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Influence of Light Regimen on the Growth and Toxicity of *Alexandrium tamarense*

by

Michael Grant Scarratt

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

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ABSTRACT

The cage-culture turbidostat allows phytoplankton to be maintained in a constant chemical environment for extended periods, and growth rate to be easily determined. The use of this system to study growth and toxin content in the marine dinoflagellate Alexandrium tamarense represents a new development in this field, since continuous culturing of toxic dinoflagellates has previously been unsuccessful. The hypothesis that the toxin content of the cells was inversely proportional to their growth rate was investigated. The effects of both irradiance reductions and daylength reductions were tested, as well as the effects of circadian growth cycles.

Moderate irradiance reductions which reduced the growth rate were not accompanied by toxin content changes. Large irradiance reductions also produced significant reductions in total toxin per cell. Daylength reduction had no effect on either growth rate or toxin content. Thus the initial hypothesis was rejected and it was concluded that light was essential for toxin synthesis. Irradiance reductions also produced measureable changes in the toxin profile, irrespective of growth rate, suggesting irradiance-dependent differential synthesis of the various toxins. Experiments with phased-dividing cultures showed that both toxin content and toxin profile were diurnally variable. Toxin content was highly correlated with chlorophyll-*a*, indicating that it is probably a direct function of cell size. Diurnal toxin profile changes were also consistent with irradiance-dependent differential synthesis.

ABBREVIATIONS AND SYMBOLS

ANOVA	analysis of variance
dcGTX	decarbamoyl gonyautoxin
dcNEO	decarbamoyl neosaxitoxin
dcSTX	decarbamoyl saxitoxin
DNA	deoxyribonucleic acid
ESNW	enrichment-solution natural water
GTX	gonyautoxin
HPLC	high performance liquid chromatography
LED	light-emitting diode
μ_{cor}	corrected growth rate
µE	micro-Einsteins (micro-mole photons)
NEO	neosaxitoxin
$^{o}/_{oo}$	parts per thousand
PSP	paralytic shellfish poisoning
R	relative culture density change
r	turbidostat culture growth rate
STX	saxitoxin
t_g	generation time

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GENERAL INTRODUCTION

Phycotoxins

Phycotoxins are substances produced by algae which are known to have toxic effects. Numerous phytoplankton species have been identified which produce phycotoxins dangerous to humans and other vertebrates (including fish and birds). Of these, dinoflagellates belonging to the genus Alexandrium (formerly classified within Gonyaulax and Protogonyaulax) are perhaps the best known. These organisms produce paralytic shellfish poisoning (PSP) toxins, and are a common problem in several regions of the world, including Atlantic Canada, New England, British Columbia, Australia, Japan and parts of Western Europe and the Mediterranean. Various other species are also known to produce phycotoxins. Pyrodinium bahamense var. compressum, another dinoflagellate, also produces paralytic shellfish toxins. The dinoflagellate Gymnodinium breve Davis (formerly Ptychodiscus brevis (Davis) Steidinger) produces brevetoxins which have caused neurotoxic shellfish poisoning (NSP) incidents in the southeastern United States and Gulf of Mexico.

Ciguatera is a poison found in tropical and sub-tropical fish which is known to have dinoflagellate origins. Diarrhetic shellfish poisoning is another dinoflagellate-produced phenomenon linked with organisms of the genus Dinophysis. It produces unpleasant but usually non-fatal gastrointestinal irritation.

In 1987, domoic acid was discovered in mussels from Prince Edward Island and ultimately linked to the diatom Pseudonitzschia pungens var. multiseries (Hasle) Hasle (formerly Nitzschia pungens Grunow var. multiseries Hasle). This toxin has since turned out to be more widespread than originally thought. It has appeared on the Pacific and Gulf Coasts of the United States where it occurs in other species of Pseudonitzschia (Taylor, 1993). In 1991, another toxic phenomenon was discovered in North Carolina associated with blooms of Pfiesteria sp. nom. prov., which kill fish in shallow estuaries of Pamlico Sound (Burkholder et al., 1992a, 1992b, Weuthrich, 1993). This organism is interesting because it blooms in response to the presence of fish (possibly triggered by ammonia or urea excretion) and releases toxins which kill the fish. The dinoflagellate then feeds on the decaying fish and reproduces. At

present, the toxins are uncharacterized, but are known to be transmitted both in the water and as an aerosol. Although the organism was identified only recently, the problem is probably not new. Sporadic reports of fish-kills have been known in the Chesapeake region for decades. However, it does seem to be getting worse, possibly due to eutrophication of the coastal waters.

The problem of phycotoxins is significant in terms of economic and public health concerns. Apart from the obvious problems of death and illness caused by eating contaminated seafood, there is the disruption to industry from both harvest closures and quarantines of contaminated fish and shellfish stocks. In addition, the substantial cost of seafood inspection and enforcement of regulations must be borne by society. Research into phycotoxins has expanded greatly in recent years as poisoning incidents have become more common. The growth of the aquaculture industry has meant that phycotoxin contamination has grown from largely a local curiosity to a major economic and public health concern. It is estimated, for example, that worldwide aquaculture production of mussels alone exceeded one million metric tons in 1989 (FAO statistics, cited in: D.J.

Scarratt, 1993). Phycotoxins will undoubtedly continue to be a problem in the future which will inspire further research and attention.

Paralytic Shellfish Poisoning

Paralytic shellfish poisoning or "PSP" is a condition suffered by humans and other vertebrates who ingest seafood (usually shellfish) contaminated with toxins from dinoflagellates of several genera including Alexandrium. The toxins block the sodium channels in neurons, causing paralysis. While the pathological condition has been known since ancient times, the causative organisms and the nature of the toxins themselves have only been discovered in this century. The link between PSP and dinoflagellates was postulated early in this century in California (Kofoid, 1911). In Canada, Alexandrium (=Gonyaulax) was identified with shellfish poisoning in the Bay of Fundy in the 1930's and 40's (see Chapter I - Review of the Literature). The basic mechanism of contamination is via the filter-feeding behaviour of bivalve shellfish. Shellfish filter large volumes of water and extract any edible particles suspended in it. If

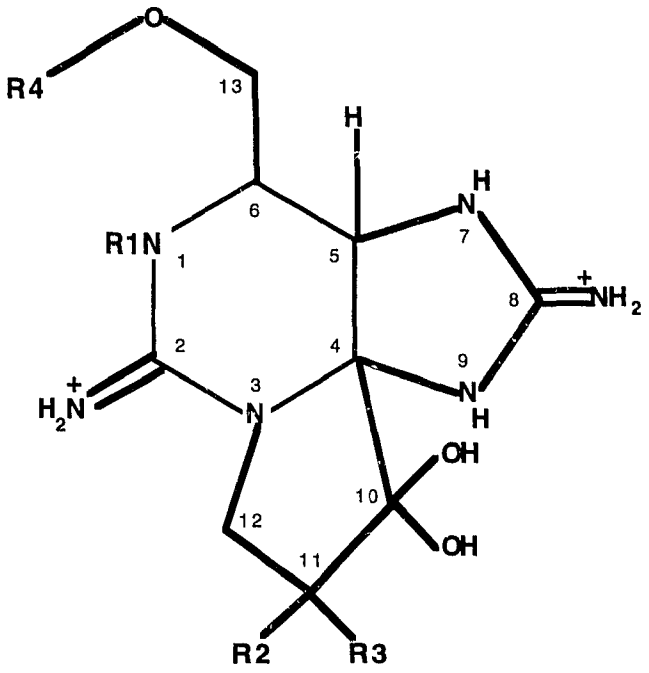
those particles include toxic phytoplankton cells, the shellfish become contaminated with the toxins. Shellfish may develop a resistance tc the toxins and although often inhibited, are not permanently harmed (Kvitek, 1993; Bricelj et al., 1993), but a vertebrate animal eating contaminated bivalves can receive a fatal dose of poison. The toxins are extremely potent. LD_{50} values in laboratory animals are in the range of 10 -100 µg toxin per kg body weight and ingestion of even a few milligrams of toxin can be fatal to humans. During serious dinoflagellate blooms, consumption of even a small amount of shellfish can be dangerous (Taylor, 1992).

In the 1950's one of the major PSP toxins was identified from the Alaska butter clam, Saxidomus giganteus Deshayes (Schantz et al., 1957). The compound was named *saxitoxin* after its source in the clam. In the mid 1960's, saxitoxin was isolated from the dinoflagellate Alexandrium catenella (Whedon et Kofoid) Balech on the Pacific coast of the U.S.A. As analytical methods improved, a variety of similar compounds was identified from dinoflagellate sources. Toxic dinoflagellate species generally contain more than one of these toxins and in some cases the mixture can be very

complex. At present, about 20 natural derivatives of saxitoxin have been characterized (Cembella and Lamoureaux, 1993; Janiszewski and Boyer, 1993), although no single dinoflagellate cell line is known to produce all of them simultaneously. Figure 0.1 illustrates the structure of saxitoxin and some of its common analogues. The compounds studied in this thesis are highlighted in bold type. One point of interest is the similarity of their structure to the purine bases of nucleic acids. This has raised much speculation about the possible origins and adaptive significance of these compounds. However, there are as yet no certain conclusions.

Morphology and Taxonomy

Saxitoxin and its derivatives are associated with several species of dinoflagellates. In addition, the taxonomic nomenclature has changed over the years which leads to some confusion in identifying the organisms involved (Taylor, 1975, 1993). In the early literature dating from the 1920's until the 1970's, the organisms were identified as Gonyaulax. However, owing to improved taxonomic methods, the generic name Gonyaulax has been dropped from more recent literature in favour of Alexandrium. For a brief period the



Toxin	R1	R2	R3	R4
STX	H	H	H	$CONH_2$
NEO	OH	H	H	$CONH_2$
GTX-1	OH	H	OSO_3^-	$CONH_2$
GTX-2	H	H	OSO_3^-	$CONH_2$
GTX-3	H	OSO_3^-	H	$CONH_2$
GTX-4	OH	OSO_3^-	H	$CONH_2$
B1	H	H	H	$CONHSO_3^-$
B2	OH	H	H	$CONHSO_3^-$
C-1	H	H	OSO_3^-	$CONHSO_3^-$
C-2	H	OSO_3^-	H	$CONHSO_3^-$
C-3	OH	H	OSO_3^-	$CONHSO_3^-$
C-4	OH	OSO_3^-	H	$CONHSO_3^-$
dcSTX	H	H	H	H
dcNEO	OH	H	H	H
dcGTX-3	H	OSO_3^-	H	H

Figure 0.1 - Structures of major PSP toxins. Bold type indicates those discussed in text. (Adapted from Oshima et al., 1990)

name Protogonyaulax was used to identify the same organisms. It should be noted that Gonyaulax is still a valid taxon and is used to name some non-toxic species with similar morphological characteristics. Around a dozen Alexandrium species have been identified as PSP producers, however, some Alexandrium isolates are known to be non-toxic. In addition, other genera besides Alexandrium contain PSP-producing species. Pyrodinium bahamense var. compressum and Gymnodinium catenatum Graham are known to produce PSP toxins, as is the freshwater cyanophyte Aphanizomenon flos-aquae. If the taxonomic complexity of the Alexandrium genus is any indication, there may be other toxic species still unidentified.

Alexandrium spp. (Figure 0.2) are thecate (armoured) dinoflagellates with cell diameters typically in the 20 - 40 μm range. The different species show some variation in general morphology. Most occur as solitary or paired cells, but A. catenella forms long chains. Alexandrium cells produce intense blooms or aggregations near the surface of the water. The cells are a characteristic reddish-brown colour and in dense concentrations can give the water a reddish tinge.

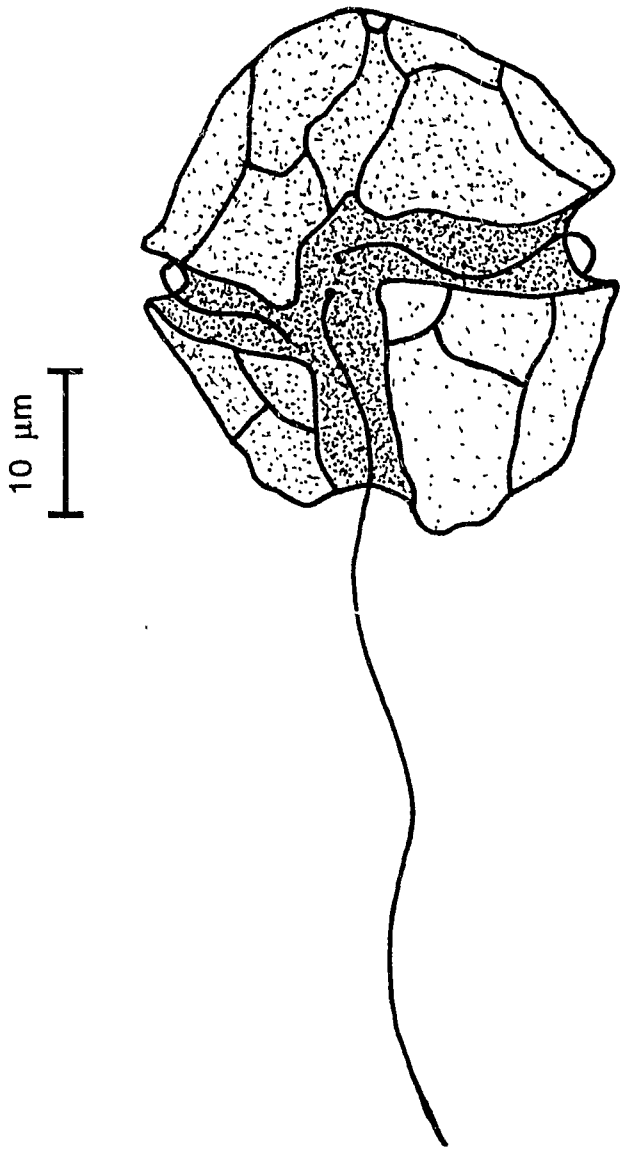


Figure 0.2 - Single cell of Alexandrium sp.. (Drawing adapted from electron micrographs - various sources)

Analytical Techniques

Analyzing PSP toxins is not a trivial task. Recent advances in chromatography have made the problem easier but it requires a considerable investment in instruments and training. Historically, the method used was the mouse bioassay (Sommer and Meyer, 1937) which, although crude, was effective enough to become the standard and is still in use for many routine monitoring purposes. It has been standardized by the American Association of Official Analytical Chemsists (Adams and Miescher, 1980). In this procedure, toxic shellfish or dinoflagellate samples are extracted in 0.1 M hydrochloric acid. The extract is then injected into laboratory mice. A suite of well-described symptoms leading to death indicates toxicity. The method has been calibrated, the standard "mouse unit" being the amount of toxin sufficient to kill a 20 g mouse in 20 min. Conversions of mouse units to toxin concentration could be made, but are only approximate due to the different toxicities of each saxitoxin analogue.

The mouse bioassay is a practical system for public health protection where the detection of net toxicity is an adequate signal.

However, its inability to resolve the different toxic compounds makes it less applicable to physiological research. In addition, the necessity of sacrificing live animals has made the method increasingly difficult to justify in recent years. Other methods have been developed including enzyme-linked immunoassays (Cembella and Lamoureaux, 1993) and even a housefly bioassay (Ross et al., 1985). However, there has long been a requirement for a purely chemical detection method which can resolve the various toxins and still be practical and inexpensive. As yet no system completely fills these requirements but considerable advances have been made in the fields of high-performance liquid chromatography (HPLC) and mass spectrometry.

The most common systems currently in use are based on HPLC. Gas chromatography is not yet practical due to the difficulty of creating specific volatile derivatives of the various toxins. Ion-spray mass spectrometry (Quilliam et al., 1989) provides very selective detection of saxitoxins, but it is too expensive for routine use in shellfish testing. The most practical HPLC system for resolving PSP toxins is the "Sullivan method", first described by

Sullivan and Iwaoka (1983). It involves HPLC separation of the toxic constituents on a reverse-phase column and post-column oxidation to fluorescent derivatives which can be detected with a fluorometer. This method is very sensitive, and toxins from as little as a few thousand dinoflagellate cells can be detected. A sample of dinoflagellate culture as small as 100 mL can give highly repeatable results. The principal disadvantage is the variability of the post-column derivatization procedure which is highly dependent on reagent flow rates and other reaction conditions. This means that comparison of results from two different instruments is difficult. Until recently, the problem was exacerbated by the lack of suitable toxin standards, although this deficiency has now largely been overcome. Development of HPLC methods has also progressed and the Sullivan system has been superseded by an improved procedure described by Oshima et al. (1989). At present, the Oshima procedure is the method of choice for physiological investigations of dinoflagellates or shellfish. It provides better resolution of the various toxic compounds than does the Sullivan method.

Current Knowledge

Current knowledge of toxic phytoplankton is highly developed in some areas and somewhat sketchy in others. Some of the basic processes leading to the formation and transport of blooms are well understood, as are many of the organism's physiological responses to environmental variables such as nutrients and temperature. The basic pathways of PSP toxin synthesis are largely known, although not all the reactions and enzymes are characterized. However, understanding of the adaptative and evolutionary significance of toxicity is generally poor. For example, there is no well-accepted explanation why phytoplankton produce toxins. Speculations include defense mechanisms, infection by (or symbiosis with) toxin-producing bacteria, plasmid or viral transmission of the "toxin genes" and relict pathways for the production of nucleic acids. All of these hypotheses have good evidence to support them but none has been conclusively established. The search for answers is complicated by the fact that not all strains of Alexandrium are toxic, which suggests that toxicity may not confer any particular competitive advantage.

Outline of Research

One of the areas of dinoflagellate research which has received relatively little attention is the effect of light on the growth and toxicity of the cells. Some basic work with batch cultures grown at different light intensities indicates that the presence of light is required for toxin production (Ogata et al., 1987a, 1989a). However, it is possible that the toxin content of dinoflagellate cells may be influenced as much by their growth rate as by anything else. For example, other intracellular compounds such as triglycerides are known to increase in concentration in diatom cells whose growth rate is inhibited by some external stress (Parrish and Wangersky, 1987, 1990). The explanation put forth for this is that the production of triglycerides occurs at a more or less constant rate while the growth rate of the cells is variable. If the cell division rate drops, triglycerides can accumulate. If the cell division rate increases, the concentration of triglycerides will drop.

WORKING HYPOTHESIS

A similar argument can be made for toxin production in dinoflagellates. There is evidence to indicate that the toxin content

of dinoflagellate cells is enhanced during periods of phosphorus and possibly also light limitation (Boyer et al., 1987; Ogata et al, 1987, 1989a). Based on this evidence, a working hypothesis was proposed that the toxin content of A. tamarense is inversely related to the growth rate through the mechanism described above. Light regimen was chosen as the environmental variable to study since it had not been widely investigated in the past. Cage-culture turbidostats were selected as the principal culture method since they afford a means of maintaining a constant environment while allowing growth rate to be determined accurately. This was a particular advantage since the organism in question usually achieves only slow to moderate growth rates. In Chapter 3, the effects of light intensity and photoperiod on the production of toxins are described for one particular strain of A. tamarense grown in turbidostats. Both the toxin content of the cells and the relative proportions of the different saxitoxin analogues (the toxin profile) were investigated. As the work progressed, it became obvious that diurnal or circadian rhythms in the dinoflagellates were significantly influencing the results. In Chapter 4, the characteristics of these rhythms and their effects on the toxin content and toxin profile of the cells is

investigated. Significant effects of light regimen on toxin production were observed as well as variations in toxin production on diurnal timescales. In addition, toxin profile changes at short time scales (hours to days) were observed which have not been noted by previous authors.

At a technical level, the application of cage-culture turbidostat techniques to the production of toxic dinoflagellates represents a new development in this field. Continuous culture techniques have not been previously used with this species and have been widely regarded as unworkable. This thesis will show that it is possible to use cage-culture culture systems with A. tamarense. In short, the results of this project represent a significant contibution to the field and should provide a basis for further research in the future.

CHAPTER 1

REVIEW OF THE LITERATURE

Growth Conditions and Toxicity

Early Investigations

The dinoflagellate species currently known as Alexandrium spp. (=Gonyaulax or Protogonyaulax spp.) have been identified with paralytic shellfish poisoning for at least half a century. Sommer et al. (1937), established the link between mussel poisoning and A. catenella (Whedon et Kofoid) Balech in California. Direct linking of the dinoflagellates with shellfish poisoning events was achieved for the Bay of Fundy populations of A. fundyense (Lebour) Balech in the years following World War II (Needler, 1949). A shellfish toxicity surveillance program was established in the Bay of Fundy in 1943 to protect the public from contaminated seafood (White, 1987).

Physiological Studies

Research into the physiological basis of toxicity began in the 1960's. In 1957, the principal toxin had been identified and purified from the Alaskan butter clam Saxidomus giganteus Deshayes and accordingly named saxitoxin (Schantz et al., 1957). Saxitoxin was also identified in two dinoflagellate species from the Pacific, Gonaulax polyedra Stein and A. catenella (=G. catenella) during this period (Schradie and Bliss, 1962; Schantz and Magnusson, 1964, Schantz et al., 1966) and preliminary chemical characterizations of the compound were made. Measurements of growth rates, toxicity, salinity and temperature tolerance described the basic conditions and behaviour of A. fundyense (=G. tamarensis) from the Bay of Fundy (Prakash, 1967). This was one of the first truly thorough investigations of the organism's physiological and ecological characteristics. In general, when growing the dinoflagellate in batch cultures, Prakash found that A. fundyense grew better in nutrient-enriched, natural seawater than in artificial seawater. The culture lag phase was shorter and generation times were faster. Also, the maximum cell yield was greater.

Salinity tolerance was found to be very wide, with cells surviving from 7 $^{o}/_{oo}$ to 40 $^{o}/_{oo}$ with an optimum tolerance of 19 - 20 $^{o}/_{oo}$ (Prakash, 1967). Temperature tolerance of these cultures was also wide, with a range of 5 °C to 25 °C and an optimum level of 15 - 19 °C. Working with A. tamarense (=G. tamarensis) collected off Monhegan Island, Maine, Yentsch (1974) found a similar temperature tolerance of 5 °C to 24 °C with optimum growth occurring between 15 °C and 20 °C. The salinity tolerance was also investigated in this study, but only over a short range. The organisms grew almost equally well at salinities between 20 $^{o}/_{oo}$ and 28 $^{o}/_{oo}$ with growth rate variations of only 20 %. White (1978) found that A. tamarense (=Gonyaulax excavata (Braarud) Balech) had a wide salinity tolerance of 11 - 43 $^{o}/_{oo}$, but that its optimum growth occurred at 30.5 $^{o}/_{oo}$. It should be noted that the different species names cited by Prakash (1967) and White (1978) do not necessarily indicate that they were working with radically different organisms. White uses the name G. tamarensis to refer only to *nontoxic* organisms and G. excavata to toxic ones, while Prakash's G. tamarensis were clearly toxic. Moreover, this taxonomic distinction is no longer considered valid.

The confusion of taxonomy in this group of dinoflagellates frequently makes interpretation of the older literature difficult.

Prakash (1967) noted that the optimum ranges for both salinity and temperature were not the same as the natural bloom conditions in the Bay of Fundy. However, the interaction of the two variables with each other was not investigated. Possibly the use of 24 h light with no night period affected the results.

The interaction of salinity and temperature in regulating the growth of A. tamarense (=G. tamarensis) was investigated by Watras et al. (1982). They found that a curve of growth rate vs. temperature for this species had a broad optimum from 13 °C to 23 °C. Observed division rates were higher at salinities of 25.5 $^{o}/_{oo}$ than at 32.5 $^{o}/_{oo}$ at all but the lowest temperature (5 °C). Salinity did not affect the general shape of the growth rate vs. temperature curve. At temperatures much above 20 °C to 25 °C the fit of the curve was erratic. However, Watras et al. (1982) concluded this was not a problem since the organisms were originally collected in Massachusetts, where ocean water temperatures rarely exceed

20 °C. Based on these findings, Watras et al. modelled the development of blooms in small estuaries on the New England coast. Using actual temperature and salinity records, their simulated blooms followed almost identical growth curves to real blooms observed in the estuaries. They concluded that salinity-dependent temperature regulation of growth is the major process governing the development of A. tamarense blooms in these estuaries.

The relationship of environmental factors to toxic blooms and shellfish toxicity in the Bay of Fundy was investigated by White (1987). Using mouse bioassay data from the Bay of Fundy Shellfish Toxicity Surveillance Program and oceanographic data for the period 1944 to 1983, White attempted to find correlations between shellfish toxicity events and environmental conditions. Water temperature, salinity, sunlight, wind speed, river discharge and the nodal modulation factor of the 18.6-year tidal cycle were examined. The most significant correlations occurred between shellfish toxicity and the conditions in the months *preceding* the bloom, especially January and February. Since the blooms do not normally occur until July, the mechanism behind this is unclear; but one

possibility is that the survival and germination of hypnozygote cysts is affected by winter conditions. A rather consistent correlation also occurred between summer toxicity and the 18.6 y tidal cycle which was observed over about 2.5 cycles. White (1987) speculated that increased tidal energy dissipation might influence the distribution of dinoflagellate aggregations, perhaps concentrating them in productive frontal zones or transporting them to shellfish-producing areas. He suggested that detailed observations should be made at the peak of the next cycle (late 1990's) to investigate this correlation further.

Prakash (1967) observed cyst formation in old cultures under nutrient-depleted conditions and under non-optimal conditions of temperature, salinity and light. He speculated on the role of cyst formation in seeding new blooms and in the "winter toxicity" of shellfish, particularly giant scallops (Placopecten magellanicus Gmelin), a phenomenon which has been observed in the Bay of Fundy and Gulf of Maine (Bourne, 1965). He did not, however, measure the toxicity of the cysts, a question which has been much debated over the years (reviewed by Anderson, 1984).

The nutrient requirements of Alexandrium spp. are well described. Prakash (1967) discovered requirements for vitamin B_{12} and thiamine. He also noted an increase in growth rates with the addition of soil extract to the medium. This was formerly a common procedure when culturing this species. However, soil extracts contain mixtures of largely uncharacterized organic compounds, so for sensitive chemical work their presence is undesirable. Improvements in defined culture media have largely eliminated this practice. Harrison et al. (1980) described an excellent defined medium which supports good growth of Alexandrium spp.. Its use has become common in physiological studies of this species. Keller and Guillard (1985) reported that their "K Medium", originally developed for oceanic ultraplankton, also supported dinoflagellates successfully. It is now enjoying widespread use.

Effects of Environmental Factors on Toxicity

GENERAL CHARACTERISTICS

Early investigations of toxicity in dinoflagellates were hampered by the lack of a high-resolution analytical technique for

detecting the toxin (General Introduction, Section 3). For many years the mouse bioassay was the only method available, so when reading the early literature it is necessary to remember that the different toxic compounds could not be resolved quantitatively. Qualitative separation was possible using thin-layer chromatography (Proctor et al., 1975). Hence, all mass or molar quantities are expressed as "saxitoxin equivalents" based on mouse toxicity, not true amounts. Nevertheless, useful information was acquired on toxin production at the cellular and bulk culture level.

Prakash (1967) found that toxin production in batch cultures was a function of culture density and levelled off when the culture entered stationary phase. A plot of toxin/mL vs. cells/mL was parabolic, indicating a lesser toxin load per cell as the culture grew older and denser. Prakash speculated that this might be due to cells rupturing and losing their toxic contents to the medium as they aged. Toxins could be detected in the medium, but only after the culture entered stationary phase. This indicated that the toxin was normally intracellular and was released into the medium by dying cells, not by growing cells. The opposite scenario had been observed in other

toxic phytoplankton such as Gymnodinium breve Davis and Prymnesium parvum Carter which are known to release their toxins into the surrounding water (Pierce, 1986, Parnas, 1963, Parnas and Abbott, 1965).

SALINITY

The relationship between toxicity and salinity is not a simple function of growth rate. White (1978) found that the optimum salinity for A. tamarense (=Gonyaulax excavata) growth was 30.5 $^{o}/_{oo}$, while the toxicity maximum occurred at 37 $^{o}/_{oo}$. At higher and lower salinities, both growth rate and toxicity were lower. White could not explain this, but speculated that osmoregulation may play a role in toxin synthesis. The implication of this is that the conditions under which blooms are likely to occur are not necessarily those which produce the highest cellular toxicities. Thus when assessing the impact of a toxic bloom, the density and duration of the bloom and the toxicity of the cells must be considered. Small concentrations of very toxic dinoflagellate cells could still render shellfish dangerous to eat, as could a prolonged bloom of weakly toxic cells.

IRRADIANCE AND PHOTOPERIOD

The effects of light intensity and photoperiod on toxin production have received relatively little attention. The earliest study which investigated the effects of light on the growth of Alexandrium sp. was that of Yentsch et al. (1974) who determined the effects of light regimen on growth rate, but not toxicity, in a Gulf of Maine strain of A. tamarense (=Gonyaulax tamarensis). Determination of the photosynthesis vs. irradiance curve showed that the response of A. tamarense to irradiance was fairly typical in comparison with other species, including diatoms such as Skeletonema sp. and Chaetoceros sp.. However, when photoperiod was investigated, it was found that the growth rate of A. tamarense was directly proportional to daylength over the range studied (8:16 L:D to 16:8 L:D). By contrast, the growth rate of two diatoms, Skeletonema sp. and Phaeodactylum sp., was inversely proportional to daylength over the same range. Although Yentsch et al. (1974) drew some interesting ecological implications from this observation, it does not match other investigations of photoperiod effects on diatoms. For example, Paasche (1967, 1968) demonstrated increasing

specific growth rates with increasing daylength in both diatoms and coccolithophores.

Even if a dinoflagellate is able to adapt to prevailing light conditions, the timescale of such adaptations will be important in determining how well the species competes with others in the same environment. A motile organism such as Alexandrium spp. might be expected to swim up or down in the water column and position itself at the optimum light intensity. However, weather patterns such as clouds will affect the amount of incident radiation available at the surface of the water. Since these changes take place on time scales of days or hours, phytoplankton must be able to adapt at a similar rate to take advantage of the available light. The response time of A. tamarense (=G. tamarensis) to changes in irradiance has been investigated in semi-continuous cultures (Maranda, 1985). Pigment content, photosynthetic rate, cellular chlorophyll, carbon and nitrogen content, cell number and cell size were measured three times per day at two light intensities (75 and 500 $\mu E\ m^{-2}\ s^{-1}$) as well as before and after a change in light intensity. The cultures were exposed to irradiance changes of 75 to 500 $\mu E\ m^{-2}\ s^{-1}$ or 500

to 75 $\mu E\ m^{-2}\ s^{-1}$ and measurements continued for four days. The results indicated that cells at low light intensity had higher pigment content, as would be expected. The cells were also smaller, and chlorophyll content normalized to carbon was larger. Photosynthetic rates (normalized to carbon) were also higher for cells at low light intensity. However, when the rates were normalized to chlorophyll, they were lower, indicating that the cells could not completely compensate for the reduced light intensity. This is likely a consequence of the package effect, which has been observed in other dinoflagellate species (Johnsen and Sakshaug, 1993). In cells exposed to the light intensity increase, the pigment content decreased almost immediately, as did the photosynthetic rate. Both parameters reached the expected high-light values within one cell generation (about three days). However, there were some indications that photoinhibition might be occurring in these cultures. In cells exposed to a light intensity decrease, pigment content increased, but rather slowly compared to the opposite case above. However, the photosynthetic rates (normalized to cell carbon) increased quite rapidly, indicating that the cells may have been photoinhibited at 500 $\mu E \cdot m^{-2} \cdot s^{-1}$, and a release of this

photoinhibition allowed photosynthetic rates to increase rapidly. In general, the cells adapted their photosynthetic rates to changes in light intensity within one cell generation, although the pigment content took longer to adjust. The cells were generally shade-adapted, showing photoinhibition at 500 $\mu E \cdot m^{-2} \cdot s^{-1}$, even in cultures maintained at that light level for several generations. Although they experienced photoinhibition, the photosynthetic rates (normalized to chlorophyll) were still higher at high light intensity, allowing A. tamarensis to take advantage of its ability to migrate vertically and select a high light level. Short exposures to high irradiance under favourable weather conditions could allow the cells to grow faster and gain a selective advantage.

Another possible implication of light adaptation concerns the role of pigments as storage compounds for nitrogen and carbon within the cell. As a cell culture or bloom ages and becomes nutrient-limited, the cellular reserves of nutrients become depleted and cell division slows. However, it has been shown that when shade-adapted cultures with high concentrations of pigments in their cells become nutrient-limited and cellular reserves of nutrients decline, the

pigments are also broken down. This can delay the onset of stationary phase in the culture by as much as one complete cell generation (Prézelin, 1982). Thus it is possible that shade-adaptation in dinoflagellates can prolong their survival under nutrient-limiting conditions. This could give them an advantage over other species during periods of nutrient limitation since they would be better able to survive until conditions improved.

To date there are only two published accounts of the effects of irradiance on toxin production (Ogata et al., 1987a, Ogata et al., 1989a). In these investigations, the relationship between growth rate and toxicity was measured under various conditions of light regimen and temperature. As expected, higher temperatures and higher irradiances produced higher growth rates. The toxin content of the cells was inversely proportional to growth rate in both cases. The relationship was stronger for temperature than for irradiance, and Ogata et al. (1987a, 1989a) concluded that light and photosynthesis were essential for toxin synthesis. In addition, they investigated the effects of a sudden decrease in irradiance or temperature on the cell toxicity. When the temperature alone was

reduced, cell division was interrupted for a few days. However, toxin production continued and the cells accumulated toxin. When growth was interrupted by reduced irradiance, the cells did not accumulate toxin, indicating that toxin synthesis had ceased. This lent support to the hypothesis that photosynthesis is essential for toxin production. However, all measurements were made during the daylight hours so no conclusions on the role of light-dark cycles could be drawn.

Apart from the work of Ogata et al. (1987a, 1989a), there has been no major investigation of the effects of light regimen changes on the toxicity of Alexandrium spp.. Since the literature appeared sparse in this area, this thesis is devoted to light regimen effects on toxin production.

GROWTH RATE

The relationship between toxicity and growth rate has been the subject of considerable debate. There is conflicting evidence that toxicity and growth rate are either inversely or positively correlated, depending on the type of experiment involved. Strong

evidence for an inverse correlation came from Proctor et al. (1975) who cultured A. catenella (=G. catenella) under continuous light and measured the toxin yield at intervals over the life of the culture. They found that at 12 to 13 °C, the yield of toxin per cell was directly proportional to cell density during the exponential phase growth. If the temperature was increased to 15 to 16 °C, the growth rate approximately doubled, but the toxin yield per cell decreased by about half. The toxin yield per litre of culture remained about the same. Thus, production of toxin at the lower temperature was much more efficient, even though the culture was growing more slowly. It is worth noting that the 12 to 13 °C temperature is about the same as the ambient seawater temperature in the region of California where the cells were originally collected.

Boyer et al. (1987) found that toxicity was inversely proportional to division rate when cells of A. tamarense (=Protogonyaulax tamarensis (Lebour) Taylor) became phosphorus limited. As the culture entered stationary phase, the toxicity increased three- to four-fold and later declined as the cells died. By contrast, nitrogen limitation did not produce this effect. The

toxicity of the cells declined as the culture entered stationary phase. The same was true in control cultures which were not specifically nitrogen or phosphorus limited. Changes in cell volume were observed but were not large enough to account for the changes in toxicity. The most plausible explanation is that the toxins, which are very rich in nitrogen, are acting as a nitrogen-storage compound. As the cells enter nitrogen limitation, nitrogen is mobilized out of the toxins and becomes available for general metabolism. Furthermore, no toxin is being synthesized under these conditions. Under phosphorus limitation, toxin synthesis can continue but cell growth and division is inhibited. Thus toxins can accumulate in the cells.

Not all information on growth rate and toxicity indicates an inverse relationship. Boczar et al. (1988) investigated toxin production in A. tamarense (=P. tamarensis) and A. catenella (=P. catenella) grown in batch cultures. They also investigated changes in toxin profile (ie. the relative proportions of the different toxins in the cells) which will be discussed later in this chapter. In terms of toxin production and cellular toxin content alone, the results showed

that the toxin content of the cells peaked in early to mid-log phase when the division rate was highest. The cellular toxin content then tailed off as the division rate diminished. This pattern was present in both species except that the increase and decline occurred more rapidly in A. tamarense. Note that this pattern of toxin production is in line with the early results from Prakash (1967), but appears to contradict some of the findings of Boyer et al. (1987). However, Boczar et al. (1988) did not specify the type of nutrient limitation experienced by their cultures, merely refering to "culture age", so direct comparisons are difficult to make. Furthermore, the difference species involved may behave in different ways.

Anderson et al. (1990b) observed a similar "high-low" pattern of toxin production and cellular toxin content with Alexandrium fundyense and A. tamarense in both nutrient-replete and nitrate-limited batch cultures. High cellular toxin content was observed during early to mid-exponential phase, declining in stationary phase. Cellular chlorophyll and protein levels followed the same pattern. Cellular arginine levels followed the opposite pattern. This is consistent with the findings of Shimizu et al. (1984; 1990) which

indicated that arginine is a precursor to the synthesis of toxins. When specific rates (d^{-1}) of toxin production and cell division were compared, a direct relationship appeared. This suggests that the highest toxin production rates occurred at the highest growth rates. Slight imbalances in the specific rates would produce fluctuations in the toxin content of the cells. Anderson et al. (1990b) speculated that if the specific toxin production rate exceeded the specific cell division rate in log phase but not stationary phase, it would produce the convex pattern of cellular toxin content observed in most batch cultures.

There is a possible resolution to the apparent contradictions of the results discussed here if we assume that there are three kinds of toxicity variability. In the cases mentioned by Proctor et al. (1975) and Ogata et al. (1987a, 1989a), cellular toxin content was compared between cultures growing under different conditions. This could be called "environmental variability". This is not the same as "growth stage variability" (Anderson et al., 1990b) in which toxicities of cells at different stages of the growth cycle are compared. The results of Proctor et al. (1975) and Ogata et al.

(1987a, 1989a) indicated a sort of environmental enhancement of toxicity which was correlated with reduced growth rate. The cells were more efficient producers of toxin at low temperature than at high temperature. The cause for this is not clear but it does not imply that at the same temperature, young, quickly growing cultures should produce more toxin (per cell) than old, slowly growing ones. Indeed, they do not, according to Prakash (1967), Boczar et al. (1988) and Anderson et al. (1990b). Boyer et al. (1987) and Ogata et al. (1987a, 1989a) also observed that cultures whose growth was interrupted by limitation of phosphate, temperature or light accumulated toxin. This is a third kind of toxicity variation which we might call "cell cycle variability" (or "environmentally-enhanced variability - Anderson et al., 1990b). It can probably be explained by assuming that the environmental change affects the cell division mechanism, but not the toxin synthesis mechanism. Cells normally produce enough toxin to provide to their daughter cells when they divide. If division is interrupted but toxin synthesis continues, toxin will accumulate in the cells. It is this type of variability which will be investigated in this thesis, using changes

in light regimen as the environmental trigger for interrupted cell division.

Toxin Biosynthesis and Dynamics

The biosynthetic pathways for saxitoxin and its various analogues have taken many years to elucidate. Apart from one study employing ^{14}C-labelled amino acids and other small compounds (Proctor et al., 1975), which revealed only sporadic incorporation of the label into saxitoxin, little progress was made on biosynthetic pathways until the 1980's. It had often been noted that the structure of saxitoxin was very similar to the purine base constituents of DNA and RNA. Proctor et al. (1975) hypothesized that known purine precursors might also be precursors of saxitoxin. They conducted experiments in which a wide variety of ^{14}C-labelled compounds was fed to cultures of Alexandrium catenella (=Gonyaulax catenella). Radiocarbon from guanine, formate and urea was incorporated into saxitoxin but only in very small amounts. The authors concluded that the pathway for saxitoxin biosynthesis must be very specific and distinct from normal purine biosynthesis. In the 1980's, more advanced studies (Shimizu et al., 1984; 1990) were able to reveal

the synthetic pathway. Isotopic labelling was used involving ^{14}C and ^{13}C-labelled arginine, acetate and glycine fed to both dinoflagellates and the saxitoxin-producing freshwater cyanobacterium Aphanizomenon flos-aquae. Using radiolabelling and NMR techniques, Shimizu et al. discovered that the toxin was synthesized from arginine, acetate and S-adenosyl-methionine which combine to form the tricyclic ring structure of saxitoxin (see Figure 1.1). A similar pathway was demonstrated for neosaxitoxin. Presumably, the other analogues are similarly synthesized.

Since the biosynthetic pathway has been determined, it is now possible to speculate on the role of saxitoxin and its analogues. One key piece of information which is needed is the location of toxin within the dinoflagellate cells. Two recent studies from the same laboratory (Anderson and Cheng, 1988; Doucette and Anderson, 1993) used immunochemical labelling techniques to identify saxitoxin within the nucleus of Alexandrium fundyense cells. Toxic strains of A. fundyense indicated strong labelling within the nucleus. Non-toxic strains of A. tamarense showed no accumulation of the immuno-label in the nucleus in some samples, but did in others. These

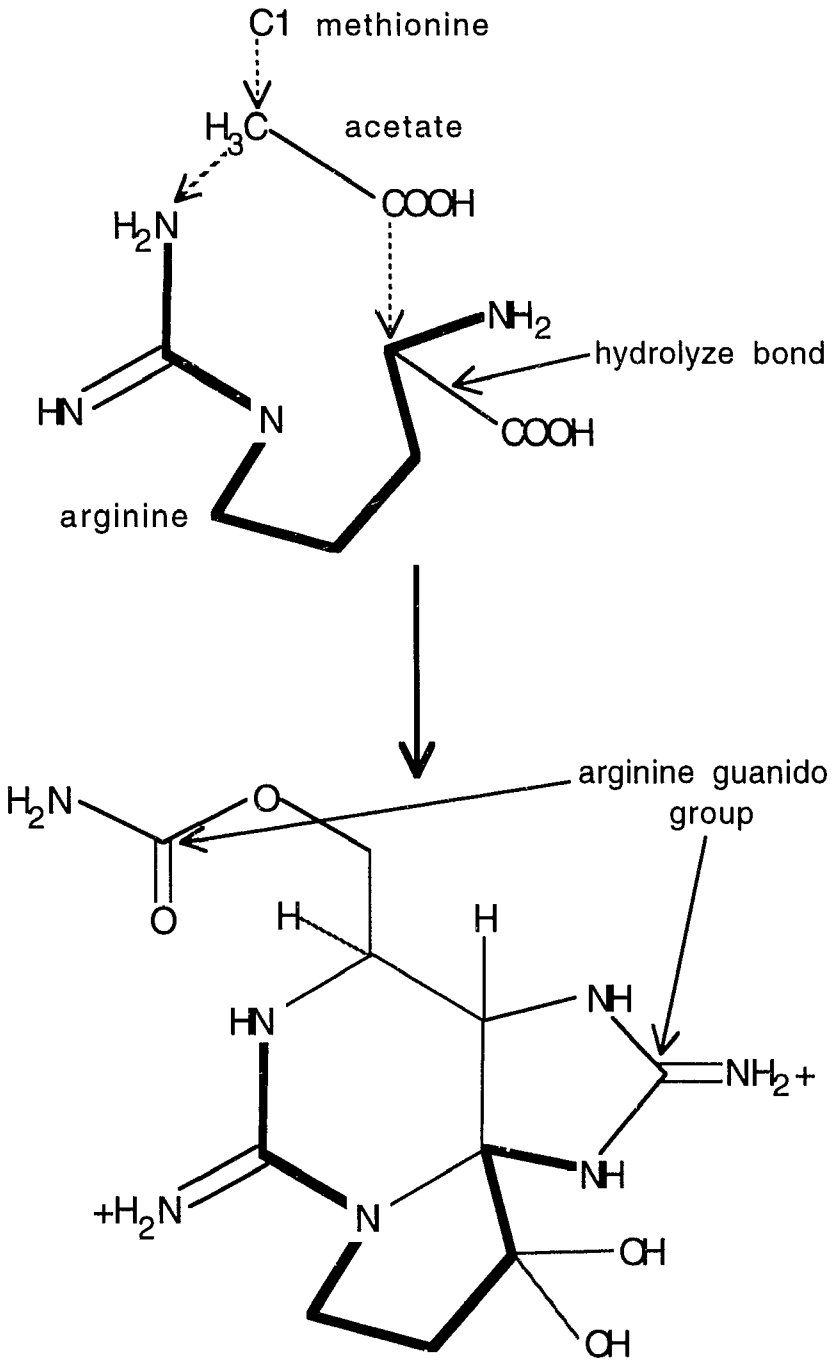


Figure 1.1 - Biosynthesis of Saxitoxin
(adapted from Shimizu et al. (1990)

staining artifacts made it impossible to conclude that the toxin was in the nucleus, but it seemed likely to be so. The toxin appeared to be associated with the chromosomes, which in dinoflagellates remain highly condensed, even during the G-phases of the cell cycle. The implication of this information is that if the toxin has a biochemical function, which is not clearly known, it is likely associated with the function of the cell nucleus. It has been suggested that saxitoxin is a nitrogen storage compound, owing to the high nitrogen content of the molecule (32.7 % by weight).

While the relationship of saxitoxin to DNA synthesis is still speculative, it is apparent that the synthesis and degradation of toxins within dinoflagellate cells are correlated with their growth cycle. Anderson (1990a) showed that toxin synthesis occurs mainly in the early S-phase of the cell cycle, but stopped completely during mitosis and cell division. Cultures of Alexandrium fundyense. were sampled at hourly intervals over the course of one cell growth cycle. During the period of mitosis, the toxin content of the cells dropped by about 50%. This might suggest that toxins are being metabolized during mitosis, but more likely represents a partitioning of toxin

between daughter cells, halving the toxin content per cell. Similar results were obtained by Taroncher-Oldenburg et al., (1994), with toxin synthesis being confined to late G1, S and early G2 phases. More detailed observations are necessary to determine if toxins and DNA synthesis are really linked or merely synchronized for some external reason. Anderson (1990a) indicated his intention to perform such experiments but at present, they have not yet been published.

Another highly detailed study, (Anderson et al., 1990b), explored the patterns of growth-stage variability and environmental variability in toxin production in both batch and semi-continuous cultures. A wide variety of cellular parameters was followed through the life of the cultures. Cell numbers, sizes, elemental composition, chlorophyll content, protein content, carbohydrate content, dissolved free amino acids and cellular arginine were all measured in addition to toxicity. In nutrient-replete batch cultures, toxin per cell followed a similar curve to cellular phosphorus, nitrogen, protein and carbohydrate. The toxin content per cell increased through early exponential phase and decreased as the cells entered stationary phase. However, arginine, which is known to be a

precursor to saxitoxin (Shimizu et al. 1984; 1990), existed in the cells at very low levels through exponential phase and increased as the cells entered stationary phase. In some cases, arginine followed almost the inverse curve of total toxin. This lends direct support to the conclusions of Shimizu et al. (1984; 1990) and also demonstrates clearly that toxin production dynamics are similar to other cellular metabolites. For the first time, clear correlations were established between toxin production and the physiological state of the cells.

Anderson et al. (1990b) also investigated toxin production dynamics in cultures exposed to nutrient, salinity and temperature stress. The results confirm the findings of the earlier studies such as Prakash (1967), Boyer at al. (1987), Proctor et al. (1975) and Ogata et al. (1987a). Under phosphate stress, the cells entered a distinct stationary phase before toxin synthesis stopped. The cellular toxin content thus rose quickly as did cellular protein, carbohydrate, chlorophyll, nitrogen and carbon. Total cellular phosphorus dropped, which is not surprising. Cellular arginine stayed low, as might be predicted from the results of the control cultures.

Under nitrate stress, the situation was reversed. Toxin synthesis stopped as the cells entered stationary phase and the toxin per cell concentration declined. Total cellular nitrogen, chlorophyll and protein declined, but phosphorus, carbon and carbohydrate increased. Cellular arginine showed little change. This pattern is reasonable if one considers that the cell is unlikely to accumulate any high-nitrogen compounds like proteins, amino acids and toxins when nitrogen is lacking. The synthesis of other compounds can continue, at least for a while. Under high salinity, the pattern was similar to the control cultures. In the low temperature culture, cell division was half the normal rate and the experiment lasted twice as long. The toxin per cell and cellular arginine concentrations were very high, but followed essentially the normal pattern. In general for all the experiments, the specific rates (d^{-1}) of toxin production and growth were positively correlated, indicating that the highest production rates of toxin occur when the cells are growing fastest.

Source of Saxitoxin - Bacteria or Dinoflagellate?

There has long been a debate surrounding the ultimate source of saxitoxins in dinoflagellate blooms. The debate divides into two

camps: those who argue for a dinoflagellate origin; and those who argue for symbiotic bacteria. The research discussed so far in this review indicates that the evidence for a dinoflagellate source is strong. Since the production of the toxin can be correlated with other physiological events in the dinoflagellate cells, it is reasonable to conclude that synthesis is occurring within those cells. However, there is a considerable amount of evidence which points to symbiotic bacteria as the source of the toxin.

The evidence in favour a dinoflagellate origin for PSP toxins is convincing. Apart from the physiological evidence linking toxin production to cell cycle events discussed earlier in this review, there is also evidence from genetic and enzyme electrophoretic experiments that the production of toxins in Alexandrium spp. is an inherited characteristic. The foundation for most of this evidence is that the toxicities and toxin profiles of A. tamarense strains are geographically variable (Alam et al., 1979; Cembella and Taylor, 1986; Cembella et al., 1987; Maranda et al., 1985). This suggests that the genetic profile of these regional strains is significantly different. However, enzyme electrophoretic evidence suggests that

the populations of Alexandrium spp. in the Gulf of Maine are relatively homogeneous (Hayhome et al., 1989). It is possible that the weakly toxic southern populations might be the product of breeding interactions between A. tamarense and A. fundyense (the two, possibly sibling, species found on the East coast), perhaps via some kind of advection and transport mechanism (Anderson, 1990b). This has been called the "dispersal hypothesis" (Anderson and Morel, 1979; Dale 1977). A physical transport mechanism has been described and modelled in the Gulf of Maine by Franks and Anderson (1990a, 1990b).

Recently, there have been some successful breeding experiments with Alexandrium spp. which indicate that the ability to produce toxins is an inherited characteristic, not an acquired one. By crossing strains of A. catenella (Whedon et Kofoid) Balech or A. tamarense with different toxin profiles, it has been shown that the inheritance of toxin profile follows a 1:1 or "biparental" Mendelian pattern, with F1 progeny showing either one of the parental toxin profiles with equal probability (Sako et al., 1992; Ishida et al., 1993). The authors concluded that the gene for toxin-producing

enzymes was located in the chromosomal DNA of the dinoflagellate and not acquired from symbiotic bacteria. A similar experiment with Gymnodinium catenatum had similar results (Oshima et al., 1993). Thus it seems that the ability to produce PSP toxins is a genetic characteristic in dinoflagellates and follows standard Mendelian patterns of inheritance.

Countering this genetic evidence are a number of controversial studies implicating marine bacteria as toxin producers. Beginning in the late 1980's, a Japanese research group found evidence indicating that the production of toxin in Alexandrium tamarense was not an inherited characteristic, but instead appeared to be acquired from symbiotic bacteria of the genus Moraxella (Ogata et al., 1987b; Kodama et al., 1988; Kodama and Ogata, 1988; Kodama, 1990a; 1990b; Ogata et al., 1989b, Ogata et al., 1990; Kodama et al., 1993, Sakamoto et al., 1993). The Japanese researchers were able to detect saxitoxin, neosaxitoxin and gonyautoxins 1 to 4 in extracts of Moraxella cultures. Ogata et al. (1990) also demonstrated the existence of PSP toxins in extracts of Bacillus sp. isolated from cultures of Gymnodinium catenatum Graham. The bacterial

production of PSP toxins has been extensively reviewed and discussed by Rausch de Traubenberg and Lassus (1991). However, there is still considerable controversy about the significance of these findings, since other researchers have not duplicated them and the toxin production rates observed in dinoflagellate blooms are much higher than can be explained by bacterial production alone.

This does not mean that the evidence for bacterial production of PSP toxins is an artifact. The production of saxitoxin and neosaxitoxin by the cyanobacterium Aphanizomenon flos-aquae is undisputed, so PSP toxins are not confined to the dinoflagellates (Carmichael, 1994). An evolutionary linkage between bacteria, cyanobacteria and dinoflagellates involving endosymbiotic associations between toxic bacteria and higher forms has been proposed (Boczar at al., 1988). Indeed, if the data of the Japanese researchers are correct (e.g. Kodama et al., 1988), endosymbiosis is almost certainly occurring. How then should one explain the very good evidence in favour of chromosomally-linked production in the dinoflagellate? It is possible that a plasmid or virus has transmitted the genes for PSP toxins to the dinoflagellates from

lower organisms, or that a very long endosymbiotic association has resulted in incorporation of the toxigenic function in the chromosomal DNA. This latter situation would be analogous to the incorporation of some mitochondrial genes into chromosomal DNA which has apparently occurred in the evolution of eukaryotes (Suzuki et al., 1986; Watson et al., 1987). In any case, bacterial and dinoflagellate production of PSP toxins are not mutually exclusive. It is entirely possible that both groups of organisms can produce toxins, and may or may not be associated with each other in toxic blooms.

Continuous Culture Methods

Apparatus

One of the principal limitations of batch culture methods is that the nutrient environment of the cells changes progressively throughout the life of the culture. The only way to avoid this problem is to use a system in which the culture medium is constantly or frequently replaced. This allows the cells to grow for prolonged periods in relatively constant conditions of nutrient

availability. There are three types of apparatus designed for this (Figure 1.2), one of which is the cage-culture turbidostat employed in this study. The other two are semi-continuous cultures and chemostats.

SEMI-CONTINUOUS CULTURES

The simplest continuous culture system is a manually-operated type which is more aptly called "semi-continuous". Phytoplankton are grown in a simple culture vessel, as for a batch culture, except that at regular intervals some culture is removed and replaced with new growth medium. The rate of replacement should be proportional to the growth rate of the culture. In principle, cultures can be grown in the same vessel indefinitely. Fay and Kulasooriya (1973) give a good description of a semi-continuous culture system.

Applications of semi-continuous systems abound. Nakamura et al. (1988) used one to investigate the effects of nutrient supplies on the growth of Chattonella antiqua (Hada) Ono. This red-tide phytoplankter causes considerable problems for aquaculture in the

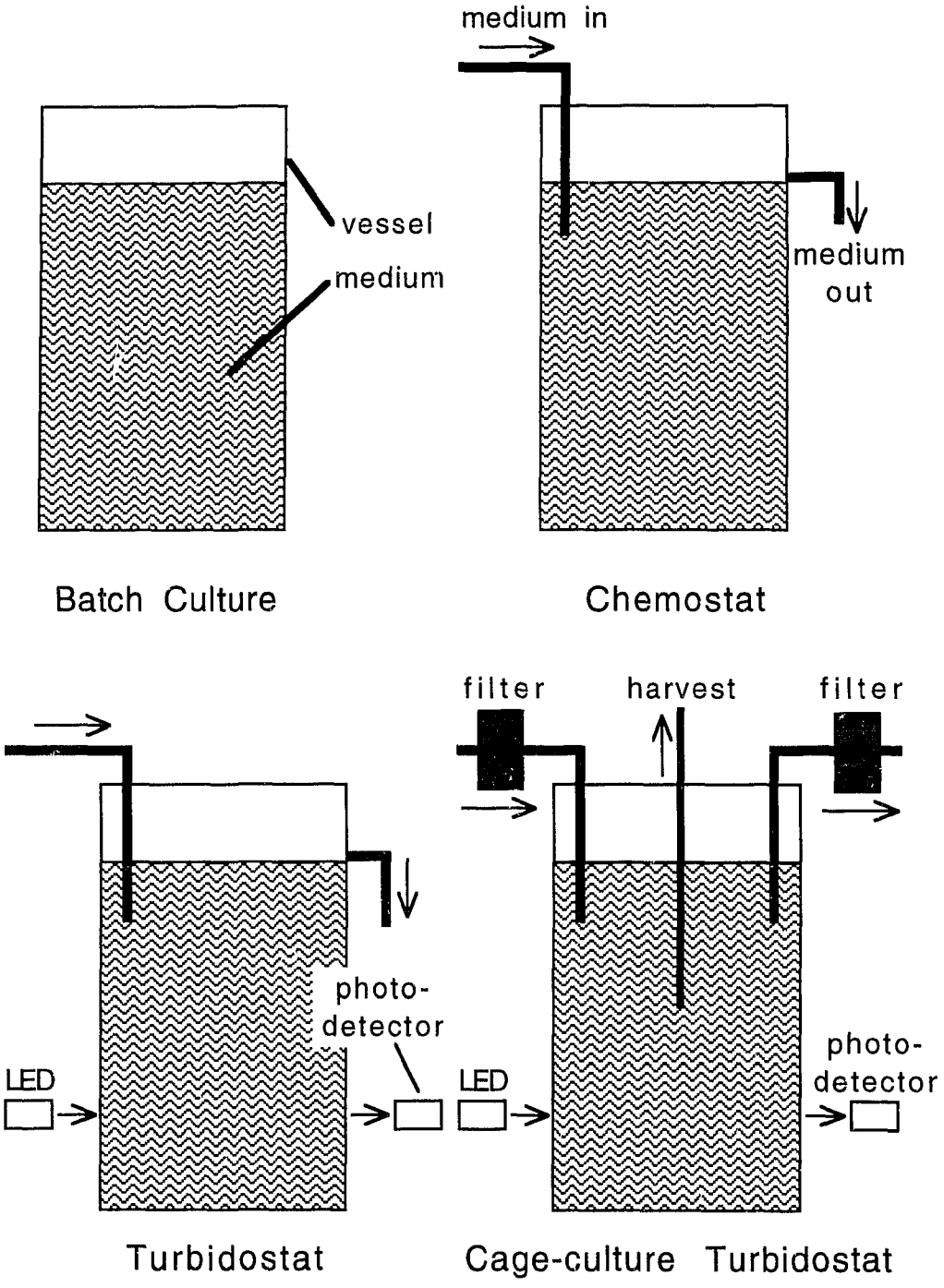


Figure 1.2 - Schematics of various culture systems

Seto Inland Sea in Japan. Another dinoflagellate, Gymnodinium sanguineum Hirasaka, has been cultured in semi-continuous systems to investigate the effects of iron and nitrogen deficiency on its growth (Doucette and Harrison, 1990). Anderson et al. (1990b) used semi-continuous cultures to investigate toxin production dynamics in Alexandrium spp..

CHEMOSTATS

The concept behind semi-continuous cultures can be automated and improved with a type of apparatus called a chemostat, which delivers new medium continuously to the culture at a rate that determines its growth rate. The supply rate of one limiting nutrient fixes the growth rate of the culture. The phytoplankton produced flow out of the culture vessel at the same rate through a constant-level overflow. Thus there is an equilibrium between the supply rate of essential nutrients and the rate of organism removal via the overflow drain. Such a system produces a continuous supply of phytoplankton cells at a constant metabolic condition. Like the semi-continuous method, this type of apparatus is useful for experiments where it is desirable to maintain the culture in a constant state for

a prolonged period. Since the dilution of the culture is continuous, the conditions in a chemostat are even more constant than in a semi-continuous culture. The penalties are increased complexity and cost, but with careful design these can be minimized.

A good, early account of chemostats can be found in Herbert et al. (1956) who described the apparatus and theory of operation as well as the application of the method to producing the bacterium Aerobacter cloacae. Droop (1968) gave an exhaustive account of the theory behind nutrient uptake experiments in chemostats and described their use in vitamin B_{12} uptake experiments with Monochrysis lutheri Droop. Dortch et al. (1991) described the use of chemostats to measure the variability of nitrate uptake rates in the diatom Thalassiosira pseudonana (Hust.) Hasle et Heimdal. Chemostats were also applied to studies of growth kinetics of phytoplankton under light limitation (Van Liere and Mur, 1979). This type of application is sometimes called a "photostat" since the limiting factor is light, not a nutrient compound (Fogg and Thake, 1987). Chemostats have even been adapted to grow filamentous green algae such as Ulothrix zonata (Weber et Mohr) Kütz in

conditions approximating those found in natural freshwater streams (Prance and Benson-Evans, 1973). The chemostat has allowed processes to be studied which could not be analyzed using traditional batch culture methods.

TURBIDOSTATS

Turbidostats represent the next stage of complexity in continuous culture systems. Instead of the medium being continuously diluted and growth being limited by nutrient supply, dilution of the culture occurs in response to increases in culture density. The density of the culture is continuously monitored using a light emitting diode (LED) shining through the culture and a photodetector measuring the transmittance (or absorption) of the light. When the culture density increases, the detector switches on a pump system which dilutes the culture by adding new medium and discharging cells via an overflow. When the culture density returns to the desired level, the detector switches off the pumps. Over the course of a day, the rate of dilution is proportional to the culture growth rate. However, unlike the chemostat systems, it is possible to supply nutrients in excess to achieve a nutrient-saturated growth

rate. It is also easier to study organisms at very low growth rates since there is no risk of "washing out" the culture, a problem which may be experienced in chemostats. In addition, since the dilution rate is directly related to the growth rate of the cells, turbidostats are very useful for determining algal growth rates under controlled, steady state conditions.

The basic theory and practice of turbidostat systems were described by Myers and Clark (1944). Apparatus and theory for precise measurements of algal growth rates were outlined by Phillips and Myers (1954a). In a complementary paper (Phillips and Myers, 1954b) they described the application of their methods to the measurement of growth rates in the green alga Chlorella pyrenoidosa Chick under different conditions of irradiance and photoperiod. A variation of the turbidostat concept called a cage-culture turbidostat was introduced by Skipnes et al. (1980). This differs from the conventional turbidostat in that the medium is pumped in and out of the culture vessel continuously through filters which prevent the cells escaping. Dilution is achieved by the LED/photodetector system described above which activates a

harvest pump to withdraw culture from the chamber. Since the flow of nutrients is continuous, the conditions in the culture vessel can be maintained almost perfectly constant. The only variable is a slight oscillation of the culture density as the harvest pump cycles on and off. This system allows nutrients and medium to be supplied at any rate desired without the risk of "washing out" the culture, regardless of culture density. Determination of growth rates is made as before, since the harvest rate is proportional to the growth rate. The cage-culture is perhaps the best system for determining growth rates since the harvest and measurement method is independent of the nutrient supply.

Cage-cultures have been successfully applied to a number of problems in algal physiology. Wangersky et al. (1989) described the use of very large (200-250 L) cage-culture turbidostats for producing phytoplankton on a near-industrial scale. Various species have been produced in this manner including Chaetoceros gracilis Schuett and Alexandrium tamarense. Smaller laboratory-scale units of similar design have been used to investigate the diurnal behaviour of Dunaliella tertiolecta Butcher (Wangersky and Maass, 1988). The

effects of physiological condition on pigment composition in Phaeodactylum tricornutum Bohlin have been investigated (Roy, 1988) as has the effect of nitrogen supply rates and light on the composition of lipids in P. tricornutum and C. gracilis (Parrish and Wangersky, 1987; 1990). Cage-cultures have been used to investigate the effects of nutrient stress on the production of lipids and pigments in C. gracilis (Lombardi and Wangersky, 1991). There has also been considerable work with cage-culture turbidostats on the effects of pollutants on phytoplankton (Ostgaard et al, 1987; Wangersky and Maass, 1991); metal ion complexation (Zhou and Wangersky, 1985) and irradiance adaptation (Claustre and Gostan, 1987).

One of the principal difficulties encountered with turbidostats and chemostats is that the culture must be homogeneously mixed in order for the mechanism to operate properly. Otherwise, the dilution rate of the culture vessel will not be proportional to the growth rate of the culture. For most diatom species, this does not present a problem since the culture can be mixed continuously with a stirrer or bubbler without damaging the cells. However, some

dinoflagellates are much more susceptible to turbulence and will be growth-inhibited or even killed under very turbulent conditions. This problem has been investigated in Alexandrium tamarense (=Gonyaulax excavata) by White (1976) who conducted simple experiments on shaker tables to demonstrate the inhibitory effect of mixing on growth rate. A more recent, quantitative study of turbulence effects showed that Gonyaulax polyedra was inhibited by turbulence stresses of 2 to 4 x 10^{-3} Pa (Thomas and Gibson, 1990). Thomas and Gibson (1990) calculate this stress to be the same magnitude as turbulence generated near the ocean surface by a light wind. The affected cells showed a loss of the trailing flagellum which prevented them from swimming in straight lines. Instead, they spun in place and could not migrate through the water column. This suggests that turbulence may be a significant factor in inhibiting red tides in nature. Dinoflagellates are generally well adapted to stratified conditions whereas diatoms will dominate in turbulent water (Estrada et al., 1988, Berdalet and Estrada, 1993). In natural systems, dinoflagellates usually bloom during periods of calm, stratified conditions caused by temperature or salinity discontinuities (Thomas and Gibson, 1990). This susceptibility to

turbulence means it is very difficult to grow dinoflagellates in continuous culture vessels without inhibiting their growth. Most successful systems rely either on very gentle air bubbling to stir the cells, or use intermittent bubbling and grow the cultures in a semi-continuous mode.

Continuous culture systems offer substantial advantages over traditional batch cultures. Each type of continuous system has its own pros and cons. Chemostats are simple to build and use but are limited in their ability to resolve accurate growth rates under some conditions. Furthermore, they depend on some variable, usually a nutrient, being growth-limiting. Turbidostats and cage-cultures are more versatile, but the added complexity and cost are penalties which must be considered. For large-scale industrial production of algae, there is no question that the higher capital cost of a continuous system is justified by better efficiency (Wangersky et al., 1989). Where accurate determination of growth rates is essential, a cage-culture offers a good solution. This is especially true for investigations of nutrient uptake rates, or when large amounts of algae at constant physiological state are required.

CHAPTER 2

PRODUCTION OF *ALEXANDRIUM TAMARENSE* IN CAGE-CULTURE TURBIDOSTATS

Introduction

Growing *Alexandrium* sp. in culture presents a number of difficult problems the experimenter must overcome. Even after a half-century of investigation of these organisms, many problems remain to be solved. A cursory glance through the literature shows that each author has developed his or her own "pet" methods. Very often the reasoning behind these methods is not explained in published manuscripts, or the information has lapsed into the realm of folklore. This makes it difficult to determine why certain things were done or what the implications of certain results might be. In this chapter, as well as demonstrating that continuous-culture production of *A. tamarense* is practical, an attempt will be made to explain the methods and rationale employed in this investigation so that the experimental results can be interpreted better.

Most previous investigations of toxic dinoflagellates have relied on simple batch culture techniques, in part because of the sensitivity of dinoflagellates to turbulence. Since continuous cultures must be stirred to some degree, this presents a problem to the investigator wishing to produce species such as A. tamarense (see Chapter 1). In this chapter, the use of cage-culture turbidostats for the continuous culturing of A. tamarense is described and the advantages and limitations of the method are explained.

Methods and Materials

Phytoplankton Cultures

Three clones (strains) of Alexandrium were used at various times during the course of this project. A. excavatum clones PR17B and PR103F (obtained from Dr. Allan Cembella, then at the Institute Maurice Lamontagne, Mont-Joli, Quebec; currently with the NRC Institute for Marine Biosciences, Halifax) were used during the development of the turbidostat culture methods. For the actual experiments, A. tamarense clone OK875-1 (obtained from Dr. Masaaki

Kodama, Laboratory of Marine Biological Chemistry, School of Fisheries Sciences, Kitasato University, Sanriku, Iwate Prefecture, Japan) was used due to its higher growth rate and toxin content.

BATCH CULTURES

Stock cultures of A. tamarense were maintained in sterile 50 mL Pyrex (Corning) culture tubes using filtered, autoclaved natural seawater (salinity approximately 30 $^{o}/_{oo}$) enriched with modified Provasoli's nutrient solution (Enrichment Solution Natural Water or "ESNW medium", Harrison et al., 1980). Seawater was obtained initially from the Dalhousie Aquatron system. However in summer, the growth rates of cultures in this water were seriously depressed. Possibly, this was because the Aquatron system draws seawater from the Northwest Arm of Halifax Harbour, Nova Scotia, which is polluted with domestic sewage. To eliminate this problem, water for the stock cultures was obtained at the National Research Council's Aquaculature Research Station at Sandy Cove, Halifax County, N.S., and from the surface waters of Mahone Bay, Lunenburg County, N.S.. These water supplies were collected in winter, filtered

through a 3 μm pleated capsule (Gelman) and aged in darkened, 20 L polyethylene containers at room temperature for several months prior to use. Following this treatment, water from both Sandy Cove and Mahone Bay supported considerably higher and more stable growth rates through the summer than did the Aquatron supply. Aquatron water collected in the winter and treated in the same manner also gave excellent results.

The aged seawater was autoclaved in 1 L batches after buffering with $NaHCO_3$ and HCl, as per Harrison et al. (1980). Harrison's enrichment solution was added after autoclaving. Silicate was omitted from the recipe. Cultures were incubated at 20 °C under cool-white fluorescent lamps (Sylvania) providing approximately 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ of illumination, and 16:8 L:D photoperiod. Culture densities were determined by counting several fields in a Fuchs-Rosenthal haemocytometer at 40x magnification. The cultures were allowed to reach 2000 - 5000 cells/mL before transferring approximately 1 mL of each culture into new medium with a sterile pipet.

CONTINUOUS CULTURES

Continuous cultures of A. tamarense were grown using three identical 10 L cage-culture turbidostats (Manna Marine Enterprises, Halifax, Nova Scotia). These instruments are based on the designs described by Skipnes et al. (1980) and Wangersky et al. (1989) (see Figure 2.1). The transparent acrylic culture vessel is an open-ended vertical cylinder closed by two end-plates. O-rings in each end-plate provide a firm seal and allow for easy disassembly and cleaning. Each end-plate has several holes which accept threaded PVC tubes or polyethylene compression fittings (Cole-Parmer) for inlet and outlet ports. There is also an overflow drain in the side of the cylinder. Filter holders on the top of the culture vessel accept 90 mm diameter filters which prevent the phytoplankton cells from escaping while the pumps are operating. For these experiments, filters were made from Nitex filter screen material with a mesh size of either 5 μm or 10 μm. Both mesh sizes provided good retention of the A. tamarense cells. The filters were replaced every week or two since they tended to clog. Filters were cleaned by soaking in 0.1 M HCl solution and could be reused several times. The filters were connected to the solenoid valves with black Poly-Flo

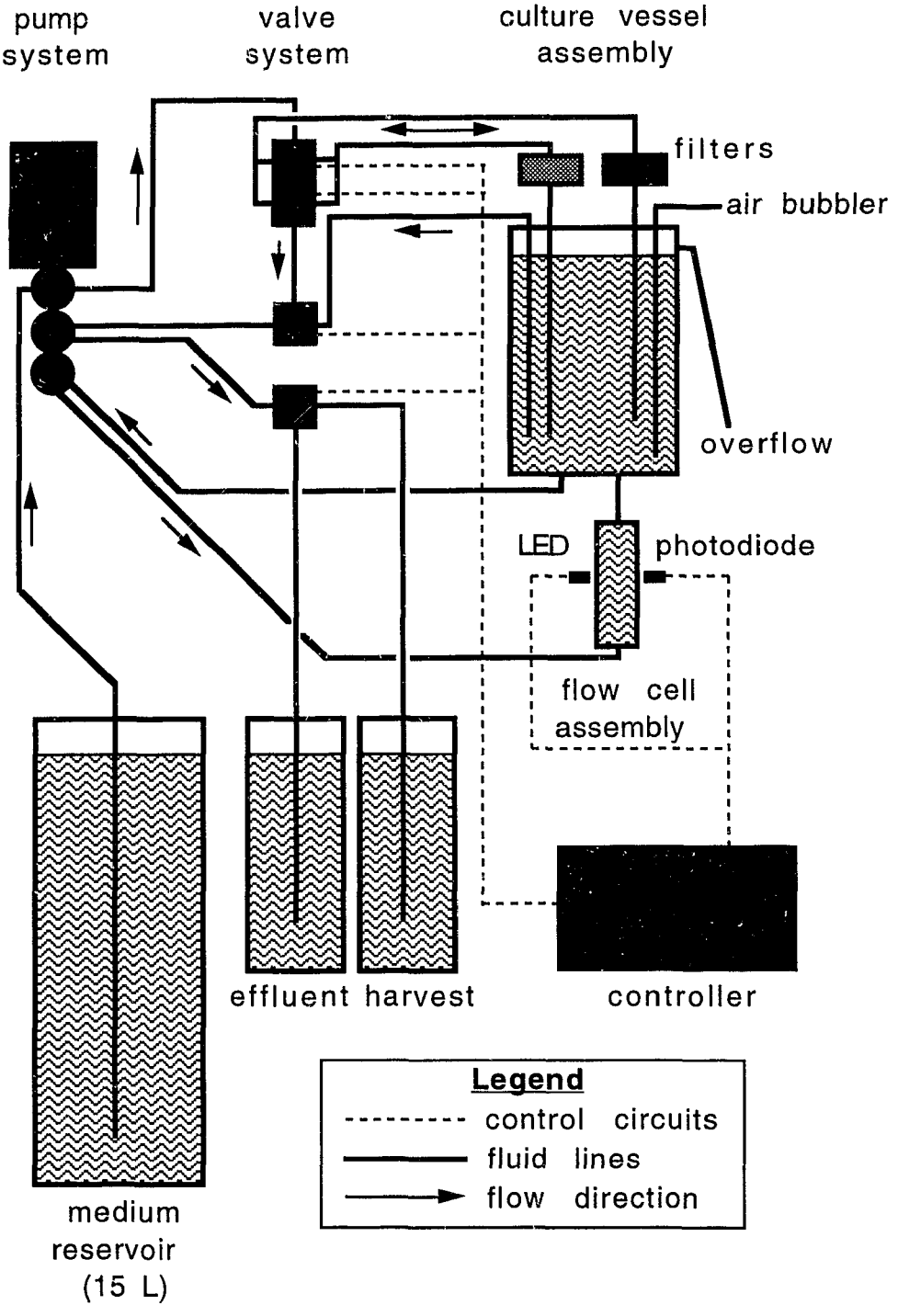


Figure 2.1 - Schematic Diagram of Cage-culture Turbidostat Apparatus

tubing (Eastman-Kodak). Where more flexible tubes were required, Viton (Cole-Parmer) tubing was used. The medium supply and effluent/harvest pumps were Masterflex (Cole-Parmer) #14 peristaltic pumps fitted with Norprene tubing. To ensure identical flow rates, all pumps were powered from a single, 10-channel pump drive (Cole-Parmer model #7568-00).

Unfortunately, the LED/photodiode turbidity sensor was affected by interference from the culture lighting. To avoid this problem, the sensor was set up to shine through a "flow cell" consisting of a 250 mL separatory funnel connected to the culture vessel through ports in the bottom end-plate. The flow cell and sensor were covered with a black plastic sheet when they were in operation. Culture was recirculated through the flow-cell with a Masterflex #15 or #17 peristaltic pump fitted with Norprene tubing and powered by the same 10-channel drive as the other pumps. Under normal operating conditions, this gave a residence time of medium in the flow cell of 3 to 5 min. However, it is possible that the residence time of dinoflagellates in the flow cell was different due to swimming behaviour. For example, the organisms might avoid the

inlet tube, be attracted to it, or accumulate in the flow cell in response to changes in the turbulence or light fields. These possible effects were not quantified, except to note that when the flow cell was opened, there was no significant accumulation of dinoflagellates inside.

The culture vessels were constructed of acrylic plastic which could not be autoclaved. This made cleaning and sterilization more difficult than for glass apparatus. The cleaning procedure was as follows. The entire apparatus was disassembled and soaked in Sparkleen (Fisher Scientific) detergent overnight, then scrubbed with a soft sponge and rinsed clean with distilled water. Tubing was just soaked and rinsed. The pumps and valve assemblies were cleaned by configuring the lines to recirculate detergent solution overnight, followed by rinsing with distilled water. From time to time, dilute hydrochloric acid (0.1 M) was pumped into the valve assembly and allowed to remain overnight before rinsing. This helped remove organic and organometallic deposits which can accumulate on the surfaces. Hydrochloric acid (0.1 M) was also used from time to time to clean the culture vessels and filter holders. To

sterilize the system, the complete apparatus was assembled and filled with a dilute bleach solution of 60 ppm sodium hypochlorite, made by dissolving 1 mL/L of Javex (6% NaClO, Colgate-Palmolive Canada) in distilled water. This solution was recirculated overnight. The apparatus was then drained and refilled with autoclaved seawater to rinse out the residual traces of bleach, recirculated overnight, and drained again. At this point, the culture apparatus was ready to be filled with medium and inoculated.

Two methods were used for inoculating the turbidostat vessels. The first involved growing large batch cultures (15 L) of A. tamarense in autoclaved Pyrex or polycarbonate carboys (Nalgene) of Harrison's ESNW medium. After they reached usable density (about 2000 cells/mL), these large cultures were pumped through autoclaved peristaltic tubing (Masterflex #17 or #15) into the turbidostat culture vessels. To ensure that each of the three turbidostats received a similar fraction of the cultures, a splitter was inserted in the line to divide the flow into three streams. Using this inoculation method, the turbidostat culture vessels could be filled with dense, log-phase cultures which were ready to use

immediately for experimentation. The second inoculation method employed smaller batch cultures (1-2 L) which were used to inoculate the culture vessels, previously filled with 8-9 L of autoclaved medium. Both inoculation methods were employed successfully. The choice depended on the time and equipment available. The cultures were then incubated under the same light conditions as the batch cultures (150 $\mu E \cdot m^{-2} \cdot s^{-1}$, 16:8 L:D, 20 °C). They reached usable density in a week or less.

The cultures were bubbled with air by means of a narrow glass tube inserted into the culture vessel (see Figure 2.1). In the experimental cultures where lower light intensity was required, the light intensity was reduced by a screen made from one or two layers of white tissue paper (Kimwipes). Light intensities were measured in an empty culture vessel using a Lambda Instruments Model LI-185 radiometer/photometer equipped with a cosine-corrected quantum sensor.

Turbidostat Operation

Once the cultures contained about 2000 cells/mL, the turbidostat control system was activated. The configurations of these control systems have been described by Skipnes et al. (1980) and Wangersky et al. (1989). The turbidostat control box provided an analogue output signal which gave a continuous record of light transmittance, and a periodic integration of the harvesting time. The baseline signal of the unit was the light transmittance. The output was in millivolts but could be calibrated in "% transmittance" or culture density units as desired. A harvest function integrator provided a periodic record of the proportion of time the harvest valve was open. Integration could be done at hourly intervals between one and eight hours. The integrator signal appeared on the plot as peaks above or below the transmittance line, spaced at the preset intervals, and calibrated in % full scale. A peak of 0% indicated that the harvest valve was not open during the previous interval. A peak of 100% meant the system was harvesting continuously over the previous interval. This record could be used to determine when the culture was growing. Since the pumping rate was known, the harvested volume could be calculated. The output

from the controller and harvest integrator was sent to a Bascom-Turner Model 8120 computerized chart recorder which allowed multiple traces to be stored on floppy disk and retrieved later as required. Simple data transformation and integration could be performed by the recorder itself. The sampling interval of the recorder was set to 14,400 ms. Each 500-point recording represented 2 h of data. This was a reasonable compromise between temporal resolution and available disk space. It allowed adequate resolution of fine features in the plots such as the harvest integration peaks, which were 30 s duration. It also allowed three turbidostats to be monitored continuously for up to eight days without the need to change floppy disks.

Daily harvest volumes were determined by collecting the harvested material. Harvest volumes could also be calculated from the integrator output as described above. Culture growth rates were calculated from the daily harvest volume according to the method of Skipnes et al. (1980) in which:

harvest rate (d^{-1}): $\mu = H/V$

doubling rate (divisions$\cdot d^{-1}$): $r = \mu/\ln 2$

where: H = volume harvested per day ($L \cdot d^{-1}$)

V = cage volume (L)

Note that the parameter "r" has units of "divisions per day" and is thus analogous to Monod's exponential growth rate "R" calculated using $\log_2$ transformations of the culture density (Monod, 1949).

Calculated growth rates were corrected for changes in culture density by adding the value of r determined above to the relative change in culture density (R) calculated as per Monod (1949):

relative density change: $R = (\log_2(N_1) - \log_2(N_0))/t$

where: N_1 and N_0 are culture densities (cells/mL), t is in days. Where finer resolution is required, these calculations can be performed with t measured in hours.

Therefore, the corrected growth rate in the culture is μ_{cor}:

$$\mu_{cor} = r + R$$

It must be noted that if:

$R < 0$ (i.e. culture density is decreasing)

and: $|R| > r$

then the value of μ_{cor} will be negative. This produces a plot which appears counter-intuitive in having negative "growth rates". However, it must be stressed that μ_{cor} represents the rate of change of the whole turbidostat culture, including any density changes caused by dilution, and not simply the cell division rate of the living plankton. The relevance of μ and μ_{cor} will be discussed later in this chapter.

The pump system delivered new medium to the culture vessels at a flow rate of 10 L/d. This rather fast rate eliminated nutrient limitation as a factor in the experiments. In practice, it was possible to operate the system at lower flow rates and for some experiments, a rate of 3 L/d was chosen in order to reduce the pressure on the filters (see Chapter 4). The pumps were supplied with 10 µm-filtered, autoclaved seawater enriched with Harrison's nutrients. Due to the large amounts of seawater required, about 30 L/d, the regular Dalhousie Aquatron supply was used. Stockpiling "winter water" was not practical due to insufficient storage space. However, for the experiments on diurnal phasing (see Chapter 4)

water was stockpiled for each experiment in order to minimize the effects of changing hydrographic conditions in the Northwest Arm.

To prevent the possible release of toxic phytoplankton into the local environment through the sewer system, effluent and waste culture from the turbidostats were treated by adding 1mL/L of Javex bleach, mixing thoroughly and allowing the mixture to stand at least 12 h before discarding. This treatment killed any A. tamarense cells which were discarded. Micro-scale tests on the effects of bleach on the cells were performed to determine the efficacy of this treatment and the results are included in Appendix 3.

The turbidostats were allowed to run for a few days to stabilize before sampling procedures began. The sampling protocol differed depending on the experiment and each will be explained in detail in later chapters. In all cases cell numbers were monitored daily using microscope counts and/or a Coulter Counter (Coulter Electronics, Model Z_B). Samples were taken from either the flow-cell recirculation line or the harvest reservoir depending on the experiment.

Results and Discussion

Culture Methods

At the start of this investigation, it was intended that the same clone of Alexandrium be used throughout to allow better comparison of results from different experiments. The PR17B clone was initially selected for its fast growth rate and high toxin yield. It had been successfully used for mass-production of PSP toxins in large scale semi-continuous cultures (P.J. Wangersky, pers. comm.). However, as time went on, this clone became less robust and more difficult to maintain, especially in the turbidostats. The same problem affected the PR103F clone as well. Many researchers offer anecdotal evidence of phytoplankton cultures, including those of Alexandrium, getting "old and tired" after lengthy periods in culture. Both of the PR clones had been in culture for a considerable time, and at temperatures well above the ambient values in their native Gaspé waters. Thus the OK875-1 clone was used for the experiments on toxin production and dynamics. This was a newer isolate and grew extremely well, producing large amounts of toxin. This made it

possible to reduce the volume of material required for toxin analysis without seriously compromising the sensitivity of the HPLC assay.

At the same time as the PR clones were becoming less reliable, it became apparent that there was considerable seasonal variation in the quality of the turbidostat cultures. In the winter, the cultures grew quickly and no problems were experienced. However, in the summer and fall, the experimental cultures grew very slowly and were especially prone to contamination from other species. The main culprits were an unidentified ciliated protozoan and a small chlorophyte, probably Oocystis solitaria Wittrock, although at times, other species appeared including tiny flagellated phytoplankton only a few microns in diameter. O. solitaria was a very persistent pest, even surviving in the 0.1 M HCl washing solution! In general, these "invaders" were a problem only in the turbidostats, although on rare occasions they appeared in the 15 L batch cultures as well. It is possible that the contaminating species were present in the original stock cultures and only became apparent at slow growth rates. It is also possible that the maintenance of turbidostat cultures under high nutrient loading for long periods

allowed the contaminating species to increase in number, whereas in batch cultures, the cultures would be transferred before any contaminant reached detectable levels. This would be especially true if the contaminants were themselves slow-growing, and initially present in only very low concentrations.

Seasonal variation in the growth rates of phytoplankton cultures is a common problem reported by many researchers. The reasons are not clear, but are possibly related to the chemistry of trace constituents in the seawater supply, or as Yentsch and Mague (1980) suggest, to endogenous cycles of activity in the phytoplankton themselves. The period of slow growth described by Yentsch and Mague (1980) was in the winter, not the summer as described in this thesis. The use of aged water for maintaining the stock cultures eliminated the problem of unhealthy inocula during the summer, which suggests that water chemistry was probably the controlling factor. No quantitative data on growth rates of stock cultures were collected, but the practical benefits of aged water were clearly visible. Unfortunately, it was impossible to store enough aged water to maintain the turbidostats. However, steps

were taken to ensure that the raw water supply was clean. Prior to the onset of contamination problems, the seawater for the turbidostat medium had been sterilized simply by filtering through a Gelman 0.2 µm in-line cartridge. This did not provide adequate sterilization and the procedures were changed so that all water was autoclaved before use in the turbidostats.

Turbidostat Operation

Because of the contamination risk, inoculation of the turbidostats had to be efficient. The cultures had to reach usable density as quickly as possible without a long lag phase. By establishing them in sterile carboys first, large high density cultures could be produced without risk of contamination. Pumping the culture aseptically into the turbidostat vessels ensured two objectives. First, it established a unialgal, healthy, high density culture in the turbidostats. Second, by using a flow splitter in the line, all three turbidostats received representative fractions of the stock cultures, which meant that the starting conditions of the three cultures were as similar as possible. Turbidostats inoculated

with individual small-volume cultures often grew at different rates, making comparisons between cultures difficult.

Two important points must be noted when activating the turbidostat system. First, the upper and lower transmittance settings for the harvest function must be far enough apart to avoid unnecessary harvesting due to signal noise. However, too great a separation will reduce the sensitivity of the apparatus. Typically, a separation of 10 % of the total transmittance was appropriate. Second, the signal amplification must be set to give an output within the range of sensitivity of the chart recorder (0-10 mV). The signal amplification required was different for each apparatus due to differences in the construction of the control units.

The response of the LED/photodetector assembly to culture density is a function of both density and path length. At very low culture densities and short path lengths, sensitivity was impaired. However, with only a 10 L culture vessel, there was a practical limit to the path length, and Alexandrium cultures do not achieve very high densities (2000 - 3000 cells/mL). Thus, the size of the

flow-cell was chosen to provide an acceptable compromise between path length and the residence time of culture in the cell. Modified 250 mL separatory funnels were conveniently-sized and had a smooth shape which prevented the sedimentation of culture material inside them. Experimentation with other flow-cell designs showed that any horizontal surface or interior corners would cause an accumulation of phytoplankton cells. The presence of the flow-cell no doubt decreased the sensitivity of the detector to culture density changes, but the compromise was the best available.

During the operation of the turbidostats, the inlet and outlet flow rates were rarely perfectly balanced. Even though the pumps were supposedly identical and running at the same speed, small differences in the tubing, valves, filters, connectors, etc. meant that the inflow and outflow rates were usually slightly different. If not corrected, the culture vessels would slowly drain or overflow. This would change the density of the culture and affect the calculation of growth rates from the integrated harvest volume. The flow rates were balanced as closely as possible by slightly crimping the tubing on the "fast" side with a hose clamp upstream of the pump. Perfect

balance was hard to achieve so the flow rates were adjusted daily to maintain culture volume as close to 10 L as possible. Observed division rates were corrected to account for the altered volumes. Typical daily volume variations were less than 10 % and usually much lower.

Mixing of the cultures was essential to allow the turbidostat to operate properly. If the cultures were not homogeneous, the transmittance measured by the photodetector would not accurately reflect the density of the culture. However, as noted in Chapter 1, A. tamarense is sensitive to turbulence and any mixing mechanism employed must be gentle. Bubbling with a slow stream of air proved the best solution. This was sufficient to keep most of the cells in the culture suspended in the medium. Some cells did adhere to the walls of the vessel in the daytime on the side nearest the lights but they became mixed again when the lights went off. Attempts to use magnetic stirrers were unsuccessful since they produced a lot of broken and non-motile cells which sank to the bottom of the culture vessel. It is worth noting that in semi-continuous production of Alexandrium in 200L cultures, bubbling with a vigorous stream of

air was found to increase production compared to unmixed cultures (P.J. Wangersky and R.L. Maass, pers. comm.). Stirring is routinely used to increase production rates in cultures of other species also. This effect may have been due to the tendency for Alexandrium cells in unmixed cultures to migrate to the surface and remain there, possibly creating a region of low nutrient concentration which inhibited growth. The conventional wisdom that stirring is inhibitory to dinoflagellates is obviously not always applicable.

Culture Growth Characteristics

The remaining section of this chapter is devoted to a discussion of growth characteristics of A. tamarense in cage-cultures. Growth rate and culture density data from several experiments will be presented. Toxin samples were collected from all these cultures, but will not be discussed until later chapters. The purpose of this section is to describe the behaviour of the cultures under various growth conditions and to illustrate some of the methodological comments made earlier in this chapter.

EXAMPLES

Figure 2.2 contains data from three cultures grown in July of 1991. These cultures were incubated under normal light and temperature conditions and were not exposed to any environmental changes. Each panel of Figure 2.2 shows division rate (r) and culture density for one of the three replicates. On days 10, 11 and 12, no samples were taken and the harvest function of the turbidostat was turned off.

Notwithstanding the three-day gap in the time series, Figure 2.2 illustrates how r provides a record of the culture growth rate. In general, when the culture density increased, r was high. Conversely, decreasing culture density co-incided with r becoming zero. Ideally, the culture should reach an equilibrium where culture density and r are constant. Such a condition indicates that the growth rate and harvest rate are balanced and the culture is in steady state. This situation was difficult, although not impossible, to achieve in these cultures. Growth rates tended to be rather low and erratic, with strong peaks and periods where no growth was recorded at all. The possible reasons for this will be discussed in detail in later

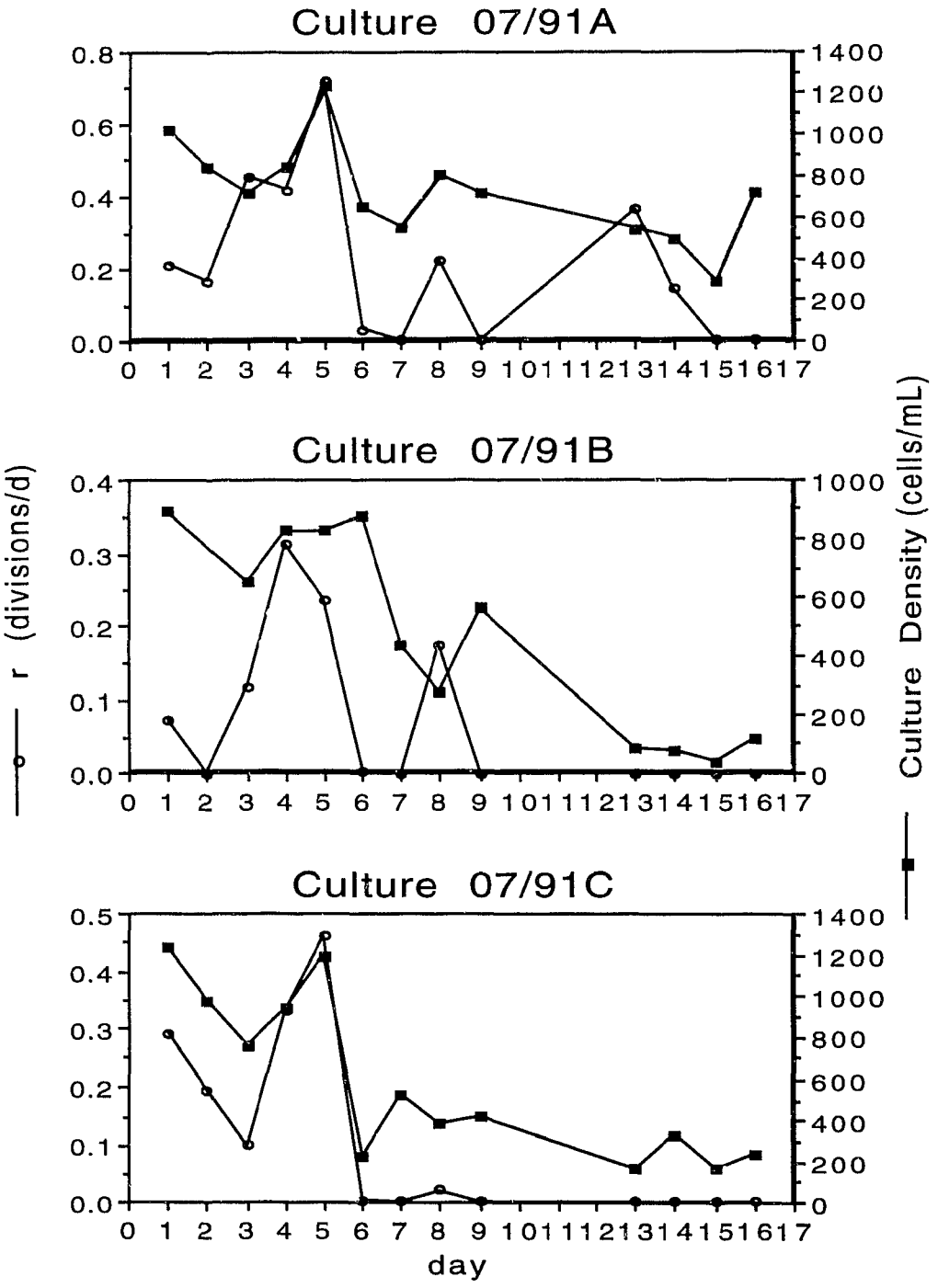


Figure 2.2 - Division Rate r and Culture Density Cultures 07/91 A,B,C

chapters, but one candidate for the periodicity is phasing or synchrony of cell division among the cells in the culture. Since on average $0.2 \leq r \leq 0.3$ divisions/d, any synchronization of division among the cells would result in most of the cells dividing simultaneously every few days, producing an erratic harvest rate in the turbidostat. Smoothing and averaging strategies for dealing with data of this type will be discussed later.

The results in Figure 2.2 also reflect the low growth rate problems experienced during the summer months. In all three cases, the culture density declined throughout the experiment and at the end, the harvest rate was effectively zero. A quick glance at the first panel of Figure 2.5 shows a much more robust culture, with a high density and more stable growth rate. This type of behaviour was typical of the strains of Alexandrium used in this study. The best growth rates and culture densities were always achieved during the colder seasons of the year.

Figures 2.3 and 2.3.1 contain data from three cultures produced in October 1991. Figure 2.3 shows r and culture density for each

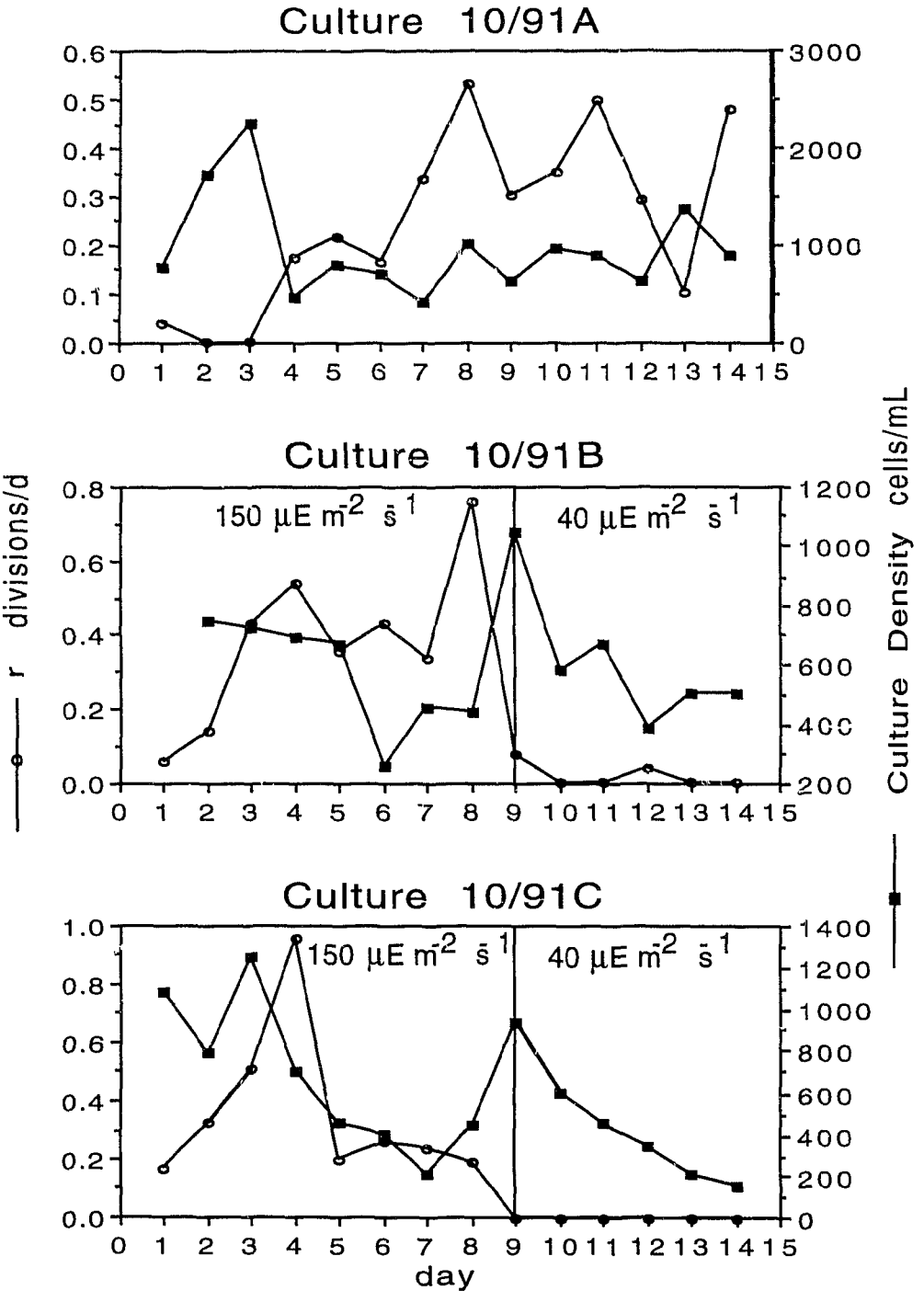


Figure 2.3 - Division Rate r and Culture Density Cultures 10/91 A,B,C

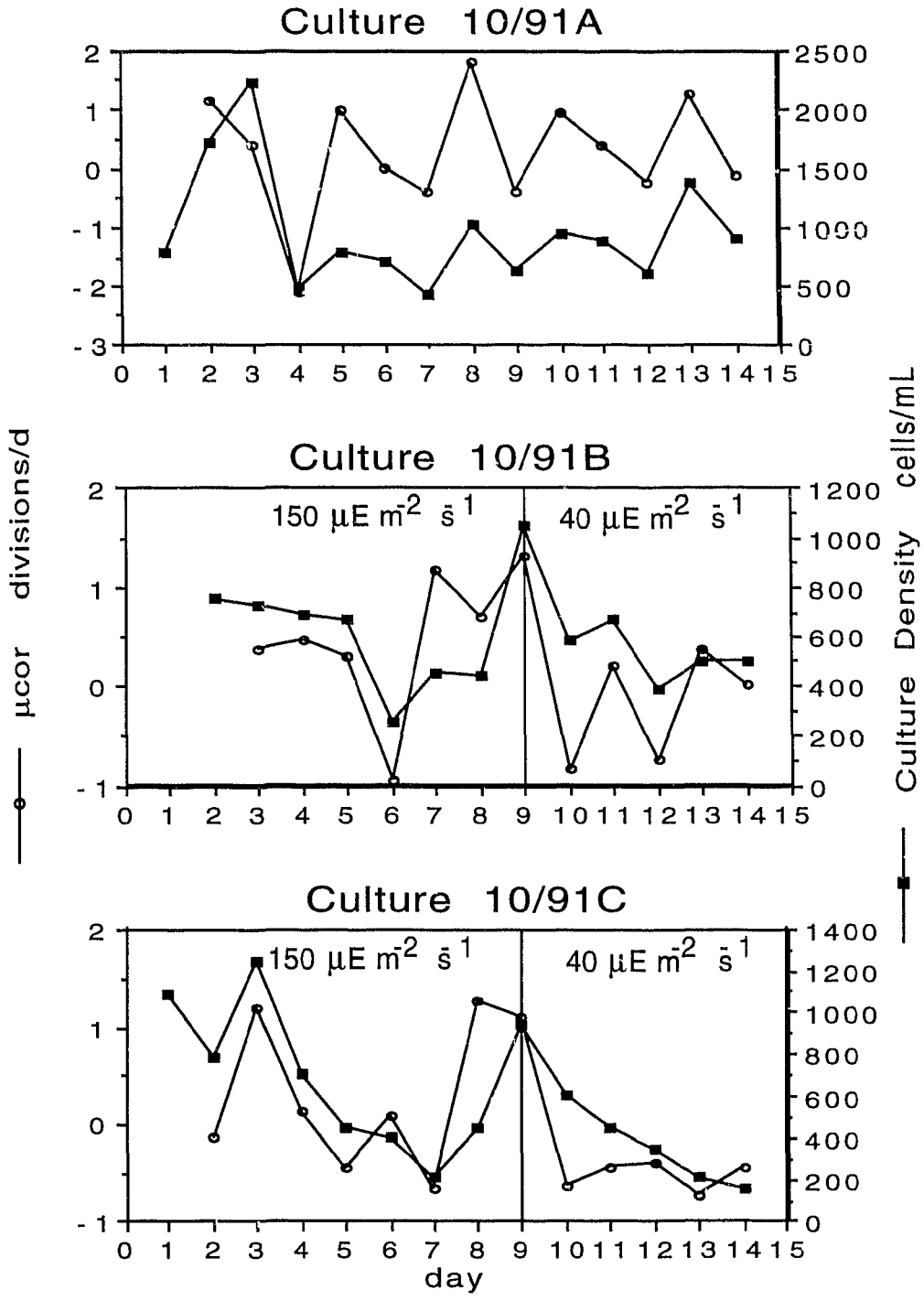


Figure 2.3.1 - µcor and Culture Density Cultures 10/91 A,B,C

culture while Figure 2.3.1 shows μ_{cor} (the sum of the division rate (r) and the relative density change (R) for each day) and culture density. In this experiment, Culture 10/91A was grown under standard irradiance (150 $\mu E \cdot m^{-2} \cdot s^{-1}$) while Cultures 10/91B and 10/91C were subjected to reduced light intensity (40 $\mu E \cdot m^{-2} \cdot s^{-1}$) from Day 9 until the end of the time series.

The 10/91 cultures (Figure 2.3) were more stable and consistent than those of 07/91 (Figure 2.2). Culture 10/91A (control) grew steadily after Day 4 with $0.2 \leq r \leq 0.3$ d^{-1} range and an average culture density around 750 cells/mL. Both curves show peaks on roughly the same days so it appears that the turbidostat was controlling culture density quite successfully. The density did oscillate, indicating that the sensitivity of the detector might be improved. However, given the rather dilute nature of these cultures, the performance of the system was quite acceptable.

Culture 10/91B showed fairly steady performance until the light reduction on Day 8. After this point r dropped to almost zero and the culture density declined slowly. This indicates that the

culture was light-inhibited and effectively stopped growing under these conditions. Similar results were obtained with Culture 10/91C, although the culture density in this case was more unstable and became very dilute at the end. The results indicate that the turbidostat system can detect growth rate changes in A. tamarense produced by lowered irradiance. They also put an upper limit on the magnitude of light intensity changes which are appropriate when studying this species. In this case, the irradiance was reduced from 150 to 40 $\mu E \cdot m^{-2} \cdot s^{-1}$, an excessive reduction for a physiological study of toxin production. Accordingly, all subsequent light-reduction experiments used smaller reductions in irradiance.

The calculation of r as an indicator of growth rate assumes that the culture stays at constant volume and constant density. If the density changes, the culture has "grown" without being reflected in the value of r. The use of μ_{cor} should better describe the real growth rate of a culture where cell density is not constant. Figure 2.3.1 shows the results of such calculations for the October 1991 cultures. Each panel gives culture density and μ_{cor} for one culture. In Culture 10/91A, both culture density and μ_{cor} stayed

fairly stable, with the peaks in the two curves roughly coincident. Cultures 10/91B and 10/91C were more erratic, but μ_{cor} obviously reflects the changes in culture density, and gives an indication of reduced growth rates caused by the irradiance reduction. The μ_{cor} curve also reflects the changes in density during the period when the culture was not growing at all and r remained unchanged at zero.

There are two important points to note when interpreting these plots of μ_{cor}. The first is that since the data are influenced by culture density reductions as well as increases, μ_{cor} is sometimes negative. This does not mean that the cells were necessarily dying during this period, just that the density of the culture decreased. Culture densities are instantaneous measurements from subsamples of harvested material at discrete points in time, while values of r are averaged over the 24 h period between measurements of the harvest volume. The cultures were continuously bubbled, but the cells were still able to clump together on the side of the vessel nearest the lights. These clumps usually formed at the surface of the water, but in some cases were near the bottom of the vessel. Since the cultures were not perfectly homogeneous, the density of

the harvested material was not necessarily the average density of the whole culture during the time interval in question. Furthermore, cells that have clumped in one region of the turbidostat are not available to the detector system and might grow without triggering the harvest cycle. Thus the change in culture density measured between two particular points might not reflect the harvesting activity which had occurred during the same period. For these reasons, values of μ_{cor} must be interpreted with caution. By comparison, r is in some ways a simpler and more straightforward parameter, although it also is subject to influences from poorly-controlled factors.

The second factor complicating the interpretation of these results is the tendency for the growth rate to oscillate. In the examples discussed so far, both r and culture density have regular peaks about three or four days apart, so that they appear to oscillate around a long-term mean. This might indicate that the cultures are partially synchronized and significant numbers of the cells undergo simultaneous division at discrete intervals. Further evidence for

this synchronization is provided by a turbidostat recorder trace taken from a culture grown in March and April of 1992 (Figure 2.4).

Figure 2.4 shows the light transmittance (in arbitrary units) of the culture over a period of 24 h. At approximately mid-day, the transmittance drops abruptly and then increases again. Harvest integration peaks before and after the transmittance change indicate that while there was no harvesting in the period prior to the transmittance reduction, the two integration periods after that show almost continuous harvesting. The light transmittance returns to its previous value during the harvesting period. This can be explained if the transmittance decrease was caused by cells in the culture undergoing synchronized division around mid-day. This would cause the turbidstat harvest function to be activated, diluting the culture and returning the transmittance to near its original level. Similar features were observed in turbidostat recordings on many other days. Synchrony and circadian cycles of growth and cell division in dinoflagellates are well described by various authors and will be discussed in greater detail in Chapter 4.

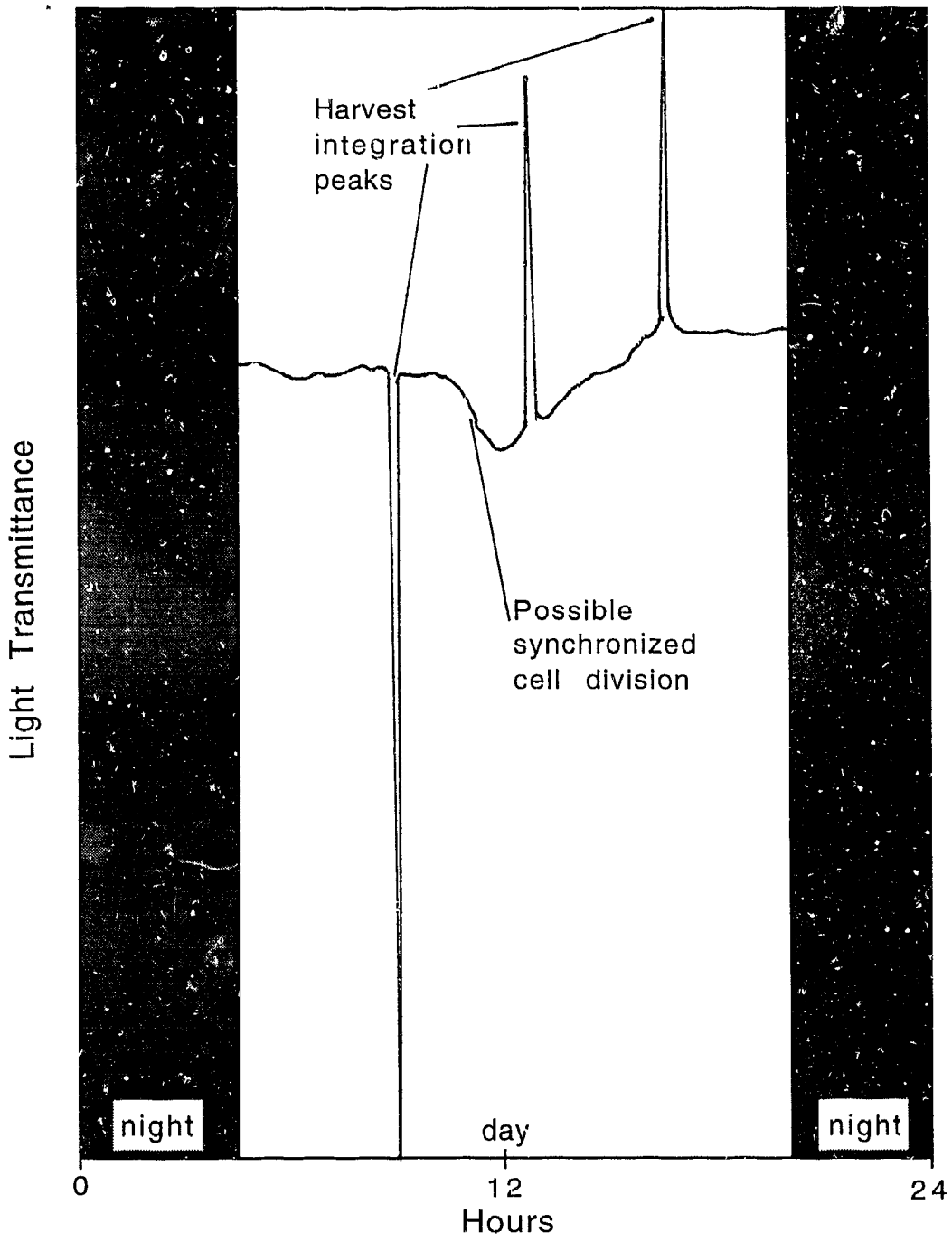


Figure 2.4 - Turbidostat recorder trace showing transmittance and harvest integration peaks. Full scale peaks indicate continuous harvesting after the transmittance reduction.

The results from these experiments indicate that A. tamarense may exhibit phased cell division in turbidostat cultures. Since the generation time of A. tamarense is typically around three days, synchronized division events will not necessarily occur every day, but may occur every few days. Thus the value of r determined on any particular day does not necessarily represent the long-term average for the culture. The simplest solution is to use a smoothing function to reduce the day-to-day variation in r and highlight the long-term average. The period between peaks in plots of r appears to be on the order of several days, so simple three or five-day moving averages should provide an acceptable means of smoothing the curves.

Figure 2.5 shows the results obtained when the division rate data taken from a culture grown in March and April of 1992 are smoothed with three-day and five-day moving averages. The upper panel shows unsmoothed culture density and r values. Both parameters show large oscillations with a period of a few days. The effect is especially noticeable in the plot of r. This oscillation makes it difficult to get an appreciation of the long-term growth rate of the culture. Three-day moving averages (Figure 2.5, lower

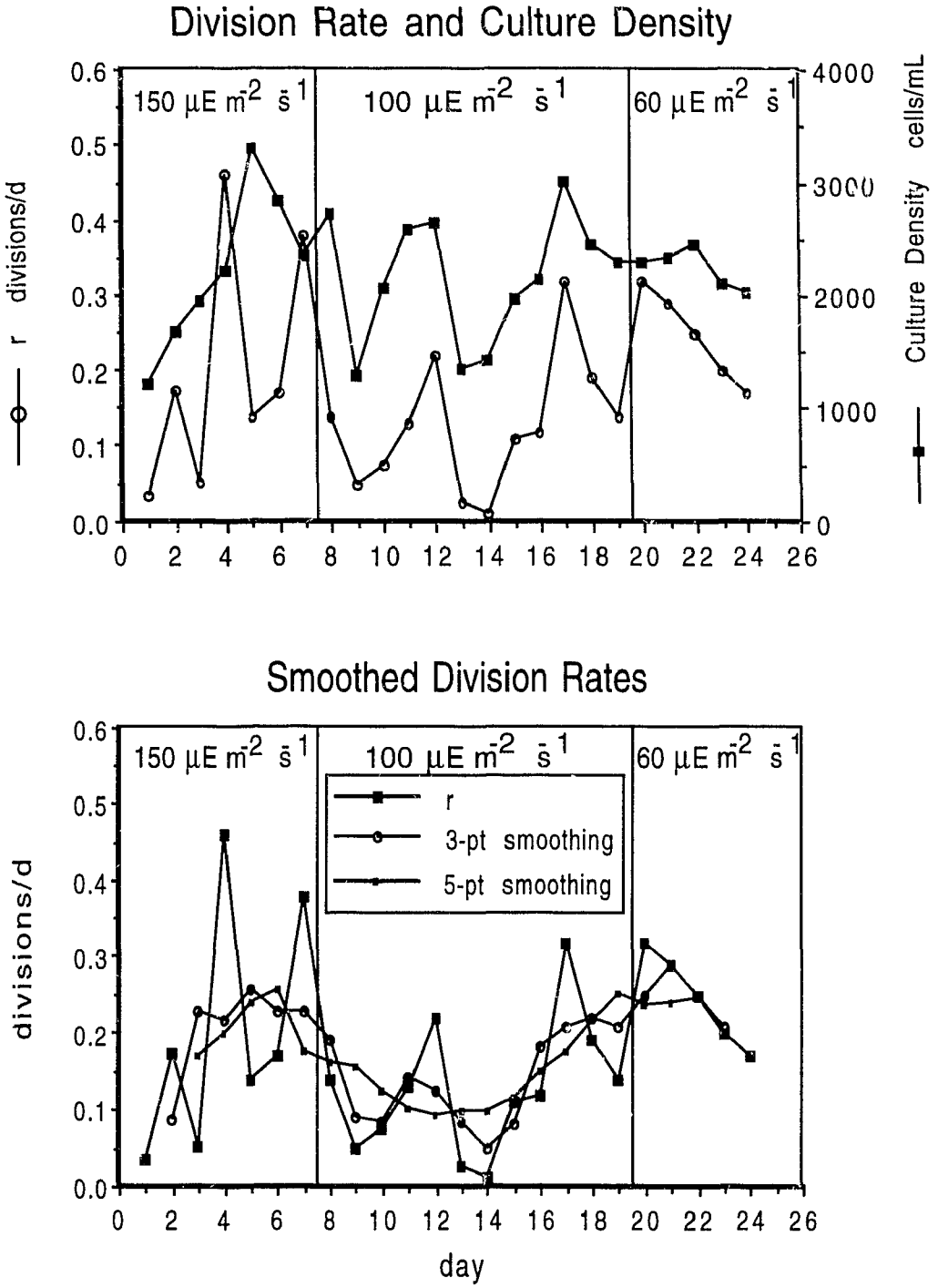


Figure 2.5 - Culture 03/92A: division rate, culture density and smoothed division rate

panel) remove most of the short-term variation and allow the longer-term variations to become apparent. The five-day smoothing function removes even more of the short-term variation and produces a curve which is more likely to represent the "steady-state" behaviour of the culture. This type of plot makes interpretation of the data easier.

The smoothing reveals a reduction in r at the point when the light intensity was reduced from 150 to 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ on Day 8. This reduction is more difficult to see in the unsmoothed data since r appears to drop and then rise again. The smoothed values drop for several days and then slowly recover, possibly indicating that the culture was adapting to the lower light intensity. The second light reduction from 100 to 60 $\mu E \cdot m^{-2} \cdot s^{-1}$ on Day 20 seems to result in another reduction in r; the time series did not continue far enough to be sure of this. However, it is obvious from this example that smoothing the curves is a useful procedure when studying cultures with a lot of short-term variation. The same technique can be applied to any measured parameter which oscillates around a long-term mean. In later chapters, moving averages will be used to

smooth cellular toxin content data from cultures thought to be undergoing phased division in order to examine long-term changes in toxin content and composition.

The choice of the averaging period depends on the growth rate of the culture. A smoothing filter with a period that is too short will not remove all of the short-term variation in the curve, while one which is too long might remove all the variation. An appropriate period to use is the average generation time of the culture. This should eliminate the oscillation in the parameter of interest without obscuring any long-term trends. Using the example of Culture 03/92A (Figure 2.5), and the formulae from Skipnes et al. (1980), the average value of μ for the culture is initially around 0.2 d^{-1} which gives a culture doubling rate of $r=0.2/\ln 2 = 0.29$ division/d or a generation time of $t_g=1/0.29 = 3.4$ d. During the period of slow growth, the average μ of about 0.1 d^{-1} would correspond to a generation time of 6.9 d. Therefore, a moving average with a period of about five days is an appropriate smoothing function to apply to this culture. In faster-growing cultures, three-day averages would be better. The smoothing period has to be an odd

number, so an exact match may not be possible, but even an approximate match should give usable results.

Conclusions

The culturing of A. tamarense in cage-culture turbidostats presents a number of problems resulting from its slow growth rate and sensitivity to environmental factors. Careful attention to water quality, cleanliness, nutrient content and sterility is essential if repeatable results are to be obtained. The cage-culture turbidostat system does work for this species, although the low density of the cultures limits the sensitivity of the light transmittance sensor. There was a significant problem with the repeatability of the method. Turbidostat cultures were useful for experimentation only if they were healthy and dense at the start of the experiment. Otherwise, there was a tendency for different cultures to exhibit different behaviours, or to stop growing entirely. This made it necessary to inoculate the culture vessels with large volumes of log-phase cultures. Good replicate cultures could only be obtained if a flow splitter was used to inoculate all replicates simultaneously.

Bubbling the cultures was essential to the operation of the turbidostats, although the sensitivity of the dinoflagellates to turbulence made complete homogenization impossible. This led to variations in culture density which caused some problems.

The variation in culture density resulted in some difficulty interpreting the culture density and harvest rate data, making it necessary to consider both relative culture density changes and harvest rate when determining the growth rate of the culture. The resulting "corrected growth rate" μ_{cor} must be interpreted cautiously due to the combination of instantaneous and integrated measurements in one parameter. The cultures also exhibited oscillating division rates, which obscure the long-term changes in growth related to light regimen. This is possibly a manifestation of phased cell division, which will be investigated in Chapter 4. The use of moving averages as smoothing functions for r allowed the long-term trends to be seen, but with an inevitable loss of short-term resolution. Selection of the averaging period was based on the approximate generation time of the culture.

CHAPTER 3

EFFECTS OF LIGHT REGIMEN ON THE GROWTH AND TOXIN CONTENT OF *ALEXANDRIUM TAMARENSE* IN CONTINUOUS CULTURES

Introduction

The light field in natural environments is subject to large variations over wide time scales. Photoperiod varies seasonally while irradiance can change on hourly, daily and seasonal scales. In temperate latitudes (45°), the photoperiod changes from the maximum daylength 15 $^{3}/_{4}$:8 $^{1}/_{4}$ L:D in June to 9 $^{3}/_{4}$:14 $^{1}/_{4}$ L:D in December (Yentsch et al., 1974). This produces a large variation in the amount of light energy available to phytoplankton over the course of a year. On shorter timescales, light availability is affected by cloud cover and water clarity. Just below the surface of the water, irradiance can be very high, between 60 and 98% of the

incident sunlight, depending on the elevation of the sun (Pickard and Emery, 1982). Surface roughness caused by waves will also increase the amount of light reflected, but this process is difficult to characterize due to the complex angular distribution of sunlight (Kirk, 1983). Photosynthetically active radiation provides about 2300 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance at the surface of the water in full-strength, vertical sun (Kirk, 1983). In practice, the value is often much lower due to cloud cover and solar angle. Below the water's surface, light is rapidly attenuated as depth increases. The attenuation constant varies with water clarity, a function of the concentration of dissolved and particulate material in the water. In a dense bloom, the attenuation is greater, and the light may be completely absorbed within the top few metres.

Clouds reduce the incident radiation in proportion to the amount of the sky which is obscured. The formula:

$$(1-0.09 \cdot C)$$

where C is the cloud cover in oktas (eighths of the sky), gives an estimate of the available light as a proportion of the clear-sky total (Pickard and Emery, 1982). Thus a completely cloudy sky would

deliver 28% as much light as a completely clear one. However, this does not account for differences in the thickness of clouds. Detailed modeling and field measurements of incident solar radiation over the oceans show large variations in temperate regions (Bishop and Rossow, 1991). For example, in data from July of 1983 and 1984 at Ocean Weather Station Bravo in the western Atlantic, the irradiance fluctuated by about an order of magnitude on timescales of days. Regional weather patterns determine how much variation is normal for a given site. In short, phytoplankton in the natural environment are exposed to large variations in incident irradiance which may affect their physiology and growth.

Experimental Hypothesis

This study attempts to relate the toxin production dynamics of the organism to changes in irradiance similar to those observed in nature. The experiments in this chapter were designed to test the effects of step-changes in irradiance and photoperiod on the growth and toxicity of A. tamarense. The working hypothesis was that toxin concentration of the cells is inversely related to their growth rate. Reductions or increases in irradiance and photoperiod could reduce

the growth rate of the cells and increase their toxin content. Experiments were designed to expose the dinoflagellate cultures to changes in light regimen which were reasonable in terms of the marine environment. Irradiance levels and photoperiod (see Chapter 2) were in the range experienced in nature and the changes in light availability were within the range experienced by blooms in natural conditions. The timescale of these experiments was on the order of days, which is similar to the timescale of irradiance changes in nature. The photoperiod experiments were less realistic, since the natural timescale of photoperiod variation is on the order of months. It is not practical to simulate this in the laboratory, but abrupt changes in daylength nevertheless should give some indication of the response of cells to photoperiod changes. Toxin content and composition were determined and their variations were related to growth rate changes induced by the light regimen.

Methods and Materials

The culture methods employed in these experiments have been discussed in detail in Chapter 2. The standard cage-culture apparatus was used in all cases. Photoperiod was controlled with a timer switch. The irradiance was 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ in the case of the control and high-light cultures. Lower light intensities were achieved by screening the lights with one or two layers of white tissue paper. Cultures were exposed to 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ for several days and then switched to lower irradiance (40 - 100 $\mu E \cdot m^{-2} \cdot s^{-1}$) for several more. The change was introduced either at the end of the light period or shortly after dawn so that the organisms experienced full day-night cycles at each irradiance level.

The cultures were sampled for toxins at daily intervals, typically around the same time each day, although this was not always the case. This introduces some problems of interpretation of results which will be discussed later. The culture vessel was continuously mixed with an air bubbler and it was assumed that the material harvested by the turbidostat was representative of the

culture as a whole. Since the harvested material was collected only once per day, a portion of it might have been in the harvest flask for up to 24 h prior to collection. The harvest flasks were kept under the same photoperiod as the culture vessel and as close to the same irradiance as possible. Some differences are inevitable since the harvest flasks were smaller than the culture vessels and of different material (4 L vs. 10 L; polycarbonate vs. acrylic). The harvest flasks were graduated to allow determination of the harvested volume. In the initial stages of the project, the entire daily harvest volume was used for toxin analysis, less a small amount needed to determine culture density. Toxin samples were collected using the procedures of Boyer et al. (1986), modified to suit the available apparatus as follows: Each harvest flask was swirled to mix the contents and a small sample was poured into a beaker for counting. The volume of the remaining material was noted, it was swirled again and poured though a 47 mm Millipore filter holder equipped with a 10 μm Nitex filter disk. In the first several experiments, suction was used to draw the sample through the filter. This was especially necessary when the sample volume was large (2 L or more), as the filters tended to clog with cells. In

later experiments it became obvious that samples of 0.5 to 1 L were sufficient to provide good results, so from November 1991 onwards, the sample volumes were standardized at 1.0 or 0.5 L depending on the harvest volume available each day. In March 1992, the volumes were standardized at 0.5 L, regardless of harvest volume. The reduced sample volume made suction unnecessary so gravity-filtering was used. In cases where the growth rate had dropped to near-zero and no harvested material was available on a given day, samples were taken directly from the culture vessel through a Y-fitting in the flow-cell recirculation line.

After concentrating the cells on the filter, they were rinsed with distilled deionized water (Millipore Super-Q) and collected in a 15 mL polypropylene centrifuge tube (Nalgene or Corning). The tubes were capped and centrifuged at 6000 rpm (RCF $\approx$ 4000g) for 20 min in a Sorval (model RC2-B) refrigerated centrifuge. The rinse-water supernatant was decanted and the pellet resuspended in 5 mL of 0.03 M acetic acid (BDH Chemicals). The suspension was then sonicated with an ultrasonic probe (Branson Sonifier or Heat Systems Ultrasonics) to break up the cells. The tubes were cooled in an ice

bath during sonication. Initially, the procedure used three 60 s bursts with 60 s intervals for cooling. Trial and error reduced this to three 30 s bursts to allow cooling. When the Heat Systems Ultrasonics instrument was acquired, the procedure was modified to use a 3 min exposure at 50 % cycling (i.e. 1.5 min actual sonication time in bursts of about 1 s each). All these procedures resulted in total disruption of the cells, insofar as could be determined under the microscope. After sonicating, the suspension was centrifuged for another 20 min at 6000 rpm. The supernatant was drawn off with a pipet and frozen at -70 °C in 1.5 mL Eppendorf micro test tubes. In some cases it was convenient to interrupt the sample processing procedure at the cell pellet stage. The pellets were frozen in the acetic acid solution at -25 °C. Sonication and final storage occurred within 48 h. There is no indication from the data that these samples were in any way altered by the interruption.

Analysis of samples for toxin concentration and composition was carried out by the NRC Institute for Marine Biosciences in Halifax, N.S.. The samples were thawed and filtered through 10 kDa molecular weight cut-off membrane filters prior to analysis.

Analysis was performed by reverse-phase HPLC with a "Sullivan train" post-column oxidation system developed from that described by Sullivan and Iwaoka (1983) and Sullivan et al. (1985). This analytical service was made available through the kind cooperation of Dr. Stephen W. Ayer, Dr. Allan D. Cembella and Mr. Joe Uher. Due to the expense of running replicate samples from long-term experiments, single samples were used with the exception of some limited time-series to test the repeatability of the measurements.

Cell counts were initially performed microscopically using Fuchs-Rosenthal or Sedgwick-Rafter counting chambers. Ten fields were counted and the average taken to calculate culture density. The Sedgwick-Rafter chamber gave more consistent results due to its greater depth and hence larger field volume. After November 1991, a Coulter Counter was used for density determinations and provided more repeatable and stable numbers. A 100 μm aperture tube was used, with an aperture current of 1 A and amplification adjusted to give a signal strength within the calibration window. Typical amplifications were 1/16 or 1/32. Three counts were made on each sample and the results averaged. A calibration of Coulter versus

visual counts was made using one week of data from Noveber 1991. When the instrument was properly adjusted, the Coulter counts fell within the range of error of the visual determinations as follows:

Average standard deviation of visual counts: 38.25% of mean

Average standard deviation of Coulter counts: 4.53% of mean

Average difference between means (Visual-Coulter): +8.7%

To determine if the irradiances used were within the linear portion of the growth vs. irradiance curve of the organism, an experiment was performed in April-May of 1992 to measure the growth rates of cultures under different irradiances. Medium-sized batch cultures (3 L) were grown in duplicate at four irradiances: 50, 100, 140 and 200 $\mu E \cdot m^{-2} \cdot s^{-1}$. The results from one of the 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ cultures were discarded due to poor growth. Cell density deteminations were performed on each culture daily for a period of 17 days. Growth rates were determined from the culture density data. No toxin samples were taken in this experiment.

Results and Discussion

Calibrations

GROWTH - IRRADIANCE RESPONSE

Above 100 $\mu E \cdot m^{-2} \cdot s^{-1}$, the growth of the cultures was practically identical, regardless of light intensity (Figure 3.1). However, the cultures maintained at 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ grew at a markedly slower rate, indicating that they were probably light-limited.

It can be concluded from this experiment that the range of light intensities supporting maximal growth in this strain of A. tamarense extends from about 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ up to at least 200 $\mu E \cdot m^{-2} \cdot s^{-1}$. Ogata et al. (1987a) grew A. tamarense successfully at irradiances of 550 $\mu E \cdot m^{-2} \cdot s^{-1}$. Maranda (1985) reported saturation of photosynthesis in A. tamarense at approximately 500 $\mu E \cdot m^{-2} \cdot s^{-1}$. It seems, therefore, that experiments conducted between 100 and 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ should provide growth rates representative of natural conditions. Since this project was

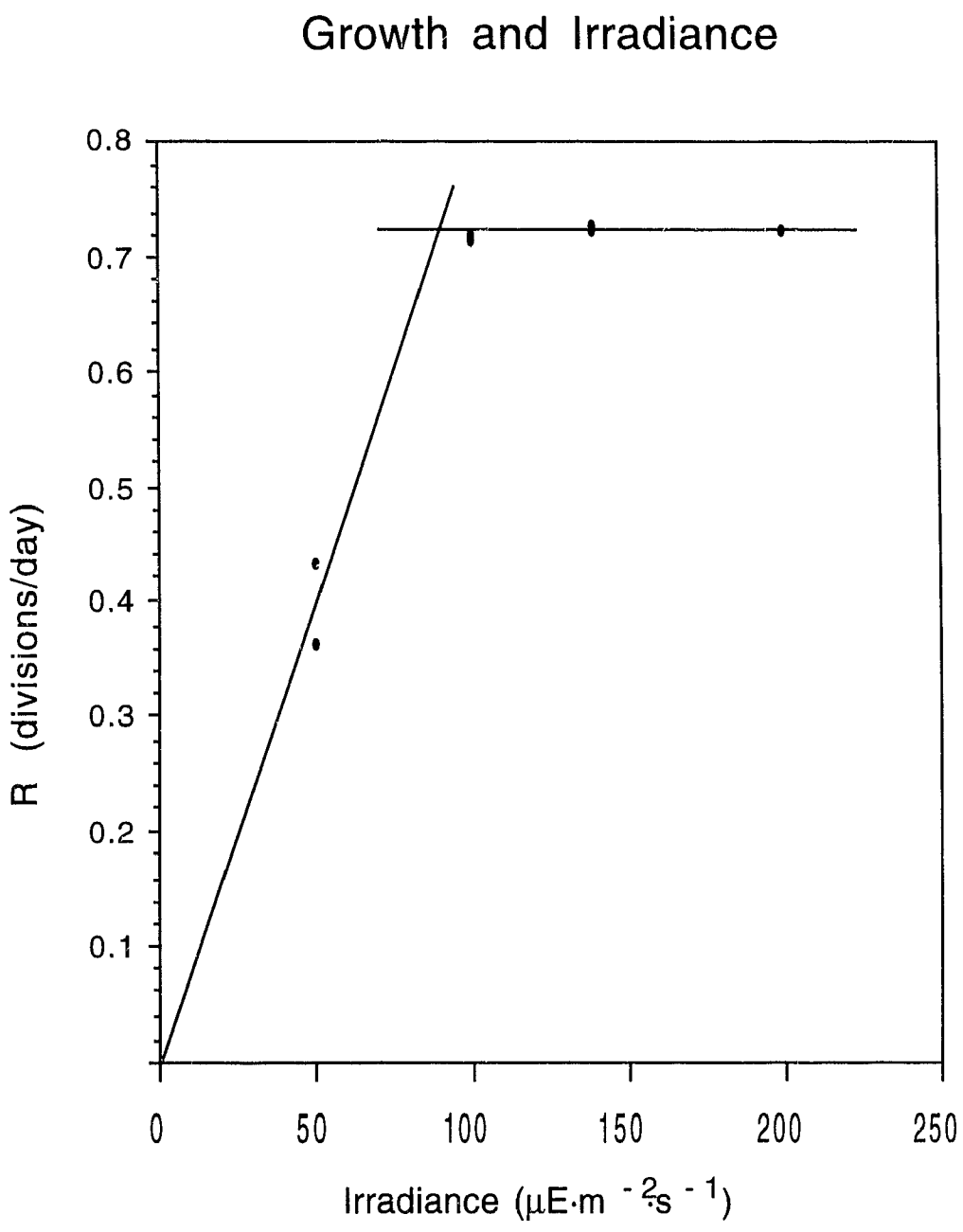


Figure 3.1 - R vs. I plot for batch cultures of A. tamarense.

designed to study the effect of growth inhibition, the lower limit of irradiance should be such that growth is merely reduced, not stopped. Thus 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ is probably reasonable, and if possible, the irradiance should be higher. In the initial light-reduction experiments, this fact was not recognized and light levels below 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ were used. This produced complete cessation of growth in some cases. In later experiments, the minimum irradiances were in the 60 to 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ range and produced good results.

TOXIN SAMPLE REPLICATION

One serious question which had to be addressed was the accuracy and repeatability of the toxin analysis. The HPLC procedure used is prone to variation due to the complexity of the post-column derivatization process (S. Ayer, pers. comm.). Differences in the flow rates of reagents, temperature and other conditions of the chromatograph operation can produce large variations in the final results. Also, the toxins themselves are thermally labile and will undergo chemical conversions such as epimerization if exposed to

high temperatures. Accordingly, care was taken to store the samples at very low temperatures and to limit the exposure of the material to potentially harmful conditions. These problems were exacerbated by the lack of quantitative standard preparations of the toxins. Qualitative standards made identification of the toxins reasonably certain, but mass and molar conversions were harder to obtain. This became less of a problem as the work progressed since the NRC was simultaneously developing good quantitative standards for most of the toxins produced by this strain of A. tamarense.

The lack of quantitative standards made it necessary to report the results as chromatogram peak areas, rather than true concentrations. It must be noted, however, that the fluorescent derivatives of the toxins are not detected equally by the HPLC system. Some give a larger response than others (Table 3.1). Thus the relative proportions of the toxins (toxin profile) indicated by peak area results do not represent the actual ratios of the toxins in the cells. When comparing the concentration of an individual toxin among different samples, this is not a problem, since the detector response is linear within the working range. However, the total toxin

Toxin	Relative Fluorescence
STX	1.0
NEO	0.136
GTX-1	0.107
GTX-2	3.0
GTX-3	3.0
GTX-4	0.107
C1 - C4	2.5

Table 3.1 - Response factors for PSP toxin detection (courtesy of J. Uher, NRC-IMB, Halifax, N.S.). These values indicate fluorescence (relative to saxitoxin) of the oxidized derivative of each toxin. The value for C1-C4 is an average, assuming an equimolar mixture of the four compounds.

content of different samples can only be compared if their toxin profiles are the same. In this study, toxin profile changes remained relatively small (10 - 20% of total toxin) so this assumption was valid. However, it is worth noting that the relative fluorescence responses of the different toxins are quite different.

The analytical procedure is rather costly and labour-intensive so it was necessary to limit the number of samples to the minimum required. Accordingly, most experiments were performed using single samples at each data point. It was necessary to know what variation was inherent in the sampling and analytical procedures. A sample replication test was performed in August 1990 to check the repeatability of the sampling method. The results (Figure 3.2) indicate good correspondence between the replicate samples. The differences which do occur likely arise from variations in the filtering procedure or incomplete mixing of the culture prior to filtering, and are not so great as to cause serious concern. This implies that the sampling procedure is sufficiently repeatable that single samples of the culture can reasonably reflect its bulk properties. Obviously, the possibility of rogue samples does exist,

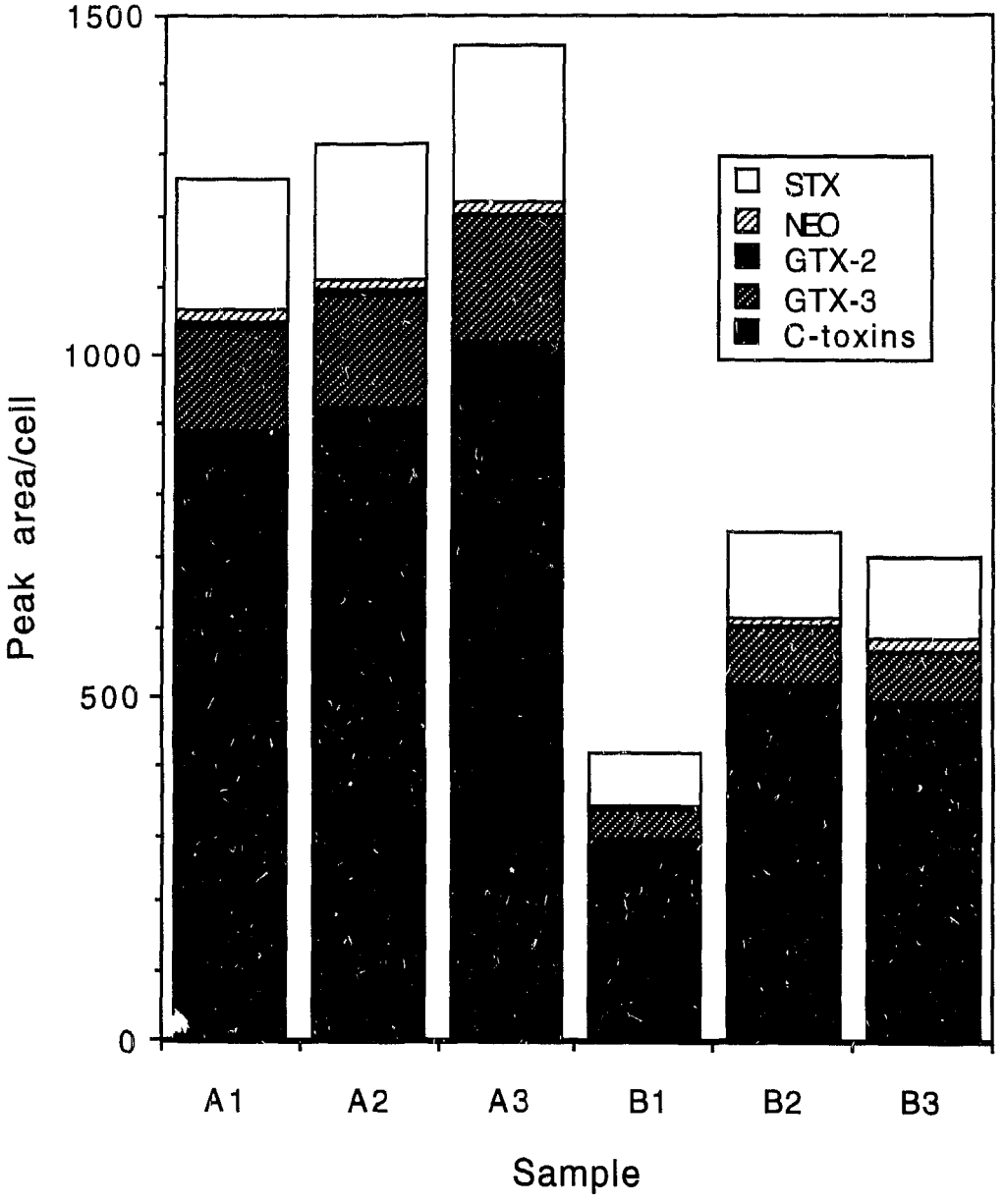


Figure 3.2 - Sample replication test from 08/90 showing results of triplicate toxin samples of two cultures (8/90A and 08/90B). Samples represent separate aliquots of a well-mixed culture which were processed independently. This test used clone PR17B. The toxin profile is different from that of clone OK875-1 used in later experiments.

but these will be isolated and probably identifiable as unusual points in the time series. The variation between the samples is usually less than 30 % so general patterns of toxin content changes should be preserved. The analytical error in the Sullivan HPLC procedure is typically less than 5 % (A.D. Cembella, pers. comm.).

COULTER COUNTER CALIBRATION

Microscopic determination of the culture density had a wide margin of error (standard deviation ≈35-40%) and its use was discontinued in favour of Coulter counting. It was necessary to be sure that the Coulter Counter was measuring cell concentrations comparable to the microscope counts. One possible source of error is the high proportion of cell doublets observed in these cultures. When cells of this species divide, the daughter cells remain attached to each other for perhaps several hours. In the microscope determinations, these doublets were counted as two cells. However, the Coulter Counter simply detects particles, whatever their shape, and might therefore count doublets as single cells. A calibration test was performed to determine whether doublets caused underestimation of the culture density and what were the

appropriate settings for the Coulter instrument controls. In spite of the presence of doublets, the Coulter Counter gave accurate results with the counts falling within the margin of error of microscope counts. The instrument may slightly underestimate the population, but the error appears to be small and its use was continued for the remainder of the project. It is possible that the relatively small aperture used (100 μm) caused sufficient turbulent shear to break up the doublets and cause them to be counted as single cells.

Variation in Toxin Content

BASIC VARIATIONS

In a study such as this, one of the major difficulties is separating the normal variations in toxin content and composition from those induced by light regimen changes. As the work progressed, it became apparent that the toxin content of A. tamarense is highly variable, even during steady growth under constant conditions. As will be shown in this chapter, variations in toxin content can be correlated with short-term changes in the culture not related to light regimen. The phased growth described in Chapter 2 is a strong candidate to explain this type of short-term

toxin variation. In these circumstances, it is very difficult to determine if an observed variation is a function of natural physiological changes or a reaction to the applied stress.

JULY 1991 DATA

An example of these variations can be seen in Figure 3.3. Data from three cultures grown in July 1991 show large changes in harvest rate and toxin content under constant conditions. Figure 3.3 illustrates r and total toxin content of the cultures. There is a loose, positive correlation, suggesting that production of the toxin is maximal during periods of rapid growth and drops when growth is reduced. Changes on the order of 50% are present in all three cultures in spite of constant and favourable growth conditions. Phased growth of the cultures is probably occurring, but there are gaps in the time series which make interpretation of the results difficult. When dealing with a system which has a large degree of short-term variation, it is best to collect data of the highest resolution practicable. Accordingly, for the remainder of the project, samples were taken every day in an attempt to produce more complete records. Where this approach was successful, it was

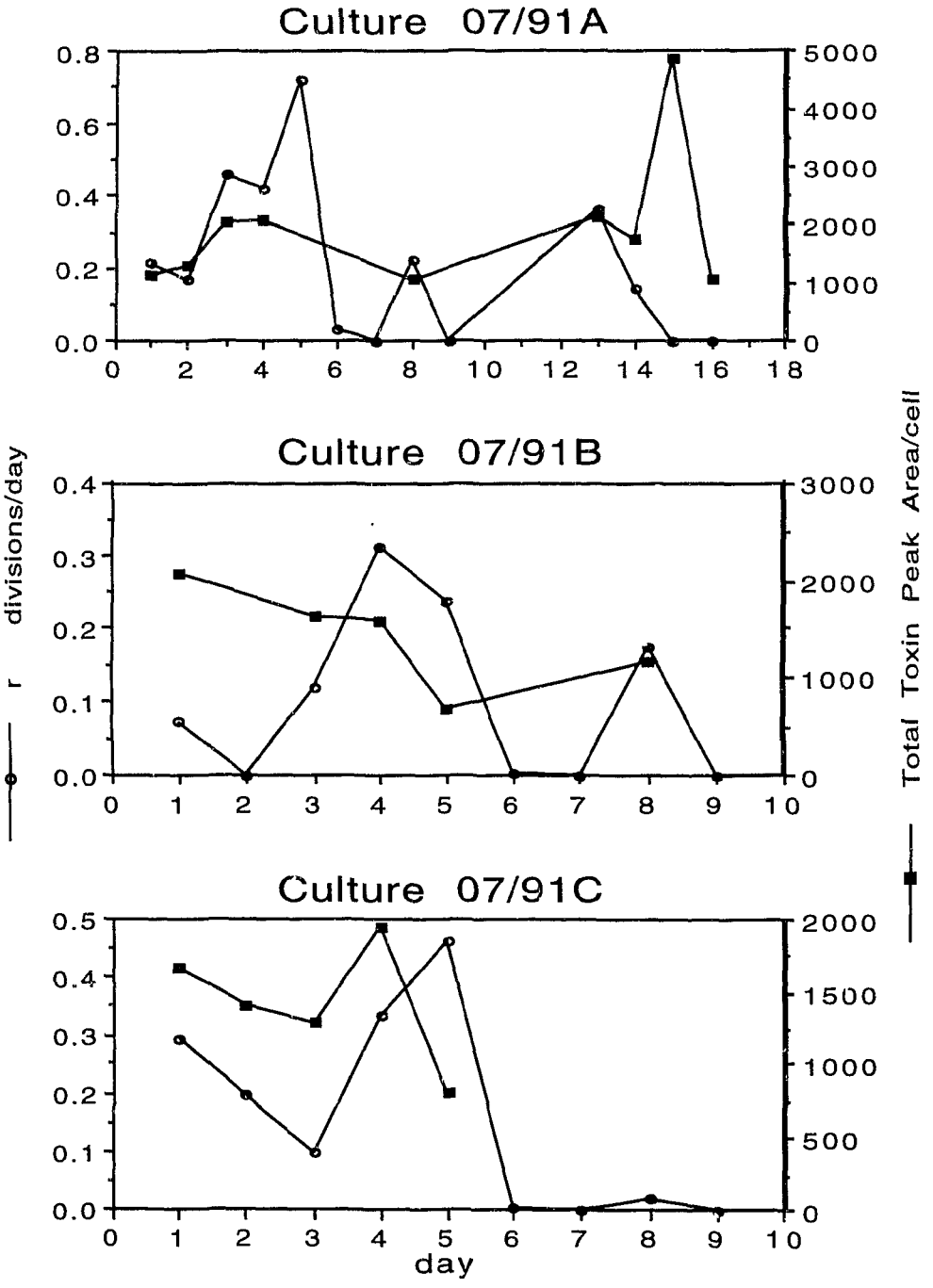


Figure 3.3 - Cell division rate and total cellular toxin for three cultures, 07/91 A,B,and C

possible to use smoothing techniques to filter out the normal variation and allow the effects of light regimen changes to be seen. The experiments which follow have benefited from this technique.

Irradiance Experiments

OCTOBER 1991 DATA

This was the first successful irradiance-reduction experiment. Considerable difficulty had been experienced up to this point getting the cultures to grow reliably in the turbidostat apparatus (see Chapter 2). Even in this case, the densities of Cultures 10/91B and 10/91C declined considerably after about Day 9. Three cultures were grown simultaneously at the normal irradiance of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$. After about one week, the irradiance on Cultures 10/91B and 10/91C was reduced to 40 $\mu E\ m^{-2}\ s^{-1}$. Culture A was retained as a control. Samples were taken daily for culture density and toxin content. Division rate was monitored continuously in the usual manner. To determine if any observed changes in division rate or toxin content were statistically significant, the mean values of each parameter before and after the irradiance change were compared using one- and

two-tailed t-tests. Statistical calculations were performed via NCSA Telnet v.2.5 using Minitab Release 9.1 on a VAX 4500 mainframe computer. Statistical results are given in table form in Appendix 2.

Figure 3.4 shows plots of r and total toxin content for the three cultures. The control (Culture 10/91A) was relatively stable throughout the experiment. For the first week, r increased and then levelled off. Average total toxin remained fairly constant, with perhaps a small decline toward the end of the experiment. Both parameters oscillated regularly around the mean with a period of about three days, indicating a likelihood of phased cell division. There is no consistent relationship between the two parameters in this culture. In the early part of the experiment, the peaks appear to be coincident while later on they are in opposite phase. It is possible that the temporal resolution of the data is too coarse to determine the exact timing of the peaks. Furthermore, they tend to consist of a single point raised above the baseline and thus may not be reliable. However, the presence of what is apparently a three-day cycle in toxin content does suggest that the production of toxins is

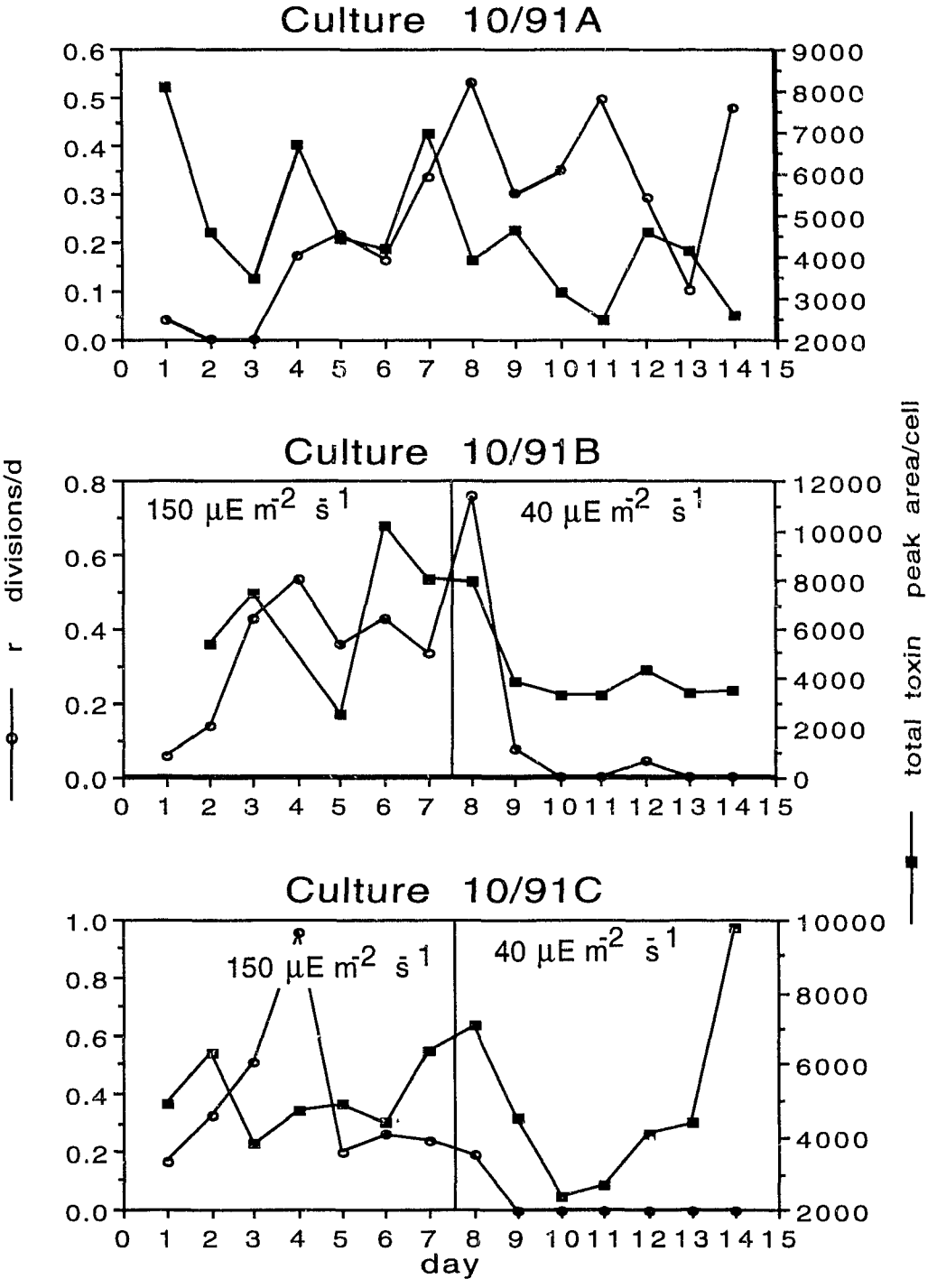


Figure 3.4 - Cell division rate and total cellular toxin. Cultures 10/91B and C experienced an irradiance reduction.

correlated with cell division in some fashion. The two experimental cultures (10/91B and 10/91C) show distinct effects of light limitation. In both cases, r decreased to zero shortly after the irradiance reduction. These reductions are statistically significant at p=0.1. In 10/91C it is significant at p=0.05. In Culture 10/91B, the toxin content also decreased and stabilized at a low level (statistically significant at p=0.1). Total toxin content and r are thus positively correlated. The results from Culture 10/91C are more ambiguous, since the total toxin was greatly reduced after the irradiance change but appeared to increase again at the end of the experiment. This might imply that photoadaptation is occurring in the culture, except that no similar response was observed in Culture 10/91B. Maranda (1985) reports that photoadaptation times in this species are on the order of one generation. The observations here are consistent with that, but since no measurements of photosynthetic rate were made, it is impossible to be certain. It is also possible that the final, raised toxin value is an artifact.

Smoothing of the harvest and toxin data with three-point running averages eliminates much of the periodic variation in the

plots and allows the relationships to become more obvious (Figure 3.4.1). In general, toxin content is high when r is high. The drop in r due to light reduction is coincident with a drop in toxin content in both the experimental cultures. Culture 10/91C appears to increase in toxicity at the end of the experiment with no associated change in r. Culture 10/91A had a very stable toxin content up to Day 8 when r stopped increasing. The toxin content then dropped slightly and stabilized. Whether or not the reduction in toxin content was caused by the stabilization of growth rate is impossible to say, but the results of this experiment do indicate that toxin content and growth rate are positively correlated.

The clone of A. tamarense used in this study (OK875-1) produces mostly N-sulfocarbamoyl toxins (C-toxins) with small amounts of gonyautoxins and trace amounts of neosaxitoxin and saxitoxin. It must also be noted that the C-toxin peak actually contains a mixture of four related compounds. Thus far, only the total toxin has been considered, but examination of the data reveals that the gonyautoxins and the C-toxins respond quite differently to changes in irradiance and growth rate. One way to visualize this is

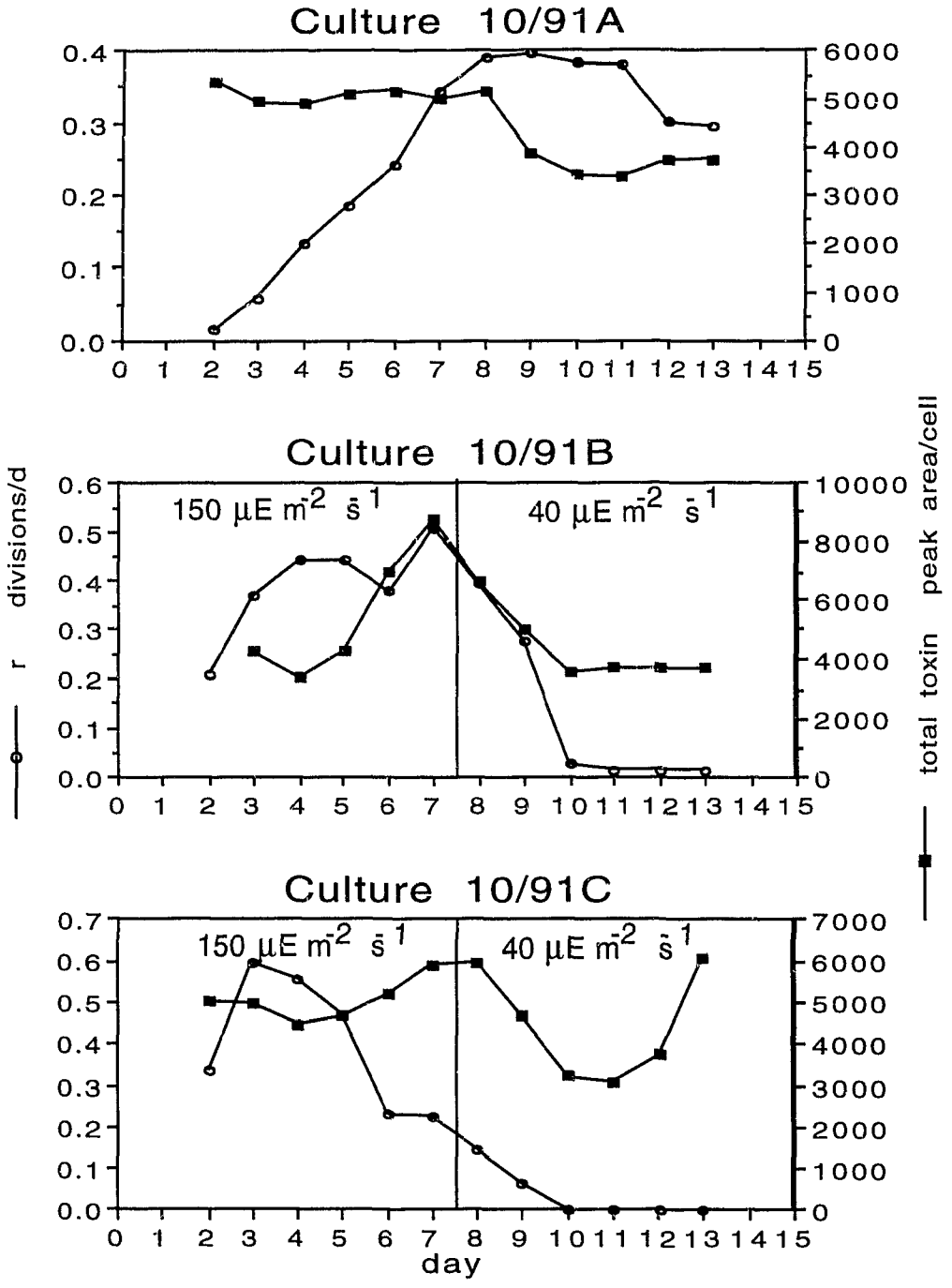


Figure 3.4.1 - Plot of division rate and total cellular toxin smoothed using 3-point running averages. Data are the same as in Figure 3.3.

to plot the peak area of each toxin as a percentage of the total peak area. Figure 3.4.2 illustrates the resulting toxin profile of GTX-3 and the C-toxins - the two largest peaks on the chromatograms. The percentages of these toxins are quite stable in Culture 10/91A and to a lesser degree in Culture 10/91C. Culture 10/91B shows a reduction in the percentage of GTX-3 and an increase in C-toxins after Day 8. This occurs at the same time as the reduction in r associated with reduced irradiance. It is possible that gonyautoxins are produced only during periods of rapid growth and are then converted to their N-sulfocarbamoyl derivatives. If growth were stopped, the ratio of gonyautoxins to C-toxins would decrease.

Toxin profile changes have been documented under nutrient limitation in semi-continuous cultures of Alexandrium fundyense (Anderson et al., 1990a). In that species (clone GtCA29) C-toxins, GTX-1 and GTX-4 declined at higher growth rates while NEO, GTX-2, GTX-3 and STX increased, under varying degrees of nitrogen limitation. Under phosphorus limitation, STX increased while GTX-2 and GTX-3 decreased with increasing growth rate. This implies toxin synthesis or conversion mechanisms which are affected differently

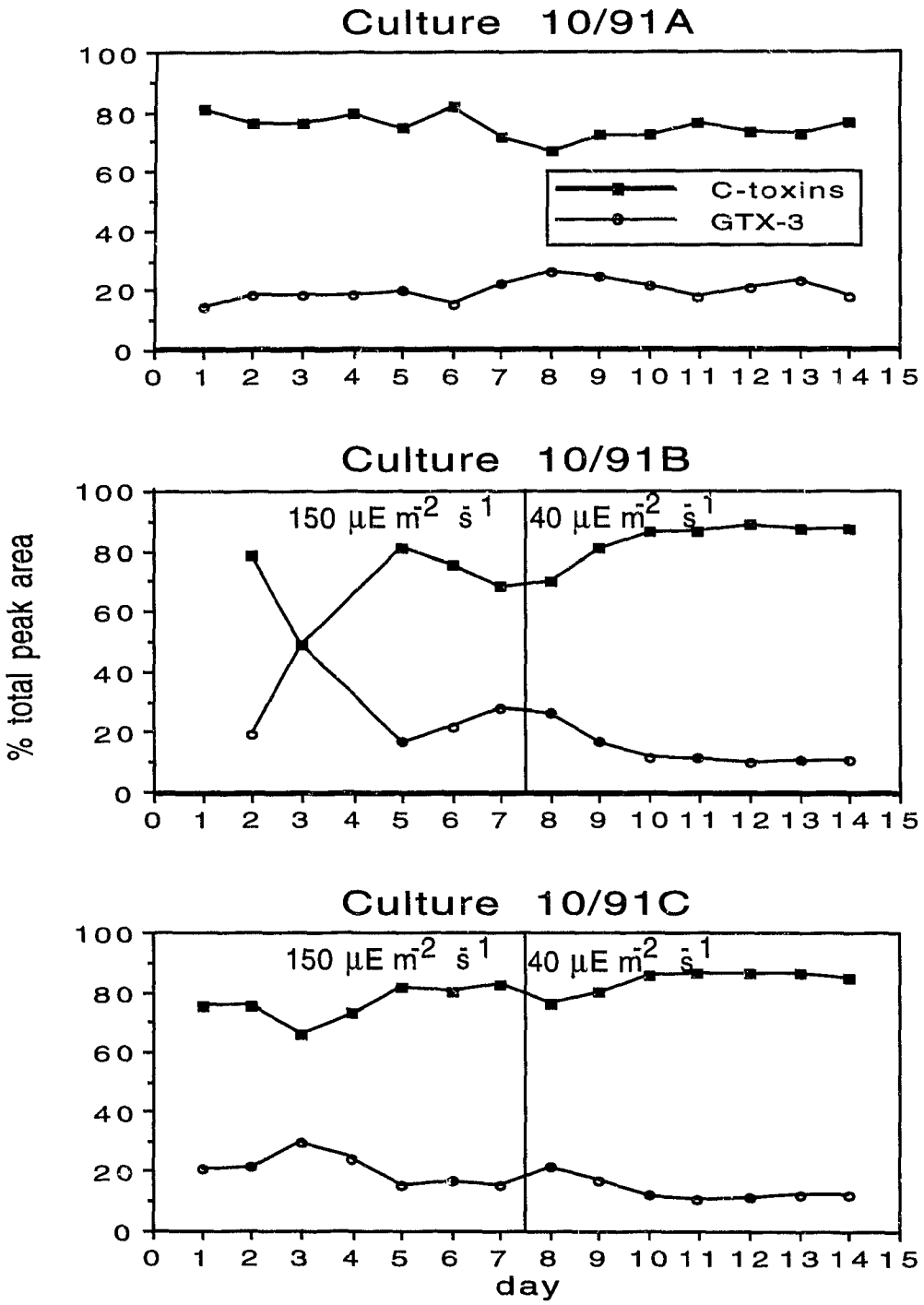


Figure 3.4.2 - Toxin profiles for October 1991 cultures showing % of total peak area for C-toxins and GTX-3

by different metabolic stresses, rather than being a simple function of growth rate. Similar mechanisms could be affected by light limitation.

It is possible therefore, that gonyautoxin production occurs during periods of high light intensity and declines or stops under light limitation. This would be possible if the enzyme synthesis mechanism producing these toxins were light-mediated in some way. Direct control by light is unlikely, but it is possible that the synthesis of a precursor or intermediate is light-dependent and results in a correlation of gonyautoxin concentration with irradiance. One-tail t-tests on the toxin percentages indicate that the observed changes are statistically significant in Cultures 10/91B and 10/91C at $p=0.05$, but not in Culture 10/91A.

NOVEMBER 1991 DATA

A duplicate of the previous experiment was undertaken in November 1991 following basically the same protocol. Due to extremely low growth rates, one of the replicates was discarded and the results of the control culture (11/91C) should be interpreted

with caution because of declining culture density. Figure 3.5 illustrates only the relative toxin proportions for Cultures 11/91A and 11/91C. All other data are in Appendix 1. Toxin samples were analysed only from Day 10 onwards since the irradiance reduction was performed close to the end of the culture life. This was necessary as the cultures required a long incubation period to reach adequate density for sampling.

The results of this experiment are not as clear as those from October 1991. In the experimental culture (11/91A), r was reduced to near-zero after the irradiance reduction, but the total toxin content of the cells did not drop as precipitously as might be expected from the October data. The pattern of toxin content in the control culture was almost identical to that of the light-reduced culture. The only difference was that the peak responses in the control culture were about twice those of the reduced-irradiance culture. Thus in this experiment there is no obvious relationship between light and either growth or total toxin content. This contradicts the results from the 10/91 cultures above, but the 11/91 time series is very short and Culture 11/91C was quite dilute

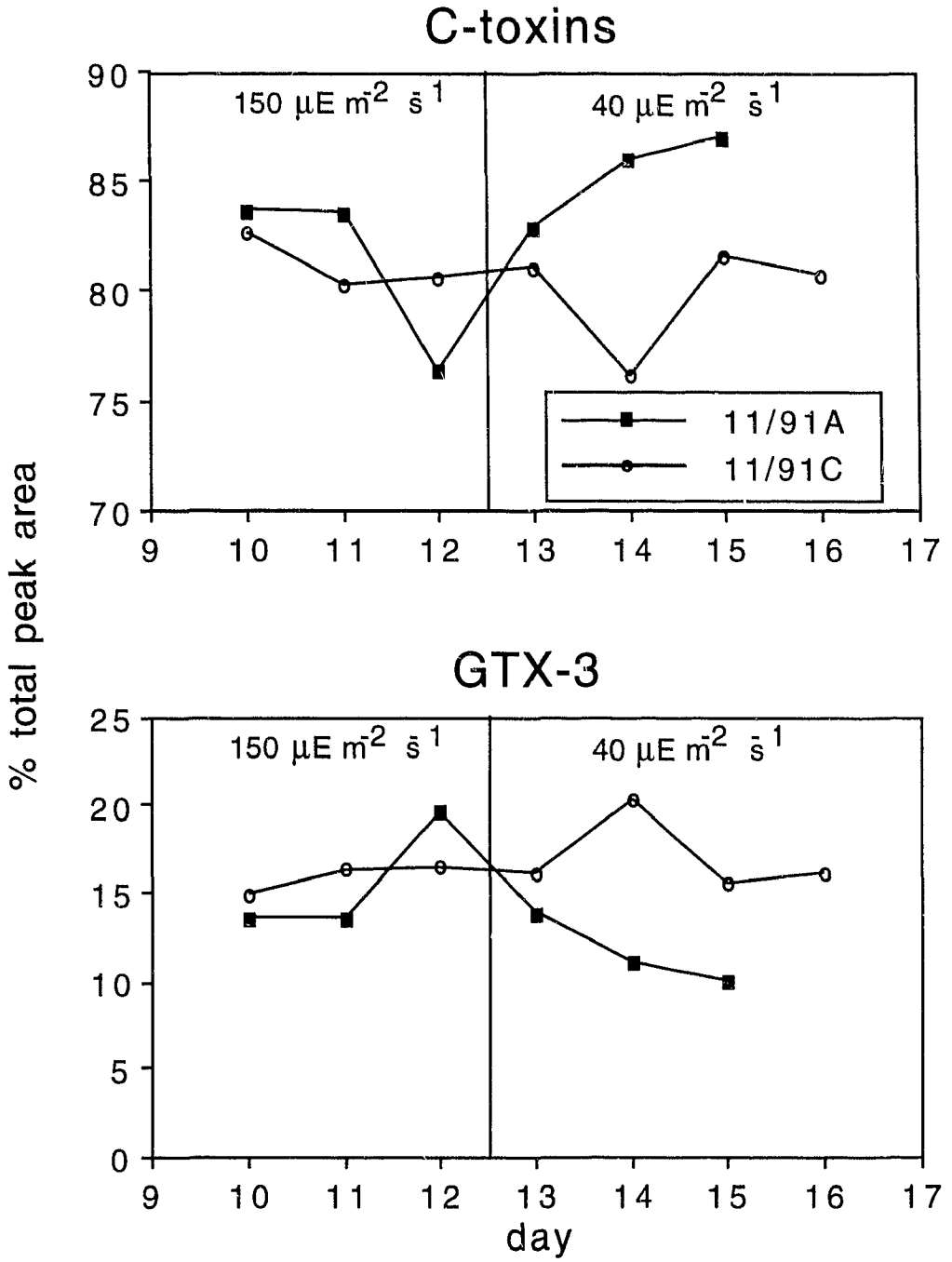


Figure 3.5 - Toxin profile data from November 1991 showing differences in C-toxins and GTX-3 between light-reduced trial (11/91A) and control (11/91C). Vertical bar indicates time of light reduction to 11/91A.

(< 500 cells/mL at termination) so the results may not be comparable. However, the toxin peak area fractions displayed virtually the same pattern of toxin profile changes in response to light as was observed in the 10/91 data. The control culture (11/91C) maintained almost uniform fractions of gonyautoxins and C-toxins throughout the test period. By contrast, the irradiance-reduced culture showed statistically significant (p=0.1) changes in the relative amount of GTX-3 and C-toxins after the irradiance change. This is consistent with the results from October 1991 and supports the earlier statement that the production of gonyautoxins is maximal during periods of high light intensity. However, the time series in this experiment is rather short. The following section discusses a long-term experiment in which light-induced changes were followed for several cell generations.

MARCH/APRIL 1992 DATA

As mentioned above, a recurring problem was the relatively short duration of each experiment. Since the data appear to indicate the presence of an endogenous phasing of cell growth and toxin production not related to environmental variables it is often

difficult to determine whether an observed change in r is caused by the light reduction or merely coincident with it. Often, cultures became contaminated with protozoans before a suitable baseline could be established. Severely contaminated cultures contained as much as 1000 protozooan cells/mL. When good growth was achieved, the cultures were monitored for as long as possible to gain insight into their long-term behaviour under steady-state conditions. Some of the best results of this type were obtained in March and April of 1992 with one culture of a three-culture trial. A second culture became contaminated and the results were not analysed. The third culture of the set did not grow very well initially, but recovered late in the experiment and gave a few days of good samples before it had to be shut down. The results from this latter case will not be discussed but are available in Appendix 1. One difference from the earlier trials is that the irradiance reduction was from 150 to 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ rather than 150 to 40 $\mu E \cdot m^{-2} \cdot s^{-1}$. This change was made to ensure that the irradiance reduction merely reduced cell division rates rather than stopping them, as occurred in the earlier experiments.

Figure 3.6 and 3.6.1 contain division rate, total toxin and toxin profile data from Culture 03/92A, including smoothed plots of all three parameters. There was a strong periodic oscillation that persisted for the entire duration of the experiment. Initially, the peaks in division rate and toxin content were coincident, but became out of phase after the irradiance was reduced. Smoothing the data through a three-day filter removed most of the oscillation and allowed overall trends to be more clearly seen. The value of r was high in the high-irradiance environment at the beginning of the experiment. After the light reduction, the average r dropped by about 50 % and remained low for about a week. It then increased to its original level. This increase could be the result of adaptation of the culture to reduced irradiance. The total toxin content of the cells remained fairly constant throughout this period. It appears that the reduction in irradiance was sufficient to depress the division rate somewhat, but did not significantly impair the production of toxins. If the last few days of possible photoadaptation are excluded (Day 16 to end), the division rate reduction is significant at $p=0.1$. The reduction in the smoothed division rate is significant at $p=0.05$. The

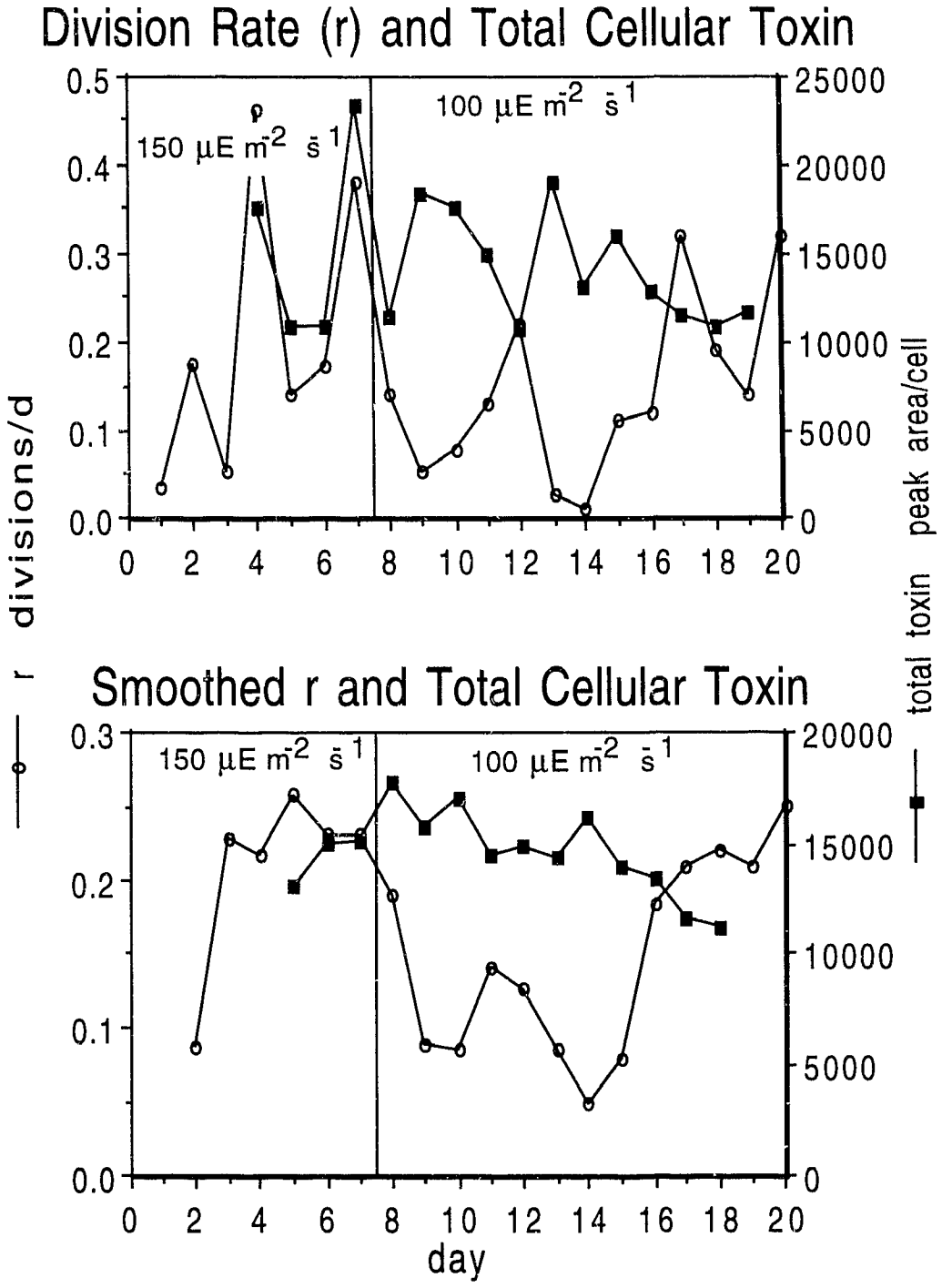


Figure 3.6 - Division rate and total cellular toxin for Culture 03/92A. Both standard and 3-point-smoothed plots are shown.

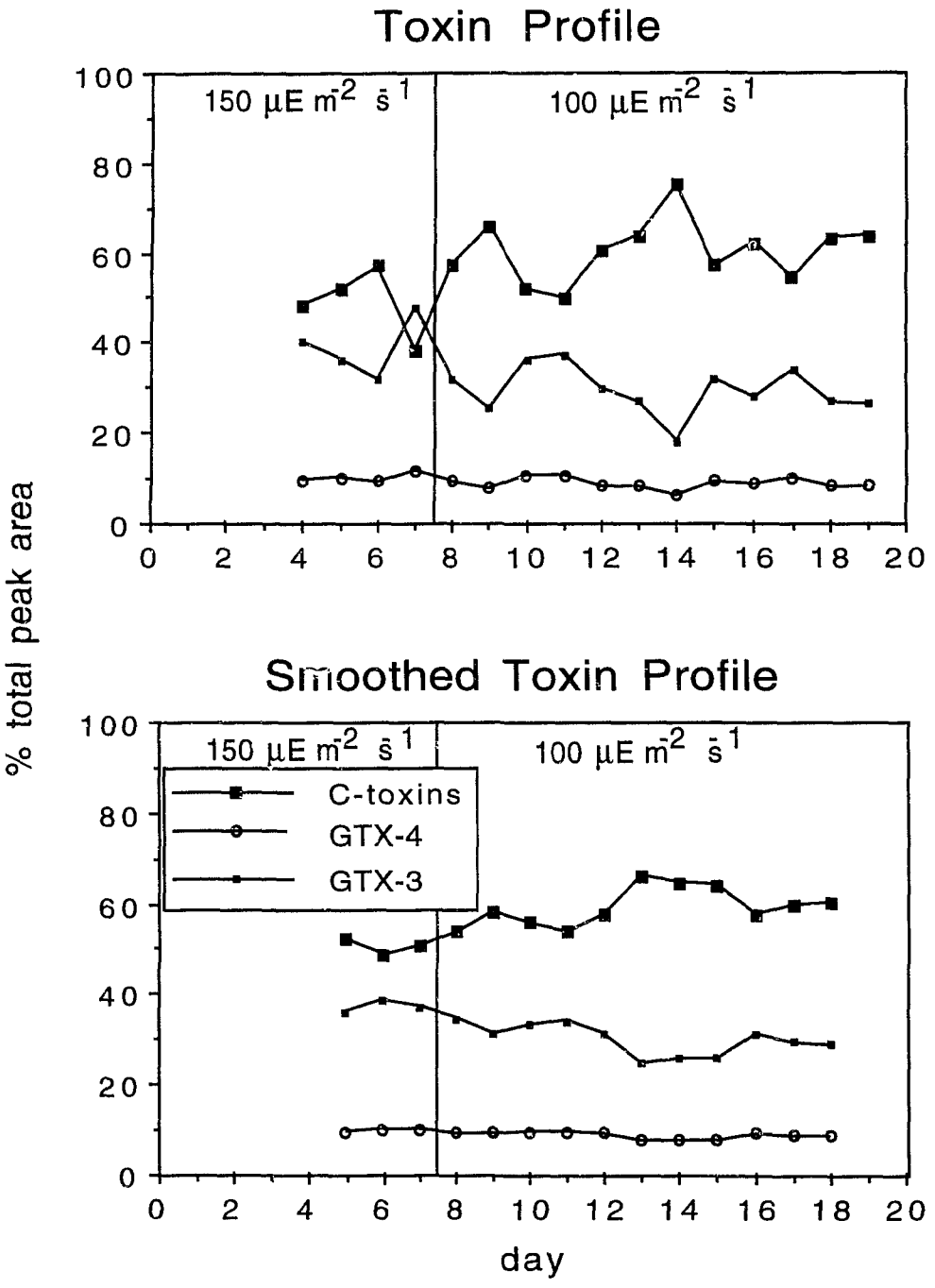


Figure 3.6.1 - Toxin profile data for March 1992 (Culture 03/92A). Standard and 3-point smoothed plots shown.

total toxin content shows no significant difference on either side of the irradiance reduction.

The proportions of the different toxins in the cells oscillated regularly over the duration of the experiment. The toxin profile data show a periodicity similar to that of r, although the oscillations were less regular and total variation is perhaps a little smaller. The percentages show a good correspondence with growth rate changes, with the proportion of gonyautoxin decreasing during periods of low growth rate. The changes in proportions of both GTX-3 and C-toxins associated with the irradiance reduction are significant at $p=0.05$. The relationships between these changes and light intensity are not as strong as in earlier experiments, but the results do support the hypothesis that gonyautoxins are preferentially synthesized at high irradiances. The oscillations also suggest that a mechanism for converting gonyautoxins to C-toxins may be favoured at low irradiances. Whatever the mechanism might be, it is apparent that the toxin profile varies on rather short time-scales. The oscillating nature of the data suggests a possible relationship to growth rate,

but the plots lack sufficient temporal resolution to determine the nature of that link.

SUMMARY OF IRRADIANCE-CHANGE DATA

The irradiance-reduction experiments generally indicate that the division rate of A. tamarense is significantly reduced by light limitation and that there also can be a significant reduction in the toxin content of the cells under severe light stress. Toxin profile also undergoes significant changes, with the proportion of GTX-3 decreasing under low irradiance while C-toxins increase. However, this effect is not as large as the cellular toxin content reduction. Contrary to the working hypothesis (see General Introduction), a reduction in division rate in these circumstances does not result in increased toxin content. Instead, the production of the toxin seems to be light-dependent. Reduced irradiance and division rate result in significantly reduced toxin content. However, small irradiance reductions do not produce a detectable decline in toxin content, even though growth is significantly inhibited. Thus toxin production and cell division are not inhibited by low irradiance to the same degree.

Photoperiod Experiments

In the natural environment, light can become limiting due to a change in photoperiod as well as irradiance. Reduction of daylength in the late summer and fall could have significant effects on the development and toxicity of blooms. Yentsch et al. (1974) observed maximal rates of dinoflagellate growth at photoperiods around 14:10, L:D, similar to summer conditions in Maine. The final set of experiments discussed in this chapter was conducted between August and October 1992 (Figures 3.7 - 3.9.1) and involved the manipulation of photoperiod and its effect on growth and toxin content.

AUGUST-SEPTEMBER 1992 DATA

Figures 3.7 and 3.7.1 contain the results of a photoperiod experiment in August-September of 1992. Three replicate cultures were grown in which photoperiod was reduced from 16:8 to 12:12 L:D. Unfortunately, Culture 08/92A grew for only a few days so it is not shown here (see Appendix 1). Figure 3.7 shows division rate, total cellular toxin content and toxin profile data for Culture 08/92B. Figure 3.7.1 shows similar data for Culture 08/92C

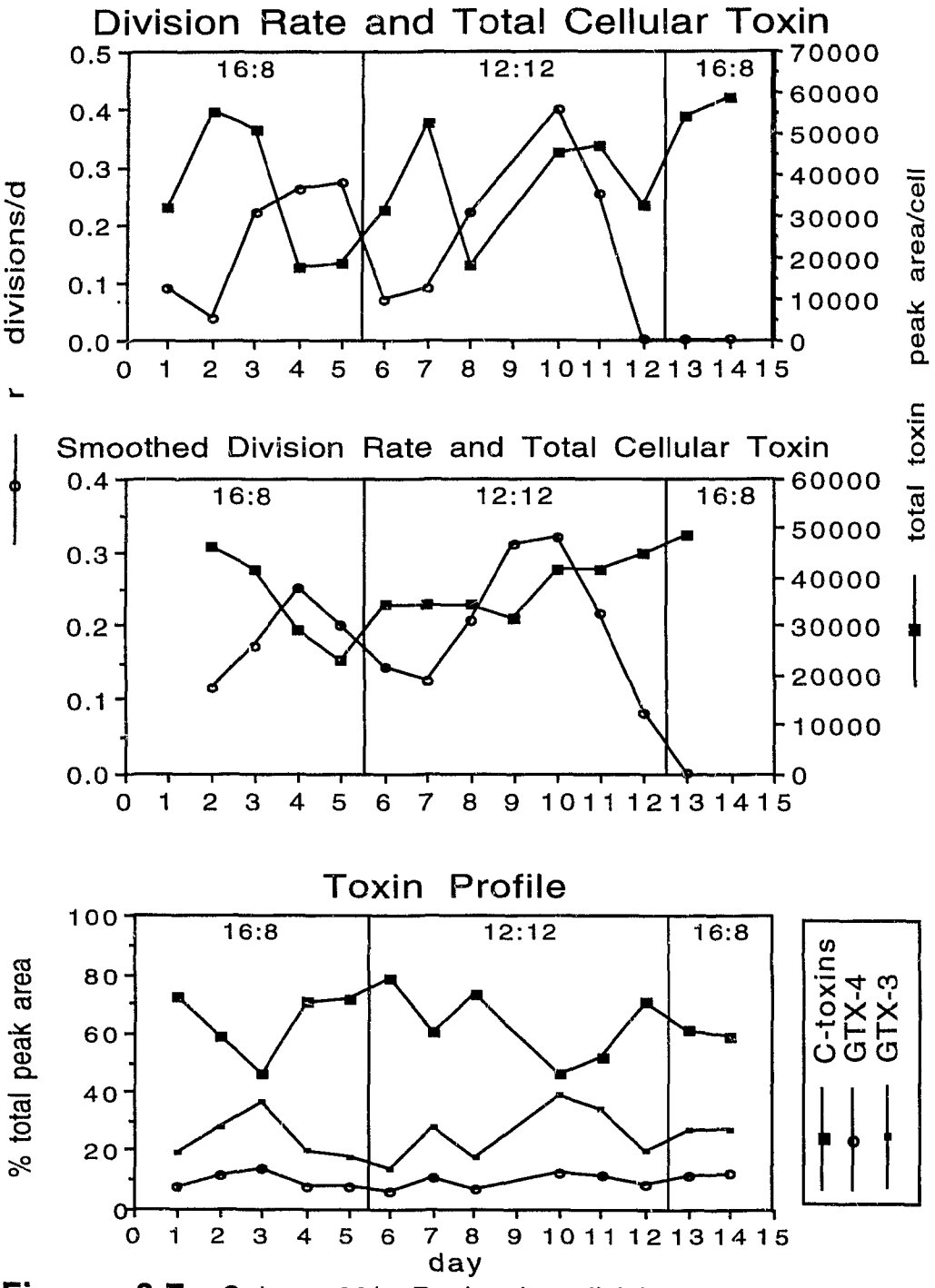


Figure 3.7 - Culture 08/92B showing division rates, total cellular toxin (standard and smoothed) and toxin profile at three photoperiods

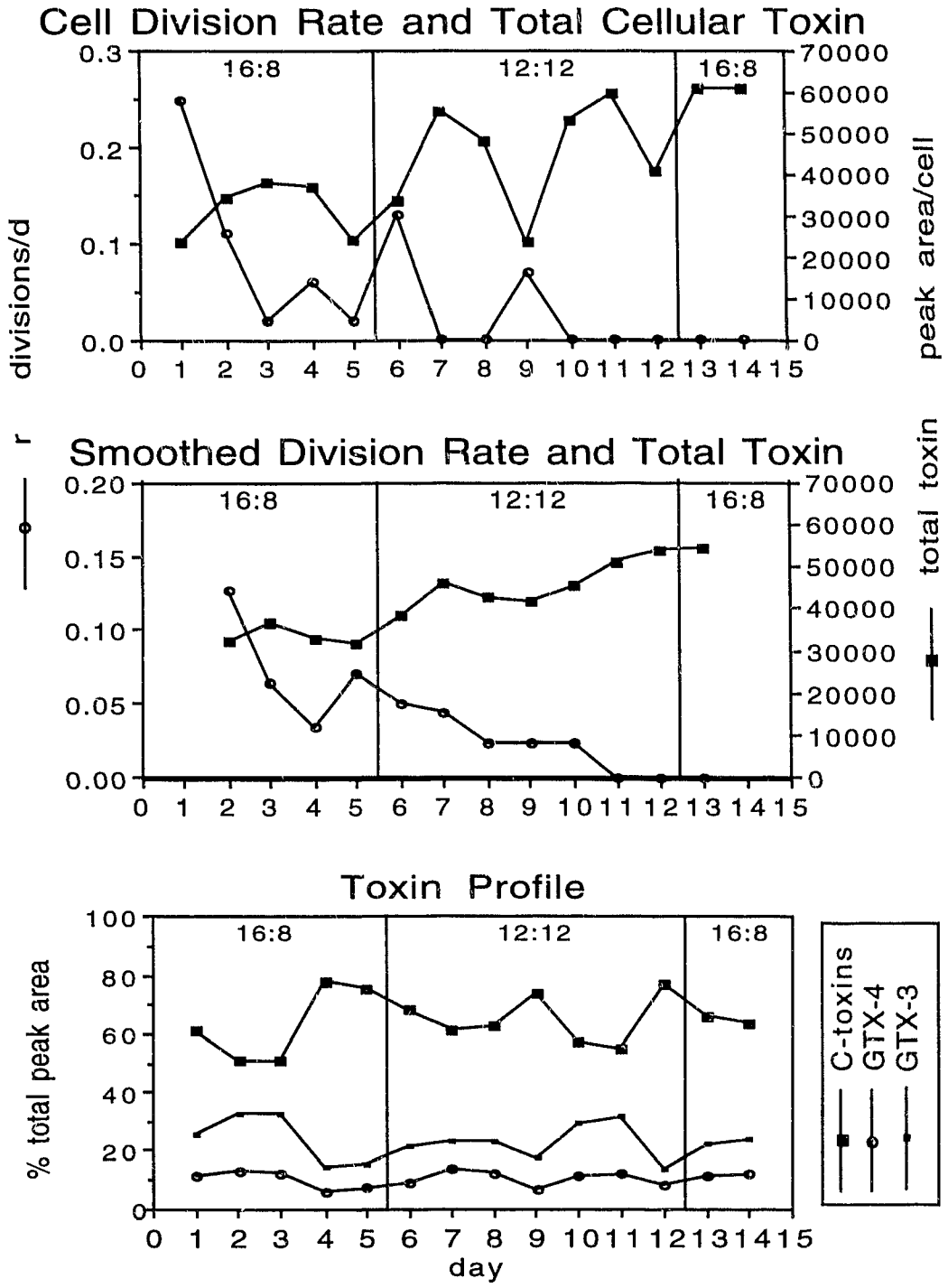


Figure 3.7.1 - Culture 08/92C showing division rates, total cellular toxin (standard and smoothed) and toxin profile at three photoperiods

Culture 08/92B shows strong regular oscillations in both division rate and toxin content. These occur on a similar period to those observed in previous experiments. However, the division rate and toxin content curves are in opposite phase, with peaks in toxin content occuring during periods when r was low. A similar relationship can be seen in Culture 08/92C, although the average division rate in this culture is somewhat lower and there are a number of days of no harvest at all. In neither culture is division rate associated with photoperiod. The harvest rate in Culture 08/92B did drop at the time of the photoperiod reduction, but recovered to normal levels shortly afterwards, which probably represents only a normal oscillation coinciding with the daylength change. Furthermore, the reduction in harvest rate was not statistically significant at $p=0.1$. Culture 08/92C appears to be similar, but the very low harvest rates make certainty impossible. In the smoothed curves, there are no variations in growth and toxin content associated with photoperiod changes. None of the parameters measured showed statistically significant changes associated with the photoperiod change with the exception of total toxin content in Culture 08/92C. This parameter increased, but the

lack of replication in the other culture suggests it may be an anomaly. The generally inverse correlation of the toxin content and division rate can be easily seen in both cultures. The toxin proportions data show the usual inverse relationship between C-toxins and gonyautoxins. As observed previously, peaks in the C:GTX ratio coincide with troughs in the culture growth rate. However, these are not apparently related to changes in photoperiod.

OCTOBER 1992

The final experiment of this series was intended as a duplicate of the previous one. The results are illustrated in Figures 3.8 to 3.8.2. Three cultures were exposed to a reduction and then an increase in daylength. Culture 10/92A stopped growing and only received the daylength reduction whereas Cultures 10/92B and 10/92C were exposed to both, with a brief hiatus between the two sets of samples. An interesting effect occurred in Culture 10/92A, where r dropped to zero after Day 1 but the total toxin content showed considerable variation over the following days. A similar situation can be seen in the early sections of Culture 10/92C. Although the decline in r did occur at the time of daylength

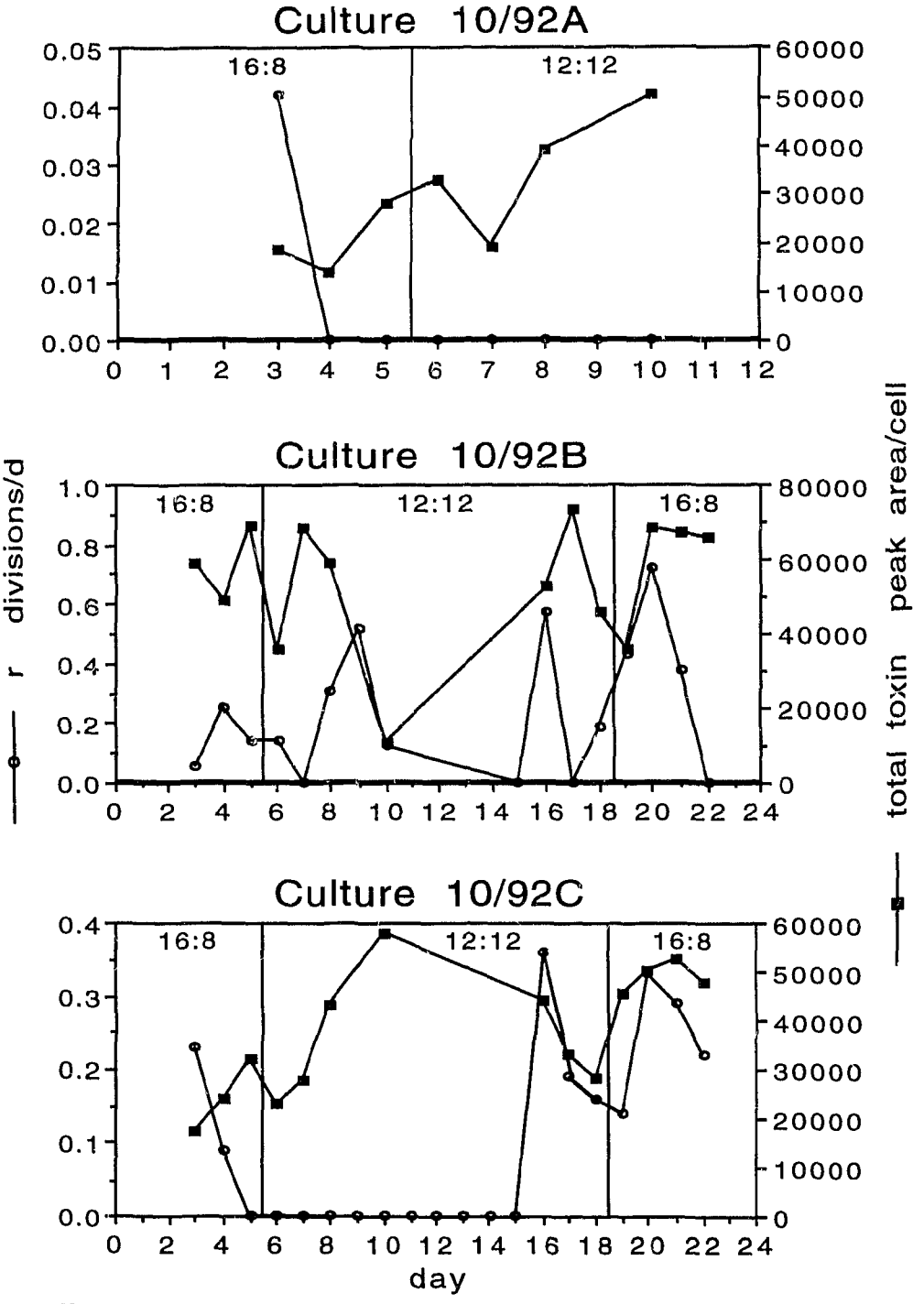


Figure 3.8 - Cell division rate and total cellular toxin content, October 1992

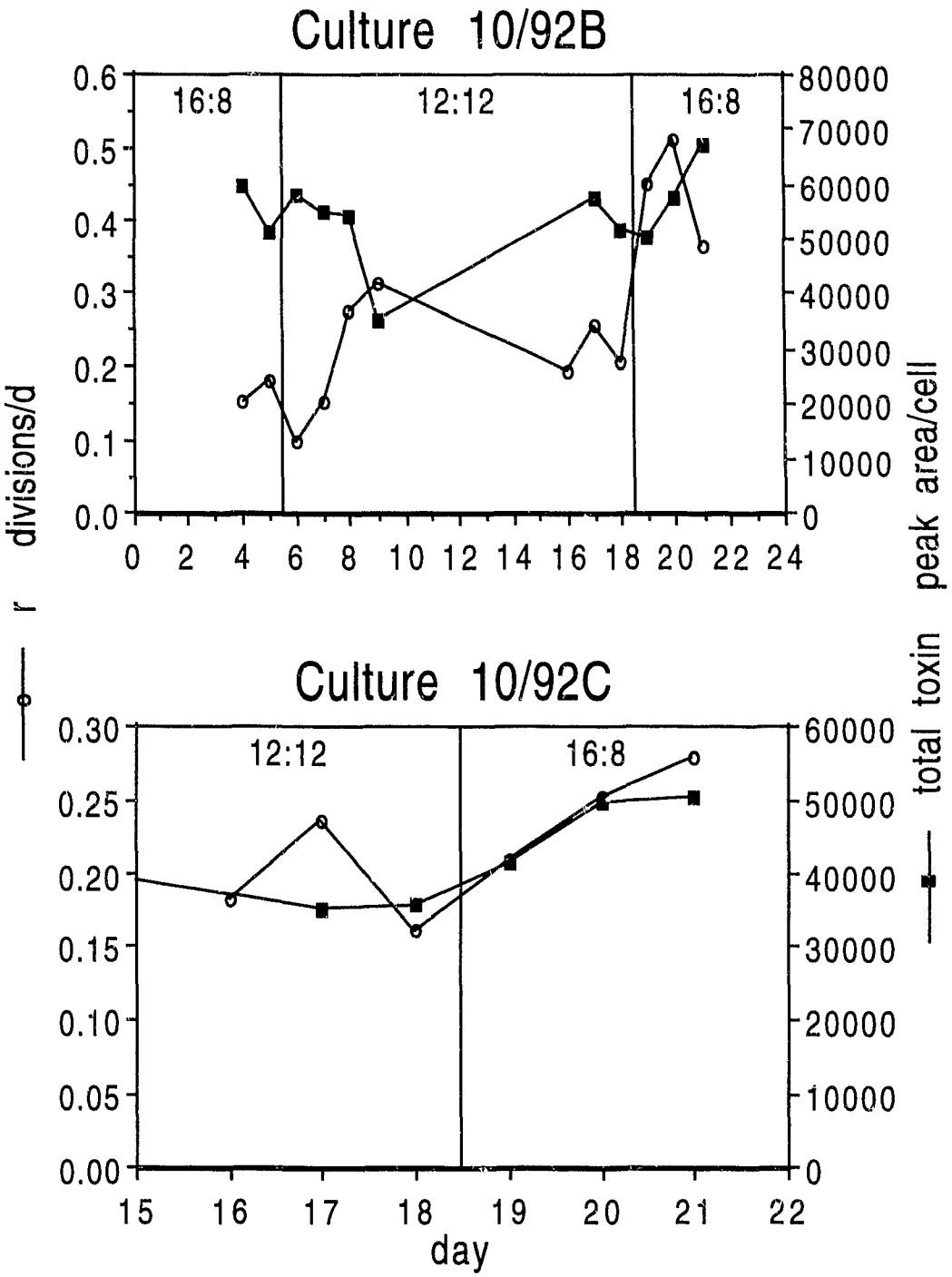


Figure 3.8.1 - Smoothed cell division rate and total toxin content, October 1992. The period of near-zero growth in Culture 10/92C has been omitted from this plot.

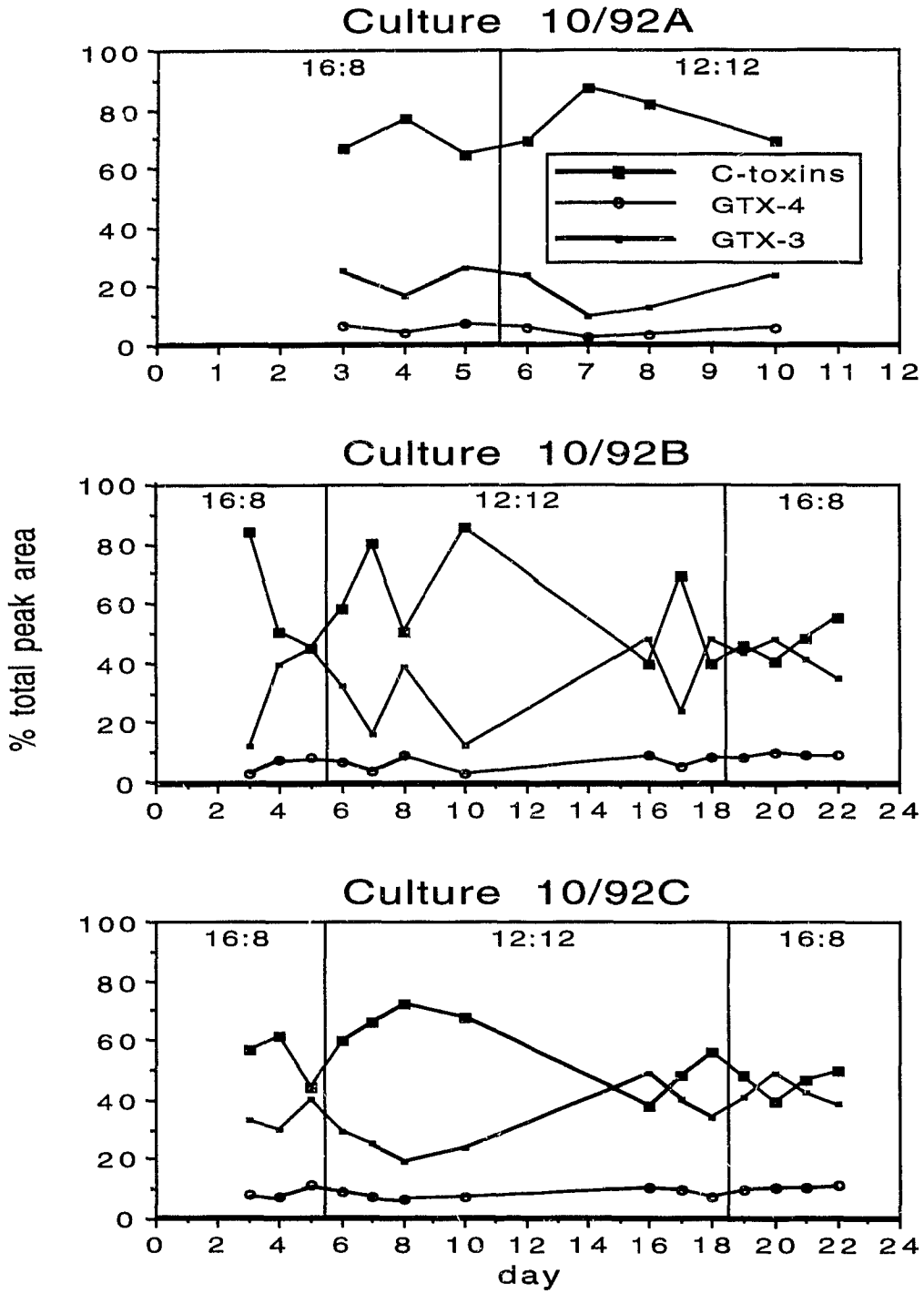


Figure 3.8.2 - Toxin profiles for three cultures grown at two different photoperiods, October 1992

reduction, it actually began a day or two prior to the photoperiod change so the correlation is probably coincidental rather than a cause-effect relationship. This implies two possibilities. Either the turbidostat mechanism is not sensitive enough to detect growth rates at very low levels, or the production of toxins within the cells is governed by mechanisms other than direct relationships to growth. If the first possibility is true, the parameter μ_{cor} can be used as an index of growth. However, as explained in Chapter 2, this technique can produce negative "growth rates" in cases where the observed culture density decreases due to washout events or migration of the cells to inaccessible parts of the culture chamber. If the second possibility is true, it suggests the involvement of an endogenous rhythm of some type in the toxin synthesis mechanism. This latter possibility led to the hypothesis tested in Chapter 4, namely that the toxins vary on a circadian cycle mediated by a biological clock or photoperiod forcing. Culture 10/92B and the latter section of Culture 10/92C show a generally positive relationship between r and toxin content. However, this relationship did not hold for the other cultures. Moreover, even in the smoothed data, there are no changes in the observed parameters which can be

directly correlated to photoperiod. None of the data collected show statistically significant differences associated with the photoperiod change. This lack of correspondence parallels that observed in the previous experiment. Similarly, the toxin profile data (Figure 3.8.2) show much the same kinds of patterns as were present in August-September 1992 also. The ratio of C-toxins to gonyautoxins increases during periods of slow growth, but any correlation these changes might bear to photoperiod is tenuous and probably coincidental.

SUMMARY OF PHOTOPERIOD EXPERIMENTS

The photoperiod experiments conducted here indicate that there is no obvious, direct relationship between photoperiod and the growth or toxin content of A. tamarense, at least under the conditions used in this study. Daylength reductions from 16:8 to 12:12 L:D were not directly correlated with reductions in r. The normal variation in r appeared to be much larger and persistent than any changes related to daylength. Toxin content was not directly related to r, being positively correlated in some cases and negatively in others. None of the parameters measured displayed

statistically significant increases or decreases associated with the photoperiod change. However, as observed in the irradiance-reduction trials, the composition of the toxin changed systematically with r. The proportion of gonyautoxins decreased at low values of r while the proportion of C-toxins increased.

The use of μ_{cor}

As explained in Chapter 2, turbidostat-determined values of r may not precisely reflect the growth rate of the culture if the LED/photocell detector is not sufficiently sensitive to record all significant changes in culture density. With dilute cultures such as those used in this study, this is a very real possibility. By calculating the relative density change of the culture from day to day and adding it to r, a "corrected division rate" (μ_{cor}) can be obtained which should be more representative of the culture growth. In the data presented above, there is no solid relationship between the r and the toxin content of the cultures. It was reasoned that if μ_{cor} were used, it might demonstrate more consistent correlations

between growth rate and toxin content. However, there was no real change in the results.

Corrected growth rates for all cultures can be determined from the data in Appendix 1. Only one example will be discussed here, but it is representative. Figures 3.9 and 3.9.1 illustrate μ_{cor} and total cellular toxin content for the October 1991 irradiance reduction experiment discussed earlier in this chapter. Examination of both normal and smoothed plots indicates no obvious correspondence between the two parameters. In Culture 10/91A the two parameters are apparently inversely correlated while in 10/91B and 10/91C, there are hints of a positive relationship. In addition, the corrected rates are not particularly well related to the light regimen changes either. The uncorrected r values presented earlier are generally better indicators of the culture condition. However, μ_{cor} does correlate quite well with the toxin profile data. Specifically, the gonyautoxin proportion curves match the general shape of the μ_{cor} curve rather closely. This again supports the conclusion that gonyautoxin production is coupled to cell growth. By

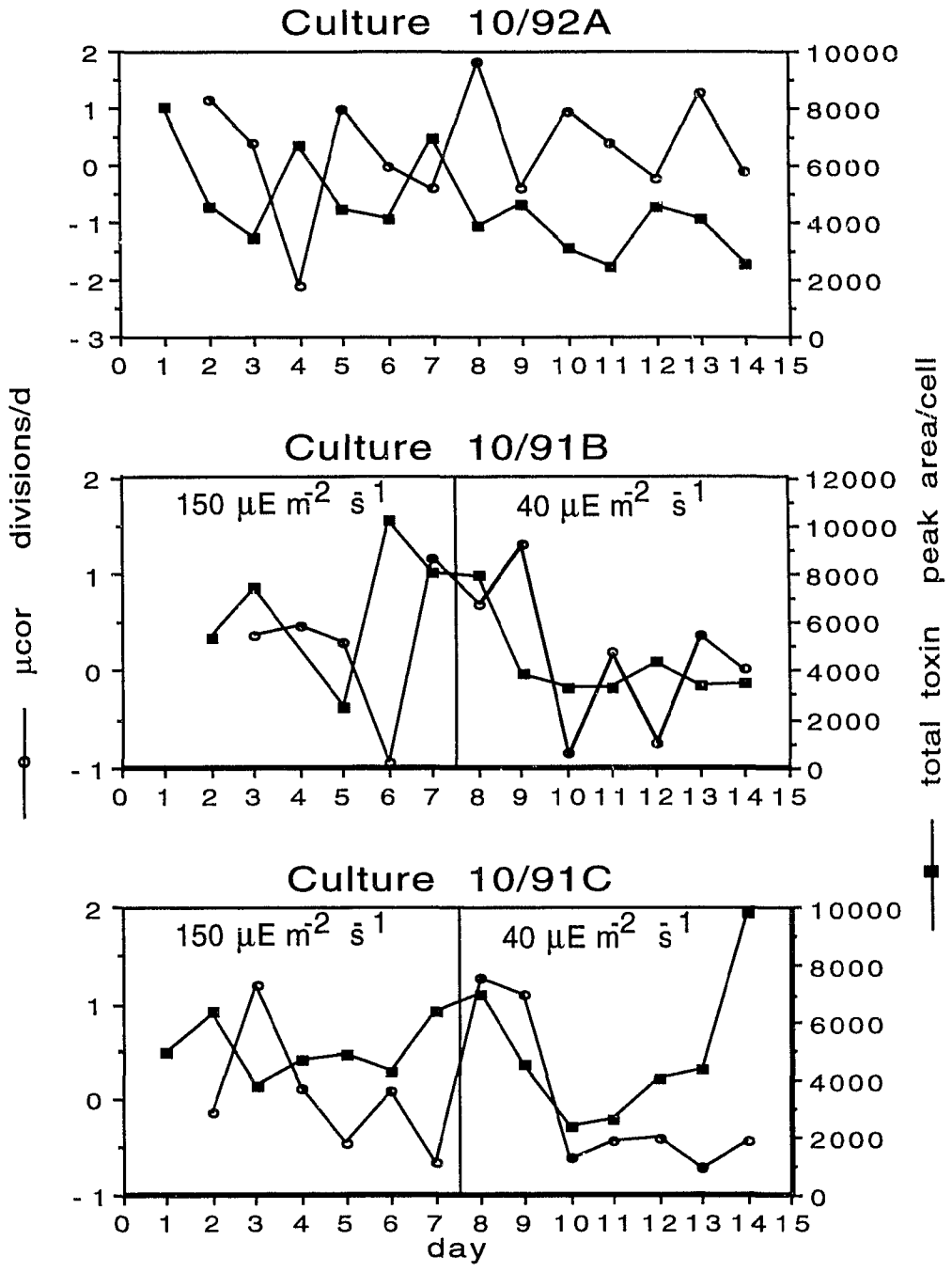


Figure 3.9 - Corrected division rate (µcor) and total cellular toxin content of three cultures, October 1991. Culture 10/91A - control; 10/91B and C - irradiance reduction trials

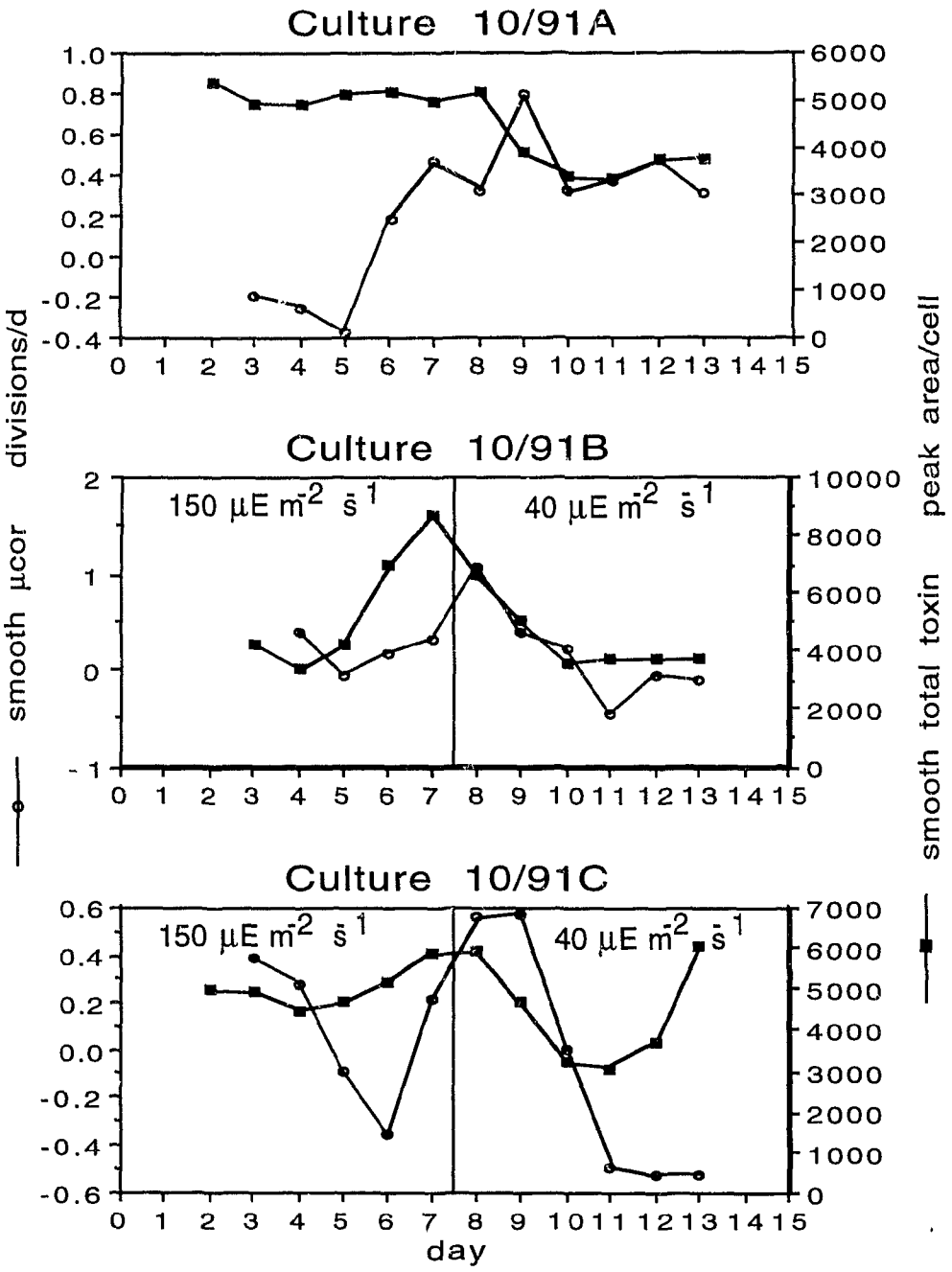


Figure 3.9.1 - Smoothed µcor and total toxin content. Cultures as in Figure 3.8

contrast, total toxin is probably influenced by chemical conversion processes not related to the light regimen.

In general, the use of μ_{cor} did not help resolve the question of toxin content and growth rate variations. The probable reason for this is the large volume and relatively low mixing rate within the culture flask. Due to migration of the cells around the culture chamber, the density of the harvested material was not necessarily a good indicator of the overall culture density. Without stirring the culture so vigorously as to risk killing the cells, it was probably not possible to overcome this difficulty. It became obvious at this point in the project that a different culture and sampling protocol was necessary if short-term variations in growth and toxin content were to be resolved. Finer time-scales and careful attention to homogenization and sampling would be necessary. The experiments which followed employed techniques to address these questions. They will be described in detail in Chapter 4.

Conclusions

The principal conclusions of this chapter are as follows. Total production of toxins was not directly related to r under the study conditions employed. However, in conditions of severe light limitation (40 $\mu E \cdot m^{-2} \cdot s^{-1}$), both r and the total cellular toxin content decreased significantly. This does not appear to be the case with moderate reductions in light availability (100 $\mu E \cdot m^{-2} \cdot s^{-1}$). Even though growth was significantly inhibited, the total toxin content of the cells did not change. It is possible that the light-inhibition of toxin production has a response threshold which is higher than that for growth rate inhibition. The original hypothesis that light-limited growth would result in increased total toxin content is not supported. Instead, the opposite is apparently true, at least under the conditions tested. However, an unexpected result of the work is the finding that the toxin profile is directly correlated with the irradiance. Low light levels correspond to reduced proportions of gonyautoxins compared to the N-sulfocarbamoyl toxins. The cause for this cannot be determined with the available data, but could be a light-mediated enzyme response such as changes

in the synthesis of a precursor molecule, or possibly conversion mechanisms transforming one toxin to another. This could also be growth-rate mediated response. Photoperiod did not have a significant effect on total toxin production, at least under the conditions employed here. There were no observable changes in total toxin content associated with photoperiod reductions. Neither r or μ_{obs} were affected by daylength changes in this study.

Although the total toxin content was not greatly affected by changes in light availability, the changes observed in toxin profiles do have implications for the toxicity of the cells. Throughout this manuscript, the words "toxin content" have been used to refer to the numerical output of the HPLC system. These numbers indicate chromatogram peak response (fluorescence) for the various toxic compounds. They say nothing about the toxic effect of those compounds on organisms which ingest them. Different toxins have different toxicities. For example, the C-toxins are generally weakly toxic while the carbamate compounds (GTX-n, STX and NEO) are highly toxic. The implications of this are obvious. A bloom which changes its toxin profile significantly can become more or less

toxic, depending on the relative proportions of the different compounds in the cells. In the case of light-limited cultures in this study, the toxicity of the cells likely decreased as the proportion of GTX-n in the mixture decreased during periods of slow growth. There is no way of determining whether this response is common to all A. tamarense clones without testing them independently but such a response is possible. It is also possible, though less likely, that the toxin profiles of different clones or species might alter in different ways, resulting in some increasing in toxicity while others might decrease in response to the same environmental change. This would have implications for monitoring and regulation of fisheries which may be geographically specific, depending on the clones and species involved.

The major difficulty encountered in this work was the large degree of variation within and between cultures with respect to toxin content and growth rate. This variation makes interpretation of the data difficult since it masks longer-term fluctuations which may be related to culture conditions such as the light regimen. Smoothing functions applied to the data solved some of the problem,

but may have obscured real dynamics and real events. The toxin content oscillates on a time scale of about three to five days. This suggests the involvement of phased cell division. If the production of toxin is linked to the cell cycle as Anderson (1990) suggests, partial synchronization of the cell cycles in a culture could produce regular fluctuations in the toxin concentration. However, the lack of correlation between these fluctuations and growth rate variations observed so far makes a direct link difficult to establish.

CHAPTER 4

DIURNAL CHANGES IN GROWTH, TOXIN CONTENT AND TOXIN PROFILE IN ALEXANDRIUM TAMARENSE

Introduction

In earlier chapters, it has been shown that the growth of A. tamarense appears to be phased. This is possibly a response to photoperiod entrainment, an internal circadian clock, or other mechanisms which synchronize the cell cycle with photoperiod. Phased growth could explain some of the variation observed in the cultures, such as the three-day cycle of growth and toxin content seen in the earlier data (Chapters 2 and 3). In some cases, cellular toxin content was in phase with the division rate changes, but in other cases was not. In addition, there were situations in which toxin content underwent large changes in the apparent absence of culture growth. This suggests that the synthesis of the toxins can be

affected by internal processes in the cell which are independent of growth rate. It is possible that these processes are governed by circadian rhythms, which are well documented in dinoflagellates. Since A. tamarense generally achieves a division rate of about 0.3 d^{-1}, synchronization of the cells in the culture could produce a cycle in growth rate and toxin content with a period of around three days. It would not be necessary for the culture to be perfectly synchronized to see this effect. Even a partial synchrony or "phasing" would be noticeable and could produce the observed cycles. Interpretation of the data in Chapter 3 was difficult since the nature of the three-day cycle was not known. In the present chapter, the existence of a diurnal cycle in growth and toxin production will be determined and some of its characteristics described.

It is well documented that the dinoflagellate Gonyaulax polyedra possesses a circadian clock which controls cell growth and division as well as photosynthesis, bioluminescence and other functions (Sweeney and Hastings, 1958; Sweeney ,1960; Hastings et al., 1961; Bode et al., 1962; Sulzman et al., 1982; Sweeney and Folli,

1984; Cetta and Anderson, 1990; Homma and Hastings, 1988; 1989; Roennenberg and Hastings, 1991). Since A. tamarense is a closely related species, it is reasonable to suppose that a circadian clock might also be present. There is some evidence for circadian cycles in A. tamarense, including diurnal variations in behaviour such as vertical migration (P.J. Wangersky, pers. comm.). Little has been published to date on the effect of endogenous cycles on toxin synthesis. Anderson (1990a) investigated the production of toxins in Alexandrium spp. over the course of one cell cycle. Most of the toxin production occurred during the S phase of the cell cycle and stopped completely during mitosis and cytokinesis (Figure 4.1). More recent evidence suggests that toxin synthesis begins in G1 phase (Taroncher-Oldenburg et al., 1994).

In an earlier study, Kodama et al. (1982) found that "young" cells of A. tamarense possessed less toxin than "old" ones. The separation of young and old cells was made on the basis of cell diameter by filter-fractionating the culture into three size categories: 20-30 μm, 30-40 μm and 40-95 μm. The smaller cells were assumed to be recently-divided ones while the large ones were

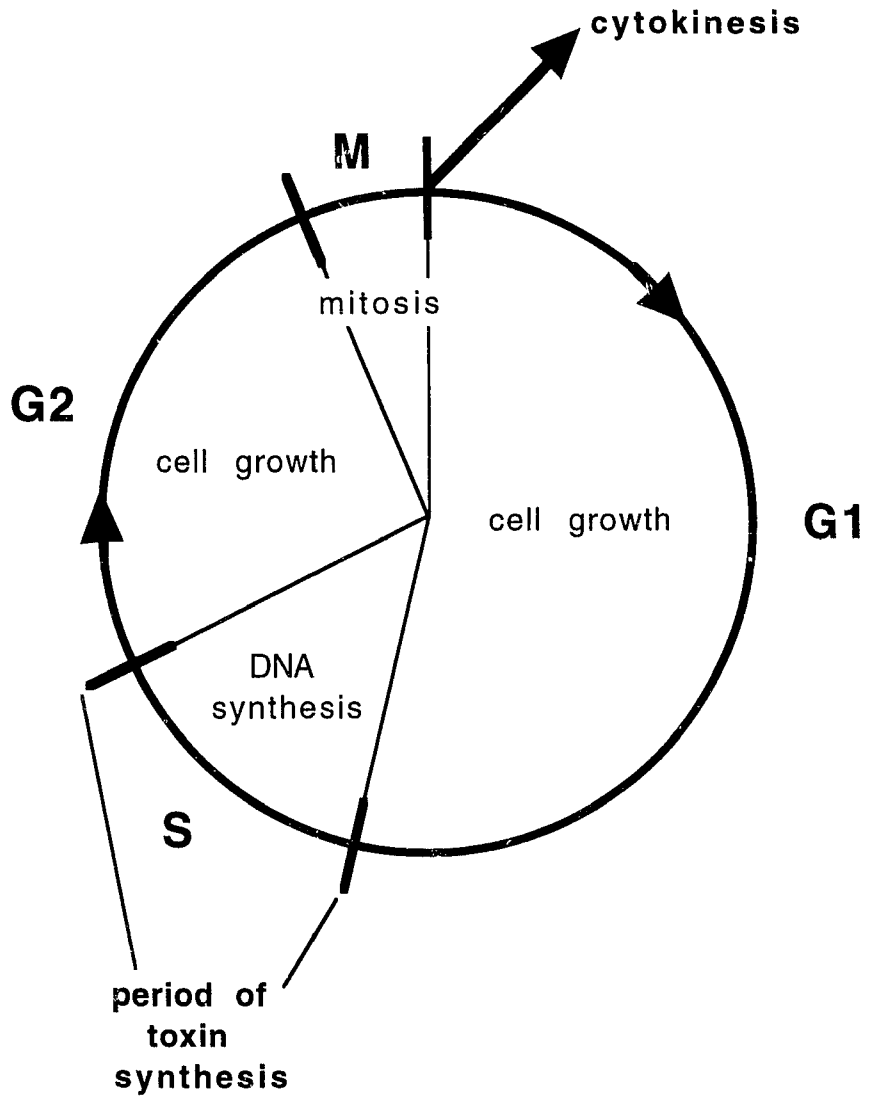


Figure 4.1 - Schematic of cell cycle in A. tamarense showing growth phases and period of toxin synthesis (adapted from Anderson, 1990)

probably old cells which had not divided for some time. The large cells were more toxic than the small ones by a factor of approximately two. This is reminiscent of Anderson's (1990) results which show toxin content decreasing around the time of cell division. The problem with these results is that Kodama et al. (1982) made no attempt to correlate the proportions of young and old cells with the photoperiod. Since cell cycle events and cell division are usually correlated with photoperiod and are probably under the control of a circadian clock, it is reasonable to suppose that toxin synthesis is also controlled by the clock. However, it is possible that the link between toxin content and cell cycle phase is a secondary effect of some other variable, such as fluctuations in cell volume or biomass. It is also possible that light is essential to the production of the toxin (Ogata et al., 1987a; 1989a). It is necessary to control for these possible effects for the full picture to be seen.

This chapter will describe two experiments in which cultures of A. tamarense were sampled at sufficiently high frequency to resolve diurnal variations. In the first (February 1993), the cultures were sampled twice per day in an attempt to resolve the apparent

three-day cycle better than was possible with daily sampling. Cell division events were detected by measuring the proportion of double cells in the culture via direct microscope counts. This provides a simple indicator of cytokinesis from which the time of mitosis can be inferred (Sweeney and Hastings, 1958). No attempt was made to introduce light regimen changes during this experiment. The results indicated that a diurnal cell division cycle existed, but that a higher-resolution sampling scheme was required to fully resolve it.

In the second (July 1993) experiment, the sampling frequency was increased to five times per day, and both cell division events and chlorophyll-*a* concentration were followed throughout the study period. The experiments were designed to identify not just the presence of diurnal cycles, but also the effects of irradiance reductions on those cycles. Also, one culture was tested for the presence of a circadian clock by placing it in 24:0 L:D photoperiod after establishing a diurnal rhythm.

Methods and Materials

The culture methods employed were essentially as described in Chapter 2. A few modifications were made to the cage-culture procedures to make them more suitable for high-frequency sampling. Also, the volume of the culture samples used for toxin analysis was reduced to allow more samples to be taken each day without greatly reducing the total volume of the culture. This meant that in some samples, especially those from the experiments of June and July 1993, the minor constituents of the toxin mixture were not resolved by HPLC. Only the major toxins (C1-C4, GTX-4 and GTX-3) could be measured. However, it should be noted that the minor toxins, GTX-1, GTX-2, NEO and STX constitute at most only a few percent of the mixture so they can be safely ignored when considering bulk toxin production.

February 1993 Experiment

This experiment was designed to better resolve any three-day cycles in toxin production which might exist, and also to indicate if a diurnal cycle was likely. Samples were taken twice per day, at

10:30 and 22:30. The photocycle was set at 16:8 L:D with lights on at 08:00 and off at midnight. Thus, samples were taken in the early morning and late afternoon. A toxin production cycle similar to that described by Anderson (1990a) should be detectable.

Duplicate cultures of A. tamarense (clone OK875-1) were grown in the turbidostats. Growth conditions were as described in earlier chapters with 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance and 10 L/d dilution rate. Toxins were extracted from 0.5 L samples in the usual manner. Since the interval between samples was only 12 h, the turbidostat did not harvest during that period in all cases. It was necessary to collect some of the samples from the culture vessel itself instead of from the harvest reservoir. In these cases, the sample was taken from the flow cell recirculation line through a Y-fitting just downstream of the culture vessel (Figure 2.1). The only other change made to the sampling procedure was the use of filtered seawater instead of distilled water for washing the cells off the Nitex collection filters. Observations of A. tamarense cells exposed to distilled water revealed cell wall ruptures ("blebs") and even lysing (Appendix 3). It is likely that such damage to the cell could result in

cytoplasm, and possibly toxins, being lost to solution or at least into particles too small to be retained on the Nitex or deposited into the centrifuge pellet. Accordingly, the extraction procedure was altered to use 0.2 μm filtered seawater for rinsing the cells off the filters. This made decanting the rinse-water supernatant more difficult since the cell pellet was more likely to be resuspended. It appeared that the presence of cell debris in the samples rinsed with distilled water had "glued" the pellet together. To avoid resuspension of the cell pellet in the seawater-rinsed samples, the rinse-water was removed using a water aspirator. If care was taken, this could be done without disturbing the pellet. It was noted that the seawater-rinsed samples appeared much cleaner than those rinsed in distilled water. The latter were often difficult to remove from the collection filters and had a cloudy appearance after centrifuging. There were usually small pigmented particles clinging to the walls of the centrifuge tubes above the level of the pellet. This material was probably debris from lysed or damaged cells which had remained in the supernatant. The seawater-rinsed samples contained no similar debris. Since it seemed preferable to have samples free of such debris, the seawater rinse procedure was

retained for the experiments of June-July, 1993 which will be discussed later.

Sub-samples (about 50 mL) were taken from the harvested culture for counting cell density and doublet frequency. Microscopic determinations of cell doublet frequency were done first. A 1 mL Sedgwick-Rafter cell was filled using a pipet and observed at 40x magnification under a compound microscope (Leitz Laborlux 12). Single and double cells were counted in ten fields and the proportion of doublet cells calculated. A typical field contained 10 - 20 cells. The remainder of the sample was then analysed with the Coulter Counter to determine culture density.

These cultures suffered more contamination than usual. Both cultures contained unidentified ciliated protozoa in significant numbers, although their concentration rarely exceeded 100 cells/mL and did not appear to increase over the life of the cultures. However, in the case of Culture 02/93B, the concentration of A. tamarense was less than 1000 cells/mL, so results from this culture should be interpreted with caution. A third culture (Culture 02/93C) was

started along with the other two, but became seriously contaminated and was scrapped without analysing any of the toxin samples. At the time of its destruction, Culture 02/93C contained nearly 1000 cells/mL of protozoa. The protozoa were much smaller than the dinoflagellates, approximately 10 μm in length. They were very active cells, swimming at much higher speeds than the dinoflagellates did. The cells were non-pigmented and presumably heterotrophic, although microscope observations revealed no indications that they were ingesting the dinoflagellate cells. Indeed, the size difference would seem to preclude that possibility. It is possible that they were utilizing cellular exudates from the dinoflagellates, but no attempt was made to determine this directly.

If the protozoa were killing dinoflagellates, it would imply that the cell division rate of the cultures was higher than the observed values. However, in the cultures used here, the contamination was minor and should not affect the results to any great degree. As explained above, seriously contaminated cultures were discarded. Furthermore, so long as the dinoflagellates were photosynthesizing and dividing as usual, predation would not likely

affect any of the toxin content data. Any observed cycles of toxin production would likely remain unchanged, even if the concentrations were reduced.

June-July 1993 Experiments

Following the results of the February experiment, another was designed in which the cultures would be sampled more frequently to better resolve the diurnal cycle. Also, the culture and sampling methods were modified somewhat to make the increased sampling possible without depleting the culture volume.

It was noted in the previous experiment and in some of the earlier, daily-sampled data, that the culture density decreased in apparently healthy cultures with numerous double cells. This might be the result of cells leaking from the culture vessel through the cage filters. In order to reduce the possibility that cells might leak through the filters, the flow rate of the medium was reduced from 10 L/d to 3 L/d. This would still provide sufficient supply of nutrients to keep the cells growing at maximal rates while reducing the pressure across the cage filters. Also, to reduce the turbulence

in the cultures and its possible effects on the health of the cells, the flow rate through the air bubbler was reduced to the minimum possible level. The cultures were bubbled vigorously for approximately 30 s immediately prior to sampling to improve homogeneity.

The turbidostat harvest mechanism was also deactivated on the rationale that since the culture was being intensively sampled, there was unlikely to be much surplus production for the turbidostat to remove automatically. Culture growth was followed simply through density increases and the cell doublet index. The LED/photodiode system was kept functioning with the output going to a chart recorder in the hopes that any synchronized cell division events might be detectable as step-changes in the light transmittance curve.

The experiment was designed to detect both the presence of a diurnal or other rhythm and also the effect of light regimen changes on that rhythm and associated toxicity changes. Three cultures were grown for five days. Culture 07/93C was kept as a control with the

normal light regimen of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance and 16:8 L:D photoperiod. Culture 07/93B was subjected to a reduction in irradiance from 150 to 85 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the mid-point of the experiment. Culture 07/93A was transferred from normal conditions to dim, constant light (30 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance, 24:0 L:D photoperiod) at the mid-point. This is a common strategy for determining if the diurnal rhythm is light-mediated or endogenous. If the rhythm persists under constant, dim light, the presence of an endogenous clock is likely. If it does not persist, the rhythm is probably light-induced (Sweeney and Hastings, 1958; Hastings et al.,1961).

This experiment was set up and run twice, with identical experimental design in each case. The first trial took place in June, 1993 and worked well, except for contamination of the cultures by ciliated protozoa (up to 1000 cells/mL). The toxin samples from the June experiment were not analysed except for those from Culture 06/93A (light reduction) which remained sufficiently uncontaminated (≈100 protozoan cells/mL) to be worth using. Samples from the other two cultures (06/93B and 06/93C) were

discarded. The cell doublets, density and chlorophyll-*a* samples were all analysed and the results are in the Appendix. The experiment was repeated in July 1993 with more success. The cultures remained sufficiently free of contamination for the data to be useful.

To resolve the diurnal cycle of cell division, the cultures in the June trial were initially sampled for doublets and culture density every two hours for a period of 24 h, and then at longer intervals for a further 36 h. No toxin samples were taken during this period. It was necessary to establish the time of cell division fairly precisely in order to plan the toxin sampling scheme. According to the results of Anderson (1990a), it would be necessary to sample the cell toxin content frequently in the period surrounding the cell division peak, but when the cells were not dividing, toxin sampling would not need to be as intensive. This initial determination of cell cycle phase was not repeated in the July experiment since it was judged to be unnecessary. The same clone (OK875-1) of A. tamarense was used for both experiments and the culture conditions were identical. It was assumed that the peak in cell division would be the same in both cases. Examination of the lower-frequency (5

samples/day) cell doublet data from the second experiment indicates that this assumption was valid.

The toxin sampling protocol was also modified to permit greater sampling frequency and different types of measurements. Samples were collected five times per day: 08:00 - lights on; 11:00 - early morning; 13:30 - late morning; 16:00 - early afternoon and 23:00 - late afternoon (1h prior to lights off). Since the results of the 24 h cell doublets-sampling trial indicated that there was no cytokinesis during the night, no toxin samples were taken between 24:00 and 08:00. The samples were withdrawn from the flow-cell recirculation line after bubbling the culture to homogenize it. Each sample consisted of approximately 125 mL of culture which was thoroughly mixed and divided into separate portions for toxin analysis, chlorophyll-*a* analysis and counting. Samples for toxin analysis were only 100 mL, instead of 500 mL. To compensate at least partially for the reduced volume of culture in the samples, the extraction volume of acetic acid was reduced from 5 mL to 3 mL. One major difference with this experiment is the use of accurate toxin standards with the usual modified Sullivan procedure, which became

available during 1993. The toxin concentrations in this experiment have been expressed not as peak areas, but as true concentrations (i.e. pg/cell). These values are based upon published concentrations of certified standards (PSP-1 Standard, Marine Analytical Chemistry Standards Program, IMB, NRC, Halifax)

Duplicate 1 mL aliquots were also taken from the 125 mL culture sample for fluorometric chlorophyll-*a* analysis. With the laboratory under subdued lighting, 1 mL samples were filtered onto Gelman GF/A glass-fibre filters using a plastic syringe and Millipore (Swinnex-25) filter holder. Each filter was extracted in 10 mL of 90% analytical grade acetone and stored in the dark at -20 °C in a glass, screw-cap vial for about two weeks prior to analysis. The samples were analysed on a Turner Designs fluorometer equipped with Corning 3-66 (emission) and 5-60 (excitation) filters. The procedure of Parsons et al. (1984) was followed. Duplicate sample sets from each culture were analysed and the results averaged.

A 20 mL aliquot of the culture sample was used for counting. Cell doublets were determined from counts of ten microscope fields in a Sedgwick-Rafter cell at 40x magnification. The remainder of the sample was run through the Coulter Counter to determine culture density.

STATISTICAL ANALYSES

To determine if the diurnal oscillations observed in the data were statistically significant, the time series of measurements for each parameter (cell doublet frequency, toxin content, chlorophyll content, division rate and percentage GTX-3) was subdivided into individual days, normalized to the mean value for each day and then averaged over the full five day experiment. The resulting "daily normalized" values were subjected to a one-way analysis of variance to determine if the daily variation was statistically different from zero. Analysis was performed using Minitab software (release 9.1, Minitab Inc.) run via NCSA Telnet 2.5 on a VAX 4500 mainframe. The computer output is given in Appendix 2.

Results and Discussion

February 1993 Experiments

GROWTH PARAMETERS

In the February experiments, the two cultures maintained reasonable culture density (≥1000 cells/mL) throughout most of the experimental period. Culture 02/93A increased its density steadily during the second week, to a maximum of approximately 2000 cells/mL. By contrast, Culture 02/93B declined steadily and reached a minimum density of 500 cells/mL (Figure 4.2). The difference may be due to the presence of ciliated protozoa in Culture 02/93B. The cell doublet curves indicate that the cultures initially grew well with approximately 30-40 % of the cells dividing each day. However, in time the doublet frequency declined and in Culture 02/93B cell division ceased entirely. Culture 02/93A fared better, with 10-20 % of the cells still dividing on the final day. Culture 02/93B was notable in displaying a fairly regular saw-tooth pattern in the cell doublet curve, in spite of declining density. The period of oscillation is about one day, although there are some inconsistencies. This indicates that a diurnal cycle in cell division is likely. However,

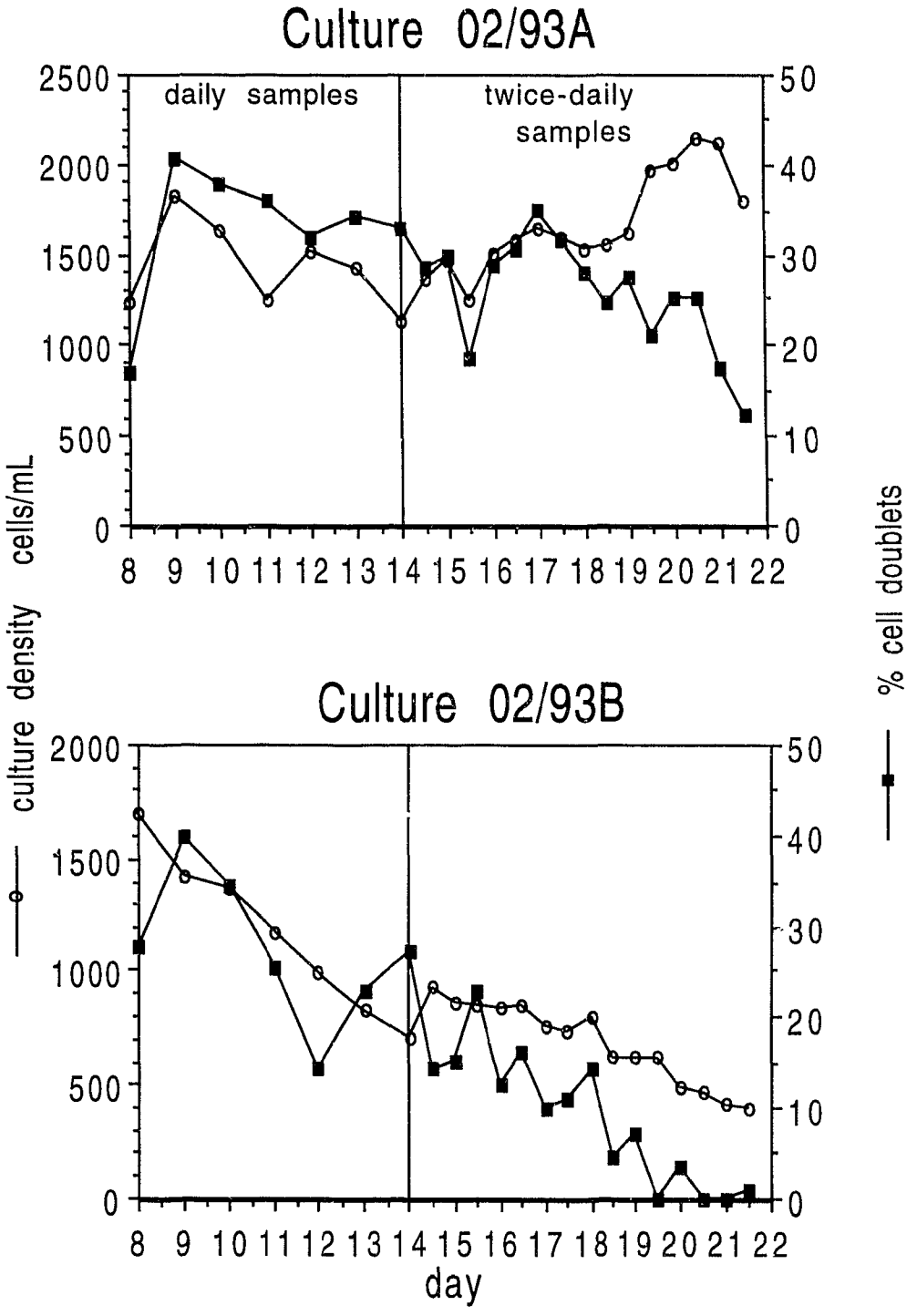


Figure 4.2 - Culture density and cell doublet frequency for February 1992

finer temporal resolution would be necessary to resolve it properly. Culture 02/93A does not show the same degree of diurnal variation. It is possible that sampling at 10:30 and 22:30 cannot resolve a diurnal cycle in cultures of this species. If cell division were to occur during the mid-day period, or mid-night as per Anderson (1990a), the peak in cell doublets would not be revealed by sampling in the morning and evening. The results from the July experiment will indicate that this was the case.

TOXIN CONTENT AND PROFILE

Figures 4.2.1 and 4.2.2 show the cellular toxin content for Cultures 02/93A and 02/93B, respectively. Again, during the early part of the experiment (up to Day 13) the samples were taken daily, while from Day 14 onwards they were taken twice-daily. The upper panel in each figure shows total toxin expressed in units of peak area/cell. The lower panel breaks down the total toxin into its three major components. Saxitoxin and neosaxitoxin have been omitted from the plots for clarity. Their concentrations are about an order of magnitude lower than GTX-4 and thus make a very minor contribution to the total toxin content. After Day 17, levels of STX and NEO in

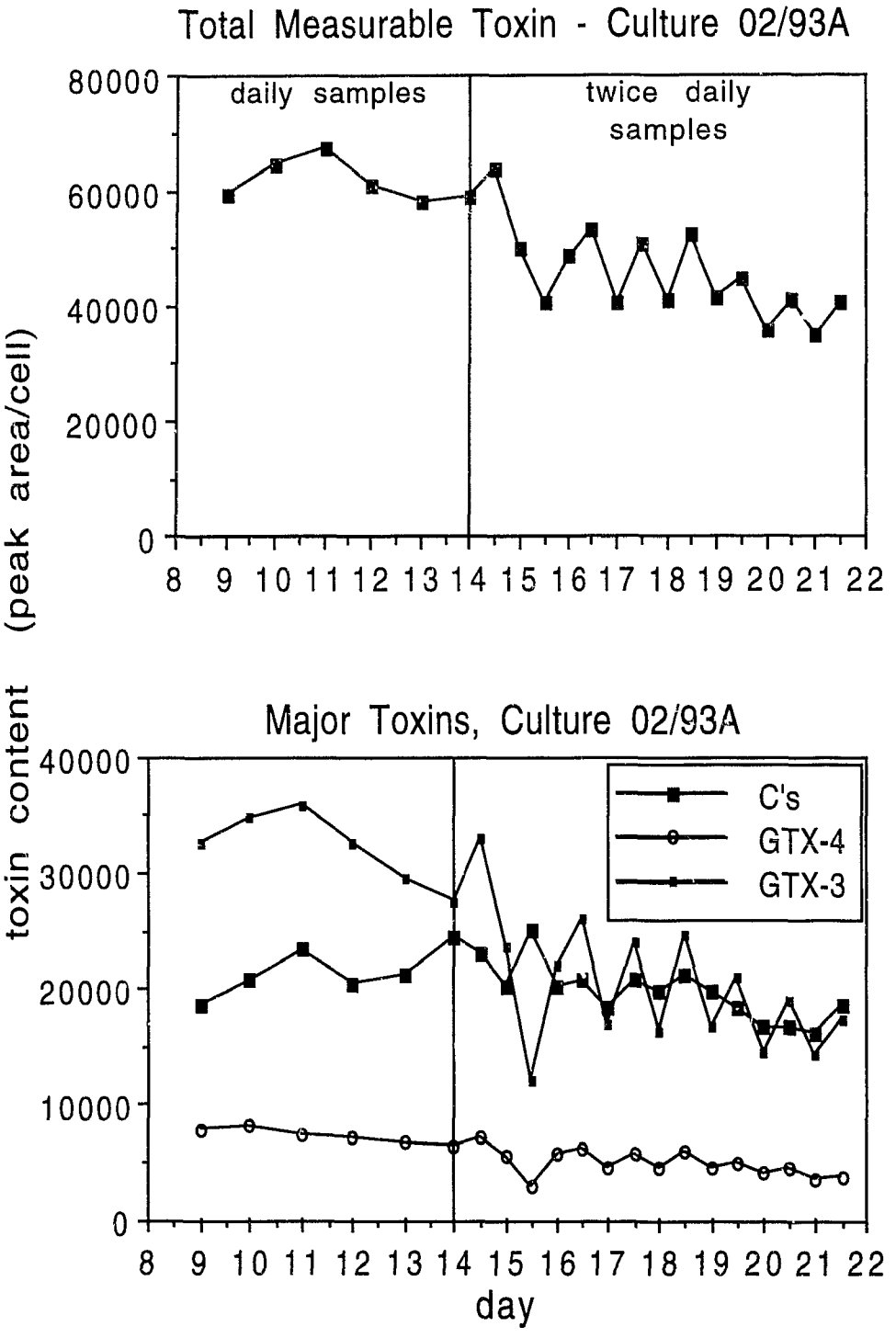


Figure 4.2.1 - Total toxin content and toxin profile of Culture 02/93A showing diurnal variation

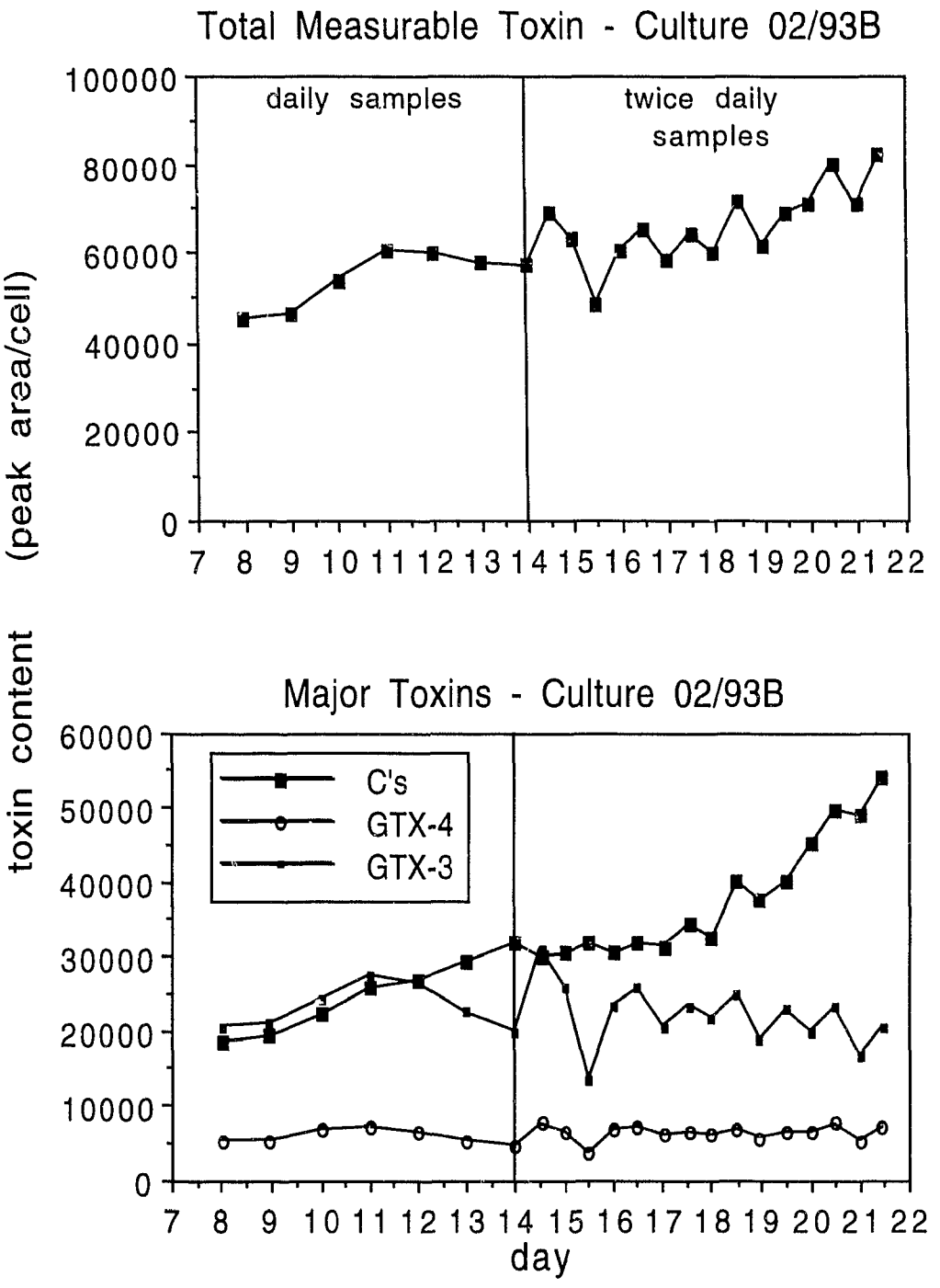


Figure 4.2.2 - Total toxin content and toxin profile of Culture 02/93B showing diurnal variation

Culture 02/93B had declined to undetectable levels. The detection limit for these two toxins is about 0.01 µg/mL in the acetic acid extract. This corresponds to a peak height of a few mV on a 1.2 V scale.

The most striking aspect of the toxin content data is the regular oscillation in the twice-daily samples from Day 14 to Day 21. This diurnal cycle in cellular toxin content is apparent in both the total toxin curves and the individual toxins, although the C-toxins show considerably less variation than the others. In general, the toxin content per cell is low in the morning samples and high in the evening ones. The only break in this pattern is the evening of Day 15 which shows a low toxin content rather than a high one. This exception is notable since it occurs in both cultures and therefore cannot easily be ascribed to sampling error alone. One possible explanation is that the samples on this day were taken at 19:10 and not at 22:30. The diurnal oscillation is present in both cultures although it appears to be of greater amplitude in Culture 02/93A. The cells are obviously accumulating toxin during the day and losing it at night. The fate of the toxin during the night period cannot be

determined from these data. Since other authors report very low extracellular toxin concentrations, except during culture senescence (Prakash, 1967, Proctor et al., 1975), it is unlikely that it is being excreted into the medium. The general timing of toxin accumulation and depletion agrees with that of Anderson (1990a) who observed toxin increasing in the afternoon and declining at night, around the time of mitosis and cytokinesis. It is likely in this case that the toxin is being depleted at night or in early morning, prior to cytokinesis at mid-day. The time of cell division is not certain in this particular case, but the evidence from the turbidostat recorder trace presented in Chapter 2 (Figure 2.4) suggests that this is the usual time of cytokinesis. In order to resolve the cycle more precisely, a finer sampling interval is required. This will be shown in the next section with the June-July 1993 experiments.

The other notable feature of these plots is the relatively large variation in the cellular concentrations of GTX-3 and GTX-4 compared to the C-toxins. As was discovered in the daily-sampled data of Chapter 3, the relative proportions of the toxins in the cells vary as do their total concentrations. The mechanism for this cannot

be determined directly from these data, but some inferences can be drawn. It is possible that GTX-3 and GTX-4 are preferentially synthesized during the day compared to the C-toxins. However, it is also possible that they are subject to chemical conversions caused by variations in the internal chemistry of the cells. For example, the toxins are known to be pH-labile (Boyer et al., 1986; Ledoux et al., 1993), resulting in conversion of toxins from one form to another. However, conversions of this type were observed at very low pH (pH=1) and high temperature (100 °C) and seem unlikely to occur either *in vivo* or in the 0.03 M acetic acid extracts. The extracts are frozen immediately after collection and should therefore be cold enough to inhibit such conversions during storage. Indeed, Boyer et al. (1986) report no detectable conversions in their 0.03 M acetic acid extracts, either during the normal extraction procedure or prolonged storage in a freezer. However, when the strength of the acetic acid was raised to 0.1 M, some limited conversions were observed and boiling during extraction produced significant changes in the toxin profiles. All samples in the present study were frozen immediately after collection and it is possible that during the freezing process, pockets of concentrated acid might form between

the ice crystals and allow some structural conversions to take place. This possibility was not tested, but since all samples were treated identically, it could not explain the periodic variation.

Another possibility is the epimerization of toxins such as GTX-1↔GTX-4 or GTX-2↔GTX-3. These conversions are much more likely, especially under basic conditions. Boyer et al. (1986) report that 0.03 M acetic acid extracts were sufficiently acidic to prevent epimerization. In any case, this process would not produce the variation in the GTX-3:C and GTX-4:C ratios actually observed. Thus, while it is not possible to be certain from these data whether non-biological conversions are responsible for the variations in toxin composition, it seems unlikely given the chemical conditions necessary for structural conversions of the toxins. It is therefore likely that the profile changes observed are the result of diurnally variable rates of toxin synthesis or conversion in the cells.

The final point of note in these experiments is the large increase in the cellular concentration of the C-toxins in Culture 02/93B toward the end of the experiment. GTX-3 and GTX-4 show no

such increase, nor do the toxins in Culture 02/93A. It is during this period that the density and cell division rate of Culture 02/93B were declining steadily. The effect of this is to increase the total toxin content of the cells and change the composition of the mixture considerably. The pattern is similar to that observed in some of the Chapter 3 examples where the C-toxins increased while GTX-3 and GTX-4 declined as the cell division rate declined. Since the reason for the decline in division rate is unknown, it is impossible to say with certainty why the toxin profile should change so radically.

June-July 1993 Experiments

GROWTH PARAMETERS

The culture density data (Figure 4.3) indicate that the four cultures were robust and healthy, with the exception of the latter stages of Culture 07/93A, where the density declined drastically after the light regimen was changed to 24:0 L:D. Cultures 07/93B and 07/93C increased initially and then levelled off in the last couple of days of the experiment. Culture 06/93A had a very stable density of around 2000 cells/mL throughout its life.

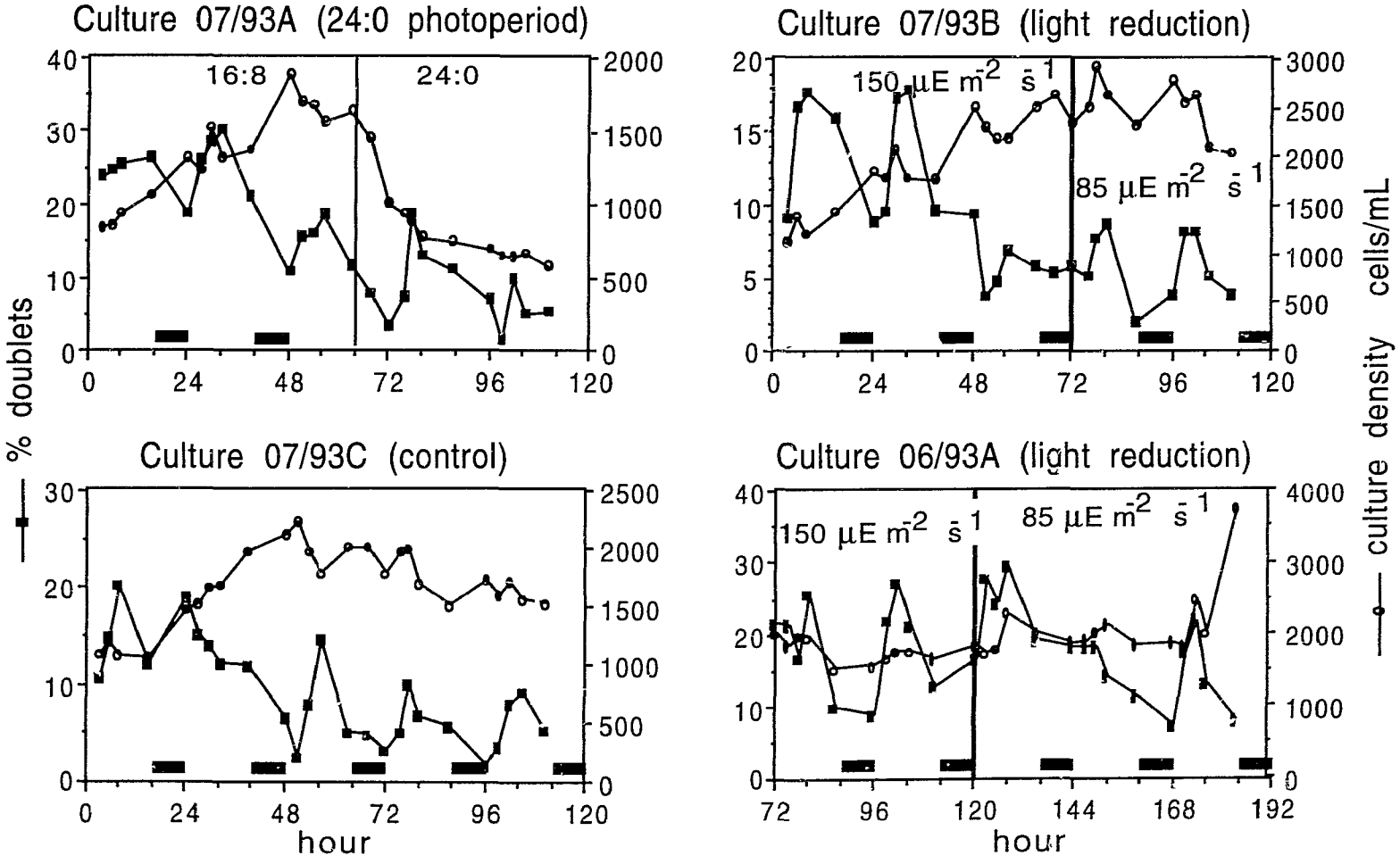


Figure 4.3 - Culture density and cell doublets showing diurnal phasing. Vertical lines indicate change in light regimen. Dark bars indicate night period.

The cell doublet data (Figure 4.3) confirm the suspicions raised by earlier experiments of a diurnal cycle in cell division. In all four cultures, the proportion of double cells increased substantially during the daylight hours and fell at night. This indicates that the cells in the culture were dividing synchronously at roughly the same time each day. The exact timing of the peak varies a bit from day to day, but in general it occurs around mid-day. The synchronization is not complete and "phasing" is a better term for it. At the most, about 30 % of the cells in the culture were dividing at the same time with the number generally falling around 20 %. This indicates a cell generation time of between 3 and 5 days. When embarking on this experiment, attempts were made to synchronize several cultures such that all the cells divided simultaneously. The usual procedure is to place the culture in the dark for a prolonged period (at least several hours) and then release it into the normal photoperiod again. In theory, the dark period should arrest the cell cycles at a point where the cells require light (a "block point", sensu Spudich and Sager, 1980). When placed in normal light again, all the cells begin growing from the same point in the cycle. This procedure has been applied to Alexandrium spp. by

Anderson (1990a) who was able to achieve a considerable degree of synchronization.

In the present experiments, it was not possible to achieve much more than 30% synchronization of cell division. Placing the cultures in the dark for more than a day resulted in the death of most of the cells, while periods less than that did not produce complete synchronization. Since the cultures appear to be about 30% synchronized without dark-period treatments, the procedure was not used for these experiments. It is possible that the relatively slow division rate of these cultures made it impossible to place them in darkness long enough to achieve synchronization without killing most of the cells. Indeed, organisms with cell division rates of around 1 d^{-1} can achieve synchrony without external treatment (Chisholm et al., 1984). However, in spite of imperfect synchronization in the present experiment, the daily peaks in cell doublet frequency are statistically significant at p=0.1 according to the ANOVA results. All but culture 07/93C are significant at p=0.05. They thus might be expected to produce detectable diurnal cycles in other cellular parameters. Figure 4.3.1 shows the daily-normalized

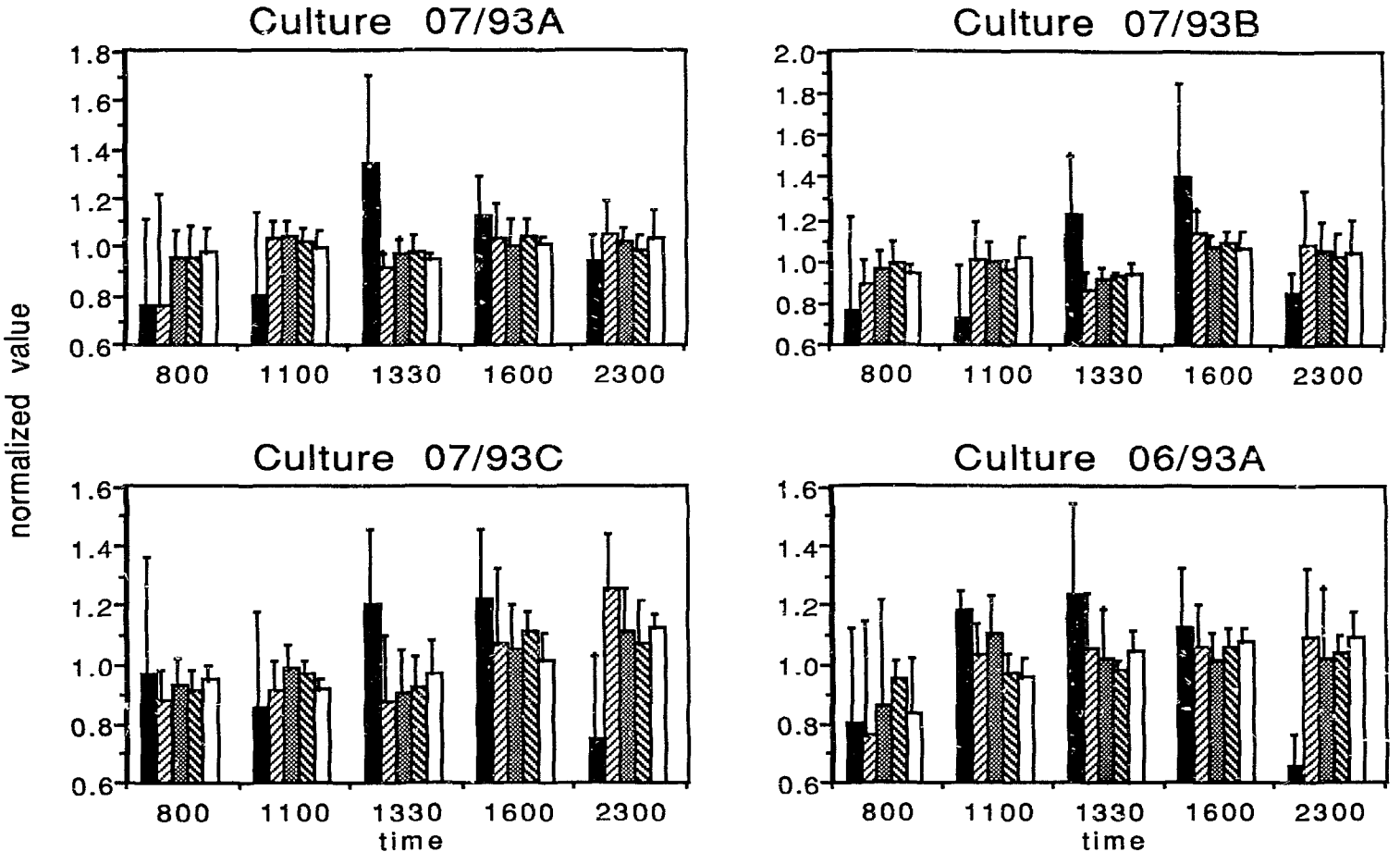


Figure 4.3.1 - Daily normalized parameters for four cultures (mean and standard deviation) ■ cell doublets, ▨ GTX-3/cell, ▩ total toxin/cell, ▧ chlorophyll-a/cell, □ GTX-3/total toxin

values (see Methods and Materials - this chapter) for cell doublets, GTX-3 (pg/cell), total toxin (pg/cell), chlorophyll-*a* (pg/cell), and GTX-3/total toxin.

The timing of the cell division peak does not correspond with that reported in the literature for this and other dinoflagellates. Anderson (1990a) observed mitosis and cytokinesis occurring in the dark hours just prior to the start of the light period. Other authors working with dinoflagellates have also observed cell division occurring at night, or shortly after dawn (Sweeney and Hastings, 1958, Sweeney, 1960, Hastings et al., 1961, Weiler and Chisholm, 1976, Weiler and Eppley, 1979, Weiler and Karl, 1979). There is some variation in the measurements, but it appears to be unusual to see dinoflagellate cell division in the mid-day period (Chisholm, 1981, Chisholm et al., 1984). It is possible that this strain of A. tamarense is unusual in the timing of its cell division, or more likely that the culture conditions have caused the division peak to shift.

One possible factor is the availability of nutrients. Phytoplankton are known to synchronize their cell cycles in response to nutrient pulses (Chisholm et al., 1984) so clearly light is not the only factor which may be involved. Most of the historical information on this topic has been collected using batch cultures. In an unstirred batch culture, dinoflagellate cells tend to migrate to the surface during the day and form a concentrated layer. At night the cells disperse throughout the culture. It is possible that the nutrients are depleted within the cell aggregation during the day, thereby limiting nutrient uptake and preventing cell division. Only at night when the cells have access to nutrients lower in the water column can they divide. This is not unlike the situation observed in stratified, natural water columns. Unpublished data indicate that other clones of Alexandrium, as well as other species of dinoflagellates, also divide near midday when grown in turbidostats (P.J. Wangersky, pers. comm.). The nutrient supply in a turbidostat is continuous and the culture is well mixed, allowing the phytoplankton cells to take up nutrients as required. Thus, cell division is not restricted to the night period and can occur whenever the cells' physiology permits. In a sense it can be argued that the cells in a

turbidostat divide when they "want" to while those in a batch culture divide when the nutrient supply permits.

The fact that the daily cell doublet peaks continue in 24 h light in Culture 07/93A is strong evidence that an endogenous clock is involved in regulating cell division. Endogenous clocks are postulated in the related species Gonyaulax polyedra (Hastings and Sweeney, 1960, Sulzman et al., 1982, Homma and Hastings, 1989, Roennenberg and Hastings, 1991). In addition, long-term endogenous clocks (circannual cycles) have been identified in toxic species of Alexandrium (Yentsch and Mague, 1980, Anderson and Keafer, 1987, Costas et al., 1990).

CHLOROPHYLL-*a* DATA

The cellular chlorophyll-*a* concentrations (Figure 4.3.2) also exhibit increases during the day and declines at night, similar to the cell doublet curves. The diurnal pattern is most noticeable and persistent for Culture 07/93C (control culture), although it is present in the other two cultures as well, especially the early stages of Cultures 07/93A and 06/93A. In all the cultures except

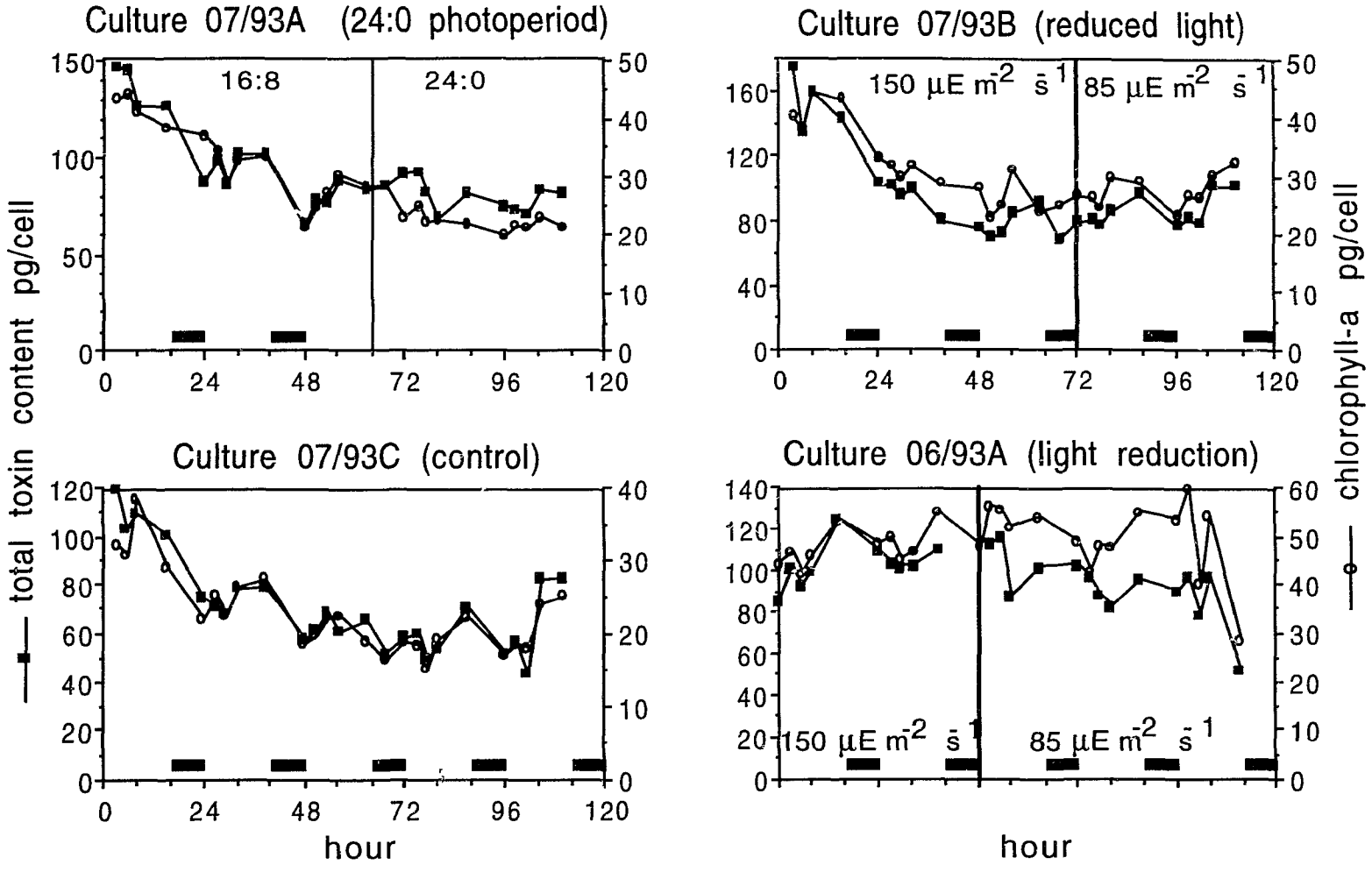


Figure 4.3.2 - Total cellular toxin and chlorophyll-a content of four cultures. Vertical line indicates change in light regimen. Horizontal bars are night periods.

Culture 07/93A, the diurnal variation (Figure 4.3.1) is statistically significant at p=0.05. The lack of significance in Culture 07/93A is likely due to the inclusion of the last two days in the normalized data. For the first three days the oscillation is strong and significant, but later during the two days in dim 24 h light, the diurnal cycle of chlorophyll-*a* content essentially disappears. Diurnal variation in chlorophyll synthesis is well documented in other species. Loeblich (1977) describes daytime increases in cellular chlorophyll content in synchronized cultures of the dinoflagellate Cachonia niei. Paasche (1968) found similar patterns in the diatom Ditylum brightwellii. The opposite pattern of cellular chlorophyll content with peaks at night was observed in Heterocapsa sp. by Latasa et al. (1992), although the authors stated that this pattern was unusual. A comprehensive review of diurnal changes in cellular properties of various phytoplankton species can be found in Prézelin (1992) showing that in general, daytime synthesis of chlorophyll is normal in these organisms. Interestingly, Hastings et al. (1961) found no diurnal variation in cellular chlorophyll content of Gonyaulax polyedra although they did detect diurnal rhythms in both photosynthetic rate and capacity.

The variation of cellular chlorophyll in this experiment could indicate two things. Either the volume (and hence biomass) of the cells is increasing and decreasing or the amount of chlorophyll in the cells is changing, without any changes in cell size. Obviously, a combination of these is also possible. In either case, the cells are responding to the day/night cycle by accumulating and losing chlorophyll (or partitioning it to daughter cells). Although the temporal resolution of the data is not sufficient to confirm it with certainty, one possibility is that the chlorophyll content increases prior to mitosis and decreases when cytokinesis occurs.

TOXIN CONTENT AND PROFILE

Total cellular toxin content (Figure 4.3.2) shows some evidence of a diurnal cycle, although the variation is not as great as in the cell doublets data. Subjecting the daily-normalized data (Figure 4.3.1) to ANOVA indicates the variation is weakly significant in Cultures 07/93B and 07/93C ($p \approx 0.1$) and not significant in 07/93A and 06/93A. The total toxin per cell peaks around the end of the daylight hours, indicating that the bulk of toxin synthesis is

occurring during the day, while a decline in toxin content occurrs at night. The cellular GTX-3 content (Figure 4.3.3) shows considerably more diurnal variation; strong peaks occur at the end of the light period. ANOVA of the daily-normalized results (Figure 4.3.1) shows significant variation in Culture 07/93C ($p=0.05$), weakly significant in 07/93B ($p=0.1$) and not significant in 07/93A and 06/93A. It should be noted here that the 120 h toxin sample in Culture 06/93A was so widely different from the others (one order of magnitude lower) that it has been discarded as an anomaly. As was observed in the February 1993 data, the percentage GTX-3 of total toxin also shows diurnal variation (Figure 4.3.4). Strong daytime peaks are apparent in all cultures. Daily normalized values for % GTX-3 (Figure 4.3.1) show diurnal variation which is significant in 07/93C and 06/93A ($p=0.05$), but not significant in the other two cultures. As explained for the cellular chlorophyll-*a* data, this may be an artifact of including data from two light regimens. In any case, the diurnal variation observed supports the conclusion that GTX-3 is preferentially synthesized during the day compared to the N-sulfocarbamoyl toxins. Since the diurnal variation in these data is better resolved than in the February examples, it is reasonable to

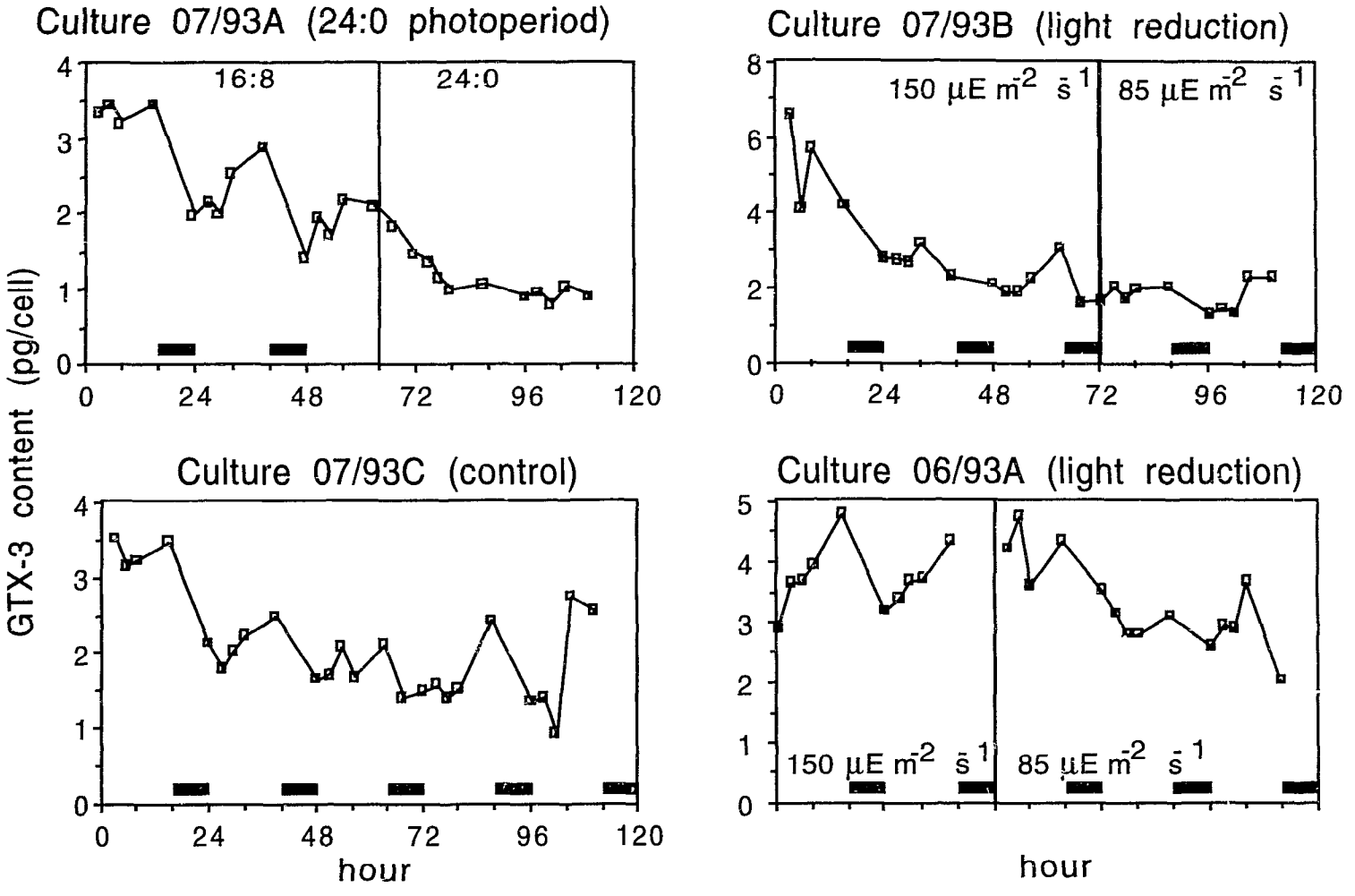


Figure 4.3.3 - Cellular GTX-3 content in four cultures showing diurnal variation and effect of light regime changes. Horizontal bars indicate dark periods.

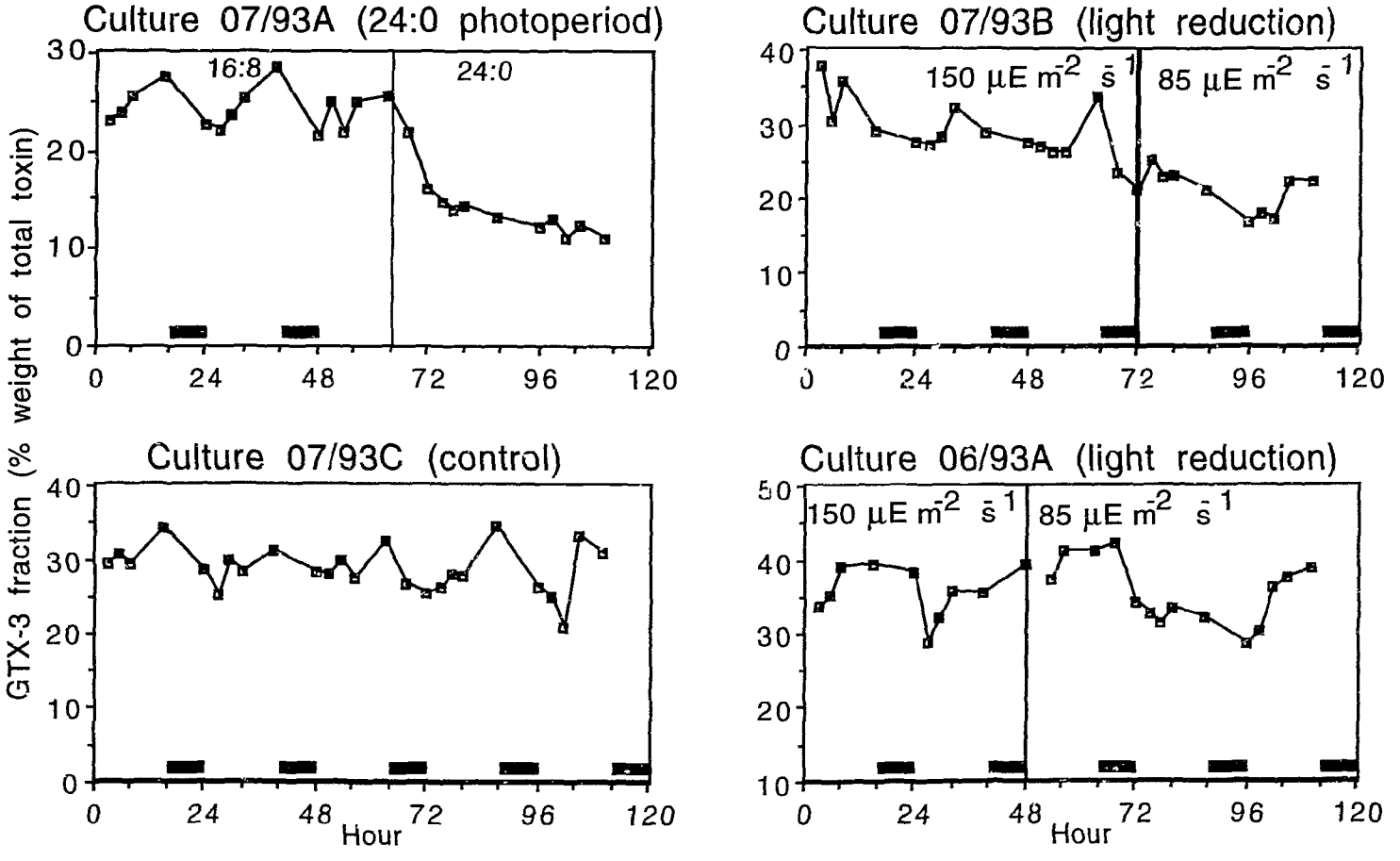


Figure 4.3.4 - GTX-3 fraction by weight for four cultures showing diurnal phasing and effects of light regimen. Horizontal bars indicate night periods.

conclude that this effect is real and not an artifact of the sampling procedure.

It is also apparent from Figure 4.3.2 that total toxin and chlorophyll-*a* content show a good correlation during the course of the experiment. Although the concentrations of the two components are quite different, the relative magnitudes of the changes are similar. This suggests a direct relationship between toxin and chlorophyll content, and therefore implies a direct relationship of toxin content to biomass under steady-state growth conditions. This can be resolved another way by plotting toxin per unit chlorophyll (Figure 4.3.5) and also the linear regression of toxin against chlorophyll-*a* (Figure 4.3.6). Figure 4.3.5 shows a curve with much less diurnal variation than Figure 4.3.2 (total toxin per cell). There is little indication that there is a daily peak in toxin per unit chlorophyll-*a* in any of the cultures. In cultures 07/93A, 07/93B and 07/93C, the regression lines (Figure 4.3.6) are well-fitted with high correlation coefficients. Since chlorophyll-*a* content is generally taken as an index of biomass, it is reasonable to conclude that cellular toxin content is linearly related to biomass, at least under

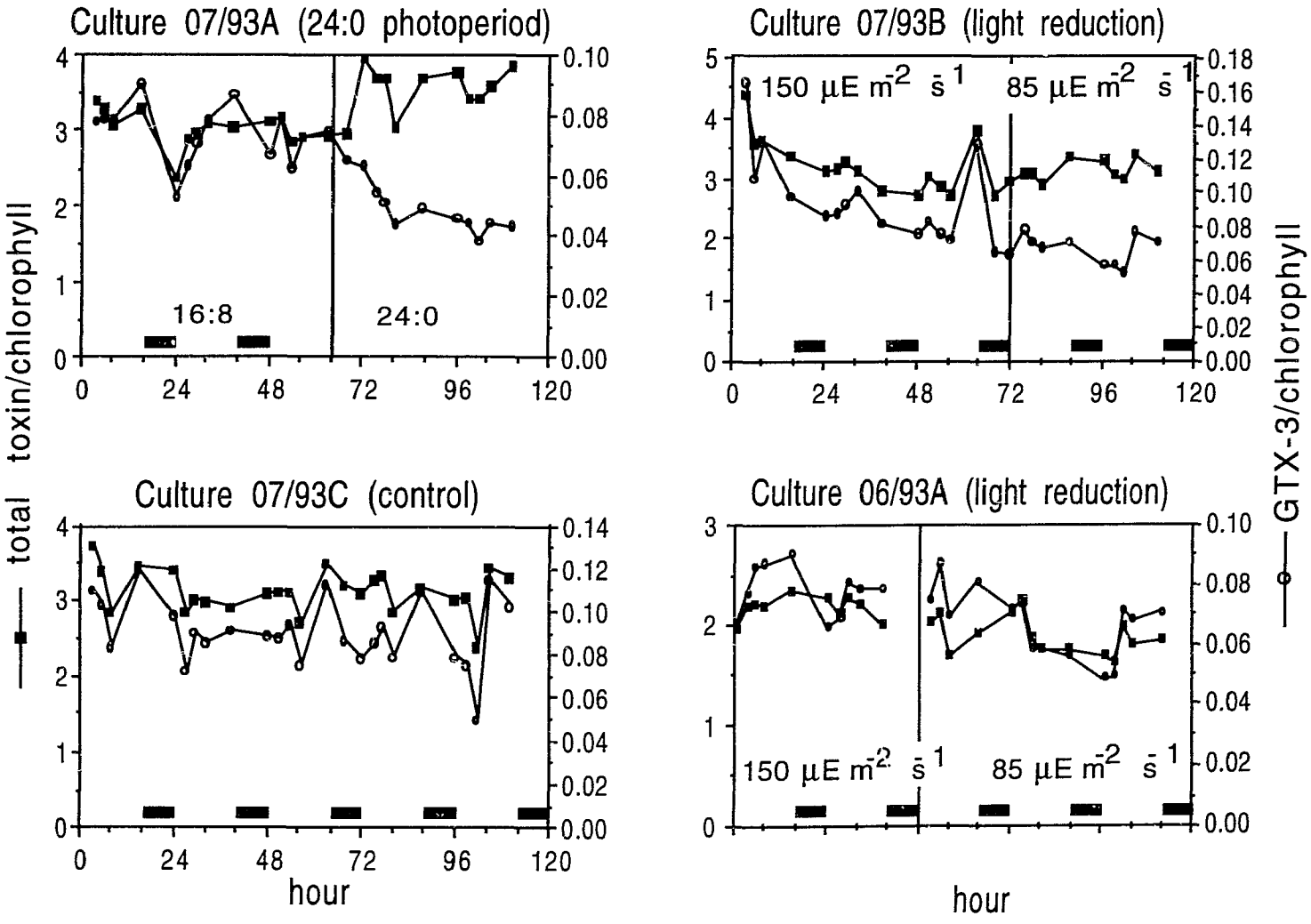


Figure 4.3.5 - Plots of GTX-3 and total toxins per unit chlorophyll-*a* (pg/cell) for four cultures showing light regimen changes. Horizontal bars indicate dark periods.

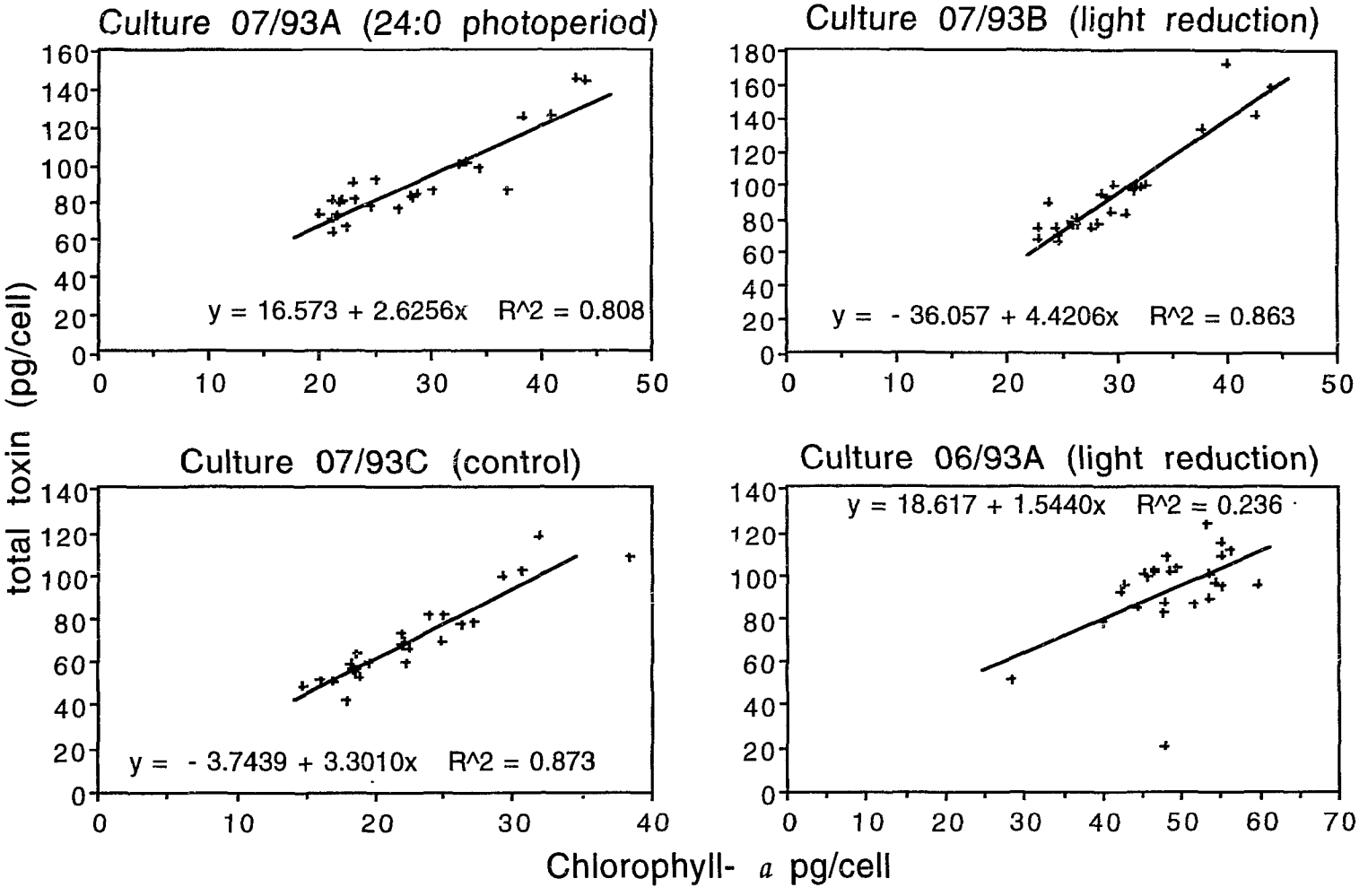


Figure 4.3.6 - Linear regressions of total cellular toxin vs chlorophyll-*a* for four cultures

conditions of steady-state growth. The exception to this is Culture 06/93A which produced essentially a scatter-plot relationship between toxin and chlorophyll-*a*. There is much less variation in both compounds in Culture 06/93A compared to the others. This may make any relationship difficult to resolve. Indeed, if the upper four points in the toxin vs. chlorophyll-*a* regression of Culture 07/93B are omitted, the correlation is a lot worse in that case also. Possibly this is a result of the fact that these two cultures are the light-reduction trials, where the environmental changes tend to influence toxin and chlorophyll-*a* in opposite directions. This would eliminate any correlation between the two which might be seen more clearly in the control culture.

LIGHT REGIMEN CHANGES

The effects of the light regimen changes on the cultures are easily seen in these time-series. The control (Culture 07/93C) displayed a regular pattern throughout the culture cycle. Cell concentration increased initially and then levelled off while the cell doublet index showed regular daily peaks (Figure 4.3). Although the average doublet frequency declined slightly during the life of the

culture, the cells were still dividing at the end and exhibiting a significant peak in cell doublets even on the final day. Culture 07/93B (light reduction trial) showed a large decrease in the cell doublet frequency halfway through the experiment which appeared to begin before the light reduction took place. However, the culture density remained fairly stable throughout this period. The other light reduction trial (Culture 06/93A) was rather similar, although the doublet frequency reduction occurred after the irradiance reduction. Whether or not these two events are linked is difficult to say, but the almost complete absence of a cell division peak on the day after the irradiance reduction suggests that division was inhibited by the drop in available light energy. The results from the 24:0 L:D photoperiod trial (Culture 07/93A) demonstrate the difficulty of designing adequate experiments to test for the presence of circadian clocks. In this culture, the photoperiod was changed from 16:8 to 24:0 L:D at 64 h. However, the resulting increase in available light energy would result in inhibiting their growth or destroying the periodic cycle (Sweeney and Hastings, 1958, Hastings et al., 1961). Accordingly, the light intensity was also reduced to low levels, similar to those used by Hastings. The

irradiance after the photoperiod change was about 30 $\mu E \cdot m^{-2} \cdot s^{-1}$, reduced from the normal 150 $\mu E \cdot m^{-2} \cdot s^{-1}$. The culture density dropped by about 50 % during the first few hours of the new light regimen. This is probably due to the reduction in light intensity. The cells did not sink to the bottom, nor lose motility and drop out of suspension. There was no visible accumulation of debris on the floor of the culture chamber the following day, nor were cells observed clinging to the walls of the culture vessel. The only remaining explanation is that they lysed and the fragments were washed out of the vessel or had lost sufficient amounts of pigment that they were not noticeable on the floor of the chamber. Either way, it is obvious that the change to 24:0 L:D dim light was too great a stress for these cells and they stopped growing. However, it is interesting to note that while the proportion of dividing cells decreased after the photoperiod change, the diurnal peaks did not disappear. Even two days after the change to 24 h light, a peak in the proportion of dividing cells occurred in the mid-day period, just as it did under the normal photoperiod. This suggests that the diurnal cycles of cell division observed in this experiment are not the result of the cells

reacting to the presence or absence of light, but rather are controlled by a circadian clock.

The plots of total cellular toxin (Figure 4.3.2) show less evidence of the influence of changes in light regimen. The shapes of the curves in Cultures 07/93A, 07/93B and 06/93A are not much different from that of the control culture. The only difference might be a slight reduction in the diurnal variation of the curve of Culture 07/93A in 24 h light. The chlorophyll-*a* curves are more variable. Cultures 07/93B, 07/93C and 06/93A are much the same but Culture 07/93A shows a distinct flattening of the curve resulting in the almost complete disappearance of the diurnal cycle. Thus, although the strong cell division peaks continued in 24 h light, the diurnal phasing of cellular chlorophyll-*a* synthesis depends on the forcing of the light/dark cycle.

Cellular GTX-3 content (Figure 4.3.3) displayed the most interesting behaviour of the cellular parameters measured. In the control culture, cellular GTX-3 had a strong diurnal cycle which persisted throughout the experiment. This was also true in the

irradiance reduction trial, Culture 07/93B. However, in Culture 07/93A, the cellular content of GTX-3 dropped considerably with the onset of the 24 h light regimen. Furthermore, the daily peaks in concentration also disappeared. Culture 06/93A experienced a similar drop in cellular GTX-3 content although there is some sign that the levels were recovering on the last day. These results lead to the conclusion that the synthesis of GTX-3 is light-dependent and apparently stops when the light intensity drops too low. It is not possible to determine with certainty whether the cessation of GTX-3 synthesis in Culture A is due to the change in photoperiod or to the reduction in irradiance, but the effect is obvious. Examination of the plots of toxin per unit chlorophyll-*a* (Figure 4.3.5) shows fairly steady levels for total toxin, but a large reduction for GTX-3 at the onset of 24 h light. The reduced light of Culture 07/93B did not appear to have much effect on this parameter. Culture 06/93A shows a reduction in both total toxin and GTX-3 per unit chlorophyll-*a*, 24 h after the light reduction. The drop is proportionately larger for GTX-3 than total toxin, supporting the conclusions deduced from Culture A.

The percentage of GTX-3 in the cells (Figure 4.3.4) exhibits a great deal of variation correlated with the light regimen changes. In Culture 07/93C, the diurnal cycle is very strong while the daily averages are quite stable throughout the life of the culture. Culture 07/93B, by contrast, shows a reduction in the proportion of GTX-3 after the light reduction. A similar reduction is visible in Culture 06/93A. Culture 07/93A displays a very large reduction in the GTX-3 proportion after the change to dim, 24 h light. The results support the conclusion that light is essential to the production of GTX-3 and that the relative rates of synthesis of the various toxins are light-dependent. The rationale for this conclusion is essentially that outlined for the diurnal changes discussed with reference to the February 1993 data, and the longer-term changes in Chapter 3. Changes in toxin composition could be the result of either differential rates of synthesis or conversion of toxin from one form to another in the cells. Since the conditions required for spontaneous conversion are rather severe, the logical conclusion is that the toxins are being synthesized at different rates under different conditions. Similar conclusions were reached by Anderson et al. (1990a) for nutrient-limited cultures. The presence of light appears

to trigger the formation of GTX-3. Whether the light directly leads to GTX-3 synthesis by activating an enzyme, or indirectly facilitates it by inducing the synthesis or mobilization of a precursor cannot be determined. However, it is significant that the synthesis of different toxins can be influenced differently by changes in culture conditions on timescales as short as a few hours.

Conclusions

The results from the February 1993 data suggest that the production of toxins in A. tamarense varies according to a diurnal cycle and that this variation influences both the overall concentration of toxins and the toxin profile. More elaborate investigations in June-July 1993 revealed that the apparent diurnal phasing of cell division is real, statistically significant and correlated with changes in toxin concentration. The diurnal variation in total toxin content of the cells was small, but was well correlated with chlorophyll-*a* content, suggesting a direct relationship between toxin and biomass. The variation in the cellular GTX-3 content was more significant and suggested diurnal phasing

of the synthesis of GTX-3 by the cells or diurnal phasing of enzymatic toxin conversion reactions. Irradiance-reduction experiments indicate that toxin synthesis is positively correlated with irradiance. Light appears to be essential to the synthesis of toxins, especially the gonyautoxins, and reductions in the available light produce changes in the toxin profiles on timescales as short as a few hours. The reasons for this cannot be determined from these data, but are likely to be the result of the influence of light on the biosynthetic mechanism producing the toxins.

REFLECTIONS AND SUGGESTIONS

If this study were to be repeated or developed further, a number of changes should be made. The most important improvement would be the use of synchronized (or phased) cultures and short sampling intervals from the outset. This would allow the diurnal cell cycle to be properly accounted for, and would also eliminate the need for data smoothing, which may obscure significant short-term variations. Daily sampling did not adequately resolve the dynamics of growth and toxin production. If this had been realized earlier in the project, the conclusions drawn could have been more definitive.

Changes to the turbidostat control system are necessary to eliminate the problem of interference from the culture lights. The need to pass the culture through an optical flow cell reduced the sensitivity of the system to small turbidity changes and also may have exposed the dinoflagellates to unnecessary shear stress as they flowed through the pump and associated tubing. Some experimentation with different shapes of culture vessels might

reduce the tendency of the cells to "hide" in the corners of the culture vessel and thus escape detection by the LED beam. Perhaps spherical or vertically tapered flasks instead of cylinders would help to keep the cultures homogeneous.

Mixing the cultures produced turbulence to which dinoflagellates are known to be sensitive. Anecdotal evidence indicates that this problem is reduced in larger-volume cultures. Increasing the volume of the culture vessels might be beneficial, except that it also means volumes and flow rates of medium are proportionately larger. One possible way of avoiding this problem is the use of a "manual cage-culture", as described in Chapter 4. This may be the most practical solution since it affords all the advantages of a true turbidostat, except automation, while eliminating the need to stir the cultures continuously. Nutrient supply rates can be kept at any level desired while mixing of the cultures is only necessary just prior to sampling.

The use of natural seawater in this study introduced an element of seasonality which is undesirable in a controlled

laboratory investigation. Attempts to use artificial seawater were unsuccessful due to poor growth rates. If an artificial seawater recipe could be found which was practical for large-scale continuous cultures, it might eliminate the problems of slow growth experienced at certain times of year. Attempts by other researchers to do this have been generally unsuccessful, so in all likelihood, large stockpiles of natural seawater would have to be maintained.

Any laboratory study should be corroborated with observations in the natural environment. Field work on natural blooms should be carried out to determine if the short-term variations in toxin content and toxin profile observed in these experiments can be detected in wild populations. Changes in toxin profile could have implications for the toxicity of dinoflagellate blooms. Cells with a higher proportion of the more toxic compounds (STX, GTX-n, NEO) will be more toxic than cells containing predominately C-toxins. Thus cells growing quickly in high-light environments could be more toxic than the same strain of cells growing under reduced irradiance. Diurnal variations should be relatively easy to detect, but the longer-term effects of light regimen may be more subtle. The ability of

dinoflagellates to migrate vertically in the water column means they have a great deal of control over their light environment. It is possible that the differences in toxin content and profile between cloudy and sunny days may not be very significant. Only direct field observations can determine if this is so.

Beyond the optimization of the cage-culture system, there are a number of questions which should be addressed by any future research involving Alexandrium in cage-culture turbidostats. One of the most important is the effect of the nutrient regimen on the timing of the cell division peak. Since variations in cellular toxin content are correlated with the cell division cycle, any effects of culture conditions on that cycle must be well understood. In this study, cytokinesis occurred during the light period, rather than at night, which suggests that the very uniform nutrient environment of the turbidostat significantly affected the timing of cell cycle events. This should be investigated by a properly controlled comparison of continuous and batch cultures under different nutrient conditions. All permutations of unstirred vs. stirred, batch vs.

continuous cultures should be investigated and the timing of cell cycle events compared.

Another important issue is that of nutrient uptake kinetics, which have not been extensively studied in Alexandrium. Continuous cultures are a good system for this type of research, since the cultures can be maintained in steady state for extended periods. An examination of growth and toxin production rates under different uptake rates would represent a major contribution to the field.

Finally, the mechanisms underlying the toxin profile changes observed here and by other authors are critical to our understanding of the function of toxins within the dinoflagellate cells. Any new knowledge of enzyme pathways, intermediates and precursors would help to resolve the biggest question of all - why are dinoflagellates toxic? Until that question is answered, the dynamics of toxin production are fascinating, but difficult to put into context.

GENERAL CONCLUSIONS

There are five major conclusions of this thesis:

I. A. tamarense can be successfully grown in a continuous culture system. Although the development of the culture method proved difficult, the potential for continuous-culture production of toxic dinoflagellates has been demonstrated clearly. In general, the turbidostats worked, and allowed cultures to be grown under sustained conditions for extended periods. This made it possible to observe changes in growth and toxin production which could not be resolved in conventional batch cultures. This represents a major advance in dinoflagellate research, since previous attempts at continuous culturing have been generally unsuccessful.

II. Contrary to the initial working hypothesis, total toxin production was not inversely correlated with division rate under most conditions. Moderate irradiance reductions had no significant effect on the total toxin content of the cells, even though the cell division rates were reduced. However, large irradiance reductions

resulted in significant reduction of both division rates and total toxin content. Photoperiod changes (16:8 to 12:12 L:D) had no significant effect on either the toxin content of the cells or their division rate. Shorter daylengths were not tested. The overall conclusion is that light is essential to the synthesis of toxins.

III. Total toxin content per cell is diurnally variable, and is probably a direct function of cell biomass. Statistically significant diurnal variations in toxin content and chlorophyll-*a* content were correlated with phased cell division. A strong relationship was observed between total toxin content and chlorophyll-*a* content. When chlorophyll-*a* was high, total toxin content per cell was high and vice-versa. This is reasonable if one assumes that the toxins behave in much the same way as other cellular components and form a relatively constant proportion of the cellular biomass under steady-state growth conditions.

IV. Toxin profile is irradiance-dependent. Under even moderately reduced irradiance, the proportion of gonyautoxins in the cells was

reduced while the relative amounts of (C-toxins) increased. The mechanism could be either preferential synthesis of gonyautoxins at high irradiance, accelerated conversion of gonyautoxins to C-toxins at low irradiance, or a combination of these processes. Photoperiod changes were not correlated with the toxin profile.

V. Toxin profile was diurnally variable, with the percentage of GTX-3 increasing during the day and declining at night. This is consistent with Conclusion IV. Preferential synthesis or accumulation of gonyautoxins during the daylight period are probably the result of light-dependent enzyme processes. It is possible that toxin synthesis is controlled via a circadian clock, as is cell division, although the experiments were inconclusive on this point.

This thesis focuses attention on light regimen-toxicity relationships which have not been widely considered in the physiology of toxic species or the development of toxic dinoflagellate blooms. Researchers have generally concentrated on nutrient supply and hydrographic conditions when studying natural blooms, as well as the growth stage when studying laboratory

cultures. The use of continuous cultures has made it possible to investigate the effects of irradiance at time scales ranging from hours to weeks. The information and culture techniques presented here are a significant new contribution to the field, and provide a good foundation from which to develop further research.

APPENDIX 1

DATA TABLES FOR ALL EXPERIMENTS

Table A.1 - August 1990

	Peak Areas:				
Sample	C-Toxins	GTX-3	GTX-2	NEO	STX
A1	12814416	2193777	157448	245832	2713851
A2	13375905	2393895	51060	258386	2833157
A3	14758960	2682367	23427	299918	3208925
B1	4825116	708434	58957	82158	1194531
B2	8532952	1394217	46885	152283	2031853
B3	8102203	1279039	64566	147216	1975409

Sample	Density (cells/ml)	Sample (ml)	Acid (ml)	Injection (ml)
A1	1797	2000	5	0.010
A2	1797	2000	5	0.010
A3	1797	2000	5	0.010
B1	2031	2000	5	0.010
B2	2031	2000	5	0.010
B3	2031	2000	5	0.010

Table A.2 - May 1991

		Harvest (l)			Densities (cells/ml)			Sample (ml)		
Date	Day	A	B	C	A	B	C	A	B	C
21	1	1.000	5.000	5.000	338	1028	1800	0	850	2000
22	2	0.100	1.000	1.100		863	875	0	1000	1100
23	3	2.600	1.740	0.950	600	813	1050	2600	1740	950
24	4	1.300	0.000	0.950	725	850	813	1300	1050	0

Table A.3 - July 1991

		Harvest (l)			Densities (cells/ml)			Sample (ml)		
Date	Day	A	B	C	A	B	C	A	B	C
11	1	1.47	0.50	2.03	1025	888	1238	1450	480	2000
12	2	1.15	0.00	1.37	838		988	1100	0	1350
13	3	3.15	0.82	0.66	713	650	763	3130	800	640

14	4	2.90	2.17	2.30	838	825	938	2880	2150	2280
15	5	4.98	1.65	3.20	1225	825	1200	0	1630	3200
16	6	0.20	0.02	0.02	638	875	225	0	0	0
17	7	0.00	0.00	0.00	550	438	525	0	0	0
18	8	1.53	1.21	0.13	800	275	375	1520	1200	0
19	9	0.00	0.00	0.00	713	563	413	0	0	0
20	10									
21	11									
22	12									
23	13	2.50	0.00	0.00	538	87	163	2480	0	0
24	14	0.96	0.00	0.00	500	75	338	950	0	0
25	15	0.00	0.00	0.00	288	38	163	4000	0	0
26	16	0.00	0.00	0.00	725	113	238	4000	0	0

	Acid	Inj.	Peak Areas: Culture A				
Day	(ml)	(ml)	C's	GTX-3	GTX-2	NEO	STX
1	5	0.020	3988894	1660228	45672	134086	816764
2	5	0.020	2687744	1279250	55294	111306	622888
3	5	0.020	9579297	5876158	122254	378931	2099816
4	5	0.020	10151616	6924275	114263	395854	2299388
5							
6							
7							
8	5	0.020	2685274	1431109	69949	100778	722712
9							
10							
11							
12							
13	5	0.020	6338465	3304796	92715	224353	1328653
14	5	0.020	1861583	1027095	25097	70480	343392
15	5	0.020	10654319	8830562	115467	501026	2357457
16	5	0.020	6340665	4016903	109051	274854	1235822

	Acid	Inj.	Peak Areas: Culture B				
Day	(ml)	(ml)	C's	GTX-3	GTX-2	NEO	STX
1	5.000	0.020	2042992	1076184	31409	69627	322553
2							
3	5.000	0.020	2035408	878101	28104	52798	386305
4	5.000	0.020	6541898	3023603	64092	194415	1366560
5	7.800	0.020	1494466	282880	202326	46701	328423
6							
7							
8	5.000	0.020	977694	287561	9022	24022	246047

Day	Acid (ml)	Inj. (ml)	Peak Areas: Culture C's	GTX-3	GTX-2	NEO	STX
1	5.000	0.020	9265252	4730835	201236	316220	2030095
2	5.000	0.020	4951186	1291315	49680	152134	1016734
3	5.000	0.020	1530523	654462	12292	36868	265534
4	5.000	0.020	9324308	4758751	55232	294648	2257125
5	6.600	0.020	6326447	1698833	159443	126477	1048725

Table A.4 - October 1991

Date	Day	Harvest (l) A	B	C	Densities (cells/ml) A	B	C	Sample (ml) A	B	C
6	1	0.284	0.381	1.171	775		1088	500		1000
7	2	0.000	0.977	2.218	1725	750	788	500	975	1000
8	3	0.000	2.981	3.549	2238	725	1250	500	2000	2000
9	4	1.220	3.729	6.640	463	692	705	1200	2000	2000
10	5	1.497	2.468	1.400	793	668	448	1500	2000	1400
11	6	1.151	2.981	1.768	702	257	396	1150	2000	1770
12	7	2.322	2.343	1.657	416	458	210	2000	2000	1660
13	8	3.667	5.247	1.317	1015	440	443	2000	2000	1300
14	9	2.100	0.527	0.000	624	1042	937	2000	1000	1000
15	10	2.419	0.000	0.000	959	580	607	2000	1000	1000
16	11	3.431	0.000	0.000	886	668	450	2000	1000	1000
17	12	2.052	0.298	0.000	617	387	340	2050	1000	1000
18	13	0.728	0.000	0.000	1365	504	206	1000	1000	1000
19	14	3.327	0.000	0.000	900	506	151	2000	1000	2000

Day	Acid (ml)	Inj. (ml)	Peak Areas: Culture C's	GTX-4	GTX-3	GTX-2
1	5	0.020	10127464	343799	1756747	297915
2	5	0.020	12033773	424301	2940670	415515
3	5	0.020	11900418	443185	2909440	391092
4	5	0.020	11751734	402396	2736226	
5	5	0.020	15774542	808862	4148088	492610
6	5	0.020	10911346	425812	2086455	
7	5	0.020	16552376	911817	5089449	599905
8	5	0.020	20991728	1431671	8208271	958713
9	5	0.020	16622208	904371	5571855	
10	5	0.020	17249692	954535	5118525	658770
11	5	0.020	13275034	601897	3147357	385929
12	5	0.020	16870212	871827	4748822	564256
13	5	0.020	16287534	903706	5253333	
14	5	0.020	14285395	642536	3396709	463166

Day	Acid (ml)	Inj. (ml)	Peak Areas: Culture B C's	GTX-4	GTX-3	GTX-2
1	5	0.020				
2	5	0.020	12270665	398739	3088646	not detectable
3	5	0.020	21028464	1122350	21028464	
4	5	0.020				
5	5	0.020	11085256	348386	2238881	
6	5	0.020	15686067	666870	4582992	
7	5	0.020	20103656	1292976	8210030	
8	5	0.020	19305270	1132853	7319274	
9	5	0.020	12977417	469136	2657099	
10	5	0.020	6641909	204063	919340	
11	5	0.020	7706722	240879	1001832	
12	5	0.020	5850166	139587	666053	
13	5	0.020	5917954	160146	730936	
14	5	0.020	6057437	158279	767068	

Day	Acid (ml)	Inj. (ml)	Peak Areas: Culture C C's	GTX-4	GTX-3	GTX-2
1	5	0.020	16339406	716017	4557168	not detectable
2	5	0.020	15118086	575444	4299111	
3	5	0.020	25052880	1574685	11308923	
4	5	0.020	19283054	868422	6487137	
5	5	0.020	10020979	299753	1931548	
6	5	0.020	9815360	329330	2062919	
7	5	0.020	7367574	163141	1377338	
8	5	0.020	12325296	389687	3484618	
9	5	0.020	13482246	549723	2881833	
10	5	0.020	4949250	135841	698037	
11	5	0.020	4161966	127569	524826	
12	5	0.020	4794890	139252	631023	
13	5	0.020	3094287	43480	455646	
14	5	0.020	10020192	324052	1502434	

Table A.5 - November 1991

Date	Day	Harvest (l) A	B	C	Densities (cells/ml) A	B	C	Sample (ml) A	B	C
18	1				1509	723	555			
19	2				1535	769	214			
20	3				1263	816	471			
21	4				392	430	405			
22	5	1.500	0.750	4.490	224	827	357	1440	690	1000
23	6	2.950	0.000	6.250	171	143	140	1000	500	1000
24	7	2.140	0.180	2.920	871	474	452	1000	500	1000
25	8	3.530	0.000	2.500	762	348	394	1000	500	1000
26	9	0.830	0.000	1.020	816	320	270	760	500	960

27	10	2.100	0.000	1.970	787	292	283	1000	500	1000
28	11	1.000	0.000	3.000	967	277	252	950	500	1000
29	12	2.420	0.000	2.610	950	247	237	1000	500	1000
30	13	8.450	0.610	4.370	837	211	225	1000	560	1000
1	14				901	223	299	1000	500	1000
2	15	0.130	0.000	7.250	935	240	350	500	1000	1000
3	16	0.000	0.000	0.000	1690	325	287	500	1000	1000

	Acid	Inj.	Peak Areas: Culture A		
Day	(ml)	(ml)	C's	GTX-4	GTX-3
10	5	0.010	11731241	392642	1897015
11	5	0.010	11040487	371555	1791295
12	5	0.010	22916041	1249353	5854146
13	5	0.010	17209973	694132	2868204
14	5	0.010	12469710	434984	1603656
15	5	0.010	5229332	181090	596177
16	5	0.010	2809131	67352	

Peak Areas: Culture A			Peak Areas: Culture A		
C's	GTX-4	GTX-3	C's	GTX-4	GTX-3
1778168	54792	165049	6034490	184703	1087283
			2888366	125462	585471
2242679	53299	250348	11295199	433439	2293955
4862031	131121	503903	8665597	310846	1711024
3765878	142340	443037	7915371	373189	2110351
7447965	223361	646885	6855601	249426	1301396
5973686	222096	557080	4018189	160168	803396

Table A.6 - March-April 1992

		Harvest (l)		Cells/ml)		Sample (ml)			
Date	Day	A	C	A	C	A	C	Acid (ml)	Inj. (µl)
11	1	0.250	2.500	1208	1394	500	500	5.00	0.010
12	2	1.200	2.000	1682	1310	500	500	5.00	0.010
13	3	0.360	4.700	1957	2968	500	500	5.00	0.010
14	4	3.200	1.000	2219	1214	500	500	5.00	0.010
15	5	1.000	0.530	3295	997	500	475	5.00	0.010
16	6	1.200	0.800	2831	1469	500	500	5.00	0.010
17	7	2.600	0.230	2349	2483	500	500	5.00	0.010
18	8	1.000	0.120	2715	2085	500	500	5.00	0.010
19	9	0.350	0.080	1288	2117	500	500	5.00	0.010
20	10	0.530	0.075	2064	1516	500	500	5.00	0.010
21	11	0.900	0.070	2593	1687	500	500	5.00	0.010
22	12	1.500	0.075	2645	1522	500	500	5.00	0.010
23	13	0.175	0.075	1357	1798	500	500	5.00	0.010
24	14	0.007	0.070	1422	1936	500	500	5.00	0.010
25	15	0.750	0.140	1980	1542	500	500	5.00	0.010

26	16	0.800	0.210	2154	1783	500	500	5.00	0.010
27	17	2.200	0.360	3005	1529	500	500	5.00	0.010
28	18	1.300	1.000	2460	1667	500	500	5.00	0.010
29	19	1.000	1.000	2298	1515	500	500	5.00	0.010
30	20	2.200	1.250	2290	1953	500	500	5.00	0.010
31	21	2.000	1.700	2339	1851	500	500	5.00	0.010
1	22	1.750	0.700	2448	1887	500	500	5.00	0.010
2	23	1.400	1.800	2105	1723	500	500	5.00	0.010
3	24	1.150	1.750	2035	1675	500	500	5.00	0.010
4	25		2.000		1463		500	5.00	0.010
5	26		2.000		2062		500	5.00	0.010
6	27		2.500		1870		500	5.00	0.010
7	29		0.455		2096		500	5.00	0.010
8	30		1.350		952		500	5.00	0.010
9	31		1.000		1345		500	5.00	0.010

Peak Areas: Culture A

Day	C's	GTX-4	GTX-1	GTX-3	GTX-2
1					
2					
3					
4	18905924	3740288	0	15705074	0
5	18346352	3503235	142343	12902313	190648
6	17395570	2842047.7	89055.609	9800129	170028.83
7	20891330	6322919	191435.25	26030852	447175.28
8	17475458	2683622.7	112990.16	9635037	166858.45
9	15606340	1743817.6	87047.234	6029928.5	95759.344
10	18755512	3785545	139864.7	13110410	123472.23
11	19177432	4071384	159922.27	14438334	221679.25
12	17058916	2319169.5	115027.15	8343857	128436.11
13	16450373	2066803.2	103048.1	6940930	95507.516
14	13967088	1102234.2	83146.109	3335046	80940.734
15	18042496	2876502.7	161325.95	10118465	173433.45
16	16998162	2277579.7	114400.7	7561313.5	112322.59
17	18793254	3414571	196706.08	11575386	200874.23
18	16776048	2105034.2	126138.95	6993483	121912.98
19	16950000	2193647.2	111101.59	6970765.5	98542.586

Peak Areas: Culture A Peak Areas: Culture C

Day	NEO	STX	C	GTX-4	GTX-1
1					
2					
3					
4	375741.19	290319.66			
5	322687.69	210099.25			
6	262511.72	185077.22			
7	499107.62	380413.59			
8	203419.95	185363.31			
9	125833.77	72818.406			
10	287755.19	154103.06			

11	319745.59	221043.86			
12	184665.34	140633			
13	152206.44	65452.465			
14	81858.648	34461.027			
15	228233.37	169087.97			
16	189071.92	95745.336			
17	281823.91	146726.98			
18	210076.59	126658.4			
19	198294.55	94353.57			
20					
21					
22					
23			15391495	1150992.4	72858.641
24			15976889	1375694.5	71396.242
25			14678585	990909.5	48038.668
26			17209982	2063772.1	119691.21
27			19120222	3241019.7	146020.37
28			15661085	1365726.7	57412.619
29			13525939	786375.5	0
30			15915754	154077	64865.957

Peak Areas: Culture C

Day	GTX-3	GTX-2	NEO	STX
23	3362327	50210.961	139779.17	64804.562
24	4014353.2	41021.414	163217.81	103462.95
25	3094401.2	32523.875	116667.77	84741.125
26	6668284.5	105623.02	212258.73	148055.58
27	10123797	78859.344	360413.84	189687.64
28	3519049.2	35259.73	169206.28	62183.152
29	2119136.7	0	79740.594	30137.9
30	3717832.5	45305.496	175617.36	55758.367

Table A.7 - Growth vs. Irradiance Experiment

April/May 1992

Daily mean and standard deviation of culture density at each irradiance. Units are cells/ml.

		200		140A		140B	
Date	Day	m	s	m	s	m	s
20	1	57	13.7	56	3.8	52	7.3
21	2	96	2.6	72	5.8	92	2.5
22	3	158	7.8	128	8.9	163	9.7
23	4	283	2.8	232	7.3	276	9.7
24	5	470	17.0	329	18.8	399	12.0
25	6	778	26.1	616	10.6	694	18.1
26	7	1461	24.9	1052	40.0	1365	20.8
27	8	1984	44.8	1646	15.8	1770	11.8
28	9	2777	79.3	2776	39.9	2896	50.6

29	10	3286	35.0	3601	56.0	3412	60.3	
30	11	3750	25.5	4058	34.1	4035	48.8	
1	12	3841	46.3	4344	9.0	4179	14.6	
2	13	4774	31.7	4776	108.2	4197	32.8	
3	14	3824	69.3	4742	12.7	4137	9.5	
4	15	3692	45.3	4771	32.3	4222	23.8	
5	16							
6	17	3476	24.6	4688	62.1	4172	26.7	

	100A		100B		50A		50B	
Day	m	s	m	s	m	s	m	s
1	56	3.9	52	6.3	26	3.3	23	3.5
2	96	7.7	93	3.7	38	0.0	38	3.5
3	170	9.5	168	12.9	42	3.2	67	1.8
4	291	15.7	288	7.4	68	7.4	64	10.1
5	449	7.5	399	17.1	46	5.8	64	8.3
6	744	4.1	718	9.2	62	1.1	81	2.7
7	1300	10.5	1132	28.9	92	5.1	134	5.3
8	1796	26.8	1809	13.8	148	2.8	170	5.1
9	3056	23.6	2996	58.3	164	7.7	288	12.8
10	3636	15.6	3914	37.8	218	6.8	406	27.5
11	3895	53.9	4616	77.2	286	9.9	566	9.8
12	3880	117.7	4710	43.8	456	15.7	718	11.0
13	3928	51.3	4787	55.9	496	14.9	1026	26.3
14	3993	74.1	4943	64.1	622	11.1	1200	30.0
15	4212	29.6	5192	57.0	793	22.1	1622	60.0
16								
17	4117	47.1	5668	147.3	1577	14.0	2746	52.0

Table A.8 - August/September 1992

			Harvest (l)			Densities (cells/ml)			Sample (ml)		
Date	Day	L:D	A	B	C	A	B	C	A	B	C
29	2	16:8	0.000	0.270	0.750	107	518	1253	1000	500	500
30	3	16:8	0.000	1.500	0.123	144	953	1120	500	500	500
31	4	16:8	0.650	1.800	0.400	428	997	547	570	500	325
1	5	16:8	1.200	1.900	0.130	606	931	618	1000	500	500
2	6	12:12	1.500	0.490	0.900	569	467	619	500	400	500
3	7	12:12	2.000	0.600	0.000	319	527	528	1000	500	500
4	8	12:12		1.500	0.000		838	570	0	500	500
5	9	12:12			0.480			579	0	0	500
6	10	12:12		2.800	0.000		1040	557	0	500	500
7	11	12:12		1.750	0.000		752	535	0	500	500
8	12	12:12		0.000	0.000		530	340	0	500	500
9	13	16:8		0.000	0.000		478	352	0	500	500
10	14	16:8		0.000	0.000		472	382	0	500	500

Day	Acid (ml)	Inj. (ml)	Peak Areas: Culture A C's	GTX-4	GTX-1	GTX-3
1	5	0.01	22433976	6893169.50	0	19198526
2	5	0.01	8564712	712914.00	0	1800030.1
3	5	0.01	8498401	643986.87	0	2082902.2
4	5	0.01	15458482	2464523.70	0	8599316
5	5	0.01	23964028	7806965.00	371311.37	27378924
6	5	0.01	18184362	3704699.70	113150.2	12661375
7	5	0.01	184911000	3818349.50	150166.17	13338694

Day	Acid (ml)	Inj. (ml)	Peak Areas: Culture A GTX-2	NEO	STX
1	5	0.01	794661.75	533471.81	579383.44
2	5	0.01	0	77638.64	163333.73
3	5	0.01	0	0	172735.78
4	5	0.01	342691	130708.84	239493.58
5	5	0.01	1149245.1	624868.94	779660.75
6	5	0.01	427150.78	242747.11	307720.06
7	5	0.01	489212.06	225950.52	325699.19

Day	Acid (ml)	Inj. (ml)	Peak Areas: Culture B C's	GTX-4	GTX-1	GTX-3
1	5	0.01	11145690	1101376.6	0	2957992.5
2	5	0.01	16926028	3168597.7	97477.16	7999595.5
3	5	0.01	22327119	6543485.38	307561.06	17757827.5
4	5	0.01	12436976	1361069.9	85285.91	3454132.2
5	5	0.01	12423314	1347646.9	76666.48	3344721
6	5	0.01	9296078	672704.37	21988.75	1631111.1
7	5	0.01	16625707	2900233	53731.21	7667687
8	5	0.01	11095762	999653.5	95636.27	2591246.2
9	5	0.01				
10	5	0.01	21341370	5900716.5	282179.16	18115338
11	5	0.01	18270474	4023573.5	189436.11	12009526
12	5	0.01	12072693	1426298.9	50619.85	3344841.5
13	5	0.01	15700351	2860153.2	45800.22	6957578.5
14	5	0.01	16390750	3242537	58766.61	7579122

Day	Acid (ml)	Inj. (ml)	Peak Areas: Culture B GTX-2	NEO	STX
1	5	0.01	67256.1	72344.56	162756.47
2	5	0.01	280910.19	124510.55	165393.37
3	5	0.01	737976.98	476044.56	362132.39
4	5	0.01	109395.7	99048.63	138939.05
5	5	0.01	189928.73	53827.77	155888.81
6	5	0.01	48632.63	41377.55	88361.52
7	5	0.01	80547.64	134878.17	138258.95
8	5	0.01	159821.53	60571.44	106098.66
9	5	0.01			

10	5	0.01	724111.87	263642.72	222879.39
11	5	0.01	519586.28	228664.31	183043.5
12	5	0.01	137723.7	60743.1	117988.6
13	5	0.01	217117.11	77104.28	109547.39
14	5	0.01	216116.94	96642.98	137552.64

	Acid	Inj.	Peak Areas: Culture C			
Day	(ml)	(ml)	C's	GTX-4	GTX-1	GTX-3
1	5	0.01	17223296	3252879.2	160186.31	7298118
2	5	0.01	21730218	5743504	278518.53	14136202
3	5	0.01	21499062	5427071	245875.16	13997905
4	5	0.01	10255776	805231.12	0	1916626.4
5	5	0.01	11266041	1065013	58530.58	2300693.7
6	5	0.01	14271123	1905904.1	54728.92	4484331.5
7	5	0.01	17987338	3961127.5	109874.92	6858524
8	5	0.01	17163224	3400469.2	145374.56	6348384
9	5	0.01	9953878	919801.12	78768.74	2295693.7
10	5	0.01	16946462	3313530	134886.83	8622459
11	5	0.01	17410546	3769863	124063.51	9977240
12	5	0.01	10529039	1120128.7	51676.04	1895829.7
13	5	0.01	14162103	2369882.5	38160.09	4743064
14	5	0.01	14759074	2722673.2	39304.5	5502048.5

	Acid	Inj.	Peak Areas: Culture C		
Day	(ml)	(ml)	GTX-2	NEO	STX
1	5	0.01	268448.62	160877.12	122685.45
2	5	0.01	535342.75	257161	217004.16
3	5	0.01	528521.62	376534.53	364842.47
4	5	0.01	0	60167.32	114781.87
5	5	0.01	36129.96	43157.34	80033.55
6	5	0.01	76023.35	78700.79	100059.52
7	5	0.01	194290.61	131465.22	88758.48
8	5	0.01	137901.33	116072.91	102200.52
9	5	0.01	83575.26	45267.35	97665.81
10	5	0.01	311720.41	114190.59	135539.8
11	5	0.01	300761.81	119978.51	215615.06
12	5	0.01	44790.51	34829.38	86675.9
13	5	0.01	54305.8	62117.59	74006.33
14	5	0.01	76997.89	67660.82	95890.77

Table A.9 - Raw Data: October 1992

Note duplicate samples from Oct. 24 (Day 16) onward.

			Harvest (l)			Densities (cells/ml)			Acid	Inj.
Date	Day	L:D	A	B	C	A	B	C	(ml)	(ml)
11	3.000	16:8	0.290	0.360	1.600	1811	211	2563	5	0.01
12	4.000	16:8	0.000	1.750	0.650	1363	474	1525	5	0.01
13	5.000	16:8	0.000	1.000	0.000	1074	792	2285	5	0.01

14	6.000	12:12	0.000	1.000	0.000	779	918	1756	5	0.01
15	7.000	12:12	0.000	0.000	0.000	476	220	1096	5	0.01
16	8.000	12:12	0.000	2.100	0.000	374	771	553	5	0.01
17	9.000	12:12	0.000	3.600	0.000					
18	10.000	12:12	0.000	0.850	0.000	224	441	469	5	0.01
19	11.000	12:12								
20	12.000	12:12								
21	13.000	12:12								
22	14.000	12:12								
23	15.000	12:12		0.000	0.000		437	861	5	0.01
24	16.000	12:12		4.000	2.500		1167	1771	5	0.01
25	17.000	12:12		0.000	1.300		271	1511	5	0.01
26	18.000	12:12		1.300	1.100		1299	1198	5	0.01
27	19.000	16:8		3.000	0.950		1339	1312	5	0.01
28	20.000	16:8		5.000	2.300		1027	1430	5	0.01
29	21.000	16:8		2.600	2.000		715	1080	5	0.01
30	22.000	16:8		0.000	1.500		574	1091	5	0.01

	Sample (ml)			Peak Areas: Culture C			
Day	A	B	C	C's	GTX-4	GTX-1	GTX-3
3	500	500	500	22601098	2240137.7	0	8532606
4	500	1000	500	14642064	820441.37	0	3215247
5	500	500	500	19587810	2127746.7	0	8008555
6	500	500	500	17587144	1537245.7	0	5905846.5
7	500	500	500	8070572	226607.36	0	865082.87
8	500	500	500	11983547	550156.94	0	1800898.6
9							
10	1000	500	500	15573586	1331583.5	0	5324684
12							
13							
14							
15							
16A		500	500				
16B		500	500				
17A		500	500				
17B		500	500				
18A		500	500				
18B		500	500				
19A		500	500				
19B		500	375				
20A		500	500				
20B		500	500				
21A		500	500				
21B		500	500				
22A		500	500				
22B		500	500				

	Peak Areas: Culture A			Peak Areas: Culture B	
Day	GTX-2	NEO	STX	C's	GTX-4
3	0	306567	261311.56	10562220	354499.56
4	156071.2	155628.94	105519.31	23640646	3642936.2
5	58693.3	224204.69	232708.48	24937104	4527426.5
6	178205.69	173474.58	149000.91	19061330	2274022.5
7	34987.34	0	0	12009727	653559.69
8	41721.47	58622.07	179076.22	23195866	4191890.5
9					
10	67778.24	106161.85	153890.87	4013428	129461.3
11					
12					
13					
14					
15					
16A				21005814	3625870.2
16B				27791376	7314802.5
17A				13224450	1018441.2
17B				14150767	1186435.4
18A				27759432	7308472.5
19A				19219708	2631068.2
19B				24961366	5443658
20A				29448394	7710558
20B				27192458	6769889.5
21A				22932890	4163653.7
21B				23287806	4413267
22A				20838258	3253745.2
22B				21241644	3454276.2

	Peak Areas: Culture B				
Day	GTX-1	GTX-3	GTX-2	NEO	STX
3	0	1582559.6	0	36311.16	37660.01
4	0	18400678	321373.41	342948.5	302449.5
5	0	24637186	0	394733	399896.44
6	0	10511750	125178.41	268153	355107.53
7	0	2443393.2	0	0	0
8	0	17744240	0	381321.97	293600
9					
10	0	582026.31	52981.38	0	0
11					
12					
13					
14					
15					
16A	141814.08	18974190	890032.75	322152.44	284071.59
16B	105247.52	40273340	840947.75	616529.06	420872.03
17A	0	4292314.5	73915.73	91420.42	134661.22
17B	0	5180339	115554.65	74188.72	190182.95
18A	273845.84	15406110	1097748.5	282480.75	285933.66
18B	87493.08	42034936	2116687.5	532690.37	491821.84

19A	101970.15	13198735	709245.31	230144.3	215925.91
19B	69075.84	27921722	901525.12	424622.97	446130.69
20A	0	33585440	0	565466.94	552007.62
20B	0	33743540	0	479806.72	570950.87
21A	0	18573374	0	320477.53	504703.59
21B	0	21051820	0	318845.56	517686.09
22A	0	12619458	0	216897.77	385527.66
22B	0	13447210	0	229133.41	405318.19

Peak Areas: Culture C

Day	C's	GTX-4	GTX-1	GTX-3	GTX-2
3	25603170	3621763.2	0	14867132	255979.94
4	22372288	2624292.7	0	10870711	192295.34
5	32593758	7940587.5	331841.75	29435144	1837323.2
6	23846580	3493336.2	0	11744315	258111.98
7	20096568	2167923.2	0	7566536	210215.48
8	17268618	1556691.7	0	4479713	42038.27
9					
10	18397872	1999349.6	0	6487186.5	38716.2
11					
12					
13					
14					
15					
16A	28895618	7279006	111855.28	35615560	744285.69
16B	30459646	8589272	128185.77	40831428	918713.06
17A	22390050	3873835.2	98082.44	16280340	611637.31
17B	25653642	5534391	57080.99	23558996	610214.56
18A	17033668	1722998.2	71761.62	7827485	355952.12
18B	21000002	3165591.2	72650.32	15081057	560388.62
19A	26499014	5983413	0	25837054	531404.12
19B	22589286	3792480.5	66239.84	16820086	559921.62
20A	27839788	7178789	0	37660624	0
20B	29345824	7551182	0	32691736	0
21A	26173150	5654743.5	0	23852762	0
21B	26416566	5825656.5	0	21765590	0
22B	25373468	5376944.5	0	18052938	0

Peak Areas: Culture C

Day	NEO	STX
3	509061	244105.2
4	318567.81	213036.86
5	982927.25	588581.62
6	435495.44	244925.95
7	247869.83	179110.2
8	187612.8	156500.36
9		
10	137599.73	156693.48
11		
12		

13		
14		
15		
16A	637014	360222.81
16B	735447.19	384790.97
17A	327666.16	205340.42
17B	470174.62	285475.91
18A	173727.3	149709.02
18B	272024.91	215582.87
19A	444741.91	401653.12
19B	314874.69	355296.78
20A	501080.81	558130.81
20B	580328.81	572901.12
21A	403916.31	482231.44
21B	448488.06	496937.41
22A	465674.47	492563.31
22B	431068	482069.91

Table A.10 - February 1993

			Harvest (l)		Density (cells/ml)		Doublets (%)			
Date	Day	Time	A	B	A	B	A	B	Sample (ml)	Acid (ml)
6	2	10:30	2.500	4.750	816	1741	19.7	14.6	500	5
7	3	10:30		4.500	340	1595	6.7	28.7	500	5
8	4	10:30		1.000	320	1381	1.4	15.3	500	5
9	5	10:30		7.500	291	1121	3.9	17.2	500	5
10	6	10:30		1.750	476	1139	15.9	29.7	500	5
11	7	10:30		2.200	473	1248	11.2	28.5	500	5
12	8	10:30		3.000	1233	1694	16.8	27.7	500	5
13	9	10:30	3.000	2.250	1821	1425	40.8	39.6	500	5
14	10	10:30	4.000	2.200	1622	1362	37.9	34.5	500	5
15	11	10:30	0.000	4.100	1244	1171	35.8	25.6	500	5
16	12	10:30	0.000	0.900	1514	995	32	14.2	500	5
17	13	10:30	6.700	0.600	1423	829	34.2	23	500	5
18	14	10:30	0.000	0.600	1129	712	32.8	27	500	5
19	15	10:30	0.000	2.000	1466	860	29.6	15.2	500	5
19	15.5	19:10	0.000	1.000	1239	853	18.5	22.9	500	5
20	16.5	21:20	0.000	0.000	1575	846	30.6	16.2	500	5
21	17	10:30	0.000	1.250	1646	766	34.9	9.9	500	5
21	17.5	22:30	0.000	4.500	1601	741	31.7	11	500	5
22	18	10:20	2.750	1.000	1537	797	28	14.3	500	5
22	18.5	22:30	0.000	1.000	1556	614	24.6	4.5	500	5
23	19.5	22:20	0.000	0.000	1960	617	21.1	0.1	500	5
24	20	10:20	0.000	0.000	2008	496	25.2	3.7	500	5
24	20.5	22:40	0.000	0.000	2149	468	25	0	500	5
25	21	10:20	4.600	0.000	2117	419	17.3	0	500	5
25	21.5	22:30	0.900	0.000	1794	396	12.3	1.1	500	5

Date	Inj. (ml)	Peak Areas: Culture A C's	GTX-4	GTX-3	NEO	STX
2	0.01					
3	0.01					
4	0.01					
5	0.01					
6	0.01					
7	0.01					
8	0.01					
9	0.01	33782320	14206040	59207364	1304704.1	1395256.7
10	0.01	33783492	12950459	56550436	1201713.2	1522174.1
11	0.01	29124066	9265984	44741976	834537.87	719177.81
12	0.01	31162352	10886826	49332044	946604.12	593024.87
13	0.01	30277932	9547515	41990840	932347.94	579295.25
14	0.01	27738074	7391547.5	31305998	776098.81	460309.47
14.5	0.01	31353772	9709337	44844464	879472.94	749586.31
15	0.01	29805206	7986744.5	34505680	59833.54	829380.87
15.5	0.01	31054502	3672259.5	15150171	0	0
16	0.01	30493038	8598850	33038748	1213211.9	615377.5
16.5	0.01	32490310	9849100	41230536	1108370	677780.62
17	0.01	30289664	7619394	27738318	-532158.7	637405
17.5	0.01	33073816	9137365	38389356	700864.87	597889.5
18	0.01	30229802	6831279.5	25362552	647527.06	414313.75
18.5	0.01	33028874	9155535	38376132	1059110.4	805658.25
19	0.01	31667370	7408884.5	26780286	-409799.9	618028.75
19.5	0.01	36160688	9753810	40996104	1119897.5	871207.81
20	0.01	33668800	7982470	29068584	1015699.1	670336
20.5	0.01	35697212	9618978	40931660	1341052.9	1278452.1
21	0.01	34339320	7753122	30013930	1263559.4	1237198.2
21.5	0.01	33448828	6995486	31292042	662379.37	925669.81

Date	Inj. (ml)	Peak Areas: Culture A C's	GTX-4	GTX-3	NEO	STX
2	0.01					
3	0.01					
4	0.01					
5	0.01					
6	0.01					
7	0.01					
8	0.01	31470588	9357496	34894480	-40724.29	833954.94
9	0.01	30424760	8793700	33677056	79975.14	863533.12
10	0.01	30295406	9044851	33315382	1165574.4	797891.44
11	0.01	30016186	8474597	32203536	-10597.71	557196.25
12	0.01	26753076	6535097	26287368	367676.66	381466.5
13	0.01	24255718	4587546.5	18605218	0	360464.5
14	0.01	22577480	3336150	14359446	0	205113.92
14.5	0.01	27660350	6974003.5	28815672	216521.66	269026.78
15	0.01	26085878	5611886.5	22065804	-515251.2	271642.84
15.5	0.01	27224272	2913529.7	11726342	0	0
16	0.01	25343842	5675562.5	19576344	23288.73	261082.69

16.5	0.01	26789926	6173273	21614896	0	836106.06
17	0.01	24505700	4596284	16027068	0	0
17.5	0.01	25449852	4813178.5	17565772	0	0
18	0.01	25779526	4732872	17239788	0	0
18.5	0.01	24516366	4152176.5	15448875	0	0
19	0.01	23209302	3525633.2	11739327	0	0
19.5	0.01	24657192	3902788.7	14189968	0	0
20	0.01	22270764	3156192.7	9964279	0	0
20.5	0.01	23176884	3507623.5	10880756	0	0
21	0.01	20403610	2288221.2	7064088	0	0
21.5	0.01	21548334	2776392.5	8147167.5	0	0

Culture Phasing Experiments:

Note that normal dark period was from 0000 to 0800 each day. The intensity of 24h light was 30μE/m²/s.

Table A.11 - Raw data: June 1993

		Light (μE/m²/s)			Density (cells/ml)			Doublets (%)		
Hour	Time	A	B	C	A	B	C	A	B	C
0.0	0800	150	150	150				24.3	13.1	11.0
2.0	1000	150	150	150				21.6	26.1	14.1
4.0	1200	150	150	150				23.9	23.6	10.3
6.0	1400	150	150	150	660	1000	1655	27.0	28.9	15.5
8.0	1600	150	150	150				21.0	11.5	6.1
10.0	1800	150	150	150	1498	2223	2108	16.0	25.3	13.2
12.0	2000	150	150	150	1755	2128	2108	15.4	16.7	8.6
14.0	2200	150	150	150	1750	1828	2125	21.1	11.1	16.1
16.0	0000	dark	dark	dark	1699	1897		21.6	14.9	5.2
18.0	0200	dark	dark	dark				14.1	15.8	13.6
20.0	0400	dark	dark	dark				18.5	12.1	13.2
22.0	0600	dark	dark	dark				15.4	15.4	6.9
24.0	0800	150	150	150				14.0	12.8	10.4
30.0	1400	150	150	150	1748	1867	2175	29.4	20.2	12.5
32.0	1600	150	150	150	1714	1880	2483	35.7	22.6	13.0
35.0	1900	150	150	150				32.5	24.0	17.1
37.0	2100	150	150	150	1810	2104	2067	30.3	22.3	14.8
39.0	2300	150	150	150				14.7	15.6	7.8
47.0	0700	dark	dark	dark				18.0	13.1	8.7
49.0	0900	150	150	150				18.1	13.6	8.0
50.0	1000	150	150	150				20.7	16.2	7.9
51.0	1100	150	150	150	1327	1639	1677	17.8	11.0	8.2
52.0	1200	150	150	150				31.7	16.9	6.9
54.0	1400	150	150	150				25.0	14.4	9.3
56.0	1600	150	150	150	1858	2085	2643	42.5	26.5	12.1
57.5	1730	150	150	150				30.4	21.1	16.9

62.0	2200	150	150	150	2065	2080	2397	27.7	14.9	14.2
72.0	0800	150	150	150	2013	2451	2758	21.6	1.3	2.1
75.0	1100	150	150	150	1851	2121	2349	21.2	11.4	3.7
77.5	1330	150	150	150	2012	2158	2185	16.8	12.7	3.4
80.0	1600	150	150	150	1964	2148	2664	25.8	10.3	2.1
86.5	2230	150	150	150	1537	2106	2381	10.0	11.9	2.7
96.0	0800	150	150	150	1596	2179	2457	8.9	6.7	2.0
99.0	1100	150	150	150	1680	2062	2123	22.1	10.8	0.6
101.5	1330	150	150	150	1755	2176	2552	27.4	20.6	3.6
104.2	1615	150	150	150	1767	2343	2462	21.2	23.6	6.5
110.5	2230	150	150	150	1655	2437	2917	12.7	15.6	10.0
120.3	0820	85	(24h)	150	1847	2376	2886	16.6	13.6	7.1
123.0	1100	85	(24h)	150	1728	2447	2676	27.9	3.4	6.0
125.5	1330	85	(24h)	150	1788	2478	3276	24.5	2.3	13.6
128.0	1600	85	(24h)	150	2292	2580	3107	29.7	4.4	12.0
135.0	2300	85	(24h)	150	2031	2436	5174	19.1	6 6	11.1
144.0	0800	85	(24h)	150	1881	1150	2642	18.3	0.0	8.4
147.0	1100	85	(24h)	150	1907	1027	2648	18.1	0.0	6.1
149.5	1330	85	(24h)	150	2020	810	2940	18.3	0.0	11.3
152.0	1600	85	(24h)		2129		2974	14.1		9.5
159.0	2300	85	(24h)		1840		2730	11.4		6.7
168.0	0800	85	(24h)		1874		2773	7.4		7.0
171.0	1100	85	(24h)		1812		2787	17.4		9.4
173.5	1330	85	(24h)		2481		3005	21.9		17.5
176.0	1600	85	(24h)		2037		3176	13.3		15.5
183.5	2315	85	(24h)		3757		2903	8.0		9.2

Chlorophyll-a (duplicate samples)
(ng/ml)

Hour	A1	A2	B1	B2	C1	C2
72.0	84.79	93.51	101.43	103.02	91.13	88.75
75.0	84.00	88.75	92.72	88.75	90.34	92.72
77.5	84.00	86.38	80.83	99.06	91.13	82.41
80.0	92.72	87.17	95.89	96.68	91.92	95.89
86.5	85.58	78.45	100.64	95.09	88.75	99.06
96.0	79.25	74.49	91.92	85.58	90.34	82.41
99.0	84.00	82.41	85.58	145.02	80.04	80.83
101.5	78.45	80.04	100.64	97.47	88.75	86.38
104.2	80.83	84.00	98.26	95.89	94.3	87.17
110.5	91.92	90.34	101.43	108.57	96.68	101.43
120.3	91.13	85.58	91.13	109.36	100.64	100.64
123.0	95.09	99.06	100.64	107.77	95.09	88.75
125.5	97.47	99.85	95.89	97.47	102.23	112.53
128.0	110.15	127.58	99.85	100.64	112.53	107.77
135.0	109.36	108.57	81.62	79.25	108.57	111.74
144.0	84.79	98.26	43.38	40.88	86.38	89.55
147.0	84.00	79.25	34.61	37.62	88.75	91.92
149.5	93.51	99.85	25.08	26.33	91.13	95.89
152.0	99.06	103.81			97.47	90.34
159.0	99.06	103.81			102.23	97.47

168.0	103.02	97.47		93.51	92.72
171.0	118.08	99.06		89.55	95.09
173.5	110.15	88.75		106.98	97.47
176.0	110.15	110.94			
183.5	103.81	109.36			

	Acid	Inj.	Sample	Peak Areas: Culture A		
Hour	(ml)	(ml)	(ml)	C's	GTX-4	GTX-3
72.0	5	0.010	100	18366896	1366814	4446135
75.0	5	0.010	100	13495113	1869712	5106899
77.5	5	0.010	100	19494808	1679991	5603753
80.0	5	0.010	100	20408886	1788772	5930905
86.5	5	0.010	100	19948559	1740747	5633071
96.0	5	0.010	100	18614080	1336086	3846303
99.0	5	0.010	100	18696630	1320975	4325745
101.5	5	0.010	100	19038191	1336414	4885791
104.2	5	0.010	100	19233630	1500495	4965491
110.5	5	0.010	100	19127567	1481694	5500201
120.3	5	0.010	100	4360297	125352	567873
123.0	5	0.010	100	19950348	1966143	5557590
125.5	5	0.010	100	21293453	1941709	6516700
128.0	5	0.010	100	20512777	1936435	6296426
135.0	5	0.010	100	21185978	2046221	6712037
144.0	5	0.010	100	20004705	1887164	5051084
147.0	5	0.010	100	19356817	1541510	4573684
149.5	5	0.010	100	18877197	1489722	4298411
152.0	5	0.010	100	18850007	1347688	4533739
159.0	5	0.010	100	18649764	1446024	4348780
168.0	5	0.010	100	18125623	1230265	3686611
171.0	5	0.010	100	18497351	1480762	4063962
173.5	5	0.010	100	20236490	1907224	5441461
176.0	5	0.010	100	20301240	2025360	5695702
183.5	5	0.010	100	20050855	1894607	5790257

Table A.12 - July 1993

		Light ($\mu E/m^2/s$)			Density (cells/ml)			Doublets (%)		
Hour	Time	A	B	C	A	B	C	A	B	C
3.00	1100	150	150	150	845	1087	1108	23.6	10.5	9.1
5.50	1330	150	150	150	854	1186	1378	24.5	14.8	16.6
8.00	1600	150	150	150	938	1065	1187	25.4	20	17.6
15.00	2300	150	150	150	1061	1057	1419	26.3	12.1	15.8
24.00	0800	150	150	150	1315	1456	1826	18.8	18.9	8.8
27.00	1100	150	150	150	1217	1523	1765	26	15	9.5
29.50	1330	150	150	150	1517	1654	2059	28.4	13.9	17.3
32.00	1600	150	150	150	1310	1671	1777	30.2	12	17.8
38.75	2245	150	150	150	1369	1959	1755	21.1	11.8	9.6
48.25	0815	150	150	150	1888	2114	2497	10.6	6.5	9.3

51.00	1100	150	150	150	1703	2224	2294	15.3	2.6	3.6
53.50	1330	150	150	150	1675	1962	2153	15.9	7.7	4.7
56.33	1620	150	150	150	1558	1778	2174	18.4	14.4	6.9
63.00	2300	150	150	150	1620	1999	2508	11.8	5	5.7
67.50	0330	(24h)	dark	dark	1442	1995	2626	7.9	4.8	5.3
72.00	0800	(24h)	85	150	1006	1792	2332	3.4	3.1	5.7
75.25	1115	(24h)	85	150	929	1959	2507	7.1	5.1	5.1
77.50	1330	(24h)	85	150	887	1981	2924	18.6	10	7.7
80.00	1600	(24h)	85	150	766	1691	2623	12.9	6.6	8.7
87.00	2300	(24h)	85	150	745	1516	2321	11	5.6	1.9
96.25	0815	(24h)	85	150	682	1719	2764	6.7	1.7	3.9
99.00	1100	(24h)	85	150	645	1590	2546	1.4	3.6	8.1
101.75	1345	(24h)	85	150	633	1701	2626	9.5	7.7	8
104.75	1645	(24h)	85	150	661	1552	2086	5	9.2	5.1
110.17	2210	(24h)	85	150	574	1533	2035	5.1	5.2	3.9

Chlorophyll-a (duplicate samples)

Hour	Acid (ml)	Inj. (ml)	Sample (ml)	A1	A2	B1	B2	C1	C2
				(ng/ml)					
3.00	3.00	0.010	100	36.1	36.9	44.1	42.9	36.4	34.4
5.50	3.00	0.010	100	39.9	35.4	47.6	41.9	40.1	44.1
8.00	3.00	0.010	100	38.4	38.6	44.4	49.4	47.1	44.1
15.00	3.00	0.010	100	42.4	39.1	43.6	46.6	40.6	42.1
24.00	3.00	0.010	100	48.6	48.4	44.1	50.9	40.4	39.4
27.00	3.00	0.010	100	41.6	42.1	45.4	50.9	44.9	42.9
29.50	3.00	0.010	100	43.4	44.1	44.9	51.4	46.6	45.6
32.00	3.00	0.010	100	42.1	43.4	49.4	55.9	47.1	45.9
38.75	3.00	0.010	100	44.6	46.6	54.4	56.7	43.9	51.4
48.25	3.00	0.010	100	43.6	36.1	61.2	55.9	44.9	46.9
51.00	3.00	0.010	100	41.1	42.6	52.2	49.4	47.4	42.1
53.50	3.00	0.010	100	44.6	46.6	51.9	44.9	50.9	43.4
56.33	3.00	0.010	100	46.9	47.1	52.4	57.4	49.6	47.1
63.00	3.00	0.010	100	44.9	47.1	47.6	47.4	46.6	46.9
67.50	3.00	0.010	100	37.6	44.1	49.1	91.1	41.6	42.9
72.00	3.00	0.010	100	21.3	25.1	48.1	46.1	41.6	45.1
75.25	3.00	0.010	100	22.8	23.8	51.9	49.6	47.4	43.9
77.50	3.00	0.010	100	20.6	18.6	48.4	48.6		43.4
80.00	3.00	0.010	100	17.6	16.8	51.9	47.9	50.2	49.1
87.00	3.00	0.010	100	16.6	16.1	43.4	43.6	55.2	47.6
96.25	3.00	0.010	100	14.8	12.3	39.1	39.6	43.6	50.4
99.00	3.00	0.010	100	14	13.8	43.1	40.6	45.1	49.1
101.75	3.00	0.010	100		13.3	43.9	44.4	46.4	47.4
104.75	3.00	0.010	100	15.6	15.1	46	46.6	49.9	49.9
110.17	3.00	0.010	100	12.2	12	50.7	47.8	49.9	51.5

	Peak Areas: Culture A			Peak Areas: Culture B	
Hour	C's	GTX-4	GTX-3	C's	GTX-4
3.00	13722291	646702	2179819	19234953	1378956
5.50	13702193	667798	2257457	16739292	933635
8.00	13293047	506733	2311538	17645198	1034002
15.00	14754982	701876	2813955	16075904	732532
24.00	12914532	492182	1991419	15574634	871533
27.00	13534479	534034	2026548	16275955	761567
29.50	14432662	676788	2353865	16500462	876793
32.00	14776717	628067	2565927	17162063	969091
38.75	15339904	731055	3027635	16428700	854981
48.25	14078557	459259	2038509	16787686	910683
51.00	14896631	628304	2543414	16198579	949053
53.50	14632028	532945	2169636	14962077	633424
56.33	15150897	704693	2608978	16158042	648334
63.00	15060844	638708	2634697	18818787	1155727
67.50	13918588	370213	2041175	14875186	409222
72.00	10555019	302622	1124721	15120365	668074
75.25	9956750	230863	973629	16620246	875305
77.50	8379443	186794	772734	15952376	815235
80.00	6026401	149198	569955	15541608	677333
87.00	6877921	231058	605683	15656762	715276
96.25	5871784	164735	473820	14139661	575376
99.00	5454128	162665	469641	14256433	412178
101.75	5435509		385279	14283943	626128
104.75	12654187	290406	1041420	16954001	726344
110.17	10786947	271100	792272	16566259	777156

	Peak Areas: Culture B	Peak Areas: Culture C		
Hour	GTX-3	C's	GTX-4	GTX-3
3.00	5220331	14333579	526088	2823935
5.50	3514737	15201661	677744	3167286
8.00	4410696	13942784	579189	2778179
15.00	3204762	15273496	628256	3574589
24.00	2958911	14670052	532415	2811451
27.00	3030409	13452850	575550	2304803
29.50	3235889	15052368	544189	3013699
32.00	3832033	14897791	657364	2882221
38.75	3265401	14878006	626242	3165948
48.25	3180509	15380409	579532	2943920
51.00	3027509	14822547	714544	2851122
53.50	2648405	15922739	609815	3223075
56.33	2871463	14369387	442366	2647309
63.00	4461227	17421471	714747	3837652
67.50	2309099	14832818	474190	2646849
72.00	2164437	14685677	487842	2477360
75.25	2873423	16069893	667440	2843869
77.50	2492791	15773708	525923	2947951
80.00	2428601	15453889	515971	2864530

87.00	2225488	17402237	759748	4094707
96.25	1592860	15189154	655829	2713344
99.00	1696376	15708936	496325	2598477
101.75	1668151	12486791	266873	1703961
104.75	2561787	18096018	903889	4147062
110.17	2531869	17880452	834458	3794888

Daily Normalized Data: Culture Phasing Experiments

This data was used for the ANOVA analyses.

Table A.13 - June 1993 (Culture A only)

Day	Time	Hour	Doubs	Chl/cell	GTX-3/cell	Total Toxin/cell
1	0800	72.0	1.13	1.03	0.76	0.85
	1100	75.0	1.11	1.00	0.96	1.01
	1330	77.5	0.88	0.98	0.96	0.92
	1600	80.0	1.35	1.04	1.05	0.99
	2230	86.5	0.52	0.95	1.27	1.23
2	0800	96.0	0.48	0.93	0.87	1.03
	1100	99.0	1.20	1.01	0.93	0.99
	1330	101.5	1.48	0.96	1.00	0.96
	1615	104.2	1.15	1.00	1.01	0.98
	2230	110.5	0.69	1.10	1.20	1.04
3	0820	120.3	0.70	0.86	0.12	0.24
	1100	123.0	1.18	0.95	1.22	1.29
	1330	125.5	1.04	0.96	1.38	1.32
	1600	128.0	1.26	1.16	1.04	1.00
	2300	135.0	0.81	1.06	1.25	1.16
4	0800	144.0	1.14	0.97	1.15	1.10
	1100	147.0	1.13	0.86	1.02	1.03
	1330	149.5	1.14	1.02	0.91	0.95
	1600	152.0	0.88	1.07	0.91	0.89
	2300	159.0	0.71	1.07	1.01	1.03
5	0800	168.0	0.54	0.95	0.92	1.08
	1100	171.0	1.28	1.03	1.04	1.16
	1330	173.5	1.61	0.95	1.02	0.95
	1600	176.0	0.98	1.05	1.30	1.17
	2315	183.5	0.59	1.01	0.72	0.63

Table A.14 - July 1993

			Doublets			Chlorophyll/cell			GTX-3/cell		
Day	Time	Hour	A	B	C	A	B	C	A	B	C
1	1100	3.00	0.95	0.73	0.62	1.04	0.97	0.98	1.00	1.29	1.05
	1330	5.50	0.98	1.03	1.12	1.06	0.92	0.94	1.02	0.79	0.95
	1600	8.00	1.02	1.39	1.19	0.98	1.07	1.18	0.95	1.11	0.96
	2300	15.00	1.05	0.84	1.07	0.92	1.04	0.90	1.03	0.81	1.04
2	800	24.00	0.76	1.32	0.70	1.11	1.07	0.89	0.85	1.02	1.00
	1100	27.00	1.04	1.05	0.75	1.04	1.03	1.02	0.94	1.00	0.84
	1330	29.50	1.14	0.97	1.37	0.87	0.95	0.92	0.87	0.98	0.95
	1600	32.00	1.21	0.84	1.41	0.98	1.03	1.07	1.10	1.15	1.05
	2245	38.75	0.85	0.82	0.76	1.00	0.93	1.11	1.24	0.84	1.17
3	815	48.25	0.74	0.90	1.54	0.80	1.07	0.91	0.75	0.93	0.88
	1100	51.00	1.06	0.36	0.60	0.93	0.88	0.97	1.04	0.84	0.93
	1330	53.50	1.10	1.06	0.78	1.04	0.95	1.09	0.90	0.84	1.12
	1620	56.33	1.28	1.99	1.14	1.15	1.19	1.11	1.17	1.00	0.91
	2300	63.00	0.82	0.69	0.94	1.08	0.92	0.93	1.13	1.38	1.15
4	800	72.00	0.32	0.51	0.98	1.01	0.97	1.00	1.22	0.88	0.88
	1115	75.25	0.67	0.84	0.88	1.10	0.96	0.98	1.14	1.07	0.94
	1330	77.50	1.75	1.64	1.32	0.96	0.91	0.80	0.95	0.92	0.83
	1600	80.00	1.22	1.09	1.49	0.98	1.09	1.02	0.81	1.05	0.90
	2300	87.00	1.04	0.92	0.33	0.96	1.06	1.19	0.88	1.07	1.46
5	815	96.25	1.21	0.31	0.67	0.93	0.84	0.83	0.99	0.74	0.75
	1100	99.00	0.25	0.66	1.40	1.01	0.96	0.91	1.04	0.85	0.78
	1345	101.75	1.71	1.41	1.38	0.98	0.95	0.87	0.87	0.78	0.50
	1645	104.75	0.90	1.68	0.88	1.09	1.09	1.17	1.12	1.31	1.53
	2210	110.17	0.92	0.95	0.67	0.99	1.17	1.22	0.98	1.32	1.43

			Total Toxin/cell		
Day	Time	Hour	A	B	C
1	1100	3.00	1.08	1.14	1.10
	1330	5.50	1.07	0.88	0.95
	1600	8.00	0.93	1.04	1.01
	2300	15.00	0.93	0.93	0.93
2	800	24.00	0.92	1.07	1.00
	1100	27.00	1.04	1.06	0.96
	1330	29.50	0.90	1.00	0.91
	1600	32.00	1.07	1.04	1.06
	2245	38.75	1.07	0.84	1.07
3	815	48.25	0.84	0.96	0.91
	1100	51.00	1.00	0.89	0.97
	1330	53.50	0.99	0.91	1.10
	1620	56.33	1.12	1.08	0.97
	2300	63.00	1.06	1.16	1.04
4	800	72.00	1.10	0.94	0.99
	1115	75.25	1.12	0.96	1.02
	1330	77.50	0.98	0.91	0.85
	1600	80.00	0.82	1.03	0.93
	2300	87.00	0.97	1.16	1.21

5	815	96.25	0.97	0.87	0.81
	1100	99.00	0.96	0.93	0.89
	1345	101.75	0.93	0.89	0.68
	1645	104.75	1.08	1.16	1.31
	2210	110.17	1.06	1.16	1.31

APPENDIX 2

RESULTS OF STATISTICAL ANALYSES

The following pages are the raw output of the test run on Minitab release 9.1 software. Column indices are provided to identify the variables. All tests performed are shown including some not quoted in the text.

October 1991 Data:

Column Directory:
C1=10/91A Divrate before
C2=10/91A divrate after
C3=10/91B divrate before
C4=10/91B divrate after
C5=10/91C divrate before
C6=10/91C divrate after
C7=10/91A ttox before
C8=10/91A ttox after
C9=10/91B ttox before
C10=10/91B ttox after
C11=10/91C ttox before
C12=10/91C ttox after
C13=10/91A %GTX-3 before
C14=10/91A %GTX-3 after
C15=10/91B %GTX-3 before
C16=10/91B %GTX-3 after
C17=10/91C % GTX-3 before
C18=10/91C %GTX-3 after
C19=10/91A %C's before

C20=10/91A %C's after
C21=10/91B %C's before
C22=10/91B %C's after
C23=10/91C %C's before
C24=10/91C %C's after

TWO TAIL TESTS:

T-test for division rate on Culture 10/91A (control).
Ho: μbefore (C1) = μafter (C2)
Ha: not equal

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	7	0.133	0.125	0.0474
C2	7	0.365	0.149	0.0564

95 PCT CI FOR MU C1 - MU C2: (-0.3940, -0.06967)

TTEST MU C1 = MU C2 (VS NE): T= -3.15 P=0.0093 DF= 11

Culture 10/91B (light reduction) Division Rates

TWOSAMPLE T FOR C3 VS C4

	N	MEAN	STDEV	SE MEAN
C3	7	0.327	0.171	0.0646
C4	7	0.125	0.280	0.106

95 PCT CI FOR MU C3 - MU C4: (-0.07899, 0.4824)

TTEST MU C3 = MU C4 (VS NE): T= 1.63 P=0.14 DF= 9

Culture 10/91C (light reduction) Division rates

TWOSAMPLE T FOR C5 VS C6

	N	MEAN	STDEV	SE MEAN
C5	7	0.379	0.279	0.105
C6	7	0.0271	0.0718	0.0271

95 PCT CI FOR MU C5 - MU C6: (0.08569, 0.6186)

TTEST MU C5 = MU C6 (VS NE): T= 3.23 P=0.018 DF= 6

Culture 10/91A Total Toxin

TWOSAMPLE T FOR C7 VS C8

	N	MEAN	STDEV	SE MEAN
C7	7	5491	1731	654
C8	7	3625	897	339

95 PCT CI FOR MU C7 - MU C8: (198.5, 3532)

TTEST MU C7 = MU C8 (VS NE): T= 2.53 P=0.032 DF= 9

Culture 10/91B Total Toxin

TWOSAMPLE T FOR C9 VS C10

	N	MEAN	STDEV	SE MEAN
C9	5	6731	2892	1293
C10	7	4225	1653	625

95 PCT CI FOR MU C9 - MU C10: (-1187, 6200)

TTEST MU C9 = MU C10 (VS NE): T= 1.74 P=0.14 DF= 5

Culture 10/91C Total Toxin

TWOSAMPLE T FOR C11 VS C12

	N	MEAN	STDEV	SE MEAN
C11	7	5065	972	367
C12	7	4980	2613	988

95 PCT CI FOR MU C11 - MU C12: (-2408, 2577)

TTEST MU C11 = MU C12 (VS NE): T= 0.08 P=0.94 DF= 7

ONE TAIL TESTS:

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	7	0.133	0.125	0.0474
C2	7	0.365	0.149	0.0564

95 PCT CI FOR MU C1 - MU C2: (-0.3940, -0.06967)

TTEST MU C1 = MU C2 (VS GT): T= -3.15 P=1.0 DF= 11

TWOSAMPLE T FOR C3 VS C4

	N	MEAN	STDEV	SE MEAN
C3	7	0.327	0.171	0.0646
C4	7	0.125	0.280	0.106

95 PCT CI FOR MU C3 - MU C4: (-0.07899, 0.4824)

TTEST MU C3 = MU C4 (VS GT): T= 1.63 P=0.069 DF= 9

TWOSAMPLE T FOR C5 VS C6

	N	MEAN	STDEV	SE MEAN
C5	7	0.379	0.279	0.105
C6	7	0.0271	0.0718	0.0271

95 PCT CI FOR MU C5 - MU C6: (0.08569, 0.6186)

TTEST MU C5 = MU C6 (VS GT): T= 3.23 P=0.0089 DF= 6

TWOSAMPLE T FOR C7 VS C8

	N	MEAN	STDEV	SE MEAN
C7	7	5491	1731	654
C8	7	3625	897	339

95 PCT CI FOR MU C7 - MU C8: (198.5, 3532)

TTEST MU C7 = MU C8 (VS GT): T= 2.53 P=0.016 DF= 9

TWOSAMPLE T FOR C9 VS C10

	N	MEAN	STDEV	SE MEAN
C9	5	6731	2892	1293
C10	7	4225	1653	625

95 PCT CI FOR MU C9 - MU C10: (-1187, 6200)

TTEST MU C9 = MU C10 (VS GT): T= 1.74 P=0.071 DF= 5

TWOSAMPLE T FOR C11 VS C12

	N	MEAN	STDEV	SE MEAN
C11	7	5065	972	367
C12	7	4980	2613	988

95 PCT CI FOR MU C11 - MU C12: (-2408, 2577)

TTEST MU C11 = MU C12 (VS GT): T= 0.08 P=0.47 DF= 7

Toxin Proportions:

Culture A:

TWOSAMPLE T FOR C13 VS C14

	N	MEAN	STDEV	SE MEAN
C13	7	18.09	2.61	0.986
C14	7	21.66	3.02	1.14

95 PCT CI FOR MU C13 - MU C14: (-6.885, -0.2433)

TTEST MU C13 = MU C14 (VS GT): T= -2.36 P=0.98 DF= 11

Culture B:

TWOSAMPLE T FOR C15 VS C16

	N	MEAN	STDEV	SE MEAN
C15	5	26.9	12.9	5.77
C16	7	13.95	5.88	2.22

95 PCT CI FOR MU C15 - MU C16: (-2.979, 28.80)

TTEST MU C15 = MU C16 (VS GT): T= 2.09 P=0.045 DF= 5

Culture C:

TWOSAMPLE T FOR C17 VS C18

	N	MEAN	STDEV	SE MEAN
C17	7	20.70	5.22	1.97
C18	7	14.03	3.86	1.46

95 PCT CI FOR MU C17 - MU C18: (1.263, 12.07)

TTEST MU C17 = MU C18 (VS GT): T= 2.72 P=0.010 DF= 11

Two-tail on Culture A:

TWOSAMPLE T FOR C13 VS C14

	N	MEAN	STDEV	SE MEAN
C13	7	18.09	2.61	0.986
C14	7	21.66	3.02	1.14

95 PCT CI FOR MU C13 - MU C14: (-6.885, -0.2433)

TTEST MU C13 = MU C14 (VS NE): T= -2.36 P=0.038 DF= 11

Two-tail test Culture B:

TWOSAMPLE T FOR C15 VS C16

	N	MEAN	STDEV	SE MEAN
C15	5	26.9	12.9	5.77
C16	7	13.95	5.88	2.22

95 PCT CI FOR MU C15 - MU C16: (-2.979, 28.80)

TTEST MU C15 = MU C16 (VS NE): T= 2.09 P=0.091 DF= 5

Two tail test Culture C:

TWOSAMPLE T FOR C17 VS C18

	N	MEAN	STDEV	SE MEAN
C17	7	20.70	5.22	1.97
C18	7	14.03	3.86	1.46

95 PCT CI FOR MU C17 - MU C18: (1.263, 12.07)

TTEST MU C17 = MU C18 (VS NE): T= 2.72 P=0.020 DF= 11

One tail tests for C-toxins:
Culture 10/91A:
TWOSAMPLE T FOR C19 VS C20

	N	MEAN	STDEV	SE MEAN
C19	7	77.01	3.56	1.35
C20	7	72.62	3.27	1.24

95 PCT CI FOR MU C19 - MU C20: (0.3586, 8.404)

TTEST MU C19 = MU C20 (VS LT): T= 2.40 P=0.98 DF= 11

Culture 10/91B:

TWOSAMPLE T FOR C21 VS C22

	N	MEAN	STDEV	SE MEAN
C21	5	70.1	12.9	5.77
C22	7	83.33	6.52	2.47

95 PCT CI FOR MU C21 - MU C22: (-29.38, 2.905)

TTEST MU C21 = MU C22 (VS LT): T= -2.11 P=0.044 DF= 5

Culture 10/91C:

TWOSAMPLE T FOR C23 VS C24

	N	MEAN	STDEV	SE MEAN
C23	7	76.36	5.91	2.23
C24	7	83.53	4.03	1.52

95 PCT CI FOR MU C23 - MU C24: (-13.19, -1.136)

TTEST MU C23 = MU C24 (VS LT): T= -2.65 P=0.012 DF= 10

November 1991 Data:

November 1991 Toxin Proportions
Test if μbefore > μafter

C1 = Culture 11/91A % GTX-3 before
C2 = Culture 11/91A % GTX-3 after
C3 = Culture 11/91C % GTX-3 before
C4 = Cutlure 11/91C % GTX-3 after
C5 = 11/91A % C's before
C6 = 11/91A %C's after
C7 = 11/91C %C's before
C8 = 11/91C %C's after

Culture 11/91A (light reduction)

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	3	15.53	3.44	1.98
C2	3	11.60	2.00	1.15

95 PCT CI FOR MU C1 - MU C2: (-3.366, 11.24)

TTEST MU C1 = MU C2 (VS GT): T= 1.72 P=0.092 DF= 3

Culture 11/91C (control)

TWOSAMPLE T FOR C3 VS C4

	N	MEAN	STDEV	SE MEAN
C3	3	15.835	0.828	0.478
C4	4	16.98	2.23	1.11

95 PCT CI FOR MU C3 - MU C4: (-4.510, 2.225)

TTEST MU C3 = MU C4 (VS GT): T= -0.94 P=0.80 DF= 4

Test for C-toxins:

Culture 11/91A:

TWOSAMPLE T FOR C5 VS C6

	N	MEAN	STDEV	SE MEAN
C5	3	81.21	4.22	2.44
C6	3	85.29	2.18	1.26

95 PCT CI FOR MU C5 - MU C6: (-15.88, 7.721)

TTEST MU C5 = MU C6 (VS LT): T= -1.49 P=0.14 DF= 2

Culture 11/91C:

TWOSAMPLE T FOR C7 VS C8

	N	MEAN	STDEV	SE MEAN
C7	3	81.13	1.27	0.736
C8	4	79.85	2.52	1.26

95 PCT CI FOR MU C7 - MU C8: (-2.771, 5.326)

TTEST MU C7 = MU C8 (VS LT): T= 0.88 P=0.78 DF= 4

March-April 1992:

One tailed tests Ho: $\mu 1=\mu 2$ Ha: $\mu 1>\mu 2$

Column Index:
C1 = division rate before
C2 = division rate after
C3 = smoothed division rate before
C4 = smoothed division rate after
C5 = total toxin/cell before
C6 = total toxin/cell after
C7 = smoothed total toxin/cell before
C8 = smoothed total toxin/cell after
C12 = edited division rate after
C14 = smoothed edited division rate after
C16 = edited total toxin.cell after
C18 = smoothed edited total toxin/cell after
C20 = %C's before
C21 = %C's after
C22 = % GTX-3 before
C23 = % GTX-3 after

Division Rate:
TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	7	0.202	0.160	0.0606
C2	17	0.1625	0.0961	0.0233

95 PCT CI FOR MU C1 - MU C2: (-0.1144, 0.1926)

TTEST MU C1 = MU C2 (VS GT): T= 0.60 P=0.28 DF= 7

Smoothed Division Rate:

TWOSAMPLE T FOR C3 VS C4

	N	MEAN	STDEV	SE MEAN
C3	6	0.2082	0.0608	0.0248
C4	16	0.1667	0.0737	0.0184

95 PCT CI FOR MU C3 - MU C4: (-0.02741, 0.1104)

TTEST MU C3 = MU C4 (VS GT): T= 1.34 P=0.10 DF= 10

Total Toxin:

TWOSAMPLE T FOR C5 VS C6

	N	MEAN	STDEV	SE MEAN
C5	4	15642	6023	3012
C6	12	13973	3121	901

95 PCT CI FOR MU C5 - MU C6: (-8335, 11673)

TTEST MU C5 = MU C6 (VS NE): T= 0.53 P=0.63 DF= 3

Smoothed Total Toxin

TWOSAMPLE T FOR C7 VS C8

	N	MEAN	STDEV	SE MEAN
C7	3	14403	1144	661
C8	11	14582	2007	605

95 PCT CI FOR MU C7 - MU C8: (-2482, 2125)

TTEST MU C7 = MU C8 (VS GT): T= -0.20 P=0.57 DF= 5

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Edited data - time series abbreviated at Day 15 to eliminate possible photoadaptation period:

Division Rate:

TWOSAMPLE T FOR C1 VS C12

	N	MEAN	STDEV	SE MEAN
C1	7	0.202	0.160	0.0606
C12	8	0.0952	0.0691	0.0244

95 PCT CI FOR MU C1 - MU C12: (-0.04813, 0.2608)
TTEST MU C1 = MU C12 (VS GT): T= 1.63 P=0.074 DF= 7

Smoothed Division Rate:

TWOSAMPLE T FOR C3 VS C14

	N	MEAN	STDEV	SE MEAN
C3	6	0.2082	0.0608	0.0248
C14	8	0.1056	0.0444	0.0157

95 PCT CI FOR MU C3 - MU C14: (0.03486, 0.1703)

TTEST MU C3 = MU C14 (VS GT): T= 3.49 P=0.0041 DF= 8

Total Toxin:

TWOSAMPLE T FOR C5 VS C16

	N	MEAN	STDEV	SE MEAN
C5	4	15642	6023	3012
C16	8	15140	3218	1138

95 PCT CI FOR MU C5 - MU C16: (-9744, 10747)

TTEST MU C5 = MU C16 (VS GT): T= 0.16 P=0.44 DF= 3

Smoothed Total Toxin:

TWOSAMPLE T FOR C7 VS C18

	N	MEAN	STDEV	SE MEAN
C7	3	14403	1144	661
C18	8	15505	1346	476

95 PCT CI FOR MU C7 - MU C18: (-3362, 1160)

TTEST MU C7 = MU C18 (VS GT): T= -1.35 P=0.88 DF= 4

% C-toxins:

TWOSAMPLE T FOR C20 VS C21

	N	MEAN	STDEV	SE MEAN
C20	4	48.67	7.78	3.89

C21 8 59.97 8.08 2.86

95 PCT CI FOR MU C20 - MU C21: (-23.10, 0.5188)

TTEST MU C20 = MU C21 (VS LT): T= -2.34 P=0.029 DF= 6

% GTX-3:

TWOSAMPLE T FOR C22 VS C23

	N	MEAN	STDEV	SE MEAN
C22	4	38.97	6.65	3.33
C23	8	29.56	6.27	2.22

95 PCT CI FOR MU C22 - MU C23: (-0.8692, 19.69)

TTEST MU C22 = MU C23 (VS GT): T= 2.35 P=0.033 DF= 5

August 1992:

Ttests on Aug/Sept 1992 data:

Two-tail tests to determine if mu before = mu after (change of L:D at Day 6)
(i.e. - is there any difference between photoperiods? - none is apparent)

Column Index:
C1 = Divrate B before
C2 = divrate B after
C3 = divrate C before
C4 = divrate C after
C5 = ttox B before
C6 = ttox B after
C7 = ttox C before
C8 = ttox C after
C9 = 5 C's B before
C10 = % C's B after
C11 = % C's C before

C12 = % C's C after
C13 = % GTX-3 B before
C14 = % GTX-3 B after
C15 = % GTX-3 C before
C16 = % GTX-3 C after

All time series have been abbreviated at Day 12 to avoid including the two final days at 16:8 L:D.

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	5	0.176	0.105	0.0468
C2	6	0.172	0.146	0.0597

95 PCT CI FOR MU C1 - MU C2: (-0.1707, 0.1793)

TTEST MU C1 = MU C2 (VS NE): T= 0.06 P=0.96 DF= 8

TWOSAMPLE T FOR C3 VS C4

	N	MEAN	STDEV	SE MEAN
C3	5	0.0920	0.0958	0.0428
C4	7	0.0286	0.0518	0.0196

95 PCT CI FOR MU C3 - MU C4: (-0.05764, 0.1845)

TTEST MU C3 = MU C4 (VS NE): T= 1.35 P=0.24 DF= 5

TWOSAMPLE T FOR C5 VS C6

	N	MEAN	STDEV	SE MEAN
C5	5	34977	17670	7902
C6	6	37768	12722	5194

95 PCT CI FOR MU C5 - MU C6: (-25159, 19576)

TTEST MU C5 = MU C6 (VS NE): T= -0.30 P=0.78 DF= 7

TWOSAMPLE T FOR C7 VS C8

	N	MEAN	STDEV	SE MEAN
C7	5	31315	7065	3160
C8	7	44874	13025	4923

95 PCT CI FOR MU C7 - MU C8: (-26795, -322.4)

TTEST MU C7 = MU C8 (VS NE): T= -2.32 P=0.046 DF= 9

TWOSAMPLE T FOR C9 VS C10

	N	MEAN	STDEV	SE MEAN
C9	5	63.7	11.2	5.03
C10	6	63.3	13.0	5.33

95 PCT CI FOR MU C9 - MU C10: (-16.49, 17.30)

TTEST MU C9 = MU C10 (VS NE): T= 0.05 P=0.96 DF= 8

TWOSAMPLE T FOR C11 VS C12

	N	MEAN	STDEV	SE MEAN
C11	5	63.1	13.2	5.92
C12	7	64.89	8.25	3.12

95 PCT CI FOR MU C11 - MU C12: (-18.14, 14.61)

TTEST MU C11 = MU C12 (VS NE): T= -0.26 P=0.80 DF= 6

TWOSAMPLE T FOR C13 VS C14

	N	MEAN	STDEV	SE MEAN
C13	5	24.23	7.93	3.55
C14	6	25.13	9.91	4.05

95 PCT CI FOR MU C13 - MU C14: (-13.30, 11.52)
TTEST MU C13 = MU C14 (VS NE): T= -0.17 P=0.87 DF= 8

TWOSAMPLE T FOR C15 VS C16

	N	MEAN	STDEV	SE MEAN
C15	5	24.32	9.00	4.03
C16	7	22.74	6.18	2.34

95 PCT CI FOR MU C15 - MU C16: (-9.803, 12.98)

TTEST MU C15 = MU C16 (VS NE): T= 0.34 P=0.74 DF= 6

October 1992:

Two tailed tests:
Test if μbefore = μafter.

Column Index:
C1 = divrate B before first L:D change (16:8 to 12:12)
C2 = divrate B after 1st change
C3 = ttox B before 1
C4 = ttox B after 1
C5 = % C's B before 1
C6 = % C's B after 1
C7 = % GTX-3 B before 1
C8 = % GTX-3 B after 1
C9 = divrate B before second L:D change (12:12 to 16:8)
C10 = divrate B after 2
C11 = divrate C before 2
C12 = divrate C after 2
C13 = ttox B before 2
C14 = ttox B after 2
C15 = ttox C before 2
C16 = ttox c after 2
C17 = % C's B before 2
C18 = % C's B after 2
C19 = % C's C before 2
C20 = % C's C after 2
C21 = % GTX-3 B before 2
C22 = % GTX-3 B after 2

C23 = % GTX-3 C before 2
C24 = % GTX-3 C after 2

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	3	0.149	0.100	0.0578
C2	5	0.218	0.200	0.0894

95 PCT CI FOR MU C1 - MU C2: (-0.3422, 0.2053)

TTEST MU C1 = MU C2 (VS NE): T= -0.64 P=0.55 DF= 5

TWOSAMPLE T FOR C3 VS C4

	N	MEAN	STDEV	SE MEAN
C3	3	59302	10066	5812
C4	4	43531	25948	12974

95 PCT CI FOR MU C3 - MU C4: (-23711, 55252)

TTEST MU C3 = MU C4 (VS NE): T= 1.11 P=0.33 DF= 4

TWOSAMPLE T FOR C5 VS C6

	N	MEAN	STDEV	SE MEAN
C5	3	0.600	0.209	0.121
C6	4	0.684	0.164	0.0822

95 PCT CI FOR MU C5 - MU C6: (-0.5485, 0.3815)

TTEST MU C5 = MU C6 (VS NE): T= -0.57 P=0.61 DF= 3

TWOSAMPLE T FOR C7 VS C8

	N	MEAN	STDEV	SE MEAN
C7	3	0.323	0.173	0.0998
C8	4	0.249	0.126	0.0632

95 PCT CI FOR MU C7 - MU C8: (-0.3017, 0.4504)

TTEST MU C7 = MU C8 (VS NE): T= 0.63 P=0.57 DF= 3

TWOSAMPLE T FOR C9 VS C10

	N	MEAN	STDEV	SE MEAN
C9	4	0.191	0.272	0.136
C10	4	0.382	0.296	0.148

95 PCT CI FOR MU C9 - MU C10: (-0.7082, 0.3262)

TTEST MU C9 = MU C10 (VS NE): T= -0.95 P=0.39 DF= 5

TWOSAMPLE T FOR C11 VS C12

	N	MEAN	STDEV	SE MEAN
C11	4	0.178	0.148	0.0738
C12	4	0.2450	0.0835	0.0417

95 PCT CI FOR MU C11 - MU C12: (-0.3029, 0.1679)

TTEST MU C11 = MU C12 (VS NE): T= -0.80 P=0.47 DF= 4

TWOSAMPLE T FOR C13 VS C14

	N	MEAN	STDEV	SE MEAN
C13	3	57275	14128	8157
C14	4	59457	15656	7828

95 PCT CI FOR MU C13 - MU C14: (-33579, 29217)

TTEST MU C13 = MU C14 (VS NE): T= -0.19 P=0.86 DF= 4

TWOSAMPLE T FOR C15 VS C16

	N	MEAN	STDEV	SE MEAN
C15	3	35085	8035	4639
C16	4	49136	3107	1554

95 PCT CI FOR MU C15 - MU C16: (-35102, 6999)

TTEST MU C15 = MU C16 (VS NE): T= -2.87 P=0.10 DF= 2

TWOSAMPLE T FOR C17 VS C18

	N	MEAN	STDEV	SE MEAN
C17	3	0.493	0.169	0.0978
C18	4	0.4737	0.0622	0.0311

95 PCT CI FOR MU C17 - MU C18: (-0.4216, 0.4612)

TTEST MU C17 = MU C18 (VS NE): T= 0.19 P=0.86 DF= 2

TWOSAMPLE T FOR C19 VS C20

	N	MEAN	STDEV	SE MEAN
C19	3	0.4746	0.0904	0.0522
C20	4	0.4576	0.0433	0.0217

95 PCT CI FOR MU C19 - MU C20: (-0.2262, 0.2602)

TTEST MU C19 = MU C20 (VS NE): T= 0.30 P=0.79 DF= 2

TWOSAMPLE T FOR C21 VS C22

	N	MEAN	STDEV	SE MEAN
C21	3	0.400	0.140	0.0809
C22	4	0.4150	0.0561	0.0281

95 PCT CI FOR MU C21 - MU C22: (-0.3833, 0.3537)

TTEST MU C21 = MU C22 (VS NE): T= -0.17 P=0.88 DF= 2

TWOSAMPLE T FOR C23 VS C24

	N	MEAN	STDEV	SE MEAN
C23	3	0.4093	0.0769	0.0444
C24	4	0.4228	0.0460	0.0230

95 PCT CI FOR MU C23 - MU C24: (-0.1725, 0.1456)

TTEST MU C23 = MU C24 (VS NE): T= -0.27 P=0.81 DF= 3

Analyses of Variance:

June-July 1993 Data

```
MTB > aovoneway c1-c5 #DAILY NORMALIZED CELL DOUBLETS - CULTURE A

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4    1.1117    0.2779     3.42    0.029
ERROR      19    1.5424    0.0812
TOTAL      23    2.6541

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  --------+---------+---------+---------
0800A       4    0.7575    0.3635  (--------*--------)
1100A       5    0.7940    0.3417    (-------*-------)
1300A       5    1.3360    0.3647                      (-------*-------)
1600A       5    1.1260    0.1596                 (-------*-------)
2300A       5    0.9360    0.1060          (-------*-------)
                                    --------+---------+---------+---------
POOLED STDEV =   0.2849                   0.70      1.05      1.40
MTB > AOVONEWAY C6-C10 #DAILY NORMALIZED CELL DOUBLETS - CULTURE B

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4     1.760     0.440     4.04    0.015
ERROR      19     2.069     0.109
TOTAL      23     3.829

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ---------+---------+---------+--------
0800B       4    0.7600    0.4465   (---------*---------)
1100B       5    0.7280    0.2531   (--------*---------)
1300B       5    1.2220    0.2901                (--------*---------)
1600B       5    1.3980    0.4572                     (--------*---------)
2300B       5    0.8440    0.1016      (--------*---------)
                                    ---------+---------+---------+--------
POOLED STDEV =   0.3300                  0.70      1.05      1.40
MTB > AOVONEWAY C11-C15 #DAILY NORMALIZED CELL DOUBLETS - CULTURE C

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4    0.8527    0.2132     2.34    0.092
ERROR      19    1.7282    0.0910
TOTAL      23    2.5809

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -----+---------+---------+---------+-
0800C       4    0.9725    0.4033         (---------*---------)
1100C       5    0.8500    0.3274      (--------*---------)
1300C       5    1.1940    0.2541                (---------*---------)
1600C       5    1.2220    0.2408                 (---------*---------)
2300C       5    0.7540    0.2834   (---------*---------)
                                    -----+---------+---------+---------+-
POOLED STDEV =   0.3016                0.60      0.90      1.20      1.50
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```
MTB > AOVONEWAY C16-C20 #DAILY NORMALIZED CELL DOUBLETS - CULTURE D

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4    1.2719    0.3180     6.38    0.002
ERROR      20    0.9967    0.0498
TOTAL      24    2.2686
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN

                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -----+---------+---------+---------+-
0800D       5    0.7980    0.3180       (------*------)
1100D       5    1.1800    0.0667                 (------*------)
1300D       5    1.2300    0.3056                   (------*------)
1600D       5    1.1240    0.1940                 (------*------)
2300D       5    0.6640    0.1122  (------*------)
                                    -----+---------+---------+---------+-
POOLED STDEV =   0.2232              0.60      0.90      1.20      1.50

MTB > AOVONEWAY C1-C5 #DAILY NORMALIZED TOTAL TOXIN/CELL - CULTURE A

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4   0.02030   0.00508     0.65    0.632
ERROR      19   0.14780   0.00778
TOTAL      23   0.16810
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -------+---------+---------+---------
0800A       4    0.9575    0.1090   (-----------*-----------)
1100A       5    1.0400    0.0632              (-----------*-----------)
1300A       5    0.9740    0.0650     (-----------*-----------)
1600A       5    1.0040    0.1254          (-----------*-----------)
2300A       5    1.0180    0.0638            (-----------*-----------)
                                    -------+---------+---------+---------
POOLED STDEV =   0.0882              0.910     0.980     1.050
MTB > AOVONEWAY C6-C10 #DAILY NORMALIZED TOTAL TOXIN/CELL - CULTURE B

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4   0.07710   0.01927     2.06    0.126
ERROR      19   0.17780   0.00936
TOTAL      23   0.25490
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  --------+---------+---------+--------
0800B       4    0.9600    0.0829     (---------*---------)
1100B       5    0.9960    0.1021           (---------*---------)
1300B       5    0.9180    0.0476   (---------*---------)
1600B       5    1.0700    0.0539                (---------*---------)
2300B       5    1.0500    0.1539              (---------*---------)
                                    --------+---------+---------+--------
POOLED STDEV =   0.0967              0.90      1.00      1.10
```

```
MTB > AOVONEWAY C11-C15 #DAILY NORMALIZED TOTAL TOXIN/CELL - CULTURE C

ANALYSIS OF VARIANCE
SOURCE     DF        SS         MS         F        p
FACTOR      4    0.1521     0.0380      2.26    0.101
ERROR      19    0.3198     0.0168
TOTAL      23    0.4720

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN      STDEV  ---------+---------+---------+-------
0800C       4    0.9275     0.0881    (--------*--------)
1100C       5    0.9880     0.0779         (--------*--------)
1300C       5    0.8980     0.1529   (--------*--------)
1600C       5    1.0560     0.1499              (--------*--------)
2300C       5    1.1120     0.1491                   (--------*--------)
                                      ---------+---------+---------+-------
POOLED STDEV =   0.1297                        0.90      1.05      1.20
MTB > AOVONEWAY C16-C20 #DAILY NORMALIZED TOTAL TOXIN/CELL - CULTURE D

ANALYSIS OF VARIANCE
SOURCE     DF        SS         MS         F        p
FACTOR      4    0.1479     0.0370      0.77    0.555
ERROR      20    0.9551     0.0478
TOTAL      24    1.1030

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN      STDEV  ---------+---------+---------+---------
0800D       5    0.8600     0.3603   (---------*---------)
1100D       5    1.0960     0.1272             (---------*---------)
1300D       5    1.0200     0.1684         (---------*---------)
1600D       5    1.0060     0.1016        (---------*---------)
2300D       5    1.0180     0.2325         (---------*---------)
                                      ---------+---------+---------+---------
POOLED STDEV =   0.2185                      0.80      1.00      1.20

MTB > AOVONEWAY C1-C5 #DAILY NORMALIZED CHLOROPHYLL/CELL - CULTURE A

ANALYSIS OF VARIANCE
SOURCE     DF        SS         MS         F        p
FACTOR      4    0.01710    0.00428      0.63    0.646
ERROR      19    0.12859    0.00677
TOTAL      23    0.14570

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN      STDEV  -----+---------+---------+---------+-
0800A       4    0.9625     0.1310  (-----------*-----------)
1100A       5    1.0240     0.0619            (----------*----------)
1300A       5    0.9820     0.0750      (----------*----------)
1600A       5    1.0360     0.0796              (----------*----------)
2300A       5    0.9900     0.0592       (----------*----------)
                                      -----+---------+---------+---------+-
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POOLED STDEV =   0.0823              0.910     0.980     1.050     1.120
MTB > AOVONEWAY C6-C10 #DAILY NORMALIZED CHLOROPHYLL/CELL - CULTURE B

ANALYSIS OF VARIANCE
SOURCE      DF        SS        MS        F        p
FACTOR       4   0.07615   0.01904     3.44    0.028
ERROR       19   0.10504   0.00553
TOTAL       23   0.18118

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ----+---------+---------+---------+--
0800B       4    0.9875    0.1090       (-------*-------)
1100B       5    0.9600    0.0534     (------*------)
1300B       5    0.9360    0.0195   (------*------)
1600B       5    1.0940    0.0590                  (------*------)
2300B       5    1.0240    0.1031           (------*------)
                                    ----+---------+---------+---------+--
POOLED STDEV =   0.0744              0.90      1.00      1.10      1.20

MTB > AOVONEWAY C11-C15 #DAILY NORMALIZED CHLOROPHYLL/CELL - CULTURE C

ANALYSIS OF VARIANCE
SOURCE      DF        SS        MS        F        p
FACTOR       4   0.15202   0.03801     4.19    0.013
ERROR       19   0.17248   0.00908
TOTAL       23   0.32450

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ---+---------+---------+---------+---
0800C       4    0.9075    0.0704  (--------*--------)
1100C       5    0.9720    0.0396         (------*------)
1300C       5    0.9240    0.1074    (------*------)
1600C       5    1.1100    0.0675                   (------*------)
2300C       5    1.0700    0.1475                 (------*------)
                                    ---+---------+---------+---------+---
POOLED STDEV =   0.0953             0.84      0.96      1.08      1.20
MTB > AOVONEWAY C16-C20 #DAILY NORMALIZED CHLOROPHYLL/CELL - CULTURE D

ANALYSIS OF VARIANCE
SOURCE      DF        SS        MS        F        p
FACTOR       4   0.04906   0.01227     3.77    0.019
ERROR       20   0.06500   0.00325
TOTAL       24   0.11406

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ----+---------+---------+---------+---
0800D       5    0.9480    0.0618   (------*------)
1100D       5    0.9700    0.0682      (------*------)
1300D       5    0.9740    0.0279       (------*------)
1600D       5    1.0640    0.0594                  (------*------)
2300D       5    1.0380    0.0589                (------*------)
                                    ----+---------+---------+---------+---
POOLED STDEV =   0.0570              0.910     0.980     1.050     1.120
```

```
MTB > AOVONEWAY C1-C5 *DAILY NORMALIZED GTX-3/CELL - CULTURE A

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4    0.0626    0.0156     0.91    0.477
ERROR      19    0.3261    0.0172
TOTAL      23    0.3887

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ----+---------+---------+---------+--
0800A       4    0.9525    0.2037    (----------*----------)
1100A       5    1.0320    0.0729              (---------*---------)
1300A       5    0.9220    0.0638   (---------*---------)
1600A       5    1.0300    0.1478              (---------*---------)
2300A       5    1.0520    0.1385                (---------*---------)
                                     ----+---------+---------+---------+--
POOLED STDEV =   0.1310              0.84      0.96      1.08      1.20
MTB > AOVONEWAY C6-C10 *DAILY NORMALIZED GTX-3/CELL - CULTURE B

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4    0.2541    0.0635     2.23    0.104
ERROR      19    0.5418    0.0285
TOTAL      23    0.7959

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -------+---------+---------+---------
0800B       4    0.8925    0.1170    (----------*----------)
1100B       5    1.0100    0.1848              (---------*---------)
1300B       5    0.8620    0.0861   (---------*---------)
1600B       5    1.1240    0.1187                       (---------*---------)
2300B       5    1.0840    0.2637                  (---------*---------)
                                     -------+---------+---------+---------
POOLED STDEV =   0.1689              0.80      0.96      1.12
```

```
MTB > AOVONEWAY C11-C15 #DAILY NORMALIZED GTX-3/CELL - CULTURE C

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4    0.5238    0.1310     3.54    0.026
ERROR      19    0.7034    0.0370
TOTAL      23    1.2272

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ---+---------+---------+---------+---
0800C       4    0.8775    0.1021  (-------*-------)
1100C       5    0.9080    0.1033    (------*-------)
1300C       5    0.8700    0.2312  (------*-------)
1600C       5    1.0700    0.2639          (------*-------)
2300C       5    1.2500    0.1651                  (------*-------)
                                     ---+---------+---------+---------+---
POOLED STDEV =   0.1924              0.75      1.00      1.25      1.50

MTB > AOVONEWAY C16-C20 #DAILY NORMALIZED GTX-3/CELL - CULTURE D

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4    0.3585    0.0896     1.65    0.201
ERROR      20    1.0868    0.0543
TOTAL      24    1.4454

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN

                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ---------+---------+---------+-------
0800D       5    0.7640    0.3871  (--------*--------)
1100D       5    1.0340    0.1130            (--------*--------)
1300D       5    1.0540    0.1870            (--------*--------)
1600D       5    1.0620    0.1441             (--------*--------)
2300D       5    1.0900    0.2310              (--------*--------)
                                     ---------+---------+---------+-------
POOLED STDEV =   0.2331              0.75      1.00      1.25
```

```
MTB > aovoneway c1-c5 #GTX-3/TTOX Culture 07/93A

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4   0.02053   0.00513     0.82    0.531
ERROR      19   0.11952   0.00629
TOTAL      23   0.14005

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+
C1          4    0.9800    0.0969      (-----------*-----------)
C2          5    0.9942    0.0806         (----------*----------)
C3          5    0.9464    0.0337   (---------*----------)
C4          5    1.0122    0.0329             (----------*---------)
C5          5    1.0308    0.1189                (---------*----------)
                                    ------+---------+---------+---------+
POOLED STDEV =   0.0793              0.910     0.980     1.050     1.120
MTB > aovoneway C6-c10 #GTX-3/TTOX Culture 07/93B

ANALYSIS OF VARIANCE
SOURCE     DF        SS        MS        F        p
FACTOR      4   0.05643   0.01411     1.43    0.261
ERROR      19   0.18702   0.00984
TOTAL      23   0.24345

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+
C6          4    0.9473    0.0439  (----------*----------)
C7          5    1.0182    0.0981            (--------*--------)
C8          5    0.9418    0.0522   (--------*--------)
C9          5    1.0584    0.0916               (--------*--------)
C10         5    1.0450    0.1568              (--------*---------)
                                    ------+---------+---------+---------+
POOLED STDEV =   0.0992              0.90      1.00      1.10      1.20
```

```
MTB > aovoneway C11-c15 #GTX-3/TTOX Culture 07/93C

ANALYSIS OF VARIANCE
SOURCE     DF        SS         MS         F        p
FACTOR      4   0.12258    0.03065      4.86    0.007
ERROR      19   0.11972    0.00630
TOTAL      23   0.24230

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+
C11         4    0.9488    0.0528     (-------*-------)
C12         5    0.9194    0.0339   (------*------)
C13         5    0.9664    0.1200      (-------*-------)
C14         5    1.0122    0.0996           (-------*-------)
C15         5    1.1214    0.0487                    (-------*-------)
                                      ------+---------+---------+---------+
POOLED STDEV =   0.0794              0.90      1.00      1.10      1.20
MTB > aovoneway C16-C20 #GTX-3/TTOX Culture 06/93A

ANALYSIS OF VARIANCE
SOURCE     DF        SS         MS         F        p
FACTOR      4    0.2300     0.0575      5.37    0.004
ERROR      20    0.2142     0.0107
TOTAL      24    0.4441

                                      INDIVIDUAL 95 PCT CI'S FOR MEAN
                                      BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -+---------+---------+---------+-----
C16         5    0.8330    0.1885  (------*------)
C17         5    0.9554    0.0628         (------*------)
C18         5    1.0450    0.0688               (------*------)
C19         5    1.0750    0.0450                 (------*------)
C20         5    1.0920    0.0854                   (------*------)
                                      -+---------+---------+---------+-----
POOLED STDEV =   0.1035           0.75      0.90      1.05      1.20
```

APPENDIX 3

EFFECTS OF BLEACH AND DILUTION ON TWO SPECIES OF PHYTOPLANKTON

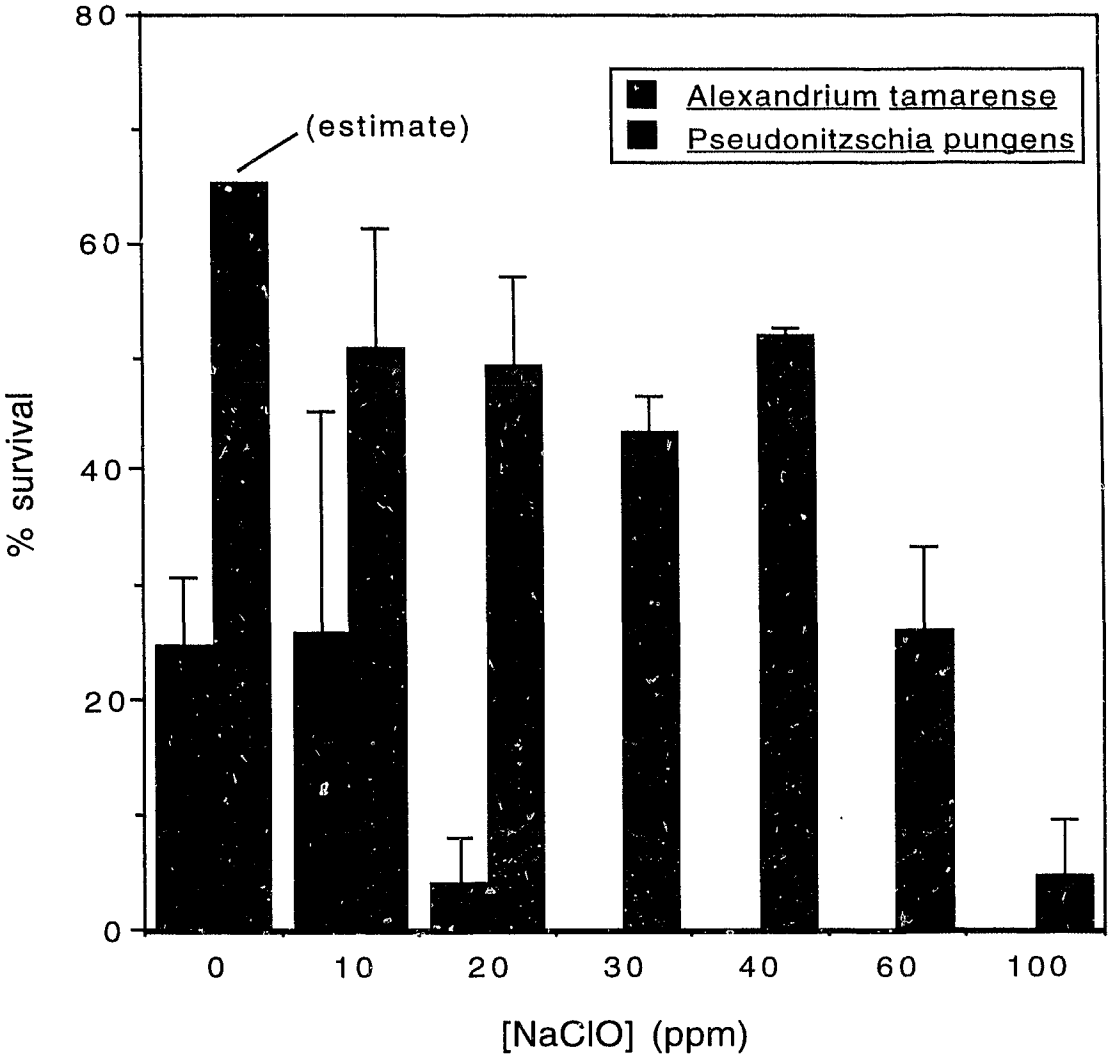

Figure A3.1: Percentage survival of phytoplankton cells exposed to bleach for 1 hour, filtered, rinsed and incubated in new medium for 96 hours. The graph shows the mean of two samples and the upper value. The value for P. pungens at 0 ppm is an estimate only, not a direct count.

Table A3.1: Effects of Dilution on Alexandrium tamarense

Times shown at top are duration of exposure to the indicate salinity.

Salinity °/oo	1 minute	5 minutes	30 minutes	14 hours
30	all motile	all motile	all motile	all motile
15	some motile	some motile	some motile	all motile
10	none motile	most blebbed	all blebbed	some motile
7.5	most blebbed	all blebbed		some blebbed
6	most blebbed	all blebbed		some lysed
3				most lysed

Table A3.2: Effects of Dilution on Pseudonitzschia pungens

Salinity °/oo	1 minute	5 minutes	14 hours
30	all alive	all alive	all alive
15	some blebbed	some blebbed	protoplasts disrupted
10	half blebbed	most blebbed	protoplasts collapsed
7.5	most blebbed	all blebbed	some blebs, protoplasts collapsed
6	most blebbed	most blebbed	some blebs, protoplasts collapsed
3	most blebbed	most blebbed	many blebs, lysis

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