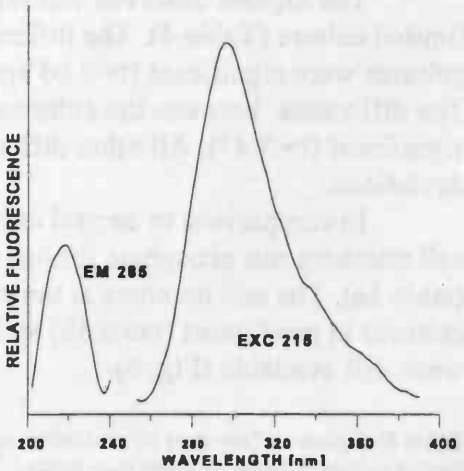

Figure 10: *Excitation and emission maxima of the three fluorescent peaks*

Toxin Analysis

Extracts were found to by quite unstable as peaks could only be measured directly after fresh extracts were applied to the HPLC analysis. Repeated analysis of the same sample within half an hour revealed a large decline in the height of all peaks.

The DAD spectrum (Fig. 8a) was used to extract a 215 nm (Fig. 8b) chromatogram. Figure 9 shows the retention times of the standards and the peaks of interest to this study (marked with arrows). Fluorescence chromatograms (Fig. 8c) revealed fluorescence of three peaks.Their excitation maximum was found at 219 nm while the emission maximum occured at 295 nm (Fig. 10). Measurement of the kinetics of these peaks showed only slight decline in fluorescence over a two hour period.

Oxidized brevetoxin(PbTx-2) eluted at 3.5 minutes, corresponding to the retention time of one of the peaks also observed in the methanol blanks. Because the brevetoxin standard did not provide an accurate calibration curve, peak areas(in Arbitrary Units (AU) squared) were used as a relative measure. All chromatograms showed patterns similar to the one shown in figures 7 and 8.

At this moment only one clonal culture has been analyzed completely. Total toxin levels (corrected for growth) increased with culture age in limited cultures. The positive correlation was significant for none of the cultures at $\alpha = 0.05$ (1 culture at $\alpha = 0.10$ and 2 more at $\alpha = 0.20$). The

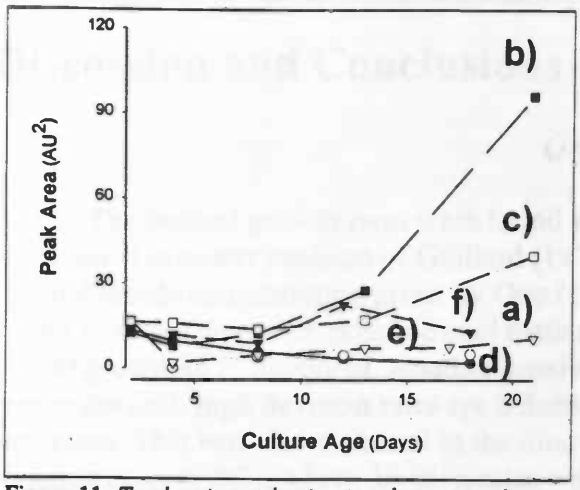

Figure 11: *Total toxin production in relation to culture age. a) control PO_4, b) 1/10 PO_4, c) 1/20 PO_4, d) control NO_3, e) 1/10 NO_3, f) 1/20 NO_3*

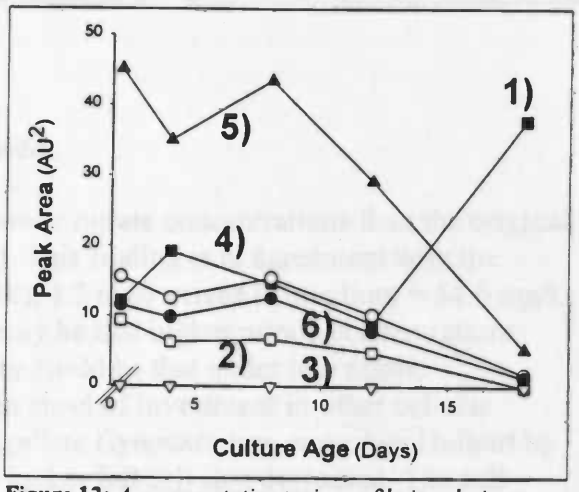

Figure 12: *A representative toxin profile in relation to culture age (days)*

control cultures showed a negative regression in both cases (significant in 1 case). Phosphate limited cultures showed higher relative peak areas at the end of the culture period than nitrate limited cultures and controls(fig 11). The difference between the slopes could not be tested statisticaly as the standarddeviation of the analysis is not known.

Control and nitrate limited cultures had a fairly constant total toxin yield during the culture period. When correlating toxin production with division rates, toxin production appears to be highest when cell numbers are decreasing. A negative slope was obtained for all 6 regression lines, the correlation was significant in 2 cases (at α=0.05, 1 more case at α=0.10). A representative toxin profile of the first 6 substances (peak areas) was plotted against culture age in figure 12. The resulting graph shows that substance number 5 is highest (observed in 4 out of 6 cultures). Most substances diminish towards the end of the culture period (Fig.12). The production of substance number 1 however, tends to increase during the culture period (observed in 6 out of 6 cases). Some erratic peak areas were obtained due to incorrect integration. Correlations of separate peaks to division rates led to no additional results, none of the correlations were significant (not shown).

Comparison of total toxin production between different culture conditions was done by Pearson regression, plotting toxin production of the limited cultures against the corresponding control cultures (Table 6). A slope higher than 1 indicates that the compared culture (horizontal) has a higher toxin production. These values confirmed the observation that phosphate limited cultures produce more toxin than control cultures. The slope of 1/10 phosphate is significantly higher than control (α= 0.05). The two controls differ little as do the nitrate limited cultures (slope close to 1)(Table 6).

Table 6: *Slopes and correlation coefficients (Rsq) of pearson plots. Shaded R squared values indicate a significant correlation.*

	1/10p	1/20p	c2	1/10n	1/20n
C1	5.89	2.66	1.39		
Rsq	0.83	0.63	0.90		
C2				0.54	1.67
Rsq				0.62	0.94
1/10p		1.41			
Rsq		0.93			
1/10n					1.80
Rsq					0.52

Discussion and Conclusions

Growth

The highest growth rates were found at lower nitrate concentrations than the original F_2 enriched seawater medium of Guillard (1975). This finding is in agreement with the optimal nitrate concentration given by Ono (1988), 1.3 mg/l versus F_2 medium = 54.6 mg/l, which is 42 fold lower. A possible explanation may be that higher nitrate concentrations inhibit growth in *F. Japonica*. Another possibility could be that under low nitrate concentrations, high devision rates are induced in stead of investment in other cellular processes. This was also observed in the dinoflagellate *Gymnodinium aureolum* Hulburt by Vrieling et al. (1997), where division rates were higher but cell size decreased. The cell morphology of *F. Japonica* is also known to be affected by unfavorable conditions (Khan et al 1996a) implicating the possibility of a similar mechanism. Whereas low nitrate concentrations seemed to stimulate growth rates, growth periods were longer in F_2 grown cultures. The F_2 grown cultures were often still growing after the observation period. Limited cultures were able to grow longer than nutrients could be measured. This may be attributed to the detection limits of the measurements.

F. japonica was found to be a poor competitor in multispecies cultures by Riegman et al. (1996). This may explain why the original, multi algal culture grew much slower than the clonal cultures.

The maximum cell numbers during the experiments remained 1 to 2 $\bullet 10^4$ cells/ml lower than the values given by Khan et al. (1996a) (3 - 4 $\bullet 10^4$ cells/ml instead of 5 - 6 $\bullet 10^4$ cells/ml). In undisturbed backup cultures, however, cell densities of up to 8 $\bullet 10^4$ cells/ml were found. These findings suggest that cultures disturbed by sampling grew suboptimaly. It is known that sampling effects the cells of Raphidophyte species (Billard 1992, Vrieling et al. 1995). Before sampling the cultures were mixed carefully, nevertheless cell agglomeration was observed ocasionally. Indicating that experimental cultures were stressed by sampling. A possible second factor that has caused the much lower cell densities was the bacterial contamination. For *Chattonella antiqua* it was found that certain species of bacteria inhibited cell proliferation, while other species promoted it (Furuki and Kobayashi 1991, Imai 1995). If assumed that bacteria also play an important role in determining growth in *F. japonica*, it is unclear whether differences in growth or toxin production between separately grown clones was due to a direct influence of the media, or by the presence bacteria in the cultures.

Toxin production; Tentative Conclusions

All the components that absorbed at 215nm and could not be attributed to any class of the standard substances used (short-chained acids, amino-acids, sugars, and pigments), were termed possible toxins. But until bio-assays are performed on these peak-fractions no conclusions can be taken about toxicity. The identified pigments fucoxanthin and diadinoxanin are known to occur in Raphidophyceae (van den Hoek et al. 1995).

Although the oxidized PbTx-2 standard failed to offer an accurate calibration curve, and eluted equally to methanol, there is a small fluctuation in retention times so it cannot be excluded that the oxidized PbTx-2 corresponds to the first possibly toxic component (identified as peak 1). As oxidized PbTx-2 elutes first in the group of 5

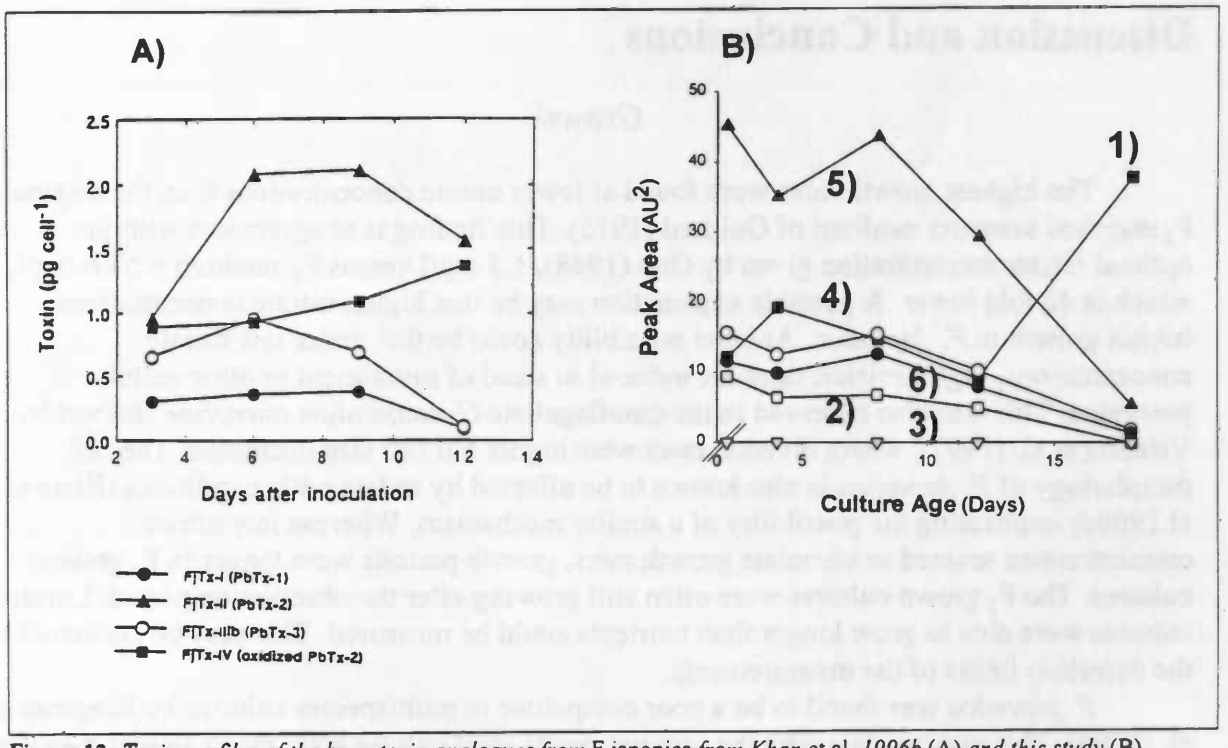

Figure 12: *Toxin profiles of the brevetoxin analogues from* F.japonica *from Khan* et al. *1996b* (A) *and this study* (B)

brevetoxins as analyzed by Khan et al. (1996b) the first peak at 215 nm and the following 5 or 6 can be suspected to be the other brevetoxin analogues. Comparing the found toxin profiles (Table 4) with the one from Khan et al. (1996b);(Fig. 13), and assuming that the first eluted substance is oxidized PbTx-2, and the substance with the highest concentration to be PbTx-2, the overall profiles are quite alike. As in the profile of Khan et al. (1996b) the general picture was that substance no 5 (FjTx-II) was highest in most cultures and diminished towards the end of the culture period. All other substances decreased towards the end of the culture period, except substance no 1 (FjTx-IV), that increased. The independent peaks act differently under the applied nitrate and phosphate concentrations. Due to a lack of additional data, conclusions are hard to draw as so far.

Table 7: *Comparison of the found peaks to Khan et al. 1996b*

Peak N°	
1	FjTx-IVb (oxidized PbtX-2)
2	*
3	*
4	FjTx-IIIb (PbTx-3)
5	FjTx-II (PbTx-2)
6	FjTx-I (PbTx-1)

Most peaks are present throughout the culture period. In ichtyotoxicity tests cells of *F. japonica* cultures were most toxic during the exponential growth phase (Khan et al.1996b). The measured total toxin yield per cell, however, was highest in senescent (decreasing cell numbers) cultures. This might be an indication that the toxin components that decrease in the stationary phase are more toxic than the increasing FjTx- IV. This is in concordance with Wright and Cembella (1998) who report a shift in side groups of brevetoxin from aldehyde groups (-CHO) to less potent primary alcohols (-CH2OH) possibly to reduce autotoxicity in senescent cells.

The toxin content in the cell-free medium of *F. japonica* and *G. breve* was reported to be low during the exponential phase and increased up to 30 % of the total toxin content in the stationary phases (Khan et al. 1996b,Wright and Cembella 1998) indicating that measured toxin concentrations may be an underestimation of the total toxin content of the culture. Errors in toxin concentrations may have occured due to eratic baselines for integration. The total toxin yields are higher than control in cultures grown under phosphate limitation and more or less identical to control under nitrate limitation. This finding is in line with the possibility proposed by Wright and Cembella (1998) that high organic- N loads may enhance toxin production. The finding that toxin yields increase under negative growth (senescence) and under phosphate limitation (which causes poor growth) indicate that toxin yield is increased under stressful conditions. This has been proposed for brevetoxin production (Wright and Cembella 1998). Summarizing the results it can be concluded that although total toxin yield is enhanced by stress and senescence, there is a shift in components. Probably to less toxic ones, but this will remain to be tested by bio-assays. This may imply that natural harmful events caused by *F. japonica* probably depend on the toxin profile and the cell density of the bloom and less on the toxin content per cell. In species like *Alexandrium* spp. Halim , *Dinophysis* spp. Ehrenberg and *Prorocentrum lima* (Ehrenberg)Dodge for example, this is not the case; these species can cause toxic effects in very low cell numbers (Peperzak 1994).

Problems and Possible Improvements

The elucidation of the structures of the brevetoxins produced serious problems as they are very labile (Collins 1978, Wright and Cembella 1998). In the development of the extraction method used here this was also the case for the toxins of *F. japonica*. From the measurement of the kinetics of the three fluorescent peaks it could, however, be concluded that separated components are stable. The method used by most other authors is the one described by Baden and Mende (1982) which probably produces more stable extracts. The great disadvantage of this technique for ecological research is that it is elaborate, involving many steps during the extraction protocol.

Recently it has become known that *F. Japonica* also possesses different toxins, tentatively termed 'fibrocapsins'. These substances have been found to be a hundredfold more toxic than brevetoxins in bioassay experiments (Liebezeit pers. com.). The advantage of the method used here is that extracts were prepared from whole cells, taking into account all methanol soluble substances. Khan et al. (1996b) used known brevetoxins as a reference on Thin Layer Chromatography for identifying related toxins in *F. japonica*, this enabled them to quantify the found toxins. New substances were expected to be found in *F. japonica,* and based on the stability of separated components it can be argued that it would be worthwhile to explore the possibility of adding a pre-separation column to the currently applied procedure. Separation may thus be improved, that is especially required for substances that co-elute with methanol.

Pending Research

At the moment of writing this report, two additional clones are being analyzed for toxin production. These data may confirm the influence of nitrate and phosphate limitation on toxin production to draw final conclusions. To check the assumption that oxidized PbTx-2 corresponds to peak number 1 new standards have to be tested. For proper quantification, internal spikes have to be applied. Finally, the toxicity of the whole extracts and that of the separated components have to be tested using both the LUMISTOX and the tumor-cell assays.

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Appendix

Contents

Legend:

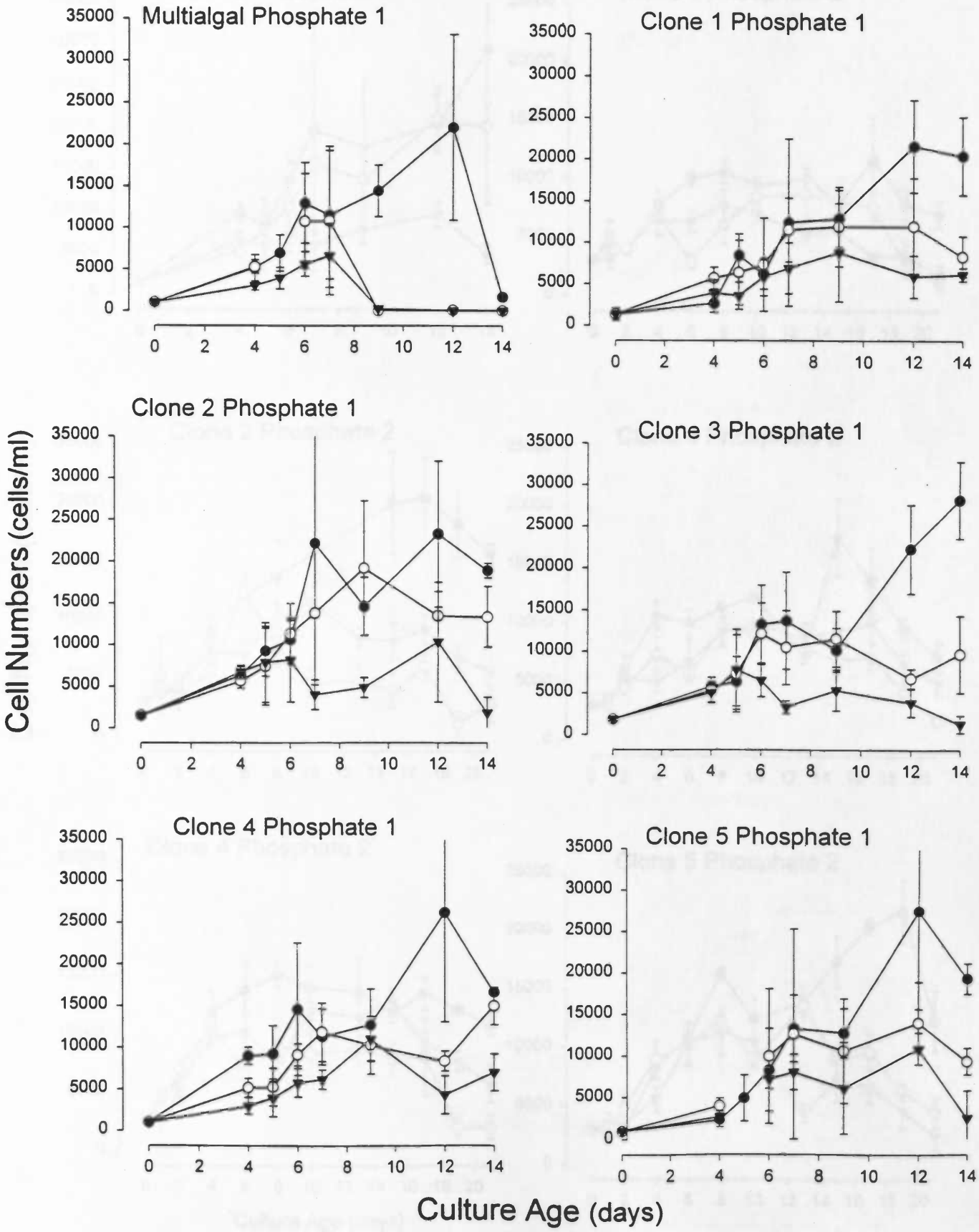
Unless otherwise indicated symbols represent:

Control culture = (Filled Circles)

1/10 Nutrient Concentration = (Open Circles)

1/20 Nutrient Concentration = (Filled Triangles)

Growth Curves First Phosphate Experiment



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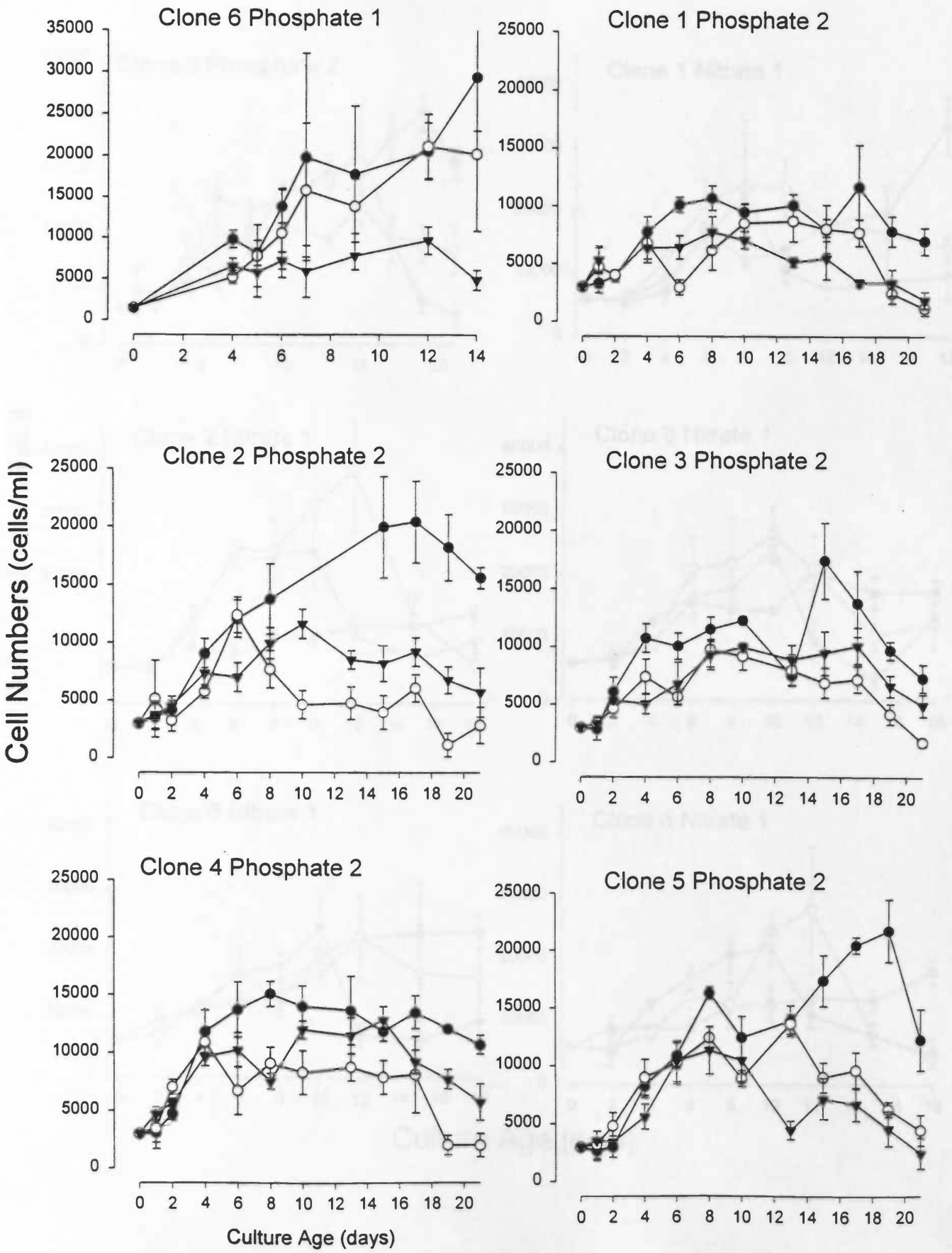

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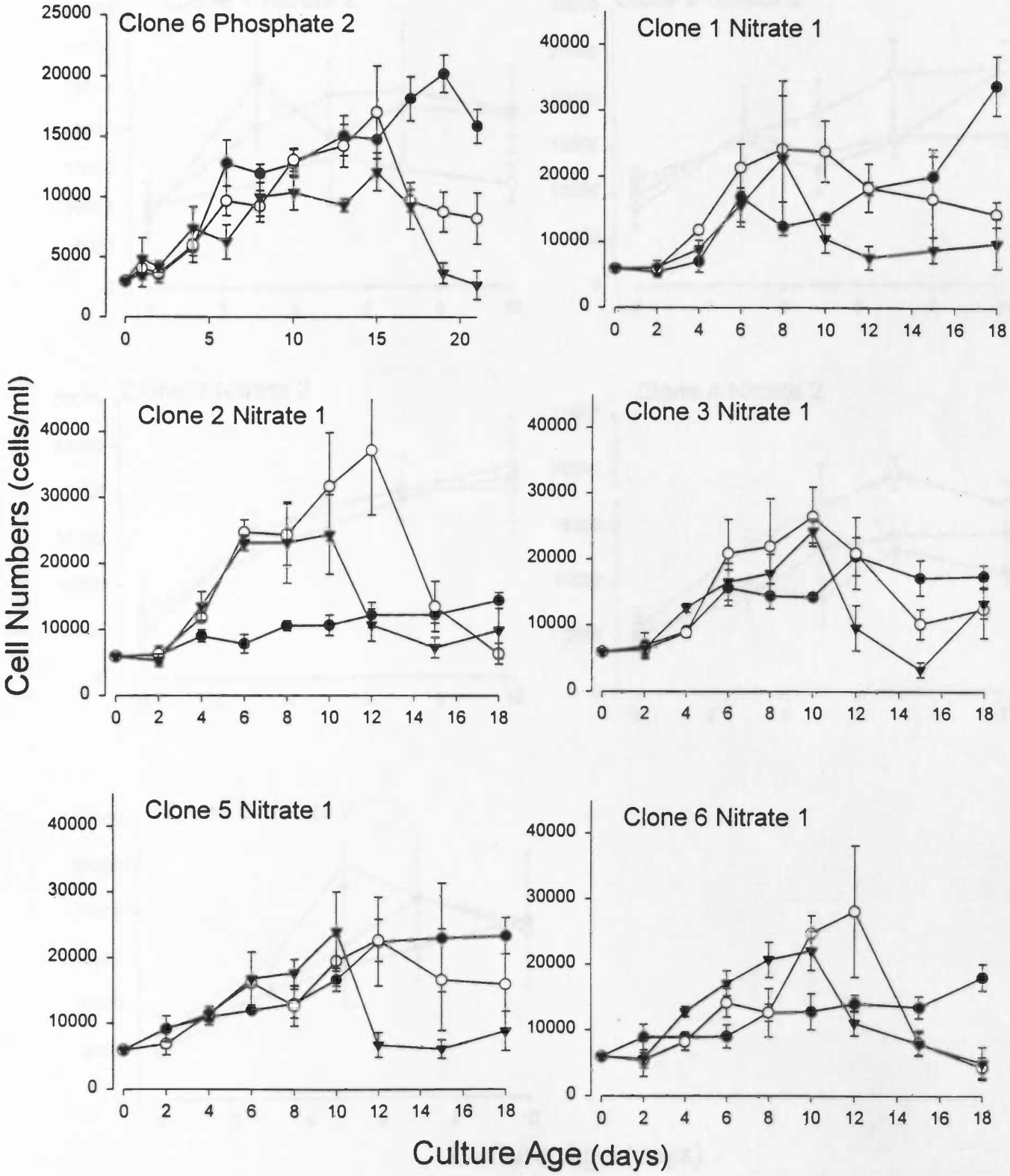

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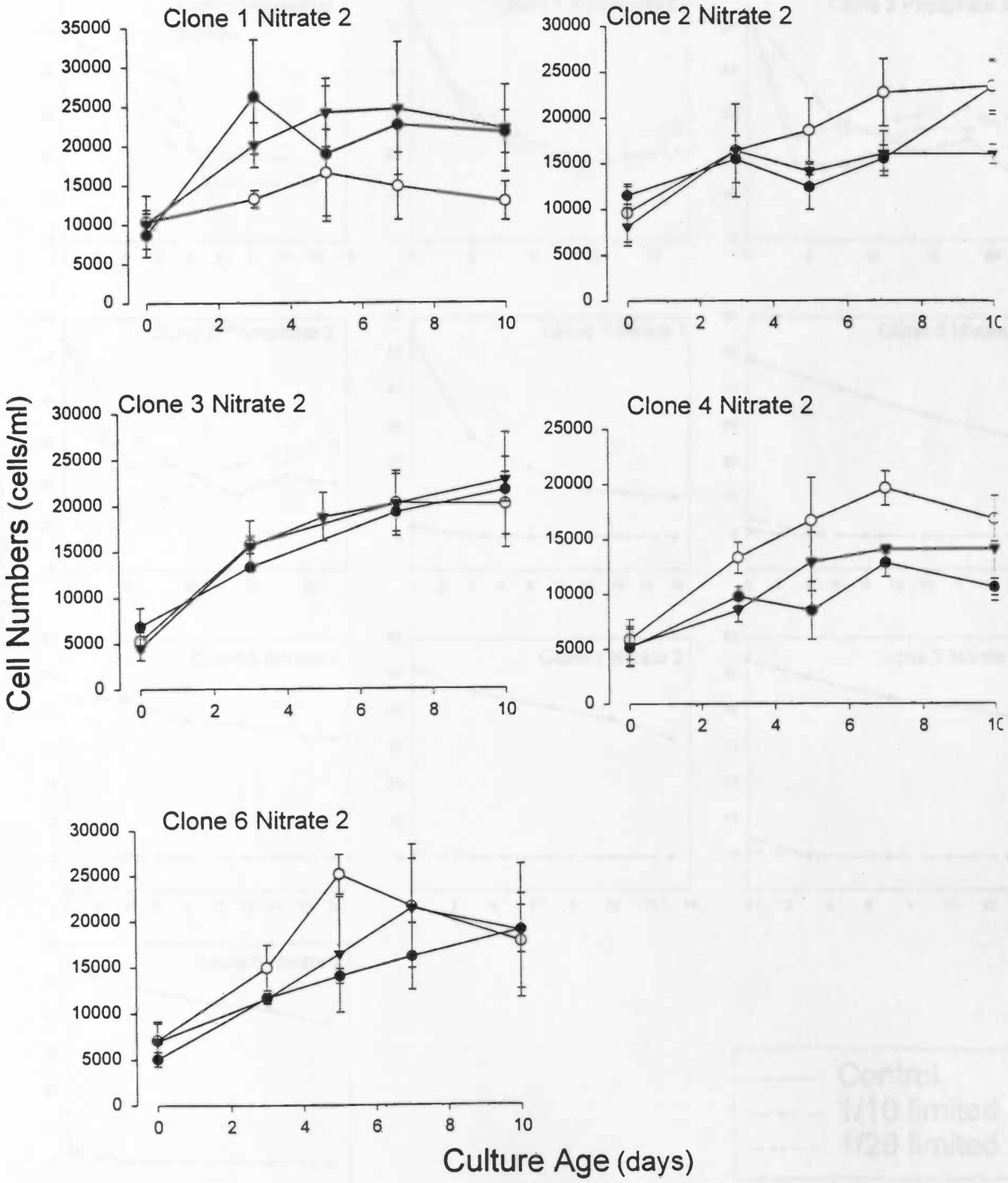

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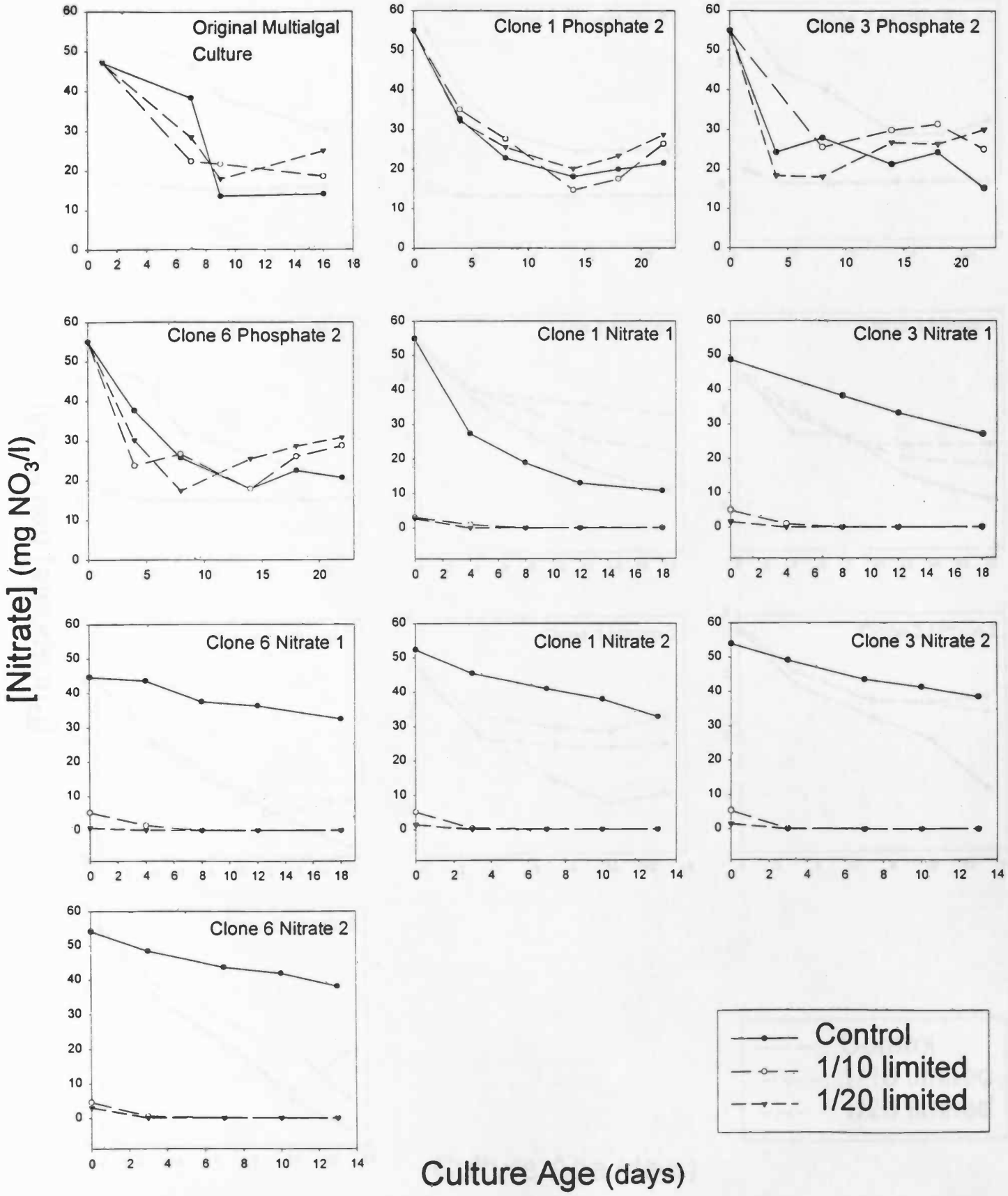

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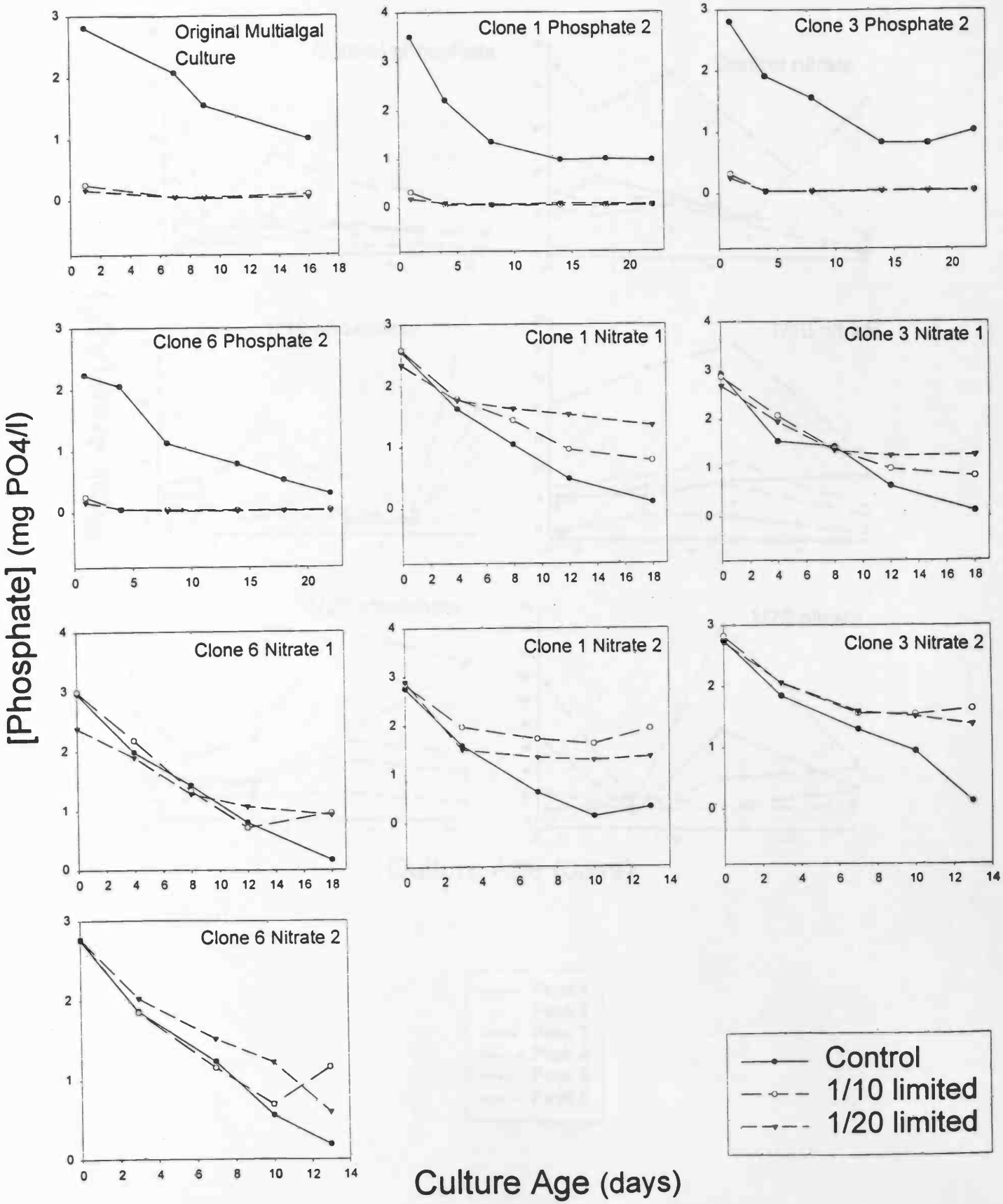
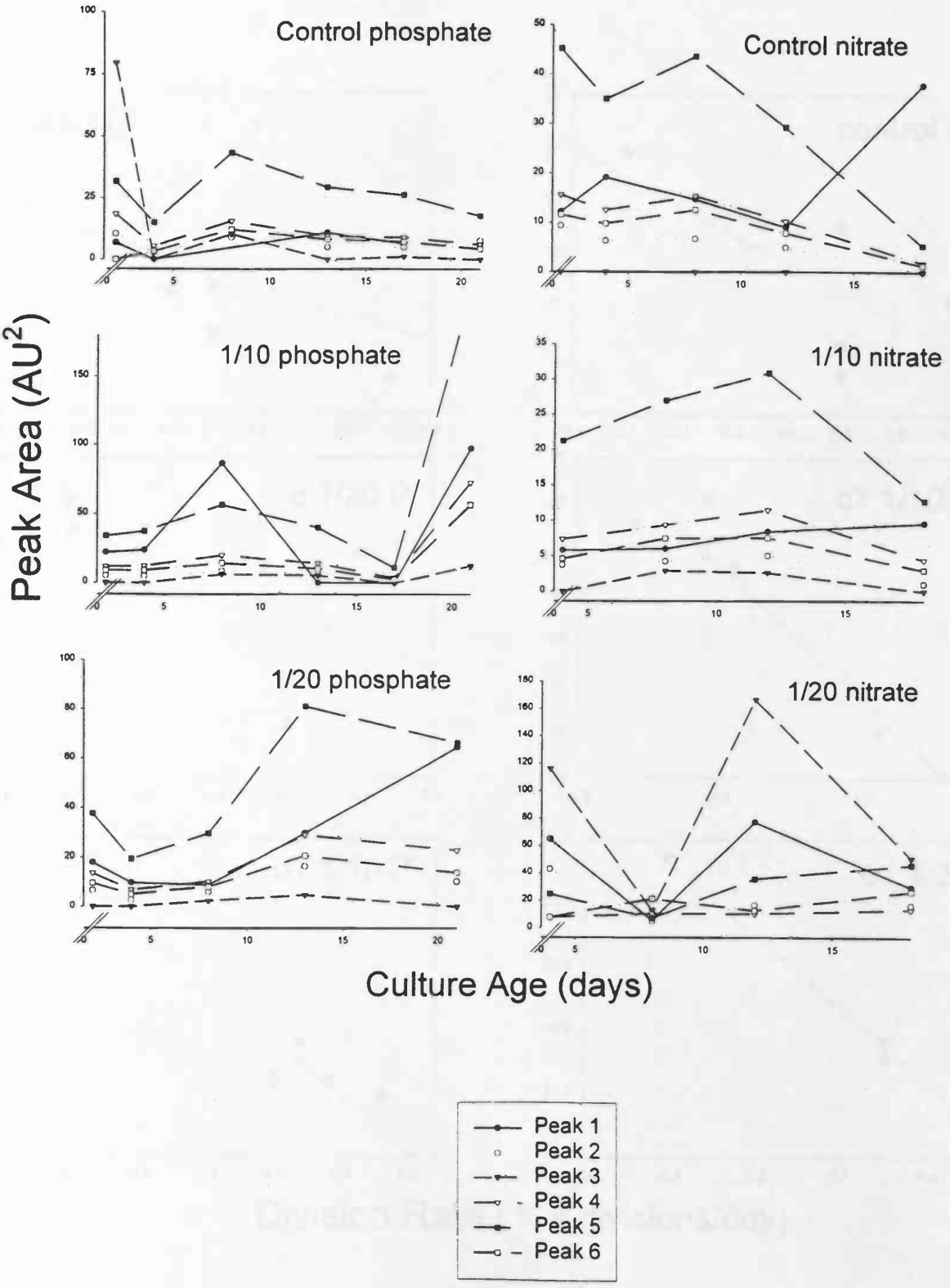


Toxin Profiles



Toxin Production in Relation to Growth Rates