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The Physiological Ecology of Diarrhetic Shellfish Poisoning (DSP) Toxin Production  
by the Dinoflagellate *Exuviaella lima* (Ehr.) Bütschli.

by  
Anthony James Windust

Submitted in partial fulfillment of the requirements  
for the degree of Ph.D.

at

Dalhousie University  
Halifax, Nova Scotia  
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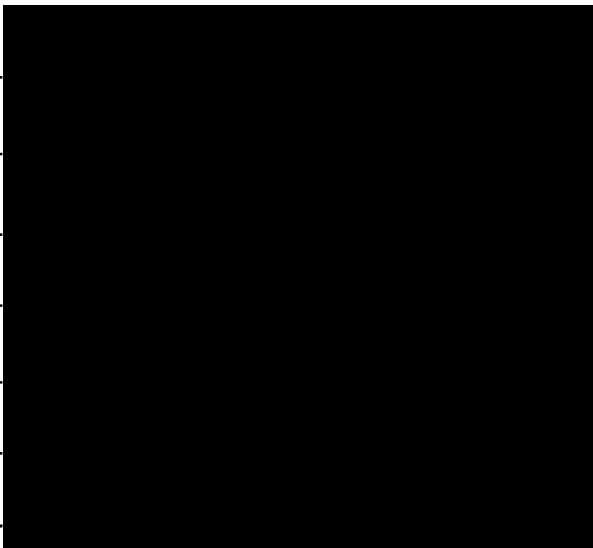
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by Anthony James Windust

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

Dated: September 8, 1998

External Examiner \_\_\_\_\_  
Research Supervisor \_\_\_\_\_  
Examining Committee \_\_\_\_\_  
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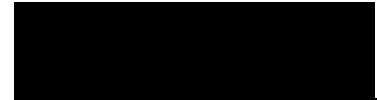
Poisoning (DSP) Toxin Production by the Dinoflagellate

Exuviaella lima (Ehr.) Butschli

DEPARTMENT OR SCHOOL: Biology

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**For Joe**

## Table of Contents

Table of Contents	v
List of Tables	vii
List of Figures	viii
Abstract	x
List of Abbreviations and Symbols Used	xi
Acknowledgements	xiii
Preface	xiv
General Introduction	1
Diarrhetic shellfish poisoning	3
Biological functions of shellfish toxins	4
Physiology of toxin production and excretion	6
Objectives and thesis overview	9
1. The effects of the diarrhetic shellfish poisoning toxins, okadaic acid and dinophysistoxin-1, on the growth of microalgae	11
Introduction	11
Methods	13
Results	18
Discussion	28
2. Comparative toxicity of the diarrhetic shellfish poisons, okadaic acid, okadaic acid diol-ester and dinophysistoxin-4, to the diatom <i>Thalassiosira weissflogii</i>	32
Introduction	32
Methods	35
Results	39
Discussion	54
3. Oxidative metabolism of a diol-ester of the diarrhetic shellfish poisoning toxin okadaic acid by <i>Thalassiosira weissflogii</i>	59
Introduction	59
Methods	61
Results	66
Discussion	83



4. The physiology of growth, toxin production and toxin release by <i>Exuviaella lima</i> in turbidostat culture . . . . .	89
Introduction . . . . .	89
Methods . . . . .	90
Results . . . . .	93
Discussion . . . . .	104
5. Simplex optimization of the growth rate of two species of <i>Pseudo-nitzschia</i> , with respect to irradiance and temperature, using a computer-controlled turbidostat culture system . . . . .	108
Introduction . . . . .	108
Methods . . . . .	110
Results . . . . .	136
Discussion . . . . .	158
General Discussion . . . . .	166
Concluding remarks . . . . .	170
Appendix . . . . .	172
Bibliography . . . . .	200

## List of Tables

Table 1.1 Disk diffusion bioassays . . . . .	19
Table 1.2 Summary of regression analyses for the growth rate bioassays . . . . .	20
Table 1.3 ANOVA table for the growth rate of <i>Prorocentrum minimum</i> exposed to okadaic acid . . . . .	25
Table 1.4 GI <sub>50</sub> values and their 95% confidence limits for the growth rate bioassays. . . . .	27
Table 4.1 <i>Exuviaella lima</i> , growth rates in turbidostat culture under different conditions . . . . .	95
Table 4.2 <i>Exuviaella lima</i> , DSP toxin production rates in turbidostat culture . . . .	101

## List of Figures

Figure 1.1 Concentration response relationships for microalgae exposed to DSP toxins .....	21
Figure 2.1 The structure of DTX-4 .....	33
Figure 2.2 The effect of OA, OA diol-ester and DTX-4 on the growth of <i>Thalassiosira weissflogii</i> in batch culture .....	40
Figure 2.3 Toxicity curves for <i>Thalassiosira weissflogii</i> exposed to OA, OA diol-ester and DTX-4 .....	42
Figure 2.4 Changes in OA and OA diol-ester concentrations when OA diol-ester and DTX-4 were incubated with and without <i>Thalassiosira weissflogii</i> .....	46
Figure 2.5 LC-MS analysis an extract of <i>Thalassiosira weissflogii</i> culture exposed to OA diol-ester .....	49
Figure 2.6 Metabolism of OA diol-ester by <i>Thalassiosira weissflogii</i> .....	52
Figure 3.1 HPLC analysis of a crude extract of <i>Exuviaella lima</i> .....	67
Figure 3.2 The effect of <i>Exuviaella lima</i> cells and culture filtrate on the hydrolysis of DTX-4 to OA via the intermediate OA diol-ester .....	70
Figure 3.3 Identification of a second metabolite of OA diol-ester in <i>Thalassiosira weissflogii</i> cultures exposed to OA diol-ester. ....	75
Figure 3.4 LC-MS analysis of the combined extracts of <i>Thalassiosira weissflogii</i> culture medium .....	78
Figure 3.5 Metabolism of OA diol-ester by <i>Thalassiosira weissflogii</i> cultures ...	81
Figure 4.1 Growth of <i>Exuviaella lima</i> in turbidostat culture .....	96
Figure 4.2 The relationship between DSP toxin production rates by <i>Exuviaella lima</i> and growth rate .....	102
Figure 5.1 Diagram of the twin turbidostat system. ....	113
Figure 5.2 Circuit diagrams .....	117
Figure 5.3 Program flowchart for the main menu of the turbidostat control program .....	124
Figure 5.4 Program flow chart for the interrupt service routine .....	126
Figure 5.5 Program flow chart illustrating the primary functions used during operation of the turbidostat. ....	128
Figure 5.6 Simplex optimization .....	133
Figure 5.7 Turbidostat operation at two different cell densities .....	138
Figure 5.8 Operation of turbidostat sensor lock-in amplification system .....	142
Figure 5.9 Turbidostat detector response as a function of cell density .....	144
Figure 5.10 A representative sample of the signals recorded during operation of the turbidostat before and at steady state with <i>Pseudo-nitzschia multiseriata</i> .....	146

Figure 5.11 Simplex optimization of the growth rate of <i>Pseudo-nitzschia pungens</i> with respect to irradiance and temperature using a variable-sized simplex . . . . .	151
Figure 5.12 The first simplex optimization of the growth rate of <i>Pseudo-nitzschia multiseriis</i> with respect to irradiance and temperature using a fixed-sized simplex . . . . .	154
Figure 5.13 The second simplex optimization of the growth rate of <i>Pseudo-nitzschia multiseriis</i> with respect to irradiance and temperature using a fixed-sized simplex. . . . .	156

## Abstract

The diarrhetic shellfish poisoning (DSP) toxins okadaic acid (OA) and dinophysistoxin-1 (DTX-1) are potent phosphatase inhibitors produced by the dinoflagellate *Exuviaella lima*. It is known that the majority of the toxins are stored in an inactive form within *E. lima* as DTX-4 and can be enzymatically hydrolysed through an intermediate, OA diol-ester, to yield OA following cell lysis. The significance of the ester intermediate and the biological function of the toxins are not known.

The physiological ecology of DSP production by *E. lima* was examined using quantitative bioassay and measures of toxin production and excretion by *E. lima* in culture. Liquid chromatography with UV or mass spectrometric detection was used for toxin quantification. A computer-controlled turbidostat was constructed to facilitate the physiological characterization of toxin production by *E. lima* during steady-state growth. Both OA and DTX-1 were shown to inhibit phytoplankton growth at concentrations of about 1  $\mu\text{M}$  in seawater, but not *E. lima*, suggesting an allelopathic role for the toxins. However, physiological data did not support this interpretation. The DSP toxins were shown to be constitutive metabolites, positively correlated with growth and not released from the cell in sufficient quantity to inhibit other phytoplankton. Quantitative, structure-activity relationships determined for DTX-4, OA diol-ester and OA using the diatom *Thalassiosira weissflogii* demonstrated comparable toxicity for both OA and the diol-ester intermediate contradicting the accepted notion that only the free-acids were toxic. Mass-spectral analysis of post assay material found that *T. weissflogii* was metabolizing the diol-ester. The relative partitioning of OA diol-ester and the metabolites between cells and medium supported the interpretation that OA diol ester, unlike OA, can readily penetrate cell membranes and once within the target cell undergo hydrolysis to release the active toxin. The sequential hydrolysis of the primary DSP toxin DTX-4 through a lipophilic ester intermediate may, therefore, play an important role in the uptake and transfer of OA in the food web and also constitute an interesting mechanism of chemical defence directed at predators.

## List of Abbreviations and Symbols Used

A	attenuation
abs	absorbance
ADC	analog to digital converter
ANOVA	analysis of variance
ASCII	American Standard Code for Information Exchange
ASP	amnesic shellfish poisoning
°C	degrees Celsius
cm	centimetre
df	degrees of freedom
DAD	diode array detection
DFO	Department of Fisheries and Oceans
DSP	diarrhetic shellfish poisoning
DTX	dinophysistoxin
fmol	femtomole
FLD	fluorescence detection
FMOC	9-fluorenylmethyl-chloroformate
g	gram
GI <sub>50</sub>	growth inhibition by 50 percent relative to a control
h	hour
HPLC	high performance liquid chromatography
Hz.	hertz
IBM	International Business Machines
ID	internal diameter
IR	infrared
IREDD	infrared emitting diode
ISR	interrupt service routine
ln	logarithm to base e
log	logarithm to base 10
L	litre
LC	liquid chromatography
m	metre
mL	millilitre
mm	millimetre
min	minute
M	molar
ms	millisecond
ns	not significant
MS	mass spectrometry
N.B.	New Brunswick
NMR	nuclear magnetic resonance
N.S.	Nova Scotia

OD	outside diameter
$\Omega$	ohm
OA	okadaic acid
P	probability or power
Pc	steady state concentration of toxin in cells
Pm	steady state concentration of toxin in medium
P.E.I.	Prince Edward Island
PSP	paralytic shellfish poisoning
PT	phototransistor
$r^2$	coefficient of determination
R	resistance
RC	resistance capacitance
s	second
SD	standard deviation
SE	standard error
SEM	scanning electron microscope
SP	setpoint
$S^2$	variance
spp.	species
t	time
T	transmittance
$\mu$	specific growth rate ( $\text{day}^{-1}$ )
$\mu\text{f}$	microfarad
$\mu\text{g}$	microgram
$\mu\text{L}$	microlitre
$\mu\text{m}$	micrometre
$\mu\text{mol}$	micromole
$\mu\text{M}$	micromolar
USA	United States of America
UV	ultraviolet
V	volt
W	watt
X	cell density
$\lambda$	wavelength

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## Preface

With permission from Springer-Verlag, the research presented in Chapter 1 is also described in: Windust, A. J., J. L. C. Wright, and J. L. McLachlan. 1996. The effects of the diarrhetic shellfish poisoning toxins, okadaic acid and dinophysistoxin-1, on the growth of microalgae. *Mar. Biol.* 126:19-25. © Springer-Verlag.

With permission from Elsevier Science the research presented in Chapter 2 is also described in: Windust, A. J., M. A. Quilliam, J. L. C. Wright, and J. L. McLachlan. 1997. Comparative toxicology of the diarrhetic shellfish poisons, okadaic acid, okadaic acid diol-ester and dinophysistoxin-4, to the diatom *Thalassiosira weissflogii*. *Toxicon* 35:1591-1603. © Elsevier Science.

## **General Introduction**

The occurrence of "blooms" of microalgae in marine and freshwater systems results from complex interactions between many physical, chemical and biotic factors that strongly favour the growth of a particular species (Paerl, 1988). Although phytoplankton production is a critical component of aquatic food webs, some species produce metabolites which are toxic and can have a severe impact on associated organisms. Toxic blooms are believed to be increasing on a global scale, perhaps owing to coastal eutrophication (Smayda, 1990), and have been particularly devastating to the aquaculture industry (Shumway, 1990). It is predicted that the impact of toxic blooms will be felt more in the future as the aquaculture industry expands to fill the void left by declining fisheries (Hallegraeff, 1993).

In laboratory studies, the physiological patterns of toxin production elicited in response to different environmental variables are complex, often contradictory. The specific values and combinations of light, temperature, salinity, nutrient composition and concentration that are optimal for growth or toxin production are poorly understood for most species (Paerl, 1988). The development of a general model to explain and predict phytoplankton toxicity remains an elusive, and perhaps impossible, goal (Flynn and Flynn, 1995). The task is made more difficult because no definite biological function has yet been assigned to any microalgal toxin, although several hypotheses have been made. The physiological patterns of toxin production by microalgae in culture, although limited in scope by experimental procedures, may

provide some clues as to their function and insight into their responses to environmental conditions. Specifically, the patterns of toxin production, storage and excretion would be expected to be consistent with any proposed function.

Quantitative physiological data on toxin production by phytoplankton are mostly biased towards a selection of marine species that render shellfish toxic to humans. Of the four recognised types of shellfish poisoning: paralytic (PSP), neurotoxic (NSP), diarrhetic (DSP) and amnesic (ASP), the majority of data encompasses PSP, ASP and DSP-producing algae. Complex analytical requirements have probably hindered work on other important toxic species, notably the NSP toxin producer *Gymnodinium breve* and *Gambierdiscus toxicus*, a causative agent of Ciguatera Fish Poisoning. Other phytoplankton species are known to be toxic to indigenous flora and fauna e.g., *Chrysochromulina polylepis*, *Prymnesium parvum*, *Heterosigma carterae*, *Pfiestria piscimortuis* and *Aureococcus anophagefferens*, but the toxic components produced by these species await identification and structural elucidation. Given the approximately 4000 extant species of marine phytoplankton (Sournia et al., 1991) it is likely many more toxins await discovery.

Recently, major advances in the chemical characterization of the DSP toxin family have been made (Hu et al., 1992a,b;1995a,b). In addition, new analytical methods for the DSP toxins have been forthcoming (Quilliam and Ross, 1996). These developments have provided a framework and means by which aspects of both the production and potential function of the DSP toxins can be explored.

### *Diarrhetic shellfish poisoning*

A variety of dinoflagellates belonging to the genera *Exuviaella* (formerly including some species of *Prorocentrum*, see McLachlan et al., 1997) and *Dinophysis* produce the diarrhetic shellfish poisoning (DSP) toxin okadaic acid (OA) or one of the related dinophysistoxins, DTX-1 or DTX-2 (Tachibana et al., 1981; Yasumoto et al., 1985). Okadaic acid has been detected in plankton collections containing species of *Dinophysis* from eastern North America (Cembella, 1989); however, the predominantly benthic *Exuviaella lima* (Ehr.) Bütschli may be the main DSP-producing species in temperate waters (McLachlan et al., 1994). Species of *Dinophysis* are not easily cultured and all the quantitative physiological data have been derived from work on species of *Exuviaella*, largely *E. lima*. Although the exact etiology of DSP remains uncertain (Cohen et al., 1990), it is probable that the toxicity of these polyether carboxylic acids results from the fact that they are potent inhibitors of the serine- and threonine-specific phosphatases, PP1 and PP2A which regulate many aspects of eukaryotic cell function (Bialojan and Takai, 1988). Because PP1 and PP2A are highly conserved proteins (Cohen and Cohen, 1989), an interesting question was posed by MacKintosh and MacKintosh (1994) as to how DSP-producing organisms avoid autotoxicity. Structural activity studies have concluded that only DSP toxins with a free acid group exhibit significant activity as phosphatase inhibitors (Holmes et al., 1990; Nishiwaki et al., 1990). Thus, the recent discovery of a number of essentially inactive, water soluble, derivatives of OA, namely: DTX-4, DTX-5a and 5b (Hu et al., 1992a,b;1995a,b) prompted the suggestion that these compounds were forms that the

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cell could safely accumulate and possibly excrete the toxins (Hu et al., 1995b). This explanation is consistent with the fact that the majority of the DSP toxin within *E. lima* cells exists as DTX-4 (Quilliam et al., 1996), and that biosynthetic studies using labelled precursors indicate that DTX-4 is the ultimate product of DSP-toxin synthesis (Needham et al., 1995). An interesting feature of DTX-4 production by *E. lima* is that, following cell lysis and release of intracellular enzymes, DTX-4 will undergo a two stage hydrolysis via an intermediate, OA diol-ester, to yield OA (Quilliam and Ross, 1996). Accepting that the inactive, DTX-4 is a means of protecting the producing cell's signalling pathways from disruption, the significance of the ester intermediate remains unknown. Most importantly, questions regarding the biological function of the DSP toxins remain unanswered.

#### *Biological functions of shellfish toxins*

Microalgal toxins are secondary metabolites, compounds synthesized by the cell using pathways distinct from those of primary metabolism and not directly involved in energy transduction, growth or reproduction (Vining, 1990, Demain, 1983). No definitive function has been assigned to them; yet, their potent biological activity has led to speculation that they are allelopathic in nature serving to combat predators, competitors or pathogens (Carmichael, 1986). The existence of suites of toxins with varying potency and the physiological patterns of toxin accumulation and excretion, in some cases, support an allelopathic role; however, other functions are possible.

The insecticidal activity of the ASP toxin domoic acid (Maeda et al., 1984) which is produced by the diatom *Pseudo-nitzschia multiseries*, prompted Bates et al. (1989) to suggest that it might function to deter zooplankton grazers. It has been shown that several amino acids, including glutamate, trigger feeding responses in copepods (Gill and Poulet, 1988) and as a glutamate agonist, domoic acid would have the potential to interfere with copepod feeding responses. The fact that copepods have been found to accumulate domoic acid without harm and to feed on toxic and non toxic *Pseudo-nitzschia* species at equivalent rates (Windust, 1992) does not support this hypothesis.

The strongest evidence for a defensive function has been derived from several studies demonstrating that herbivorous zooplankton avoid consuming PSP toxin-producing dinoflagellates (Turriff et al., 1995; Huntley et al., 1986; Ives, 1985). Unfortunately, not all tests of this phenomenon are consistent, as zooplankters have been shown to ingest dinoflagellates containing PSP toxins and can act as vectors transferring the toxins to fish (Robineau et al., 1991; White, 1981). In addition, field data have confirmed active grazing on toxic dinoflagellates by zooplankton with no apparent ill effects (Turner and Tester, 1989). That toxins can perform a direct role in dinoflagellate biology has been demonstrated by Burkholder (1995) in revealing the remarkable life history of the "ambush predator" *Pfiestria piscimortuis*, which uses neurotoxins to kill fish and gain nutritional benefit from the carcass.

As a broad spectrum phosphatase inhibitor, OA is also a possible candidate for a defensive agent. Phosphatase inhibitors are not unique to dinoflagellates. They are

also produced by organisms as diverse as cyanobacteria and beetles and as such, they could represent a general strategy in chemical defense (MacKintosh and MacKintosh, 1994). Evidence of a functional role for the DSP toxins is indirectly supported by the fact that as phosphatase inhibitors, the DSP toxins have obvious potential to poison the producing cell (MacKintosh and MacIntosh, 1994). Given that intracellular phosphatases typically exist at a concentration of  $\sim 1 \mu\text{M}$  (Haystead et al. 1989) and the fact that intracellular concentrations of the DSP toxins within *Exuviaella lima* greatly exceed these levels (100-1000  $\mu\text{M}$ ) it is difficult to reconcile such excessive production and storage with any internal regulatory role. In *E. lima* autotoxicity may be avoided through the chemical modification of OA to sulfated esters such as DTX-4 (Hu et al., 1995b). Chemical modification of active toxins to avoid suicide is a common theme with bacterial toxins (Demain, 1983; Vining, 1979) and has been postulated by Baden et al. (1989) to explain the multiple forms of the NSP toxins produced by *Gymnodinium breve* and which act as sodium channel activators. Parallels with PSP toxin suites could also be drawn, but it is not known if plant cells have receptors sensitive to PSP or NSP toxins, although it is possible (Strichartz and Castle, 1990).

#### *Physiology of toxin production and excretion*

The physiological patterns of toxin production and excretion are important from a functional viewpoint. Excretion of secondary metabolites infers an extracellular function (Vining, 1990), and the production of exotoxins by microalgae has been

associated with the elimination of potentially competing phytoplankton (Gentien and Arzul, 1990; Paerl, 1988) or the inhibition of bacteria (Trick et al., 1984). Conversely, endotoxin production is more consistent with herbivore deterrence (Paerl, 1988). Exotoxins surrounding phytoplankton cells could be important as signals directed at zooplankton predators that respond to chemical cues (Targett and Ward, 1991). However, feeding studies suggest otherwise, because copepods ingest toxic cells first and then either reject such cells on future encounter (Turriff et al., 1995) or are incapacitated (Ives, 1985). There is no evidence to suggest excretion of the PSP toxins (Proctor et al., 1975). Domoic acid (Bates et al., 1991) and OA (McLachlan et al., 1994) have been detected in the medium of batch cultures. Unfortunately, the degree to which cell lysis, rather than actual excretion, contributes to medium levels is difficult to assess in batch cultures. Thus, despite the speculation that the water soluble DTX-4 may facilitate the excretion of OA (Hu et al., 1995b), definitive evidence is still lacking.

Environmental factors such as light, temperature, salinity, nutrient concentration and nutrient composition could all potentially influence the patterns of toxin production, accumulation and excretion in ASP, DSP and PSP-producing algae; their effects may also be complex and interactive. However, direct relationships between these variables and toxin production are generally not found with the dinoflagellates which produce the PSP (Flynn et al., 1995; Anderson et al., 1990; Boyer et al., 1987) or DSP toxins (McLachlan et al., 1994; Jackson et al., 1993). A possible exception is the production of the ASP toxin domoic acid in response to silicate (Bates et al., 1991)



or phosphate limitation (Pan et al., 1996a). Even so, field data contradict such simple explanations (Smith et al., 1990a,b) and there is little evidence to suggest that either silicate or phosphate are limiting in coastal environments (Harris, 1986).

In evaluating the effects of environmental variables on toxin production it is particularly important to factor out the indirect effects that these variables have on the growth rate of the producing organism. The toxin content of a cell is the net result of the rates of several processes: toxin production, toxin catabolism, toxin excretion and cell division (Anderson et al., 1990). The rate of cell division is an important consideration, because following mitosis the intracellular toxin content is effectively halved. Thus, increased rates of cell division, without a concomitant rise in the rates of toxin production will effectively "dilute" intracellular toxin concentrations. Conversely, conditions which reduce or halt cell division, even without influencing toxin metabolism directly, may result in an elevated cellular level of the toxin. For these reasons, it has been recommended that a more objective assessment of the physiology of secondary metabolite production be realized by measuring an instantaneous rate per unit biomass ( $Q_p = \text{mol cell}^{-1} \text{time}^{-1}$ ) to cancel the effect of cell division (Anderson et al., 1990; Trilli, 1990). However, from an ecological or functional perspective intracellular concentrations may be important; therefore, viewing the data from both perspectives may be advantageous.

### *Objectives and thesis overview*

The overall objective of this thesis was to examine the allelopathic potential of the DSP toxins produced by the dinoflagellate *Exuviaella lima*. Part 1 of this thesis examines the DSP toxins in terms of their biological activities, transformations, metabolism and their physiological patterns of production and excretion. These data are evaluated within an ecological context and the efficacy of DSP production by *E. lima* as a chemical defense is discussed. The physiology of toxin production by *E. lima* was examined in continuous culture; this required the design and construction of a computer-controlled, continuous culture system. A technical description of this system and its application to the optimization of the growth rates of phytoplankton is provided in Part 2.

Chapter 1 describes bioassay experiments showing that the DSP toxin OA, dissolved in seawater, inhibited the growth of a variety of phytoplankton species at the micromolar level, but not *E. lima*, which produces the toxin. This suggested an allelopathic role for the toxins whereby DSP toxins released by *E. lima* could inhibit the growth of competing phytoplankton.

Chapter 2 extended the bioassay experiments reported in Chapter 1 to include the recently discovered DSP toxins DTX-4 and OA diol-ester. Although non toxic these derivatives are significant because DTX-4, the dominant DSP toxin produced by *E. lima*, can be hydrolysed through the lipophilic intermediate OA diol-ester to yield OA. As expected DTX-4 exhibited very low toxicity but, surprisingly, OA-diol-ester was nearly as toxic as OA to the diatom *Thalassiosira weissflogii*. Further, it was

discovered that the diatom was metabolizing a large proportion of the diol-ester to a more water soluble-product. Measurements were also made of the spontaneous hydrolysis of DTX-4 and OA diol-ester to OA in seawater.

Chapter 3 reports experiments describing the metabolism of OA diol-ester by *T. weissflogii* in detail. Multiple oxidation products of the diol-ester were discovered including a carboxylic acid derivative. The relative partitioning of DSP toxins and metabolites between cells and medium suggested that OA diol-ester, unlike DTX-4 or OA, can readily enter cells, and thus may function to transfer OA across cell membranes.

Chapter 4 presents measurements of DSP toxin production and excretion by *E. lima* made at steady state in continuous culture. These data allow the potential role of the DSP toxins as allelochemicals to be evaluated within a physiological context.

Chapter 5 (Part 2) is primarily a technical description of a computer-controlled, continuous culture apparatus and its application to the optimization of the growth rate of two species of *Pseudo-nitzschia* with respect to light and temperature. A novel turbidostat sensor system using a lock-in amplifier implemented in software is described as well as the use of a simplex procedure to direct the optimization process.

## **Part 1**

### **Chapter 1**

#### **The effects of the diarrhetic shellfish poisoning toxins, okadaic acid and dinophysistoxin-1, on the growth of microalgae**

##### **Introduction**

Marine natural products chemistry is rapidly expanding, and numerous new and unusual compounds have been reported in the last 15 years. Photosynthetic microalgae have proved to be a particularly rich source of novel compounds, many of which display potent biological activity. Indeed, the microalgal toxins that cause the paralytic (PSP), neurotoxic (NSP), diarrhetic (DSP) and amnesic (ASP) shellfish poisoning syndromes are among the most biologically active natural products known. The considerable impact that these toxins have had on public health and the aquaculture industry has prompted the development of sensitive analytical procedures for their detection, and detailed studies of their biological origin and chemistry have been undertaken. Paradoxically, little is known about the effects of these compounds on other marine organisms or their biological function, although there is compelling evidence that PSP toxins may act to deter herbivorous zooplankton (Ives 1985; Huntley et al. 1986; Targett and Ward 1991). Unlike the PSP, NSP and ASP toxins which apparently only target specific receptors in the plasma membranes of animal cells (Biscoe et al. 1975; Strichartz and Castle 1990), the DSP toxins okadaic acid (OA) and dinophysistoxin-1 (DTX-1) are powerful inhibitors of the serine and

threonine specific phosphatases 1 (PP1) and 2a (PP2a) and therefore have the potential to influence a broad range of cellular processes in eukaryotic cells including plants (MacKintosh et al. 1990). Although OA and DTX-1 production is restricted to a few dinoflagellate species within the genera *Exuviaella* or *Dinophysis*, DSP is a global problem (Shumway 1990; Hallegraeff 1993), and extensive outbreaks have occurred in Europe and Japan. More recently, a DSP outbreak was reported for North America (Quilliam et al. 1993). In addition, OA has been implicated as a secondary toxin in ciguatera poisoning (Yasumoto et al. 1985; Morton et al. 1994) which is pervasive throughout the Pacific.

It is possible that the unique and broad activity of the DSP toxins make them ideally suited to fulfil an allelopathic role in the biology of the producing organisms. The intriguing concept that chemically mediated, species-species interactions, or allelopathy, between microalgae can influence community structure has been the subject of much experimentation (Pratt 1966; Keating 1977; Reynolds et al. 1981) and discussion (Smayda 1963, 1980; Lefèvre 1964) since Lucas (1947) developed his "ectocrine theory" as one explanation for the function of biologically active algal metabolites. Despite this history, it is interesting to note that recent texts on phytoplankton ecology either do not mention allelopathy (Harris 1986) or skirt the issue (Sommer 1989). In part, this is because of the acceptance of non-equilibrium models to explain phytoplankton dynamics, which preclude competitive interactions (Harris 1986) or the use of resource competition models (Titman 1976) which deal primarily with physiological principles related to nutrient uptake. However, the

primary reason that biotic interactions are not considered is simply due to the lack of specific and quantitative evidence (Maestrini and Bonin 1981). Apart from a few notable exceptions (e.g. McCracken et al. 1980; Gross et al. 1991), most reports of allelopathic effects are based on the use of crude culture filtrates where the active compounds responsible are not known or quantified; this makes further examination of the phenomenon impossible. In contrast, DSP production by certain dinoflagellates presents a clear opportunity to study a potential allelopathic agent on a defined and quantitative basis. This is for several reasons: OA is commercially available in pure form, it has a defined mode of action, analytical methods have been developed permitting its detection at trace levels (Lee et al. 1987; Pleasance et al. 1990) and importantly, several species of the benthic dinoflagellate *Exuviaella* that produce DSP toxins are easily grown in laboratory culture. As an initial step in assessing the possibility that the DSP toxins are allelopathic in nature, a series of quantitative bioassays were performed that test the effect of OA, and in one instance DTX-1, on the growth of a variety of different microalgae including an isolate of *E. lima* confirmed to produce both OA and DTX-1.

## **Methods**

### *Phytoplankton cultures*

*Thalassiosira weissflogii* (Grun.) Fryxell et Hasle (CCMP #1336), *Isochrysis galbana* Parke (CCMP #1323), *Pavlova lutheri* (Droop) Hibberd (CCMP #1325), *Prorocentrum minimum* (Pavillard) Schiller (CCMP #1329), *Dunaliella tertiolecta*

Butcher (CCMP #1320), *Dunaliella salina* Teodoresco (CCMP #1303) and *Amphidinium carterae* Hulburt (CCMP #1314) were obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton (West Boothbay Harbor, Maine U.S.A.). The culture of *Exuviaella lima* (Ehrenberg) Bütschli (CCMP#1743) used was initially isolated from a mussel farm shortly after an outbreak of DSP and confirmed to produce both OA and DTX-1 (Marr et al. 1992). The culture medium was f/2, prepared according to McLachlan (1973). Seawater was membrane filtered (0.3- $\mu\text{m}$ ) at the time of collection and again immediately prior to use. Stock cultures were maintained at 22°C on a 16:8 h light:dark cycle under an irradiance of 80  $\mu\text{mol-photon m}^{-2}\text{s}^{-1}$  provided by 40-W cool-white fluorescent lamps.

#### *DSP toxins*

Stock solutions of OA and DTX-1 were prepared from pure crystalline material isolated from mass cultures of *E. lima* (Hu et al. 1992a).

#### *Growth assays on solidified medium*

Preliminary OA-sensitivity tests were done, using a technique similar to that described by Chan et al. (1980), against those species capable of luxuriant growth on solidified medium: *Dunaliella tertiolecta*, *D. salina*, *Thalassiosira weissflogii*, *Amphidinium carterae* and *Exuviaella lima*. *Pavlova lutheri*, *Prorocentrum minimum*, and *Isochrysis galbana* grew poorly on solidified medium which precluded their use in disk diffusion assays. Petri dishes containing f/2 medium solidified with 1% agar

(Difco, Bacto-Agar) were inoculated by pipetting 0.1 mL of an actively growing culture onto the surface and then spreading the inoculum evenly using a sterile L-shaped glass rod. Aliquots of a 1.0 mg mL<sup>-1</sup> stock solution (1.24 mM) of OA in 95% ethanol were pipetted onto sterile 1-cm diameter filter paper disks to provide a range of doses from 5 to 500 µg OA disk<sup>-1</sup>. Control disks were prepared with 95% ethanol only. After preparation all disks were allowed to sit overnight in a laminar flow hood to ensure the complete evaporation of the solvent. One control disk and one or two treated disks were placed, equidistantly, on the surface of the freshly inoculated Petri dishes and incubated under the same conditions as the stock cultures. The diameters of inhibition zones were measured with a ruler after sufficient growth had occurred, which for most species was usually within one week; however, colonies of *E. lima* required at least two weeks to be clearly visible.

#### *Growth assays in small-scale batch culture*

To test the effect of OA on algal growth in liquid medium, *Thalassiosira weissflogii*, *Isochrysis galbana*, *Pavlova lutheri* and *Prorocentrum minimum* were grown in 1-cm path-length polystyrene spectrophotometer cuvettes (Fisher) to facilitate optical density measurements as an index of growth and to limit the amounts of toxin required to a minimum. *Thalassiosira weissflogii* was also challenged with DTX-1 alone and by an equimolar combination of OA and DTX-1 to test for possible antagonistic or synergistic effects. For each experimental treatment, 4 or 5 replicates were prepared by pipetting aliquots of stock 95% ethanol solutions of OA or DTX-1,



or for controls 95% ethanol, into the cuvettes. The solvent evaporated overnight and then 1 mL of sterile medium was added to each cuvette followed by a 20  $\mu$ L inoculum of cells and then additional sterile medium for a final volume of 3.0 mL. The final concentrations of OA and DTX-1 tested against *T. weissflogii* were: 12.4, 6.2, 3.1, 1.2, 0.6 and 0.1  $\mu$ M. The final concentrations of OA used against *P. lutheri*, *I. galbana* and *P. minimum* were: 20.6, 4.1, 2.0, 1.0 and 0.5  $\mu$ M. The cuvettes were sealed with Parafilm (American National Can), thoroughly mixed by repeated inversion, and incubated upright in front of vertical, fluorescent-light banks under the same conditions as the stock cultures. The polystyrene cuvettes and the Parafilm were not sterilized; however, microscopical examination revealed no significant bacterial contamination during the short incubations. All treatment groups were haphazardly arranged to cancel the effects of any light or temperature gradients. Cell density was monitored daily by recording the optical density (OD) of the cultures in the cuvettes at 750 nm using a LKB Ultrospec 4050 spectrophotometer until the control cultures entered stationary phase. The OD measurements were converted to cell densities using standard curves prepared for each species (Sorokin 1973). The mean growth rate of each culture was calculated over the interval from inoculation to the point when the control culture reached the stationary phase. The growth rates of cultures exposed to toxins were expressed as the percentage inhibition with respect to the control group, and then subjected to probit analysis (Hubert, 1984). Comparisons between experiments were made by calculating the concentration of toxin required to reduce the growth rate by 50% ( $GI_{50}$ ) and their confidence intervals (Sokal and Rohlf, 1981).

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The effect of OA on the growth rate of *Exuviaella lima* in liquid culture was assessed initially at 3.1  $\mu\text{M}$  OA and then, in a later experiment, at 12  $\mu\text{M}$  OA. The inherent difficulties of measuring the cell density of *E. lima* using OD, because of its tendency to aggregate, necessitated the use of 4-chamber tissue culture slides (Lab Tek) that permitted direct cell counts with an inverted microscope. The chamber slides were prepared with toxin in the same manner as described above for the cuvettes. The inoculum culture was first collected on a sterile 20  $\mu\text{m}$  Nitex mesh screen, rinsed twice with filtered seawater to remove any exogenous toxins and then resuspended in filtered seawater. A 10- $\mu\text{L}$  inoculum of cells was added from an appropriately diluted washed culture to provide approximately 100 cells per chamber; sterile medium was then added to give a final volume of 1.5 mL per chamber. There were 6 replicates per treatment in the first experiment and 8 in the second. The cultures were allowed to settle for a few hours before initial cell counts were made. The chamber slides were placed within Petri dishes, sealed with Parafilm to retard evaporation and incubated under the same conditions as the stock cultures. After 10 days of growth, a second cell count was made, and the mean relative growth rate per day calculated for each replicate. Treatment effects were assessed using Students' t-test.

## Results

### *Growth assays on solid medium*

With the exception of *Exuviaella lima*, the algae tested on solid medium were inhibited by OA (Table 1.1). The most sensitive species was *Thalassiosira weissflogii* which was inhibited with 5 µg OA disk<sup>-1</sup> and failed to grow anywhere on the plates incubated with 100 or 500 µg OA disk<sup>-1</sup>. However, *E. lima* was not inhibited by 500 µg OA disk<sup>-1</sup>; in fact, it was observed that colonies proliferated even underneath the test disks at this dose level. In no case was inhibition of growth noted around the control disks.

### *Growth assays in small-scale batch culture*

In the small-scale batch culture assays, OA and DTX-1 inhibited the growth rates of all the species tested except *E. lima*. Complete inhibition of growth of *Thalassiosira weissflogii* occurred at a concentration of 6.2 µM OA or 3.1 µM DTX-1. The concentration of toxin and response elicited for *T. weissflogii*, *Pavlova lutheri* and *Isochrysis galbana* were highly correlated and the linear regressions made on the transformed data were highly significant (Table 1.2). These concentration-response relationships (Fig. 1.1) indicated that the toxins were indeed responsible for the observed effects on growth. In contrast, regression analysis showed no significant correlation ( $p > 0.05$ ) between OA concentration and growth inhibition of *Prorocentrum minimum*. Further, the linear model fitted to the transformed data was non-significant (Table 1.2).

**Table 1.1** Disk diffusion bioassays; the diameter (mm) of the inhibition zone around test disks indicated by species and dose. nt: not tested, C: 95% ethanol control, CI: complete inhibition.

Species	Dose of okadaic acid ( $\mu\text{g disk}^{-1}$ )					
	500	100	50	20	5	C
<i>Dunaliella tertiolecta</i>	58	40	nt	0	0	0
<i>Dunaliella salina</i>	68	52	nt	0	0	0
<i>Thalassiosira weissflogii</i>	CI	CI	nt	44	20	0
<i>Amphidinium carterae</i>	nt	nt	22	nt	nt	0
<i>Exuviaella lima</i>	0	0	nt	nt	nt	0

**Table 1.2** Summary of regression analyses for the growth rate bioassays. ns: non significant, \*\*\*  $p < .001$ , df: degrees of freedom for  $F$ , Int: intercept. The dependent variable is probit transformed percent inhibition of the mean growth rate and the independent variable is the  $\log_{10}$  of the toxin concentration in  $\mu\text{M}$ .

Experiment	Slope	Int	$r^2$	df	$F$	p
<i>Thalassiosira weissflogii</i> vs. OA	5.23	3.89	.83	1,10	47.2	***
<i>Thalassiosira weissflogii</i> vs. DTX-1	5.32	5.78	.82	1,10	45.6	***
<i>Thalassiosira weissflogii</i> , OA+DTX-1	3.83	4.65	.92	1,14	162.6	***
<i>Pavlova lutheri</i> vs. OA	2.44	4.00	.90	1,18	154.0	***
<i>Isochrysis galbana</i> vs. OA	1.70	3.28	.96	1,18	421.3	***
<i>Prorocentrum minimum</i> vs. OA	0.18	4.32	.06	1,18	1.2	ns

**Figure 1.1** Concentration response relationships for microalgae exposed to DSP toxins. Percent inhibition of growth rate is probit scaled, OA + DTX-1 indicates an equimolar combination of the two toxins.

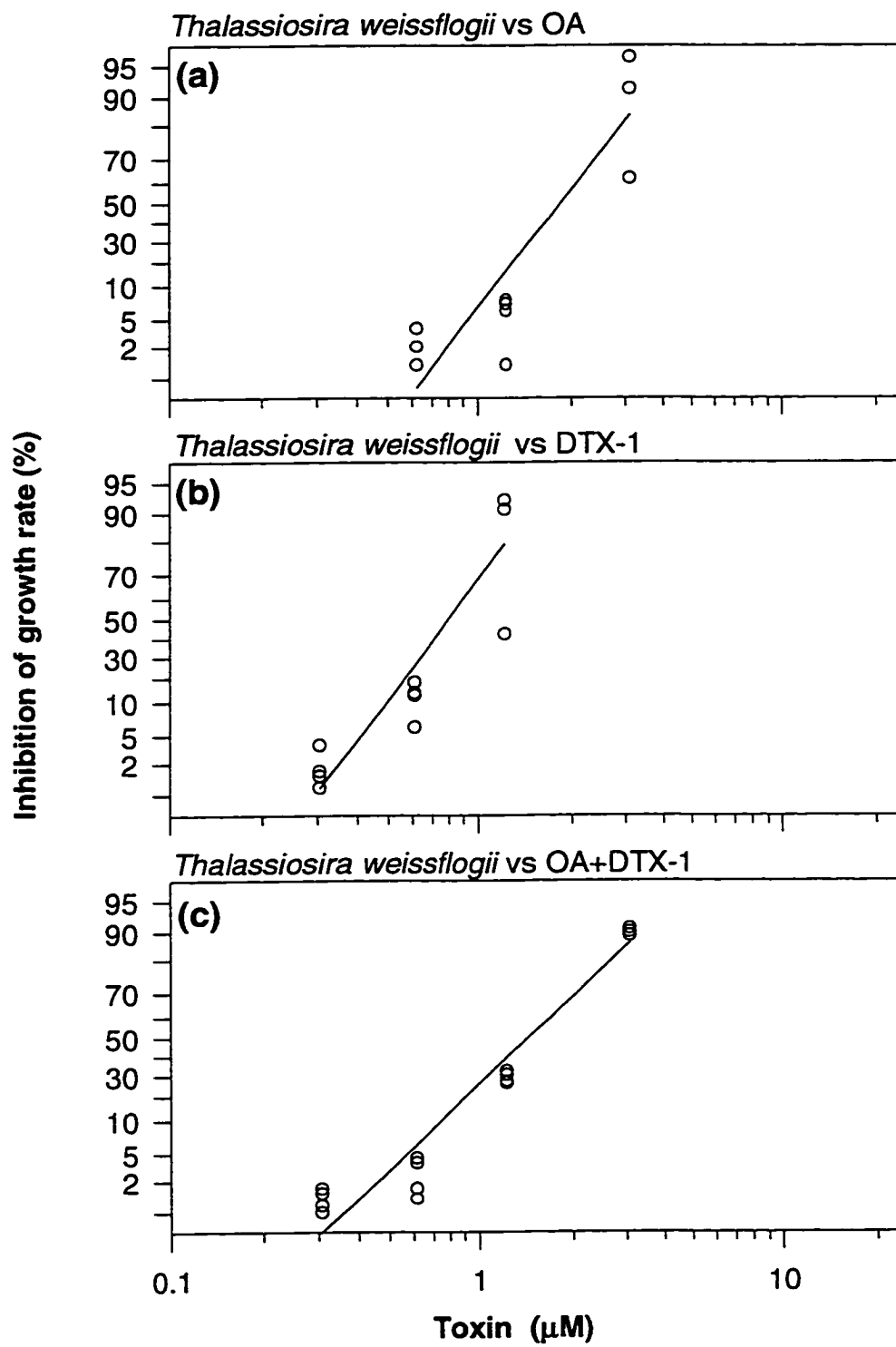


Figure 1.1

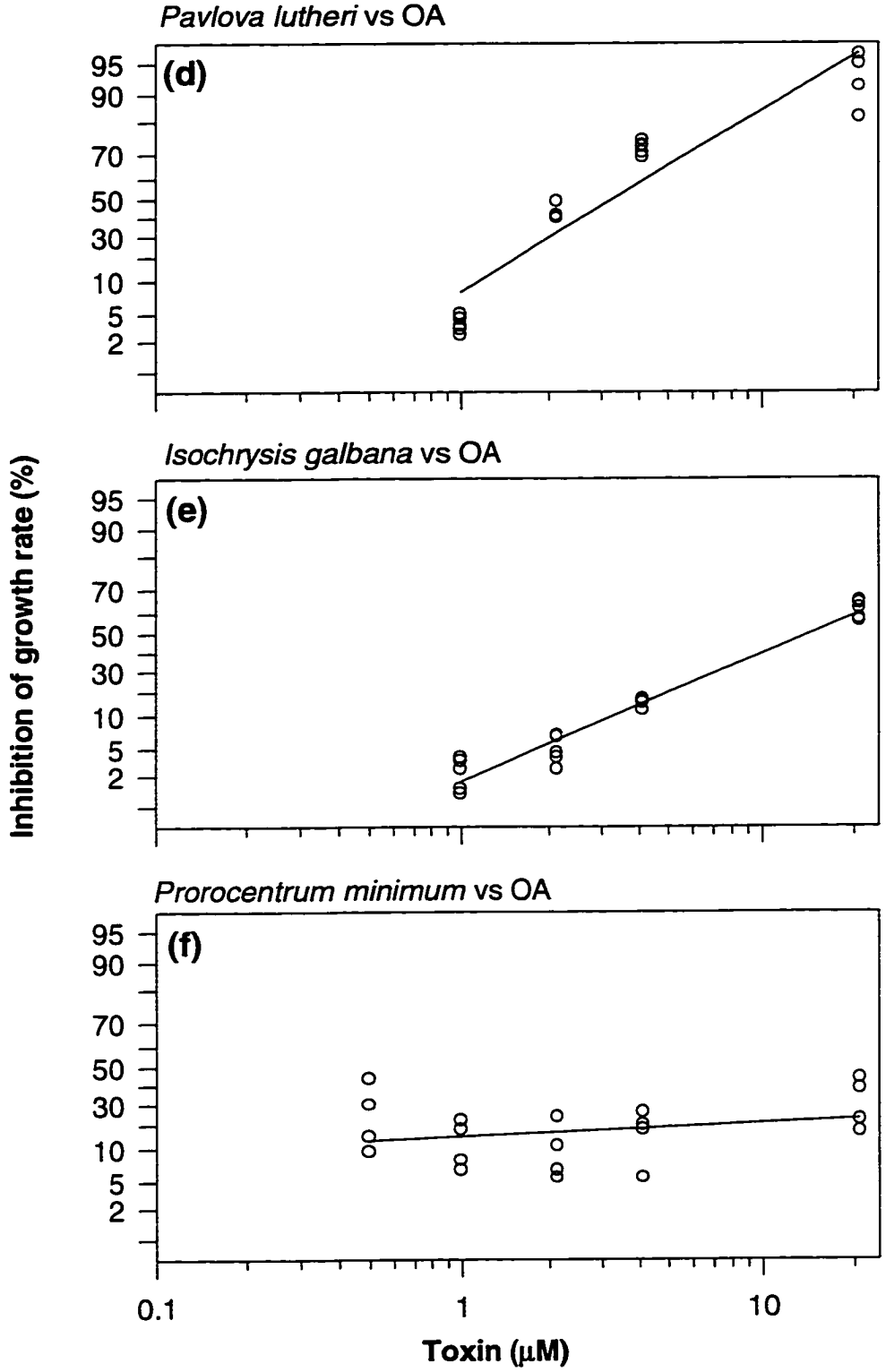


Figure 1.1 (cont.)



As there was no linear concentration-response relationship found for *P. minimum* challenged with OA, the mean growth rates measured for each treatment group were directly compared by ANOVA which indicated a significant treatment effect (Table 1.3). Multiple comparisons of treatment means with the control group revealed that only the 20.6  $\mu\text{M}$  OA group had a significantly lower growth rate when compared to the control ( $t_s=2.95$ ,  $df=6$ ,  $.05>P>.01$ ). The apparent inhibitory effects calculated for *P. minimum* at toxin concentrations below 20.6  $\mu\text{M}$  OA (Fig. 1.1) are non-significant and are due to a high variance within the control group caused by the unusually high growth rate of one culture. Microscopical examination of the control group revealed that they primarily consisted of single cells with relatively few in the final stages of cell division (mean cell density  $\pm$  SE =  $56,300 \pm 2,250 \text{ mL}^{-1}$ , number of these cells dividing  $\pm$  SE =  $8,300 \pm 850 \text{ mL}^{-1}$  = 15%) yet, in the 20.6  $\mu\text{M}$  OA treatment population a large proportion of cells were found paired, in the final stages of cell division (mean cell density  $\pm$  SE =  $6,450 \pm 1,250 \text{ mL}^{-1}$ , number of these cells dividing  $\pm$  SE =  $6,030 \pm 1,130$  = 93%). Examination of *P. minimum* cultures incubated at concentrations of OA less than 20.6  $\mu\text{M}$  revealed that few cells were in pairs, although direct cell counts were not made on these groups. The large differences in final cell concentrations found by direct counts between the control cultures and those incubated at 20.6  $\mu\text{M}$  OA clearly support the significant effect on growth rate found above. In contrast, the final cell yields calculated from optical density measurements for all groups except that at 20.6  $\mu\text{M}$  OA were similar, at about  $70,000 \text{ cells mL}^{-1}$ .

**Table 1.3** ANOVA table for the growth rate of *Prorocentrum minimum* exposed to okadaic acid. \* =  $0.05 \geq P > 0.01$

Source of variation	df	SS	MS	F
Between groups	5	.3216	.06432	3.622 *
Within groups	18	.3197	.01776	
Total	23	.6413		

Cultures of *I. galbana*, *P. lutheri* and *T. weissflogii* inhibited by the DSP toxins were also examined microscopically for incomplete cytokinesis, but the small size and varied orientation of these cells precluded unambiguous observations.

Based on the regression coefficients, the concentration of toxin required to cause a growth inhibition of 50% with respect to the controls ( $GI_{50}$ ) was interpolated for each assay for which a significant linear regression was found (Table 1.4). Comparison of these values suggests that *I. galbana* was more resistant to OA than either *P. lutheri* or *T. weissflogii*. Reference to the 95% confidence intervals associated with each  $GI_{50}$  point support this finding as those for *I. galbana* do not overlap any other set (Table 1.4). The lower  $GI_{50}$  value calculated for *T. weissflogii* exposed to DTX-1 as opposed to OA would argue that DTX-1 is more toxic than OA; however, the considerable overlap of the confidence intervals calculated for these values imply no real differences. Moreover, the combined effect of challenging *T. weissflogii* with an equal combination of both toxins appears to be no different than any one toxin alone (Table 1.4), a conclusion supported by the close similarity between the regressions (Fig 1.1), and their parameters (Table 1.2) derived from each experiment with *T. weissflogii*.

The effects of OA at 3.1  $\mu$ M on the growth of *E. lima* in liquid culture revealed a slightly significant enhancement of growth rate ( $t_s=2.33$ ,  $df=10$ ,  $.01 < p < .05$ ); however, when the experiment was repeated at a higher dose level of 12  $\mu$ M OA, with increased replication, no significant differences between the control and treatment groups were found ( $t_s=0.68$ ,  $df=14$ ,  $p > .05$ ).

**Table 1.4**  $GI_{50}$  values and their 95% confidence limits for the growth rate bioassays.

Experiment	$GI_{50}$	95% confidence limits	
	( $\mu$ M)	(μM)	
<i>Thalassiosira weissflogii</i> vs. OA	1.63	3.78	0.74
<i>Thalassiosira weissflogii</i> vs. DTX-1	0.71	1.48	0.36
<i>Thalassiosira weissflogii</i> vs. OA+ DTX-1	1.23	2.27	0.68
<i>Pavlova lutheri</i> vs. OA	2.57	6.12	1.04
<i>Isochrysis galbana</i> vs. OA	10.27	17.88	6.15

## Discussion

Okadaic acid is known to cause hyperphosphorylation of numerous animal (Haystead et al. 1989), fungal (Kinoshita et al. 1993) and plant proteins (MacKintosh et al. 1990). The structures of PP1 and PP2a are apparently more highly conserved in eukaryotes than those of either histone or tubulin (Cohen and Cohen, 1989). It is therefore likely that the DSP toxins have the potential to disrupt normal cell function in all eukaryotes. Here it is demonstrated that at micromolar concentrations OA is a potent microalgal growth inhibitor, effective against several different species of microalgae. The concentration-response relationships calculated for both OA and DTX-1 added, singly or in equal combination, to *Thalassiosira weissflogii* cultures were not statistically different, indicating that there were no significant antagonistic or synergistic effects. The equivalence of DTX-1 and OA as inhibitors is not surprising in view of their close structural similarity. Therefore it is important to quantify the concentration of both these forms of the toxin when assessing the potential effects of the DSP toxins in natural situations.

Typically, *in vitro* assays employing OA-sensitive enzymes require only nanomolar concentrations of the toxin to cause demonstrable effects (Haystead et al. 1989). The higher micromolar concentrations required to inhibit the growth of microalgae in culture can be attributed to both the higher levels of OA-sensitive enzymes *in vivo* (~1  $\mu$ M) and to the diffusion barriers to OA presented by cell walls and membranes (Klumpp et al. 1990). Furthermore, interspecific differences in these

factors might explain the differential susceptibility to OA observed in both the disk-diffusion and batch-culture assays.

The ubiquitous nature of DSP-sensitive phosphatases within eukaryotic cells makes it difficult to suggest a specific cellular process that is disrupted by the toxins causing the observed inhibition of growth. It is possible that there is disruption of multiple cellular processes, although when *Prorocentrum minimum* was inhibited by OA, a high percentage of cells were found in the final stages of cell division, just prior to cytokinesis, suggesting a more specific effect. This is consistent with the findings of Schönthal and Feramisco (1993) who reported that OA arrested development of mammalian cell cultures at specific points during the cell cycle, G<sub>1</sub> and G<sub>2</sub>/M, dependant on the timing of OA addition. They conclude that OA acts as a cytostatic drug by interfering with the control and expression of cell cycle regulatory proteins.

Okadaic acid, even at the extremely high doses used in the disk diffusion assays, failed to inhibit the growth of a clone of *E. lima* known to produce both OA and DTX-1. Significantly, the related species *P. minimum*, which does not produce DSP toxins (Cembella 1989), was inhibited by OA in liquid culture, albeit at a higher concentration (21 µM) than that found for the other species affected. The insensitivity of *E. lima* to very high levels of exogenously supplied OA suggests specific adaptations to defend itself. Using immuno-labelling techniques, Zhou and Fritz (1994) have obtained evidence suggesting that, in both *E. lima* and *P. maculosum*, intracellular OA is associated with the chloroplast. This location is considered insensitive to OA since only trace amounts of PP1 and PP2A are associated with

chloroplasts and therefore these enzymes are unlikely to be directly involved in the regulation of chloroplast metabolism (Siegl et al. 1990). However, toxin compartmentalization is but one of a variety of strategies used by organisms to avoid autotoxicity. Others include modification of their own target receptors and the conversion of the toxin to an inactive form (Demain et al. 1983). The recent discovery of DTX-4 (Hu et al., 1995a) prompted Hu et al. (1995b) to suggest the latter possibility.

DSP toxins are secondary metabolites whose presence is obviously inimical to normal eukaryotic cell function; why then do dinoflagellates such as *E. lima* produce these toxins? This evidence demonstrating that OA inhibits the growth of several microalgal species but not *E. lima* itself, supports, but does not prove, the hypothesis that DSP toxins may play an allelopathic role. Okadaic acid is released into the culture medium during batch culture of *E. lima* and has been reported by Rausch De Traubenberg and Morlaix (1995) to comprise about 30% of total toxin production. The remarkably low levels of cell lysis noted in *E. lima* cultures even after 6 months (McLachlan et al. 1994) would suggest that the OA found in the medium is due in part to excretion. The concentrations of OA reported in the medium at low culture densities ( $\sim 3,000 \text{ cells mL}^{-1}$ ) are  $\sim 0.01 \mu\text{M}$  (Rausch De Traubenberg and Morlaix, 1995) and are well below those found in this study to inhibit microalgal growth, but the direct extrapolation of these data to natural situations is not straight-forward. Firstly, estimates of toxin excretion derived from batch culture are difficult to interpret because they are integrative measures. Secondly, not only are relevant ecological

designs capable of studying subtle interactions of microalgae *in situ* lacking (Smayda, 1980), but correlating the numerical abundance of a particular species with the production of a secondary metabolite to the exclusion of other factors is difficult. Even though microalgal allelopathy is not a generally accepted phenomenon, both those favourable to the concept (Maestrini and Bonin, 1981), and those who dismiss it (Lewis, 1986), put forth similar criticisms of the existing evidence: the chemical agents have not been identified and definitive, ecologically relevant experiments have not been done. The discovery that OA and DTX-1 are potent microalgal inhibitors, yet ineffective against a producing organism such as *E. lima*, satisfies the basic prerequisites of an allelochemical agent and indicates that further research is warranted.



## Chapter 2

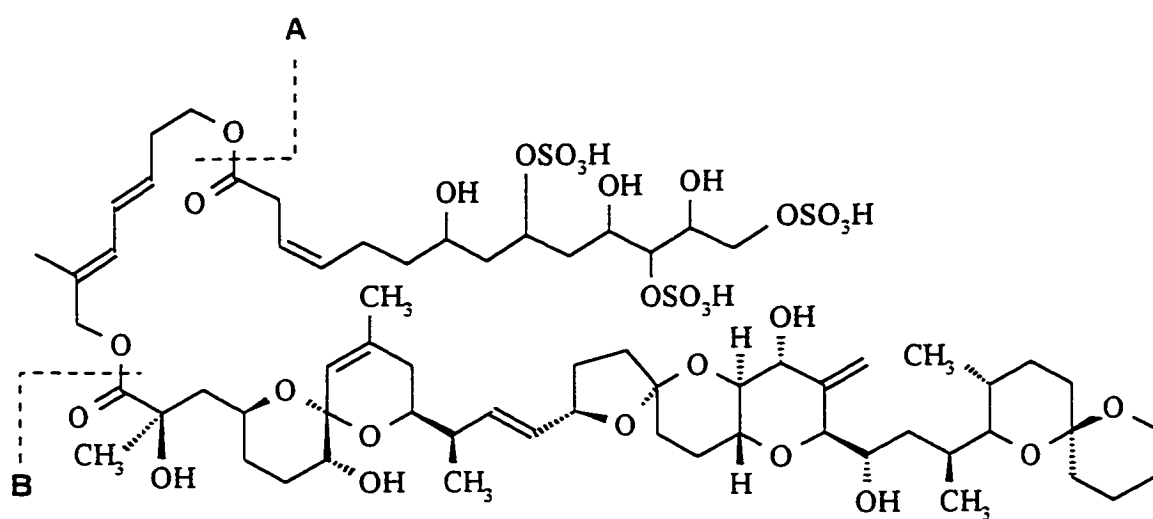
### Comparative toxicity of the diarrhetic shellfish poisons, okadaic acid, okadaic acid diol-ester and dinophysistoxin-4, to the diatom

#### *Thalassiosira weissflogii*

#### Introduction

It has been demonstrated that OA inhibits the growth of a variety of microalgae in a concentration-dependent manner, but not that of a DSP-producing dinoflagellate *Exuviaella lima*, suggesting an allelopathic role for the toxin (Chapter 1; Windust et al., 1996). This activity of OA is consistent with an ability to inhibit serine and threonine specific phosphatases (Bialojan and Takai, 1988), thereby disrupting normal eukaryotic cell functions. Indeed, OA is extremely potent and enhances overall levels of protein phosphorylation when present at nanomolar levels within the cell (Haystead et al., 1989). Recently, additional derivatives of DSP toxins have been recovered from cultures of *E. lima*. These include OA diol-ester (Hu et al., 1992a) and the water soluble DTX-4 (Hu et al., 1995a). Importantly, both OA diol-ester and DTX-4 can be hydrolysed to yield the parent toxin OA (Fig. 2.1). *In vitro* enzyme assays have shown that DTX-4 is ~ 50 times less effective as a phosphatase inhibitor than OA (Hu et al., 1995a) and that OA diol-ester is relatively inactive (Hu et al., 1992a). The low activity of these ester derivatives of OA is consistent with previous studies which have concluded that high activity against phosphatases is correlated with a free carboxyl group (Holmes et al., 1990; Nishiwaki et al., 1990).

**Figure 2.1** The structure of DTX-4. DTX-4 is water soluble because of the presence of the sulfated acyl chain. Hydrolysis of the ester bond at A yields an uncharged lipophilic intermediate, OA diol-ester. Hydrolysis of OA diol-ester at B yields OA, a powerful phosphatase inhibitor.



**Figure 2.1**

Given the relative inactivity of DTX-4 and OA diol-ester as phosphatase inhibitors *in vitro*, it was of particular interest to evaluate their toxicities *in vivo* using a bioassay procedure utilizing the diatom *Thalassiosira weissflogii* (Grun.) Fryxell et Hasle (Chapter 1). The data for structure-activity relationships determined *in vivo* were expected to reinforce the accepted view that phosphatase inhibition by these polyether compounds requires an exposed carboxylic acid group. This in turn would support the earlier contention that the toxic effects of OA on *T. weissflogii* were due to phosphatase inhibition. Unexpectedly, the results indicated significant activity by the diol-ester of OA towards *T. weissflogii*, but post-assay analyses using liquid chromatography-mass spectrometry (LC-MS) showed that some hydrolysis of the ester to OA had taken place. Although this provided one possible explanation for the toxicity of the diol ester, it also raised the possibility that target cells could hydrolyse OA diol-ester to OA, thereby metabolically activating the toxin. Because this behaviour could be of considerable ecological significance, a second experiment was conducted to test whether either DTX-4 or OA diol-ester could be metabolised to the lethal toxin OA by *T. weissflogii*.

## **Methods**

### *Bioassay and culture procedures*

An axenic culture of *Thalassiosira weissflogii* (CCMP# 1336) was obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME., USA). Growth conditions, culture medium and bioassay

methods were as previously described (Chapter 1). In brief, however, measured volumes of toxin dissolved in methanol were pipetted into polystyrene spectrophotometer cuvettes, and the solvent was allowed to evaporate. Fresh seawater medium was then added to the cuvettes followed by an inoculum of *T. weissflogii* cells to give a total volume of 3 mL at a cell concentration of approximately  $5 \times 10^3$  cells mL<sup>-1</sup>. Four or five replicates were prepared for each concentration of toxin. Cell densities were measured over one week by recording absorbance at 750 nm with an LKB Ultrospec 4050 spectrophotometer, and transforming these readings to cell densities using standard curves calculated by the method of Sorokin (1973). At the end of the experiment, all bioassay cuvettes were frozen at -20°C.

#### *Post-assay analysis*

Five replicate bioassay cultures exposed to 3.2 M OA diol-ester were thawed, combined (15mL), extracted as described below and then analyzed for OA and OA diol-ester using LC-MS by the method of Quilliam (1995).

#### *Toxins*

Stock solutions of OA, DTX-4 and OA diol-ester were prepared from pure crystalline material obtained from large-scale cultures of *Exuviaella lima* (Hu et al., 1995a,b). The initial concentrations of toxins in the stock solutions were calculated based on the weighed samples of original material; these were later corrected, based on analytical determinations.

### *Toxin Metabolism Experiment*

Toxins were incubated in sterile seawater medium with or without *Thalassiosira weissflogii* cells. For each toxin, appropriate volumes of toxin dissolved in methanol were added to two sets of 12 sterile glass centrifuge tubes (50 mL, conical-bottomed). The tubes were held overnight in a laminar flow hood to allow the solvent to evaporate. The following day, an exponentially growing culture of *T. weissflogii* was diluted 1:2 v/v with fresh medium to give a cell density of  $114 \times 10^3$  cells mL<sup>-1</sup>. Aliquots of this suspension (15 mL) were added to half the tubes prepared with each toxin, and sterile medium (15 mL) was added to the remainder. The final toxin concentrations in the spiked tubes were 2.4  $\mu$ M for DTX-4 and 1.8  $\mu$ M for OA diol-ester. After preparation the tubes were capped with parafilm, haphazardly arranged in a test tube rack and incubated at 22°C on a 14:10 light:dark cycle at an irradiance of 150  $\mu$ mole-photons m<sup>-2</sup>s<sup>-1</sup> as measured with a 2 $\pi$ -quantum sensor (LI-COR, LI-189) and provided by 40-W Cool-White fluorescent lamps situated below the tubes. Immediately after the setup was completed a single tube from each treatment group was taken for extraction to test the efficacy of the extraction procedures; thereafter samples were taken each day for 5 days. Before extraction of medium containing diatom cells, a sample (3 mL) was taken to measure cell density as described above and returned to the centrifuge tube prior to extraction.

### *Extractions*

To reduce handling to a minimum, extractions were performed directly in the centrifuge tubes. Each tube was immersed for 2 min in an ultrasonic bath (B220H, Branson Ultrasonics Corp., Danbury, CT) and then extracted thrice with  $\text{CH}_2\text{Cl}_2$  (5 mL) by vortex-mixing for 30 s (Fisher Vortex Genie 2, Scientific Industries Inc., Bohemia, NY.). The organic layers, withdrawn using a glass pipette, were combined and evaporated to dryness under nitrogen using a Turbovap concentrator (Zymark Corp., Hopkinton, MA). Each sample was then redissolved and serially transferred in about 1.5 mL 80% MeOH (3 x 0.5 mL) to a volumetric vial and brought to a final volume of 2 mL with 80% MeOH, then held at  $-20^\circ\text{C}$  until the end of the experiment. All extracts were spin-filtered through a  $0.45\mu\text{M}$  membrane filter (Ultrafree-MC: Millipore Corp., Bedford, MA.) using a centrifuge (MP4R, International Equipment Co., Needham Heights, MA) prior to analysis by LC-MS.

### *Chemical analyses*

Analyses of OA and OA diol-ester were by ion-spray liquid chromatography-mass spectrometry (LC-MS) as previously reported (Quilliam, 1995). Routine analytical procedures for DTX-4 were not available at the time of these experiments. However, DTX-4 is reported to be only slightly soluble in chloroform when partitioned against water (Hu et al., 1995a) so efficient extraction by dichloromethane from aqueous medium was not expected.

## Results

### *Bioassay experiments*

At concentrations of 3  $\mu\text{M}$  or greater, both OA and OA diol-ester almost completely inhibited the growth of *Thalassiosira weissflogii* (Fig. 2.2a and b). In contrast, substantially higher concentrations of DTX-4 were required to detect any effect on the growth of *T. weissflogii* (Fig. 2.2c). At concentrations of OA or OA diol-ester where growth was nearly completely inhibited, some increase in cell biomass occurred at the beginning of the experiment (Fig. 2.2a and b). This may reflect the time lag required for the toxin deposited on the bottom of the cuvettes to become fully dissolved and then be taken up by the diatom cells. The large standard errors noted for the 1.6  $\mu\text{M}$  OA diol-ester concentration (Fig. 2.2b) were the result of one of the five replicates demonstrating high growth, even greater than those within the control group. Each DSP toxin can be described by a unique concentration-response curve (Fig. 2.3).



**Figure 2.2** The effect of OA, OA diol-ester and DTX-4 on the growth of *Thalassiosira weissflogii* in batch culture. Cultures were grown with different concentrations of toxin in spectrophotometer cuvettes. Cell densities were calculated from absorbance readings at 750nm. For clarity, some concentration groups that completely overlapped each other or the control are not shown. Four replicates were used for the OA and DTX-4 assays and 5 for the OA diol-ester assay, vertical bars represent  $\pm 1$  SE.

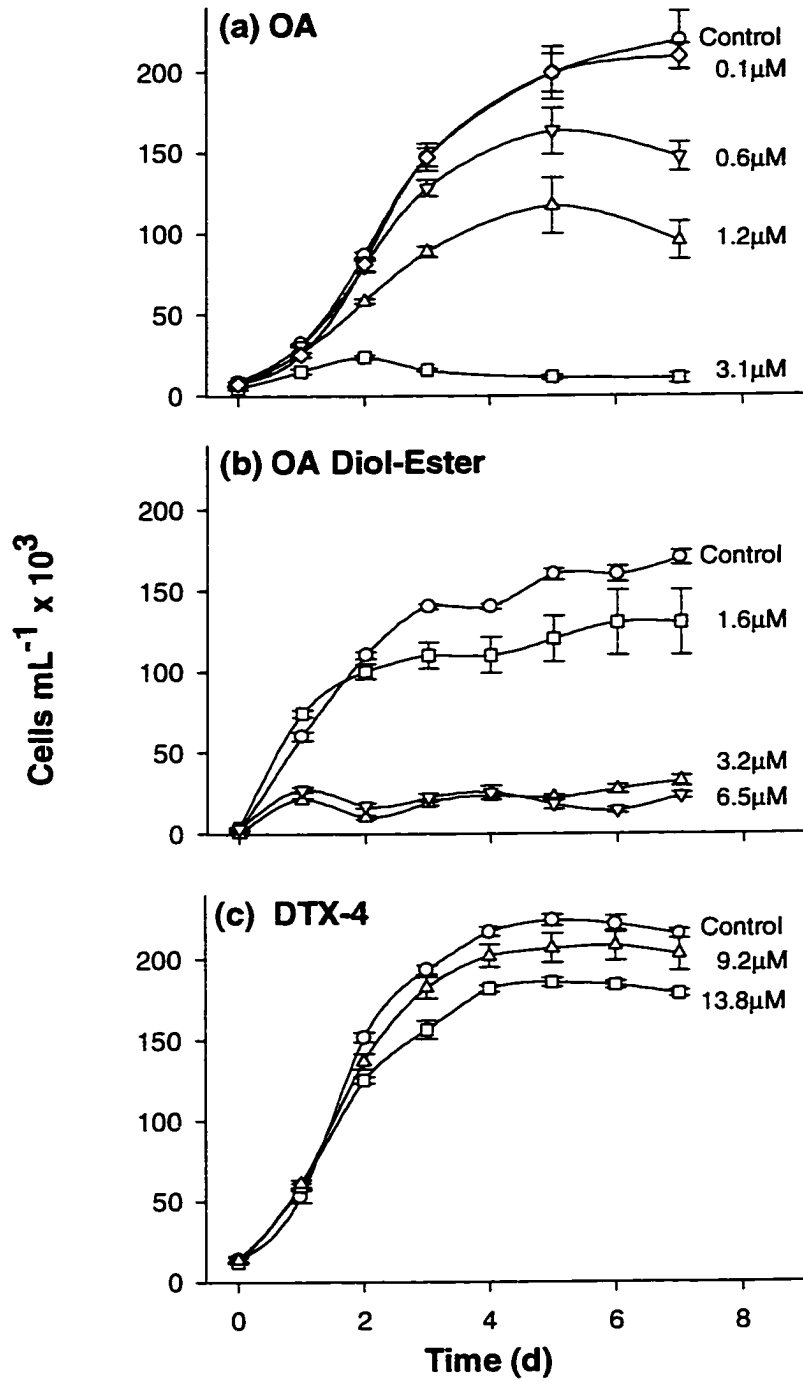


Figure 2.2

**Figure 2.3** Toxicity curves for *Thalassiosira weissflogii* exposed to OA, OA diol-ester and DTX-4. The final cell yield of cultures after 7 days of growth was calculated as a percentage of the control and graphed as a function of toxin concentration. Four replicates were used for the OA and DTX-4 assays and 5 for the OA diol-ester assay, vertical bars represent  $\pm 1$  SE.

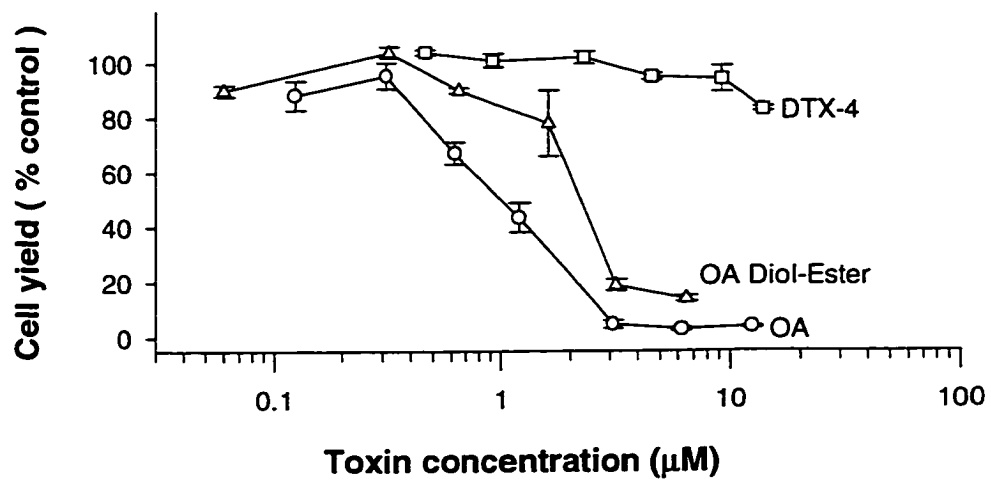


Figure 2.3

Okadaic acid was the most potent while DTX-4 was virtually inactive at the concentrations tested. Interestingly, the response curve for OA diol-ester is merely displaced slightly from that plotted for OA. The  $GI_{50}$  values found by direct interpolation were only about two-fold higher for OA diol-ester ( $GI_{50} = 2.2 \mu\text{M}$ ) than for OA ( $GI_{50} = 1.0 \mu\text{M}$ ).

#### *Post-assay analysis*

LC-MS analysis using selected ion monitoring showed that cultures initially exposed to  $3.2 \mu\text{M}$  OA diol-ester contained  $0.65 \mu\text{M}$  OA diol-ester and  $0.23 \mu\text{M}$  OA at the end of the experiment. Thus, of the original  $9.6 \text{ nmol}$  of ester added to each cuvette in the  $3.2 \mu\text{M}$  concentration group, 7% had been converted to OA, 20% remained as OA diol-ester and 73% was unaccounted for. Analysis of the original stock solution of OA diol-ester used to prepare the bioassay treatments revealed no trace of OA.

#### *Toxin transformation and metabolism*

The concentrations of OA diol-ester and DTX-4 used in the metabolism experiment were below those expected to completely inhibit the growth of *Thalassiosira weissflogii*. Indeed, cell numbers in both toxin treatments increased from  $114 \times 10^3 \text{ cells mL}^{-1}$  at the start of the incubations to  $170 \times 10^3 \text{ cells mL}^{-1}$  in the OA diol-ester treatment and to  $188 \times 10^3 \text{ cells mL}^{-1}$  in the DTX-4 treatment by day 2 and thereafter declined only slightly (growth curves not shown).

The concentration of OA increased over time at an approximate rate of  $0.01 \mu\text{M day}^{-1}$  in both the DTX-4 and OA diol-ester treatments regardless of the presence of *T. weissflogii* cells (Fig. 2.4a and b). Thus, the amount of OA liberated by hydrolysis over 5 days accounted for 2.0% of the DTX-4, and 2.7% of the OA diol-ester, initially added. In contrast, the difference in concentration of OA diol-ester during treatments with and without *T. weissflogii* cells were dramatic (Fig. 2.4c). In the presence of *T. weissflogii* cells, the total concentration of OA diol-ester declined rapidly to near zero by day 5. In the sterile medium however, the concentration remained more or less constant. The total mass of toxin recovered at the start of the experiment was only 60% of that initially added, indicating an incomplete recovery by the extraction procedure. Even so, all samples were measured by the same procedure and a much greater proportion of toxin was unaccounted for after OA diol-ester was incubated with *T. weissflogii* cells. The large proportion of OA diol-ester that could not be accounted for following incubation with *T. weissflogii* cells in this experiment paralleled the findings from the analysis of the bioassay material and strongly suggested that OA diol-ester was being metabolized by the diatom.

Little, if any, difference was seen between the levels of OA diol-ester when DTX-4 was incubated in sterile medium or in the presence of *T. weissflogii* cells except that on day 5 the level of OA diol-ester in the sterile medium was much higher than when incubated with the diatom (Fig. 2.4d). As this change was only seen at the endpoint of the experiment, it is not conclusive, but is consistent with the assumption that *T. weissflogii* can metabolise OA diol-ester.

**Figure 2.4** Changes in OA and OA diol-ester concentrations when OA diol-ester and DTX-4 were incubated with and without *Thalassiosira weissflogii*. Serial and independent incubations of toxins were made in centrifuge tubes with either sterile seawater medium or medium freshly inoculated with *T. weissflogii* at a cell density of  $114 \times 10^3$  cells mL<sup>-1</sup>. Each day for five days one tube from each group was extracted and analyzed for OA and OA diol-ester using LC-MS. Open circles represent incubations with *T. weissflogii* cells, solid circles represent incubations in sterile seawater medium. All points are independent and no replicate measurements were made. Note the differences in scale of the Y axis for c.

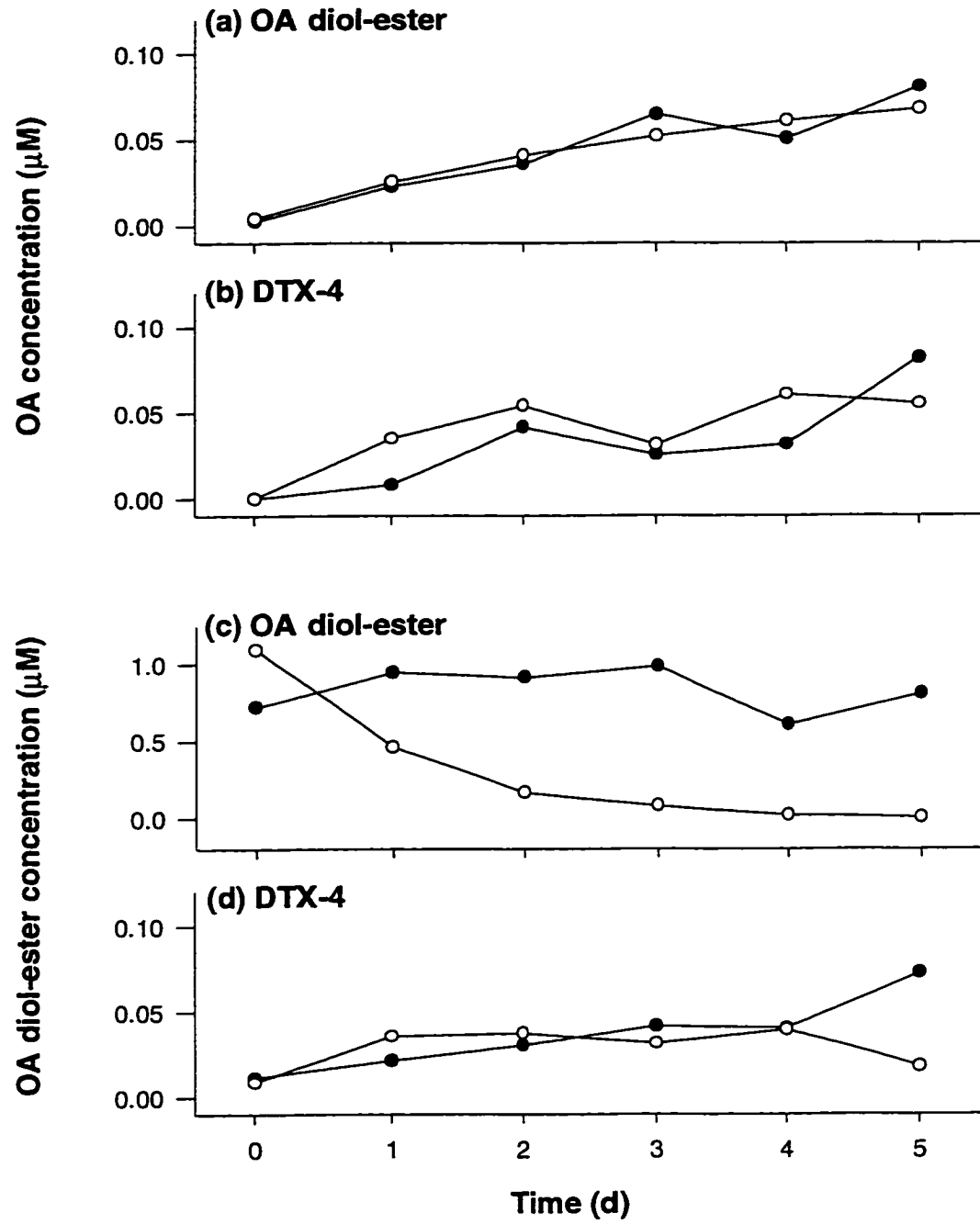


Figure 2.4



*Identification of an unknown metabolite of OA diol-ester*

OA and OA diol-ester were detected by LC-MS using selected ion monitoring at  $m/z$  805.5 and  $m/z$  929.5 respectively. A principal ion chromatogram derived from the LC-MS analysis of an extract of a *Thalassiosira weissflogii* culture exposed to OA-diols is shown (Fig. 2.5a). OA eluted at 4.8 min (peak 2, Fig. 2.5a) detected by a single  $[M+H]^+$  ion at  $m/z$  805.5. OA diol-ester eluted at 5.8 min (peak 3, Fig. 2.5a) and was detected by two ions: one, the primary  $[M+H]^+$  ion at  $m/z$  929.5, the other a fragment ion at  $m/z$  805.5 due to the OA part of the molecule. Examination of data from extracts of *T. weissflogii* cultures incubated with OA diol-ester revealed the presence of an additional peak at 4.5 min (peak 1, Fig. 2.5a) in the  $m/z$  805.5 chromatogram. Since single ion monitoring was being used for these analyses and the concentrations were too low for full-scan analysis, extracts containing the unknown compound were pooled, concentrated, and then analyzed by full-scan LC-MS. The mass spectrum for the unknown compound at 4.5 min (peak 1, Fig. 2.5a) displayed abundant ions at  $m/z$  805.5 and  $m/z$  945.5 (Fig. 2.5b). Because the latter ion was 16 mass units higher than the OA diol-ester  $[M+H]^+$  ion at  $m/z$  929.5, it suggested that peak 3 was due to an oxygenated metabolite of OA diol-ester and given the persistence of the  $m/z$  805.5 ion, that the addition must have taken place on the diol-ester side chain.

**Figure 2.5** LC-MS analysis an extract of *Thalassiosira weissflogii* culture exposed to OA diol-ester. a) Principal ion chromatogram. The extract comprised cells and medium. Peak 2 is OA, peak 3 represents the parent and fragment ions of OA diol-ester and peak 1 is a fragment ion of an unidentified metabolite of OA diol-ester. Separations were performed on a Vydac 201TP52 C<sub>18</sub> column, 5µl of sample was injected and eluted with 75% MeOH containing 0.1 % TFA at a flow rate of 0.2 mL min<sup>-1</sup>. Effluent from the column was analyzed by ion-spray MS on a SCIEX API-III LC-MS system with selected ion monitoring. b) The mass spectrum of the metabolite of OA diol-ester (peak 1 in a) produced by *Thalassiosira weissflogii*. The ion at  $m/z$  805.5 corresponds to the OA part of the molecule, but the second ion at  $m/z$  945.5 is 16 mass units higher than the [M+H]<sup>+</sup> ion of OA diol-ester at  $m/z$  929.5, identifying the metabolite as an oxygenated diol-ester. The minor ions, at  $m/z$  751.5, 769.5, 787.5, 909.5 and 927.5 are the result of the sequential loss of water molecules from the two major ions.

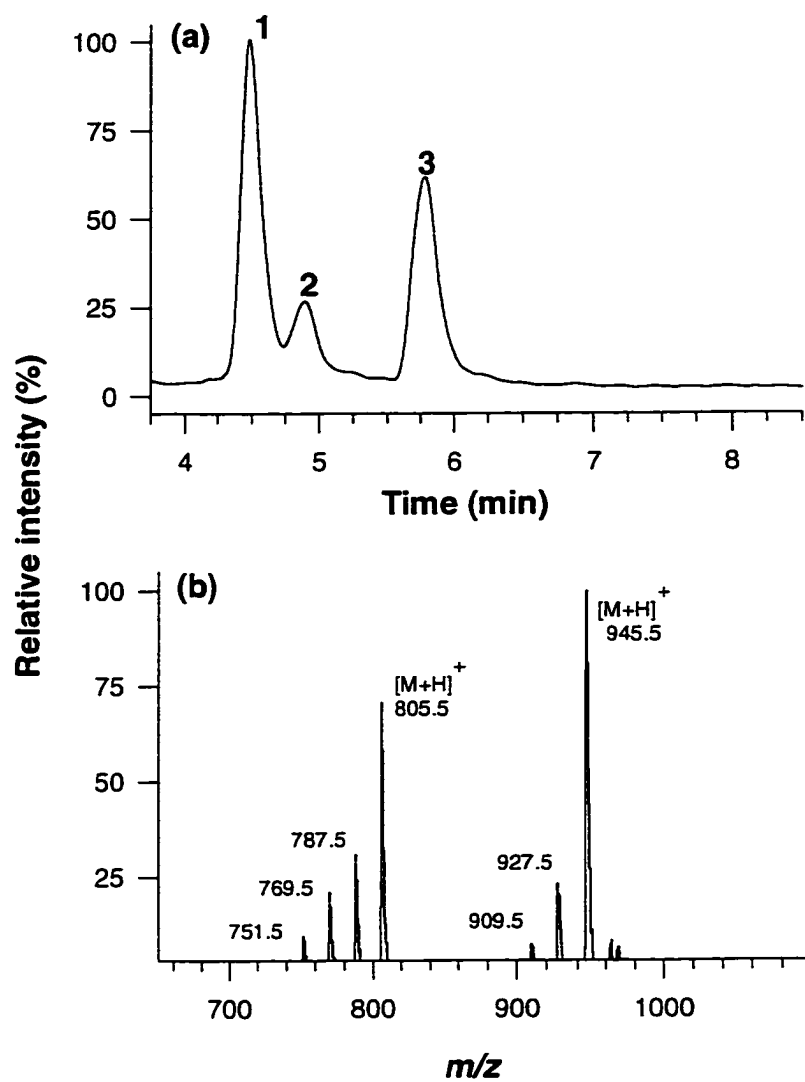


Figure 2.5

The contention that OA-diol-ester was metabolised by *T. weissflogii* was supported by other observations. Firstly, the unknown peak was found only when OA diol-ester, or to a much lesser extent DTX-4, was incubated in the presence of *T. weissflogii* cells, and never when these compounds were incubated in sterile medium. Secondly, the production kinetics of the unknown (Fig. 2.6a) corresponded to the disappearance of OA diol-ester when incubated with *T. weissflogii* cells (Fig. 2.4c). Thirdly, there was an order of magnitude greater production of the compound when OA diol-ester, rather than DTX-4, was exposed to the diatom (Fig. 2.6a and b), implying that the limited production of the unknown in the DTX-4 treatment resulted from the low levels of OA diol-ester produced by hydrolysis of DTX-4 (Fig. 2.4d).

**Figure 2.6** Metabolism of OA diol-ester by *Thalassiosira weissflogii*. The changes in the concentration of the metabolite of OA diol-ester over time are shown when OA diol-ester (a) and DTX-4 (b) were incubated with *T. weissflogii* cells. The values are based on the peak area of the fragment ion (e.g., Fig. 2.5, peak 1) and the assumption that the degree of fragmentation of the parent ion was similar to that of OA diol-ester. Note the change in scale of the vertical axis in b.

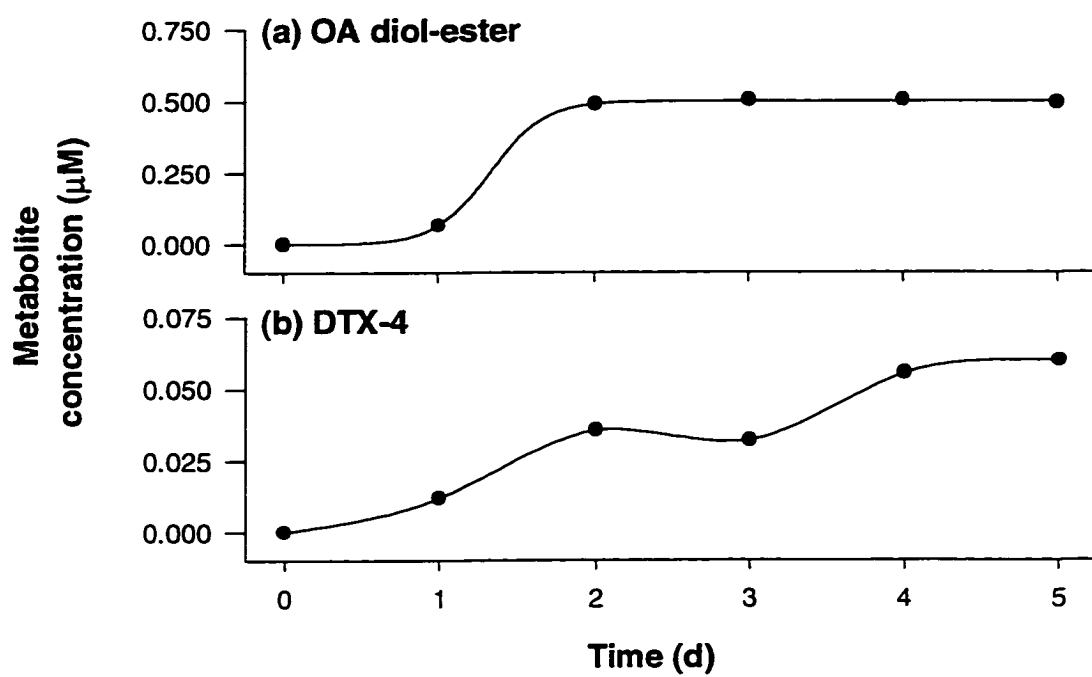


Figure 2.6

## Discussion

The free-acid DSP toxins, OA and DTX-1, are highly active phosphatase inhibitors effective against the serine- and threonine-specific phosphatases PP1 and PP2A (Bialojan and Takai, 1988; Nishiwaki et al., 1990). Phosphatases are highly conserved proteins and OA has been found to interfere with phosphatase-kinase based control mechanisms in a broad range of eukaryotes (MacKintosh et al., 1990). An allelopathic role for OA has been suggested because of its ability to inhibit the growth of a wide range of marine microalgae, but not that of *Exuviaella lima*, a toxin-producing dinoflagellate (Chapter 1). Although the importance of phosphatases to cell function make them ideal targets for allelopathic agents, producing cells must also avoid autotoxicity (MacKintosh and MacKintosh, 1994). This function in DSP toxin-producing dinoflagellates may be fulfilled by the synthesis of weakly active sulfates, such as DTX-4 (Hu et al., 1995b). This contention is supported by the fact that the majority of intracellular DSP exists as DTX-4 in *E. lima* (Quilliam et al., 1996) and by biosynthetic evidence that implicates DTX-4 as the initial product of DSP synthesis in *E. lima* (Needham et al., 1995). The allelopathic potential of OA towards microalgae has been demonstrated when the toxin is dissolved in seawater (Chapter 1), and the biosynthesis of water-soluble DSP compounds such as DTX-4 by *E. lima* has been proposed as a means of facilitating excretion (Hu et al., 1995b). DTX-4 is rapidly hydrolysed to OA diol-ester and OA by enzymes released following lysis of *E. lima* cells (Quilliam and Ross, 1996), therefore the DSP compounds may also be effective against predators that rupture producing cells during feeding.

That DTX-4 was only slightly toxic towards *Thalassiosira weissflogii* is consistent with *in vitro* phosphatase assay results which indicate that the carboxyl group is essential for high activity (Holmes et al., 1990; Nishiwaki et al., 1990). Furthermore, it is possible that the weak activity of DTX-4 observed in the bioassays with *T. weissflogii* resulted from OA released by the spontaneous hydrolysis of DTX-4 (Fig. 2.4b). Holmes et al. (1990) report an  $IC_{50}$  of 0.2 nM for OA against PP2A *in vitro*, with the methyl-ester 4,500 times less potent. However, in the *in vivo* bioassays reported here with *T. weissflogii*, it was found that OA diol-ester was only 2 times less potent than OA with the  $GI_{50}$  for the ester at 2.2  $\mu$ M versus 1.0  $\mu$ M for OA. This discrepancy between the relative activities of OA and OA esters *in vivo* and *in vitro* is significant.

Some toxicity in the diol-ester treatments may be explained by hydrolysis to OA, perhaps catalyzed by non-specific esterases within *T. weissflogii* cells. Indeed, the metabolic activation of toxins is a well-known phenomenon in plants and animals (Harborne, 1993). Interestingly, no evidence was found of enhanced hydrolysis of OA diol-ester or DTX-4 to OA in the presence of *T. weissflogii* cells (Fig. 2.4a and b). Both DTX-4 and OA diol-ester will hydrolyse spontaneously to give OA, but the rates observed were low, resulting in only a 2 to 3% conversion respectively during the course of the experiment. Although these rates would account for the minor toxicity of DTX-4 observed at high concentrations, they do not account for the high toxicity of the diol-ester at low concentrations. Specifically, when *T. weissflogii* was challenged with OA diol-ester at 3.2  $\mu$ M, only 0.23  $\mu$ M OA was measured at the end of the



experiment. From the dose-response data for OA, this concentration of toxin would cause only a slight inhibition of growth, even if present at the start of the assay and cannot account for the high level of inhibition observed. One possibility is that OA diol-ester is toxic to *T. weissflogii* in its own right with a mode of action distinct from that of OA. It must be pointed out that the toxicity of OA to whole organisms is only assumed to result from phosphatase inhibition, and this has not been conclusively demonstrated *in vivo* (Cohen et al., 1990). A second possibility, which is worth considering, is that the relative toxicity of the DSP compounds *in vivo* may be primarily explained by their widely differing solubilities. This is indirectly supported by several lines of evidence: the much higher concentrations of OA required to cause an effect *in vivo* versus *in vitro*, the propensity of the esters to hydrolyze spontaneously (albeit at a slow rate), and the oxidative metabolism of OA diol-ester by *T. weissflogii*.

The concentrations of OA required to inhibit phosphatases in extracts (~1 nM) are three orders of magnitude less than those required to affect the biochemistry of whole cells (~1 μM). This is believed to be due to the barriers imposed by cell walls and membranes to the diffusion of OA (Cohen et al., 1990; Klumpp et al., 1990). In general, the lipid solubility of a toxin will predict its propensity to accumulate within cells and is therefore a significant factor in determining potency. OA, although often described as a lipid-soluble polyether, still possesses a carboxyl group likely to be ionized at physiological pH; this would hinder diffusion across membranes. In contrast, OA diol-ester has no ionizable group and would be expected to move across cell

membranes with relative ease. It is therefore suggested that the minor hydrolysis of OA diol-ester to OA observed in these experiments (~ 3%) is in fact very important in determining *in vivo* toxicity as the diol-ester could provide an effective carrier for transporting OA into a cell. OA liberated by hydrolysis within the cell would be a thousand times more potent than that outside the cell and this could explain why, contrary to data derived from *in vitro* experiments, a high relative toxicity of OA diol-ester to *T. weissflogii* was observed.

The ability of OA diol-ester to enter cells readily is indirectly supported by the rapid and inducible hydroxylation of this molecule by *T. weissflogii* (Fig. 2.6a). Hydroxylation of xenobiotics by plant and animal cells is typically carried out by the cytochrome P<sub>450</sub> complex present in the endoplasmic reticulum of cells, and P<sub>450</sub> induction by toxins is a well-documented detoxification mechanism in animal cells and higher plants (Higashi, 1988) as well as microalgae (Gentile et al., 1990). If true in this instance, all of the OA diol-ester metabolized by the diatom must have first entered the cell and, since essentially no diol-ester remained at the end of the incubations with *T. weissflogii*, this probably comprised the majority of the toxin.

If OA diol-ester serves as an effective intermediate for the transport of DSP toxins across cell membranes, it could prove to be a significant factor in DSP transfer and toxicity within the food web, including human consumers. This is because DTX-4 is the major DSP toxin found within *E. lima* (Quilliam et al., 1996) and it will be hydrolysed in seconds to OA diol-ester, but then only slowly to OA, following cell lysis (Quilliam and Ross, 1996). Thus as *E. lima* cells are ingested and lysed by

predators, OA diol-ester will be the primary DSP toxin available for uptake and potential conversion to OA. It is interesting to note that the enzymatically-catalyzed release of toxins from precursors following tissue disruption is a well-documented defense mechanism in terrestrial plants, e.g., the cyanogenic glycosides in clover (Jones, 1972), and DSP production by dinoflagellates may have a similar defensive function.

In conclusion, structure-activity relationships determined *in vivo* and *in vitro* may provide conflicting results. This is particularly important in the case of the DSP toxins because the primary toxin DTX-4 can be sequentially hydrolysed through uncharged, lipophilic intermediates to ultimately yield the active, free acid toxin OA.

## Chapter 3

### Oxidative metabolism of a diol-ester of the diarrhetic shellfish poisoning toxin okadaic acid by *Thalassiosira weissflogii*

#### Introduction

Okadaic acid will inhibit the growth of a variety of phytoplankton species at low micromolar concentrations in seawater, but not the producing organism *Exuviaella lima* (Chapter 1; Windust et al., 1996). Although these data support an allelopathic function, it is not at all clear to what extent such inhibition takes place in nature. However, a second line of evidence is emerging that also supports a defensive role for the DSP toxins. There are two plausible routes by which DSP toxins contained within *E. lima* cells may escape and come into contact with other organisms: excretion as the sulfated esters, followed by hydrolysis to OA as suggested by Hu et al. (1995b), or release following cell destruction through predation or degeneration. *Exuviaella lima* cells contain enzyme(s) capable of hydrolysing DTX-4 to OA diol-ester and OA (Quilliam and Ross, 1996). The enzymatic decomposition of DTX-4 following cell disruption is a two-stage process; firstly, DTX-4 is hydrolyzed in seconds to OA diol-ester; then slowly, over a period of hours, the ester is hydrolyzed to OA (Quilliam and Ross, 1996). Therefore predators rupturing *E. lima* cells during feeding and digestion would likely be challenged by DSP toxins primarily in the form of OA diol-ester. This was observed by Bauder et al. (1996) who found that OA diol-ester was the dominant form of DSP in bay scallop (*Argopecten irradians*) fed *E. lima* cells. Although OA

diol-ester is essentially inactive as a phosphatase inhibitor *in vitro* (Hu et al., 1992a), it is nearly as toxic to the diatom *Thalassiosira weissflogii* as OA when added to the culture medium (Chapter 2; Windust et al., 1997). Further, when *T. weissflogii* cultures were challenged with sub-lethal concentrations of OA diol-ester, the majority of the ester was oxidized to a more water-soluble product. This implies that the diol-ester must have first entered the diatom cell because the oxidative metabolism of toxins is typically mediated by cytochromes located within internal membranes. Reasonably, as the most lipophilic form of these DSP toxins, the diol-ester would be expected to penetrate cell membranes readily and be taken up by cells. Accordingly, the toxicity of the ester to *T. weissflogii* could be explained by the partitioning of the diol-ester into the cell and subsequent hydrolysis, possibly mediated by intracellular esterases, to yield the active free-acid OA (Chapter 2). Thus the enzymatic hydrolysis of DTX-4 to an uncharged lipophilic intermediate OA diol-ester, could play an important role in the transfer and toxicity of DSP to other organisms including man. It may also represent a specific biochemical adaptation to enhance the effectiveness of this putative chemical defence by facilitating the entry of the toxin into the cells of the attacking organism.

Reported here are the results of additional investigations into the metabolism of OA diol-ester by *T. weissflogii*. The primary goals were to isolate and further identify the oxygenated metabolite of the diol-ester by *T. weissflogii* cells and to investigate the relative partitioning of OA diol-ester and metabolite between cells and medium to test the previous hypothesis (Chapter 2) that OA diol-ester was entering the diatom cell,

being metabolised via oxidative pathways and then excreted. In addition, the enzymatic hydrolysis of DTX-4 to OA diol-ester and OA as mediated by *E. lima* cells and culture filtrate was examined to determine the fate of DTX-4 should it be excreted by the toxin-producing cell.

## Methods

### *Cultures and toxins*

The sources of *Thalassiosira weissflogii* (Grun.) Fryxell et Hasle (CCMP # 1336) and *Exuviaella lima* (Ehr.) Bütschli (CCMP # 1743), culture maintenance, medium preparation and source of toxins were as previously described (Chapter 1).

### *The fate of DTX-4 exposed to Exuviaella lima cells and culture filtrate*

An 80% methanol/water extract (100 mL) of a boiled *Exuviaella lima* cell pellet ( $\sim 9 \times 10^7$  cells) was partially evaporated to reduce the methanol concentration (25 mL), filtered (0.45  $\mu\text{m}$ ) and applied to a preconditioned (6 mL  $\text{CH}_3\text{CN}$ , 6 mL  $\text{H}_2\text{O}$ ) C8-solid phase extraction cartridge (Millipore Corp., Marlborough, MA.). Following washing (6 mL  $\text{H}_2\text{O}$ ), a fraction containing DTX-4 and related compounds was eluted (3 mL 25%  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ ) free from more lipophilic components. A portion of this fraction (100  $\mu\text{L}$ ) was then evaporated to dryness, redissolved in sterilized *f/2* seawater medium (100  $\mu\text{L}$ ) then diluted 1:2 either with sterile seawater medium or *E. lima* culture filtrate (0.22  $\mu\text{m}$ ). These solutions were incubated for 40 min at room temperature, boiled to halt further enzymatic activity and then analysed by LC-UV.

To study the longer term fate of DTX-4 and OA diol-ester exposed to *E. lima* cells and medium over time, samples of DTX-4 were incubated with washed *E. lima* cells, *E. lima* culture filtrate and sterilized f/2 medium. Incubations were done in sterile, transparent micro-centrifuge tubes to each of which was added 50  $\mu\text{L}$  of an 8  $\mu\text{g mL}^{-1}$  DTX-4 solution in methanol and the solvent allowed to evaporate while held in a laminar flow hood. Cells of *E. lima* were prepared by filtering 25 mL of a 10 day-old culture containing  $36 \times 10^3$  cells  $\text{mL}^{-1}$  on to a 10- $\mu\text{m}$  nylon mesh using gentle suction. The cells were washed (3 x 25 mL with sterilized seawater) and then re-suspended in fresh culture medium (25 mL). Culture filtrate was prepared by passing an aliquot of culture medium through a syringe filter (0.45  $\mu\text{m}$ ). Twelve tubes for each of three treatment groups were prepared by adding 1 mL of sterile medium, filtered *E. lima* culture medium or washed *E. lima* cells to the micro-centrifuge tubes containing DTX-4. The initial concentration of DTX-4 in the assay tubes was therefore 0.4  $\mu\text{g mL}^{-1}$  (0.27  $\mu\text{M}$ ). In addition, two tubes containing only washed cells and two with culture filtrate but no added toxin, were prepared to allow appropriate corrections to be made later for toxins contained in the medium or excreted by the cells. All tubes were gently vortexed, laid on their sides in a growth chamber at 20°C on a 12-h light:12-h dark cycle. Irradiance was 55-65  $\mu\text{mole-photons m}^{-2} \text{s}^{-1}$ , as measured by a 4 $\pi$ -quantum sensor (LI-COR, LI-185B meter with a SPH.Quantum sensor), and provided by 40-W cool-white fluorescent bulbs. Immediately after the setup (which gave an incubation time of 30 min), and then every 24 h for 4 d, two replicate tubes from each treatment group were removed and toxins extracted from the medium for

analysis by liquid chromatography-mass spectrometry (LC-MS). Tubes containing cells were first centrifuged; the supernatant was transferred to a fresh micro-centrifuge tube and only the medium extracted. Liquid-to-liquid extractions were made into  $\text{CH}_2\text{Cl}_2$  (2 x 1 mL) with vigorous vortex mixing and brief centrifugation each time to ensure complete phase separation. The organic layers were combined, evaporated to dryness in glass vials, then sealed and stored at  $-20^\circ\text{C}$ . Before analysis the extracts were redissolved in methanol (2 x 500  $\mu\text{L}$ ) and transferred by syringe through a 0.22  $\mu\text{m}$  filter into high recovery HPLC sample vials, evaporated to dryness again, then redissolved in methanol (25  $\mu\text{L}$ ) and quickly sealed. This procedure therefore provided a 40-fold concentration increase relative to the assay volume of 1 mL.

*Metabolism of OA diol-ester by Thalassiosira weissflogii, control experiment*

Micro-centrifuge tubes (12, sterile, transparent polypropylene) were prepared with OA diol-ester (1.0  $\mu\text{g}$ ) as described above. To each of three tubes was added an aliquot (1 mL) of one of the following: a three-week-old culture of *Thalassiosira weissflogii* ( $2.3 \times 10^5$  cells  $\text{mL}^{-1}$ ), a cell homogenate prepared by 2 min pulse sonication of the culture on ice, 0.22  $\mu\text{m}$ -filtered medium from the same culture or sterile f/2 medium. The tubes were then incubated for 24 h under the same conditions as described above. After incubation,  $\text{CH}_2\text{Cl}_2$  (250  $\mu\text{L}$ ) was added to each tube, the tube vortexed for 30 s, briefly centrifuged to ensure complete phase separation and then a portion (225  $\mu\text{L}$ ) of the organic layer was removed, evaporated to dryness, redissolved in methanol (100  $\mu\text{L}$ ) and analysed by LC-UV.



To ensure that bacteria were not present in the cultures of *T. weissflogii*, and responsible for the oxidation of OA diol-ester, samples of the diatom culture were plated on marine agar (Difco 2216) and incubated under the same culture conditions (above) for one week and then examined for the presence of bacterial colonies.

*Metabolism of OA diol-ester by Thalassiosira weissflogii, main experiment*

Aliquots (80  $\mu\text{L}$ ) of a stock solution of OA diol-ester ( $2.5 \text{ mg mL}^{-1}$ ) dissolved in methanol were dispensed into Erlenmeyer flasks (20, sterile, 250 mL-volume) held in a laminar flow hood and the solvent allowed to evaporate. Exponentially growing *Thalassiosira weissflogii* cells (100 mL at  $0.27 \times 10^6 \text{ cells mL}^{-1}$ ) in f/2 medium were then added to each flask giving a final concentration of diol-ester of  $2.0 \mu\text{g mL}^{-1}$  (2.2  $\mu\text{M}$ ). This design avoided committing a large mass of valuable toxin to a single culture and allowed independent, replicate measures of the metabolism of OA diol-ester to be made throughout the course of the experiment. Cell densities at the start and end of the experiment were taken by measuring the absorbance at 750 nm of aliquots (3 mL) taken from four separate flasks and comparing the readings to a standard curve (Chapter 1). The cultures were then incubated under the same conditions as described above.

Every 12 h, at the beginning and end of the light period, two flasks were selected haphazardly, swirled to ensure even mixing and a 5 mL sample containing cells and medium taken from each using a serological pipette. To maintain independence of the samples, a given flask was only sampled once. Following each

sampling all flasks were swirled and replaced in a haphazard order in the incubator to cancel the effects of any light or temperature gradients. Samples were filtered (15 mm diameter, glass fibre) and the filtrate extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 5 mL). Extracts were evaporated to dryness under nitrogen, redissolved in methanol (250  $\mu\text{L}$ ) and pipetted into LC sample vials (300  $\mu\text{L}$  capacity) and tightly sealed. The diatom cells on the filters were rinsed twice with sterile f/2 medium (2 x 5 mL), placed in a micro-centrifuge tube (1.5 mL) and extracted by sonication (30 s with a 2 mm diameter probe) in methanol (250  $\mu\text{L}$ ). The wet, pulped filter and methanol were then placed in the chamber of a spin filter (0.45  $\mu\text{m}$  Ultrafree-MC: Millipore Corp., Bedford, MA) and the methanol removed by centrifugation and pipetted into LC sample vials.

At the end of the experiment all the cultures were pooled, exhaustively extracted with  $\text{CH}_2\text{Cl}_2$  and the combined organic layers taken to dryness by rotary evaporation. Details of the isolation, purification and structural elucidation of the metabolites of OA diol-ester will be given elsewhere (Hu et al., unpubl.).

### *Chemical analyses*

A fast-running isocratic separation using LC-UV was used to track the metabolism of OA diol-ester during the course of the experiment. This prevented premature termination of the experiment and thus ensured maximum conversion of OA diol-ester. All the samples were later analysed using gradient elution for more accurate quantitation. In addition, LC-MS was used to quantify the concentration of OA as well as provide mass spectral data on the metabolism of OA diol-ester.

All LC analyses were performed on a model HP1090M LC system (Hewlett-Packard, Palo Alto, CA) fitted with a diode array detector. Separations of OA diol-ester and the metabolites were made on a 2.1 x 150 mm column packed with 5 $\mu$ m Zorbax Rx C8 (Chromatographic Specialties, Brockville, Ont.), 0.2 mL min<sup>-1</sup> flow rate, 5  $\mu$ l injection volume and UV detection at 238 nm. Isocratic elutions were made with a mobile phase of aqueous 55% CH<sub>3</sub>CN. The mobile phase for gradient elutions was aqueous CH<sub>3</sub>CN-ammonium acetate (1mM, pH 7.0) programmed from 20% to 50% CH<sub>3</sub>CN over 15 min, then 50% to 90% CH<sub>3</sub>CN over 1 min and held at 90% CH<sub>3</sub>CN for 5 min. A 15 min re-equilibration time under the starting conditions was used between injections.

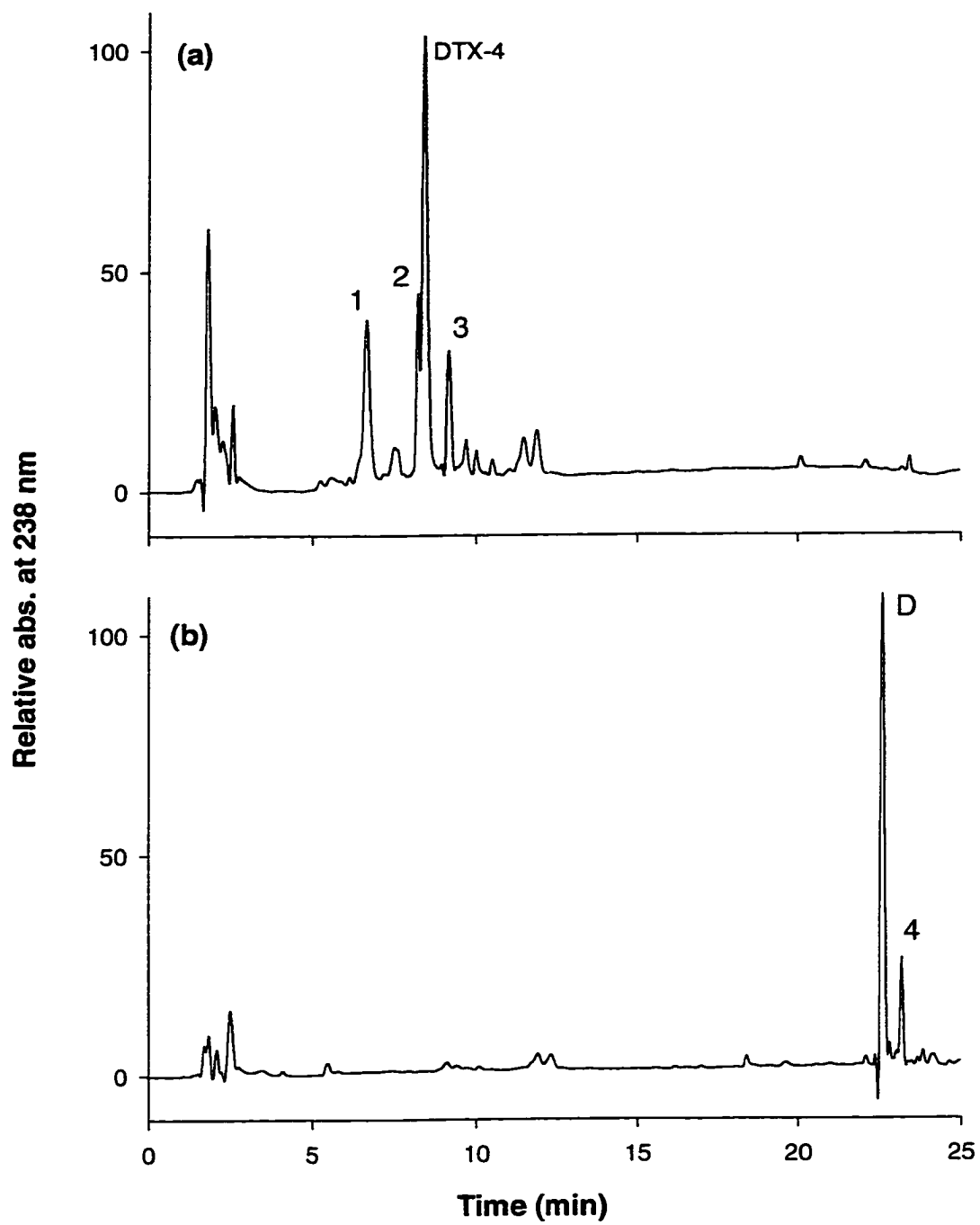
LC-MS determinations were made by coupling the HP1090M to an API-III+ triple-quadrupole mass spectrometer (Perkin-Elmer/Sciex, Concord, Ontario, Canada) equipped with an ion-spray interface. Full details of the LC-MS of DSP compounds can be found in Quilliam et al. (1996).

## Results

### *DTX-4 incubations with Exuviaella lima cells and culture filtrate*

The rapid hydrolysis of DTX-4 to OA diol-ester is illustrated graphically by the chromatograms shown in Figure 3.1. UV detection was at 238 nm to match the absorbance maxima of the conjugated dienes present in the side chains of both DTX-4 and OA diol-ester. The peak identities for DTX-4 and OA diol-ester are based on their absorbance spectra and co-incident retention times with authentic material.

**Figure 3.1** HPLC analysis of a crude extract of *Exuviaella lima*. (a) Before exposure to *E. lima* culture filtrate. (b) After 40 min exposure to *E. lima* culture filtrate. The LC-UV chromatogram in Figure 3.1a is of a crude methanolic extract of *E. lima*. DTX-4 and three related sulfated-esters of OA (peaks 1,2 and 3) are shown. The sample was evaporated to dryness, re-dissolved in seawater and then exposed to *E. lima* culture filtrate for 40 min, boiled and reanalysed. All of the DTX-4 and related compounds had been hydrolysed to OA diol-ester (peak D) and one other related compound (peak 4, Fig. 3.1b).

**Figure 3.1**

The upper chromatogram (Fig. 3.1a) shows the analysis of a methanolic extract of boiled *Exuviaella lima* cells and is dominated by DTX-4; also present are a number of related, sulfated-esters of OA (peaks 1, 2 and 3) as previously observed in negative ionspray LC-MS analyses of extracts of the same isolate (Quilliam and Ross, 1996). Following exposure to *E. lima* filtrate for 40 min, this collection of sulfated esters had been completely hydrolysed, primarily to OA diol-ester, but also to a later-eluting compound (Fig. 3.1b, peak 4). Diode-array acquired spectra for DTX-4, OA diol-ester and compounds 1 and 2 gave a  $\lambda_{\max} = 238$  nm, but compounds 3 and 4 exhibited a  $\lambda_{\max} = 240$  nm, suggesting that 4 is obtained from hydrolysis of 3.

When DTX-4 was incubated in the presence of washed *E. lima* cells or *E. lima* culture filtrate, the same rapid hydrolysis of DTX-4 to OA diol-ester observed in Figure 3.1 took place within the first 30 min as no DTX-4 could be detected in either treatment after this time. In the presence of washed *E. lima* cells (Fig. 3.2a) or *E. lima* culture filtrate (Fig. 3.2b) maximal levels of the diol-ester were observed at 30 min, followed by a gradual hydrolysis of OA diol-ester to OA over the next 4 d. In contrast, very low levels of OA ( $0.9 \times 10^{-9}$  M) and OA diol-ester ( $3 \times 10^{-9}$  M) were observed after 30 min when DTX-4 ( $270 \times 10^{-9}$  M) was incubated in fresh, sterile seawater medium. These levels only rose slightly after 96 h of incubation, with CA measured at  $7 \times 10^{-9}$  M and OA diol-ester at  $9 \times 10^{-9}$  M. This low level of spontaneous hydrolysis of DTX-4 (about  $1\% \text{ d}^{-1}$ ) in sterile seawater medium was expected (Chapter 2). These results indicated the presence of active esterases in the culture medium and possibly on the surface of *E. lima* cells.

**Figure 3.2** The effect of *Exuviaella lima* cells and culture filtrate on the hydrolysis of DTX-4 to OA via the intermediate OA diol-ester. DTX-4 was completely hydrolysed to OA diol-ester (solid line) within 30 min when exposed to washed *E. lima* cells (Fig. 3.2a) or culture filtrate (Fig. 3.2b). Concentrations of OA (dotted line) and OA diol-ester were determined by LC-MS. Regressions (concentration [ ] in  $\mu\text{M}$ ,  $n = 2$  at each sampling time, all points are independent, vertical bars represent  $\pm 1$  SE): Fig. 3.2a,  $[\text{OA diol-ester}] = -1.26 \times 10^{-3} \times h + 0.16$ ,  $r^2 = 0.97$ ;  $[\text{OA}] = 0.80 \times 10^{-3} \times h - 0.00$ ,  $r^2 = 0.92$ . Fig 3.2b,  $[\text{OA diol-ester}] = -1.31 \times 10^{-3} \times h + 0.17$ ,  $r^2 = 0.93$ ;  $[\text{OA}] = 1.48 \times 10^{-3} \times h - 0.01$ ,  $r^2 = 0.88$ .

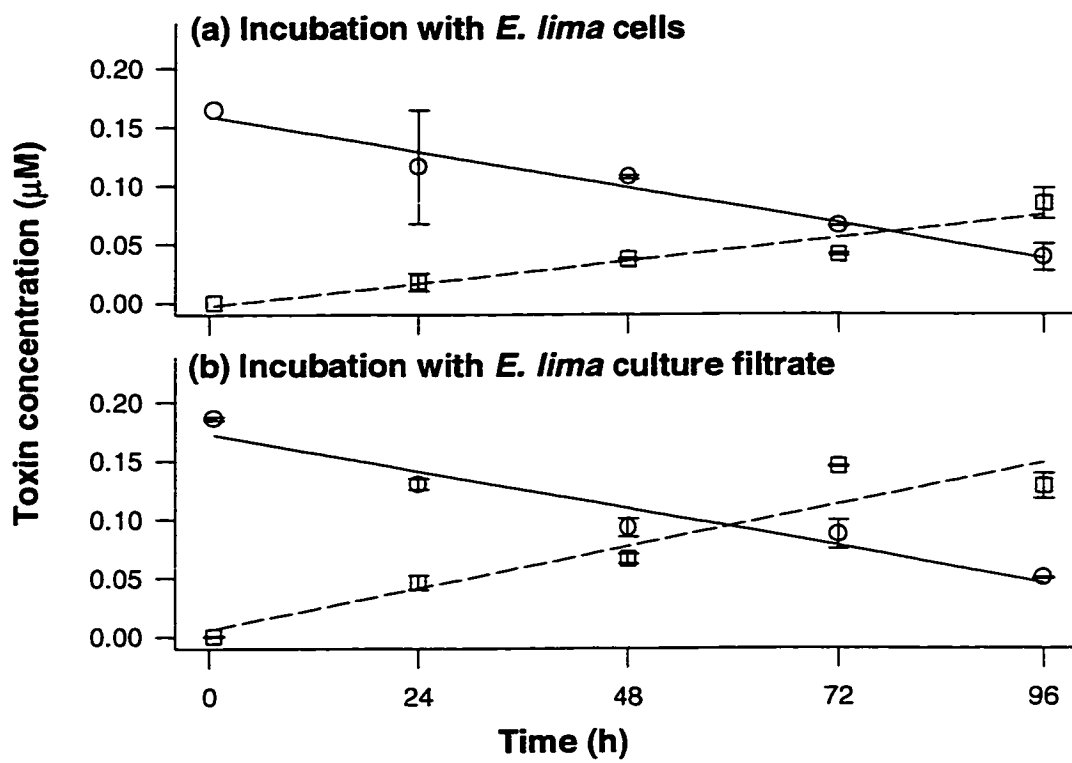


Figure 3.2



The linear regressions presented in Figure 3.2 were highly correlated and the constant terms (Y-axis intercepts) representing the initial concentrations of OA or OA diol-ester were very similar between treatments, as was the rate of decrease in the concentration of OA diol-ester ( $1.26 \times 10^{-3} \mu\text{M h}^{-1}$ ) when incubated with *E. lima* cells versus that observed with *E. lima* filtrate ( $0.31 \times 10^{-3} \mu\text{M h}^{-1}$ ). However, the rate of increase of OA was much lower ( $0.80 \times 10^{-3} \mu\text{M h}^{-1}$ ) when *E. lima* cells were present than with *E. lima* culture filtrate ( $1.48 \times 10^{-3} \mu\text{M h}^{-1}$ ) even though equivalent amounts of the diol-ester had decomposed in both treatments. Thus, while there is no evidence of OA excretion during the experiment, or of the metabolism of OA diol-ester by *E. lima* cells, there remains the suggestion that some uptake of OA by the *E. lima* cell fraction had occurred.

In examining the fate of DTX-4 exposed to either *E. lima* cells or filtrate derived from *E. lima* cultures, it was necessary to measure the concentrations of OA diol-ester and OA already present in the filtrate or released by the dinoflagellate during the experiment to ensure that the rates of DTX-4 hydrolysis, as measured by the increase in OA diol-ester and OA, were not overestimated. OA diol-ester was not found in either the filtrate derived from the *E. lima* culture or in the medium following incubation with washed *E. lima* cells to which DTX-4 had not been added. OA was detected in the *E. lima* filtrate used in the experiment at  $34 \pm 4 \times 10^{-9} \text{ M}$  (mean  $\pm$  1 SE) and in the medium to which washed *E. lima* cells were added, but not DTX-4, at  $4.0 \pm 0.8 \times 10^{-9} \text{ M}$  (mean  $\pm$  1 SE). Although relatively insignificant, these values were subtracted from the measurements of OA concentration as appropriate.

*Metabolism of OA diol-ester by Thalassiosira weissflogii: preliminary control experiment*

No bacterial growth was observed on the marine agar plates inoculated with *Thalassiosira weissflogii* culture. However, the diatom grew well on the marine agar plates; thus streak-plate isolation methods could be used to readily re-isolate this clone into axenic culture should contamination occur.

OA diol-ester was detected by LC-UV in all experimental treatments in the control experiment, eluting at 9.4 min. However, a second peak observed at 4.9 min was seen only in extracts derived from tubes incubated with intact algal cells. A peak with this retention time was given by samples containing the hydroxylated metabolite in previous experiments (OA diol-ester + O, hereafter referred to as metabolite I or M1) and characterised by LC-MS (Chapter 2). The concentration (mean  $\pm$  1 SE) of OA diol-ester in the sterile control group was  $0.58 \pm 0.04 \mu\text{M}$ , representing a 55% recovery of the initial diol-ester added; this moderate level was expected given the single-step extraction procedure used. The concentration of diol-ester in the culture filtrate was  $0.56 \pm 0.02 \mu\text{M}$  and not significantly different from the control ( $t = 0.4$ , where  $t_{0.05|41} = 2.776$ ). The concentrations of diol-ester in the intact cell ( $0.22 \pm 0.02 \mu\text{M}$ ) and cell homogenate ( $0.43 \pm 0.07 \mu\text{M}$ ) incubations were both significantly different from the control ( $t = 8.28$ ,  $.001 > P > .01$  and  $t = 3.91$ ,  $0.5 > P > 0.1$  in that order).

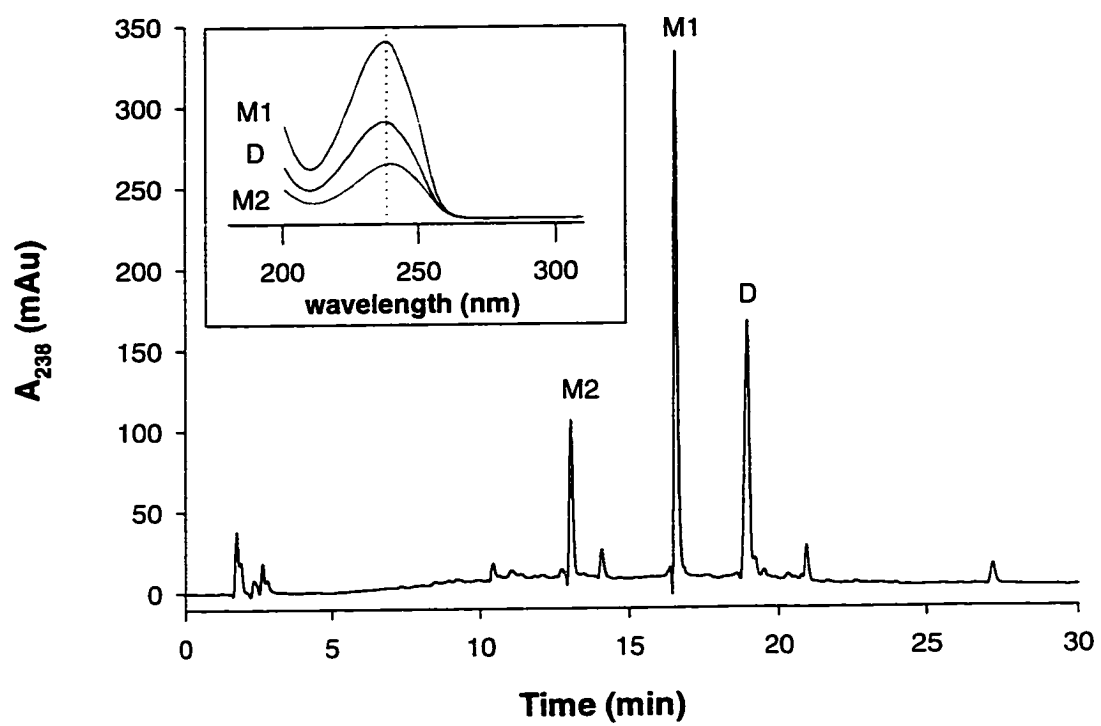
*Metabolism of OA diol-ester by Thalassiosira weissflogii*

The cell density in the *Thalassiosira weissflogii* cultures increased from  $270 \times 10^3 \pm 6 \times 10^3$  cells mL<sup>-1</sup> at the start of the experiment to  $380 \times 10^3 \pm 1 \times 10^3$  cells mL<sup>-1</sup> (mean  $\pm$  1 SE, n = 4) at the end.

Isocratic LC analyses made during the course of the experiment showed that the concentration of OA diol-ester in the medium declined rapidly from the initial level of 2.2  $\mu$ M to essentially zero after 72 h. It was also apparent, based on the retention time and characteristic UV spectrum, that a substantial concentration of the previously observed metabolite (M1) had accumulated in the medium during this time. Therefore at the 72-h time point the experiment was terminated and bulk extraction of the culture made.

Gradient LC analyses performed on the sample extracts to quantify accurately the concentrations of OA diol-ester and the metabolite revealed the presence of an earlier-eluting peak not evident in the isocratic runs (Fig. 3.3). This compound increased in concentration in the medium throughout the course of the experiment in inverse proportion to OA diol-ester, possessed a UV spectrum similar to OA diol-ester and the first metabolite but, with a  $\lambda_{\text{max}}$  shifted from 238 nm to 240 nm (Fig. 3.3), and was therefore presumed to be a second metabolite of OA diol-ester (M2).

**Figure 3.3** Identification of a second metabolite of OA diol-ester in *Thalassiosira weissflogii* cultures exposed to OA diol-ester. LC-UV gradient analysis of combined extracts of *T. weissflogii* culture medium that were taken every 12 h during the course of the metabolism experiment. In addition to OA diol-ester (peak D), and the previously observed metabolite (M1), an earlier-eluting peak was observed at 13.1 min (M2). DAD acquired spectra for this compound (inset) showed it possessed an absorbance spectrum similar to those of OA diol-ester and M1 ( $\lambda_{\text{max}} = 238$  nm) but, with a slightly higher absorbance maximum ( $\lambda_{\text{max}} = 240$  nm). It was also noted that the retention time of M2 increased by 20% when 1% TFA was added to the mobile phase, suggesting M2 had acidic functionality.

**Figure 3.3**

*Metabolism of OA diol-ester by Thalassiosira weissflogii: LC-MS analysis*

Additional information on the metabolism of OA diol-ester by *Thalassiosira weissflogii* was gained by combining and concentrating all of the analytical samples taken throughout the course of the experiment and analysing this sample by LC-MS in scan mode ( $m/z$  from 780 to 1000). The resultant principal-ion chromatogram (Fig. 3.4a) therefore represents an integrated measure of the metabolism of OA diol-ester by the diatom during the experiment. Figure 3.4b-f provides the individual mass chromatograms for the principal ions observed in Figure 3.4a. Although both  $[M+H]^+$  and  $[M+NH_4]^+$  signals were observed, the  $[M+H]^+$  ions were only a minor constituent of the total signal and are not shown in Figures 3.4b-f.

Figures 3.4b-d show OA, OA methyl-ester and OA diol-ester in that order. Metabolite 2 (Fig. 3.4e) is seen at  $m/z$  960.6. The fact that this is 14 mass units higher than OA diol-ester suggests the oxidation of a primary hydroxyl group to a carboxylic acid and is consistent with the observation that the retention time of M2 increased when 0.1% trifluoroacetic acid (TFA) was present in the mobile phase. Metabolite 1 eluted at 14.9 min and was seen at  $m/z$  962.6, consistent with previous MS data (Chapter 2) identifying it as a hydroxylated derivative of OA diol-ester. However, the additional peaks on either side of M1 of the same mass indicate the presence of isomeric oxidation products and were denoted M3, M4 and M5. As these were not seen with LC-UV the conjugated diene function in the side chain had clearly been disrupted in these metabolites.

**Figure 3.4** LC-MS analysis of the combined extracts of *Thalassiosira weissflogii* culture medium. The presence of numerous compounds transparent to UV detection were detected (compare Fig. 3.4a with Fig. 3.3). Extracted mass chromatograms for the principal ion observed  $[M+NH_4]^+$  are given in Figure 3.4b-f. Figure 3.4b-d show OA, OA methyl-ester (peak Me) and OA diol-ester (peak D) respectively and Figure 3.4e and f the oxidative metabolites. The asterisk denotes a signal from an isotope peak.

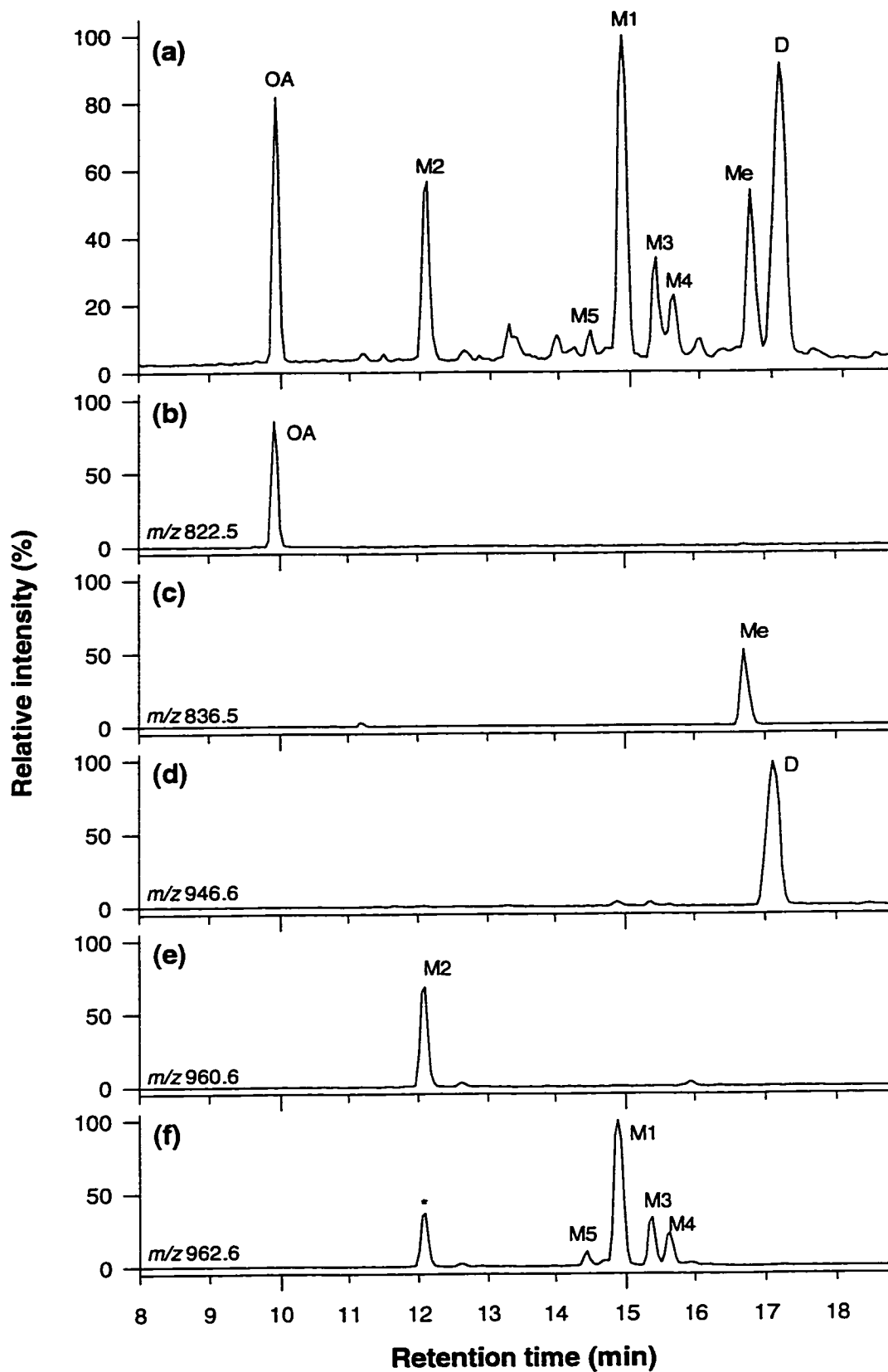


Figure 3.4



Ultimately NMR and high resolution MS data were used to derive the structures of M1, M2 and M3. The details of the structural elucidation will be reported elsewhere (Hu et al., unpubl.), however, they show that the primary sites of oxidative attack were the terminal (M2) and sub-terminal (M1) carbons in the diol-ester side chain.

*Metabolism of OA diol-ester by Thalassiosira weissflogii: medium fraction*

Figure 3.5a illustrates the changes in OA diol-ester and the total metabolite concentration (including OA) in the culture medium. Within the first 48 h of the experiment about 90% of the toxin initially added to the cultures as OA diol-ester could be accounted for in the medium fraction, but this figure declined to 70% by the end of the experiment.

The predominant form of the toxin at the end of the experiment was clearly M1 followed by OA and then M2. The rate of formation of the metabolites slowed noticeably between 24 and 36 h, coincident with the light period, and then increased again during the following dark period (Fig. 3.5a). Although the data are limited, this suggests that metabolism of OA diol-ester was primarily taking place in the dark period. The rates of formation of M1, M2, M3 and OA closely paralleled each other (Fig. 3.5b); after 48 h no further increase in concentration of the metabolites including OA was noted. OA methyl-ester (Fig. 3.5b) was also consistently detected at low, but very constant levels throughout the experiment ( $1.25 \pm 0.07 \times 10^{-7}$  M, equivalent to about 6% on a molar basis of the initial concentration of OA diol-ester).

**Figure 3.5** Metabolism of OA diol-ester by *Thalassiosira weissflogii* cultures. The change in concentration of OA diol-ester is shown in relation to the total concentration of metabolites (Fig. 3.5a). The changes in concentration of each metabolite are shown separately (Fig. 3.5b). The black bars on the time axis represent the 12 h of darkness during each photoperiod which, over the first 48 h, coincide with the highest rates of metabolism of OA diol-ester. The levels of OA diol-ester, M1 and OA in the cell fraction are presented as a percentage of the total initial toxin added to the cultures (Fig. 3.5c and 3.5d). In Figs. 3.5b and 3.5d log scales were used to accommodate wide differences in toxin concentration. Vertical bars represent 1 SE, n = 2, all points are independent.

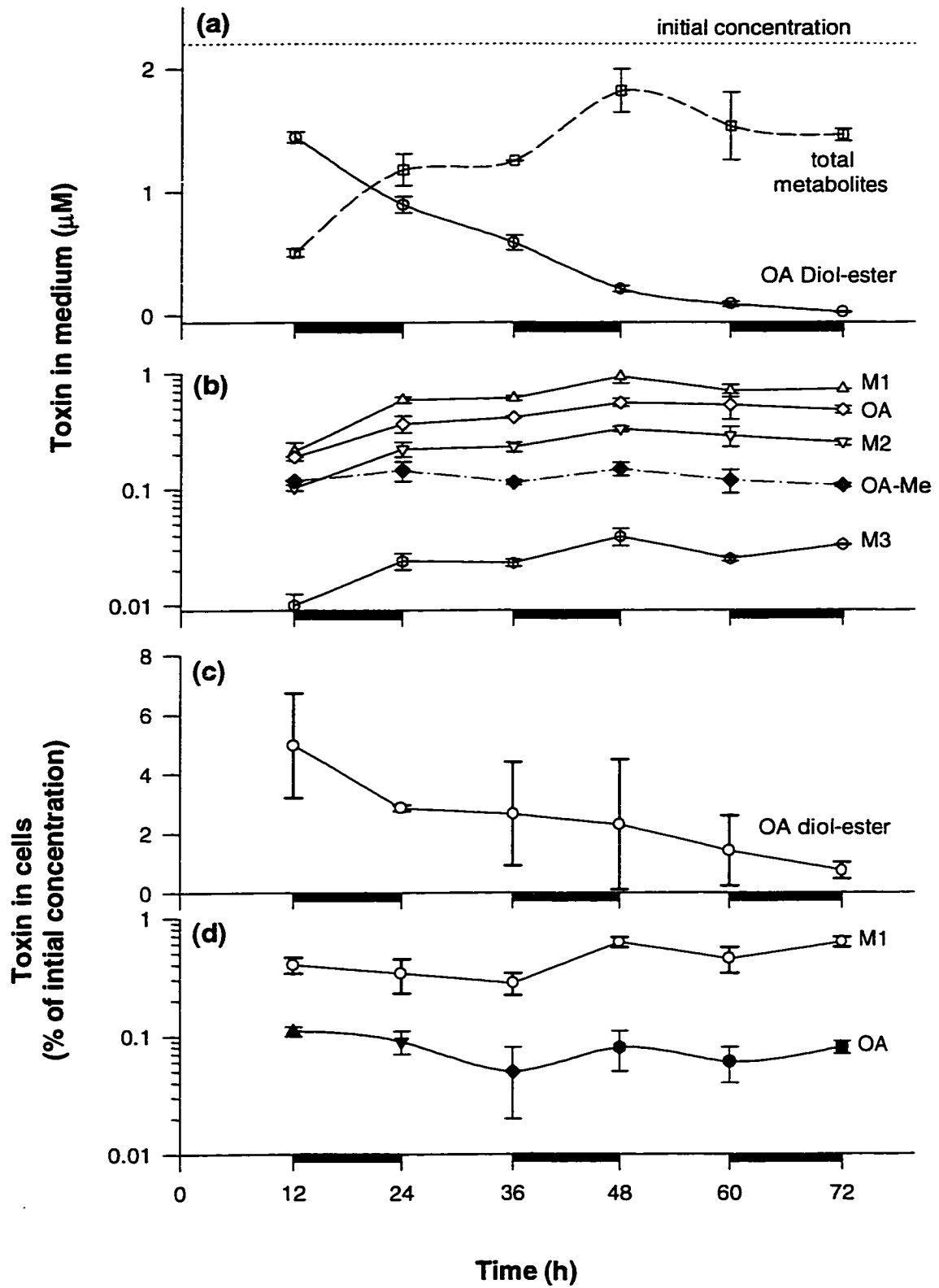


Figure 3.5

LC-MS analysis of a sample of the OA diol-ester used to initiate the experiment confirmed that OA methyl ester was present as a 7% impurity and explained the source of this compound.

*Metabolism of OA diol-ester by Thalassiosira weissflogii: cell fraction*

Only OA diol-ester, M1 and OA were detected in the cellular fraction.

Although the majority of OA diol-ester was recovered from the medium, about 5% of the initial amount added was associated with the cellular fraction within the first 12 h of incubation as OA diol-ester, but this level declined over 72-h of incubation to less than 0.5% (Fig. 3.5c). The concentrations of M1 and OA were low and relatively constant during the experiment, accounting for about 0.5 and 0.1 % of the initial concentration respectively (Fig. 3.5d).

## **Discussion**

Dinophysistoxin-4, the primary product of DSP toxin synthesis, is rapidly hydrolysed to OA diol-ester and thence to OA following cell rupture owing to the release of esterases from intracellular compartments (Quilliam and Ross, 1996). The experiments reported here in which DTX-4 was incubated with either washed *Exuviaella lima* cells or culture filtrate demonstrates that the same fate would befall DTX-4 if, as Hu et al. (1995a) suggested, it is the form excreted from the cell. This observation explains why DTX-4, although highly water-soluble, has never been

detected in the medium of *E. lima* cultures despite persistent efforts by several groups to do so (J.L.C Wright and M.A. Quilliam unpubl.). Quilliam and Ross (1996) reported complete hydrolysis of DTX-4 to OA within a day following cell rupture by freezing and thawing cell pellets of *E. lima* cells. In the current experiments DTX-4 was also rapidly hydrolysed but the diol-ester persisted for several days. OA diol-ester was also found to remain for up to one week in the digestive tissues of scallop (*Argopecten irradians*) after feeding on *E. lima* cells (Bauder et al., 1996). It is probable that the faster hydrolysis of OA diol-ester observed in pellets of ruptured cells is the result of unnaturally high concentrations of enzymes in such preparations. Therefore, not only will OA diol-ester become the predominant form of the DSP toxins following release from the producing cell through excretion or cell destruction, it may also persist for sufficient time to be a factor in the subsequent partitioning of DSP toxins in the environment.

Despite being inactive as a phosphatase inhibitor, the toxicity of OA diol-ester to *Thalassiosira weissflogii* was explained as a result of the lipophilic, uncharged OA diol-ester facilitating the entry of the toxin into the cell, followed by partial hydrolysis to the lethal toxin OA once inside the cell (Chapter 2). The data presented here, with respect to the specific nature of the oxidative metabolism of OA diol-ester and subsequent partitioning of OA diol-ester and metabolites between cell and medium fractions, add further credence to these arguments.

It is clear from the current study that oxidation of OA diol-ester by *T. weissflogii* only occurs when intact cells are exposed to the toxin. All the

transformation products of OA diol-ester identified thus far involve oxidation of the ester side chain, not the OA portion of the molecule. The most abundant oxidation product (M1) resulted from hydroxylation of the allylic methylene group in the side chain (Hu et al., unpubl.). Such a hydroxylation step points to the involvement of microsomal enzymes like the cytochromes P-450 (Müller et al., 1984) and parallels the similar hydroxylation of fatty acids at the omega-1 position by wheat microsomes (Zimmerlin et al., 1992) and of P-450 enzymes isolated from *Bacillus megaterium* (Miura and Fulco, 1975). P-450 enzymes in eukaryotes require NADPH, are found within the endoplasmic reticulum, and represent the terminal component of an electron transport chain. This strongly suggests that metabolism of OA diol-ester occurs within the diatom cell. Interestingly, another class of marine polyether toxins, the brevetoxins, has been shown to elicit cytochrome P-450 activity in exposed fish (Washburn et al., 1996). However, in comparison to animal systems, little is known about the activities of plant microsomal enzymes with respect to toxin metabolism and studies involving microalgae are both rare and limited to the metabolism of polycyclic aromatic hydrocarbons (Warshawsky et al., 1995). Plant P-450 enzymes are recognised to catalyse unique reactions involved in plant secondary metabolism (Russell, 1970). Given the enormous diversity of secondary metabolism in plants, it has been speculated by Bolwell et al. (1994) that metabolism of xenobiotics by plants is non-specific and may not represent a general detoxification mechanism as does the mixed-function oxygenase system of vertebrate liver cells. In the case of *T. weissflogii*, definitive proof of cytochrome P450 involvement in the metabolism of DSP toxins will

require isolation of active microsomal fractions, demonstration of blue light-reversible inhibition by carbon monoxide and a requirement for NADPH (Bolwell et al., 1994).

When OA diol-ester was added to the medium of *T. weissflogii* cultures, a substantial proportion of the ester was initially found in the washed cells, but after 12 h the levels of the ester in both cellular and medium fractions declined in unison. This implies a concentration-dependent flux of OA diol-ester from the medium into the cells. In contrast, the levels of OA and M1 found in the cellular fractions were low and independent of their medium concentrations, and M2 and M3 were not detected. These facts all support the interpretation that OA diol-ester enters the diatom cell, where it is oxidised and then excreted as more water-soluble products. Interestingly, OA methyl-ester, present as a contaminant in the starting material, was detected only in the medium fraction, not the cell fraction and remained at the same concentration throughout the experiment. OA methyl-ester, like OA diol-ester, is also uncharged and would therefore be expected to penetrate the diatom cell. The failure to detect OA methyl-ester in the cells could be the result of the low concentration of the methyl-ester with respect to the diol-ester (~ 7%) or indicate that the ester side chain is important in determining the ability of the toxin to enter the diatom cell.

Okadaic acid was detected in both cellular and medium fractions but the level of OA in the cellular fraction was low and did not noticeably increase over time even though the level in the medium increased to 0.5  $\mu\text{M}$  over the first 48-h of the experiment. As the metabolites of OA diol-ester result from the oxidation of the diol-ester side chain, they would yield OA if hydrolysis of the ester occurred. As a

consequence, the increase in concentration of OA in the culture medium results from the hydrolysis of OA diol-ester or its oxidised products. The fact that no further accumulation of OA was noted after 48 h, coincident with the virtual disappearance of OA diol-ester and despite the persistence of the metabolites after this time, suggests that the source of the OA in the medium was most likely derived from the hydrolysis of OA diol-ester. The plateau in levels of OA and the metabolites of OA diol-ester following 48-h incubation is therefore a result of substrate limitation.

The metabolism of the DSP toxin OA diol-ester by the diatom *T. weissflogii* probably does not occur in nature, but it is relevant as a model system with which the flux of DSP toxins across a cell membrane and into a cell can be inferred and compared. The amount of OA measured in the cell fraction was low and independent of its concentration in the medium. This corresponds to the conclusions of Klumpp et al. (1990) that whole cells are relatively impermeable to OA because high medium concentrations of OA (~35  $\mu\text{M}$ ) were required to invoke even non-lethal physiological responses from *Paramecium*. In our experiments the concentration of OA diol-ester decayed exponentially in the medium fraction coincident with a rapid increase and then decline in the cellular fraction. This is indicative of a concentration dependent influx of this toxin into the cell. However, this influx must be severely attenuated by the addition of polar substituents to the diol-ester side chain as the metabolites of OA diol ester do not partition with the cellular fraction. Thus the presence of the side chain of OA diol-ester, which is exposed by the hydrolysis of DTX-4, confers its ability to enter cells readily. Interestingly, spectroscopic analysis has determined that



the formation of the ester bond in DTX-4, ultimately giving rise to the diol-ester, is a specific, enzymatically mediated event which occurs after the synthesis of the complete carbon backbone of the DTX-4 molecule (Wright et al., 1996). It is difficult to know if the ability of OA diol-ester to penetrate cell membranes has adaptive significance as a chemical defence, but it very likely has functional significance in terms of the transport and toxicity of DSP toxins in the food web.

## Chapter 4

### The physiology of growth, toxin production and toxin release

by *Exuviaella lima* in turbidostat culture

#### Introduction

Based on the work presented in Chapters One and Two, the DSP toxins have the potential to inhibit the growth of other phytoplankton cells at concentrations of about 1  $\mu\text{M}$  in seawater. An objective assessment of the ecological importance of this measure of toxicity requires some understanding of the rates of DSP toxin production by *Exuviaella lima*, and most importantly, the rate of release of the toxins from the producing cells into seawater. Specifically, it is important to consider the following questions: are micromolar levels of the DSP toxins in seawater to be expected in nature and what proportion of total DSP production is released from the cell?

The measurement of secondary metabolite production and release rates for phytoplankton cells is not straight-forward. Firstly, batch cultures are often used, this confounds the measurements as the concentrations of metabolites within the cells or medium represent those accumulated over time and are therefore integrative measures (Rhee, 1980; Trilli, 1990). Secondly, batch cultures consist of cells in all stages of growth and it is impossible to assess to what degree cell lysis is contributing to medium levels as opposed to excretion or release from healthy cells. Finally, because the volume of medium is much larger than that of the cells, the concentration of metabolites in this fraction may be very low and difficult to detect.

Continuous culture offers some advantages over batch culture because the specific rates of toxin production (Trilli et al. 1990), toxin excretion and cell growth (Skipnes et al., 1980) may be measured directly at steady state provided the dilution rate of the culture is known. However, a fundamental prerequisite of continuous culture is that the cells can be maintained in uniform suspension and do not adhere to the walls of the growth vessel. In batch culture, *E. lima* is notorious for clumping and sticking to the bottom of culture vessels because of its extensive production of mucus (Heil et al., 1993; McLachlan et al., 1994). Even so, attempts were made to culture *E. lima* in turbidostats designed for growth optimization studies (reported in Part 2). Surprisingly these attempts were successful and, although the characteristics of the *E. lima* cell suspension were less than ideal, consistent, robust and stable turbidostat cultures could be maintained for months at a time. This chapter describes the turbidostat culture of *E. lima* and its application to the measurement of the production and release rates of the DSP toxins at steady state. In light of this information the two questions posed above are considered further.

## Methods

### *Turbidostat culture*

The source and maintenance of *Exuviaella lima* (clone Pa) are as described in Chapter 1. The design, setup and operation of the turbidostats are as presented in Part 2 with one difference, full strength f/2 medium (McLachlan, 1973) was employed throughout as attempts to operate at low cell densities ( $\sim 2 \times 10^3$  cells mL<sup>-1</sup>) and

nutrient levels (f/20) were not successful. The levels of nitrate and phosphate were slightly adjusted from that described for f/2 by McLachlan (1973) to provide a 16:1 ratio giving concentrations of 1 mM nitrate and 62.5  $\mu$ M phosphate. A 12 h light:12 h dark photoperiod was used throughout. Eleven different steady states were achieved under a variety of different irradiance and temperature levels, and the growth rates, toxin production and release rates were measured as described below.

Cultures were considered to be at steady state when growth rates became constant (within 5%) for three successive days; typically this required up to seven days after changing the culture conditions. At steady state the specific growth rate ( $\mu$ ) was taken to equal the dilution rate (D). Culture volumes were maintained at 1.0 L.

The instantaneous rates of DSP production ( $Q_p$ ) or release ( $Q_r$ ), in terms of mols cell<sup>-1</sup> day<sup>-1</sup>, were calculated by first measuring the steady state toxin concentration in cells ( $P_c$ ) and medium ( $P_m$ ) in terms of mols L<sup>-1</sup> and then using the following relationships (Trilli, 1990):

$$Q_p = [(P_c \times \mu) / X] + Q_r$$

$$Q_r = (P_m \times \mu) / X$$

where: X is the steady state cell density in cells L<sup>-1</sup>.

#### *Extraction of cells*

A 40-mL aliquot of culture was placed into a glass, conical-bottomed centrifuge tube and the cells pelleted by centrifugation for 60s at low speed. The culture medium was decanted and the cell pellet partially re-suspended by adding 0.2

mL of seawater and gently vortex mixing the cells. The tube was then placed in a boiling water bath for 3 min to inactivate enzymes that would otherwise hydrolyse DTX-4 to OA diol-ester and OA. The extraction was started by adding 0.8 mL of 100% methanol to the tube and placing the tube in an ultrasonic bath for 2 min. After sonication the tube was centrifuged to pellet the cellular material and the supernatant transferred by pipette to a 2.0-mL volumetric flask. A second round of extraction was then made by adding 1.0 mL of 80% methanol and repeating the sonication and centrifugation steps. The supernatant was added to that in the volumetric flask and the volume brought to the mark with 80% methanol. After thorough vortex mixing a portion of aqueous methanoic extract was passed through a 0.45  $\mu\text{m}$  filter and pipetted into an HPLC autosampler vial and capped tightly and stored at  $-20^{\circ}\text{C}$  prior to analysis by LC-UV for DTX-4 and LC-MS for OA, DTX-1 and OA diol-ester as described in Chapters Two and Three.

#### *Extraction of medium*

A 100-mL sample of culture was passed through a glass-fibre filter to remove the cells and detritus. The medium samples were then stored at  $-20^{\circ}\text{C}$  until all the samples to be extracted had been obtained. At this point, the medium samples were thawed and thoroughly mixed. A 25-mL aliquot was placed in a 60-mL separatory funnel. The medium was then partitioned against 5 mL  $\text{CH}_2\text{Cl}_2$  by vigorously shaking the funnel 40 times. Following phase separation the organic layer was decanted into a glass scintillation vial. This procedure was repeated three more times and the

combined organic extracts evaporated to dryness. The extract was then redissolved in 0.5 mL of 100% methanol and transferred to a high recovery HPLC sample vial. The scintillation vial was then rinsed with a second 0.5 mL aliquot of 100% methanol which was added to the HPLC vial. The extract was again evaporated to dryness and then finally re-dissolved in 25  $\mu$ L of 80% methanol prior to analysis for OA, DTX-1 and OA diol-ester. This procedure provided a 1000 times concentration relative to the original sample. To assess the efficiency of this procedure to recover OA from seawater, three 25-mL samples of seawater were spiked with 10  $\mu$ g of OA and extracted using the same procedure.

## Results

### *The growth of Exuviaella lima in turbidostat culture*

*Exuviaella lima* grew consistently in turbidostat culture over long periods. No systematic attempt was made to determine the ideal conditions for turbidostatic growth, but the following general observations are offered as guidelines. Growth rates were not affected by pH between 7.5 and 9.0 but were markedly reduced in continuous light. Steady state growth was not attained at either low cell densities or low nutrient concentrations. Cell clumping would occur following inoculation and other disturbances such as the cessation of stirring for more than a few minutes, the direct addition of nitrate to the culture vessel and in response to transitions in the photoperiod. The clumping of the cells was a transitory phenomenon and the clumps would disperse over time. Intermittently stirring the culture, sufficiently to overcome

the tendency of the cells to settle, but no more, was important in maintaining a consistent cell suspension. Although some of the population of cells within the turbidostat existed as single cells the majority existed as small clumps of a few cells.

Eleven steady states were obtained under a variety of different growth conditions and the growth rates at each steady state measured (Table 4.1). Growth rates were influenced by the cell density employed (steady states 1 to 4, Table 4.1). Although higher growth rates were measured at cells densities below  $20 \times 10^3$  cells  $\text{mL}^{-1}$  the correspondence between cell density and beam attenuation was best using a set point attenuation of 1.00 abs. units which, over 8 different steady states, maintained a cell density of  $20.2 \pm 0.5 \times 10^3$  cells  $\text{mL}^{-1}$  (mean  $\pm$  1 S.E.). Depending on the cell density employed, the highest growth rates for *E. lima* were obtained between 22 and 25 °C and 200 to 240  $\mu\text{mol-photon s}^{-1}$ . A substantial drop in growth rate was noted when the temperature was increased to 28 °C (Table 4.1).

The general characteristics of a turbidostat culture of *E. lima* can be seen in Figures 4.1a to d. Growth only occurred during the light period apparently starting an hour or two after the lights were turned on and terminating with a burst of growth when the lights were extinguished (Fig 4.1a., arrow). The apparent delay and burst in growth at the start and end of each light period, although very consistent, were likely artifacts caused by the responses of cell suspension to transitions in the photoperiod and the subsequent reaction of the turbidostat control mechanism. Dark to light transitions caused a noticeable (to the eye), but transitory, clumping of the cells.

**Table 4.1***Exuviaella lima*, growth rates in turbidostat culture under different conditions

Steady state	Irradiance ( $\mu\text{-mol photons m}^{-2} \text{ s}^{-1}$ )	Temperature ( $^{\circ}\text{C}$ )	Beam Attenuation (Abs. Units)	Cell Density ( $\times 10^3 \text{ mL}^{-1}$ ) *	$\mu$ ( $\text{day}^{-1}$ )
1	200	22	0.40	9.1 (0.4)	0.17
2	200	22	0.52	10.7 (0.3)	0.26
3	200	22	0.70	10.8 (0.5)	0.29
4	200	22	1.00	18.7 (2.2)	0.22
5	240	25	1.00	21.0 (3.2)	0.22
6	240	19	1.00	20.2 (1.6)	0.15
7	200	16	1.00	17.5 (0.5)	0.12
8	160	19	1.00	22.4 (1.4)	0.13
9	160	13	1.00	21.1 (1.0)	0.10
10	160	25	1.00	19.7 (0.7)	0.14
11	200	28	1.00	21.0 (0.6)	0.16

\* Direct microscopic counts of triplicate 10 $\mu\text{L}$  aliquots; mean and standard errors are shown.



**Figure 4.1** Growth of *Exuviaella lima* in turbidostat culture. (a) Inflow volume or dilution over time. (b) Beam attenuation at 880nm over time. (c) Culture pH over time. (d) CO<sub>2</sub> injection over time. Note: the black bars represent the dark part of the photoperiod.

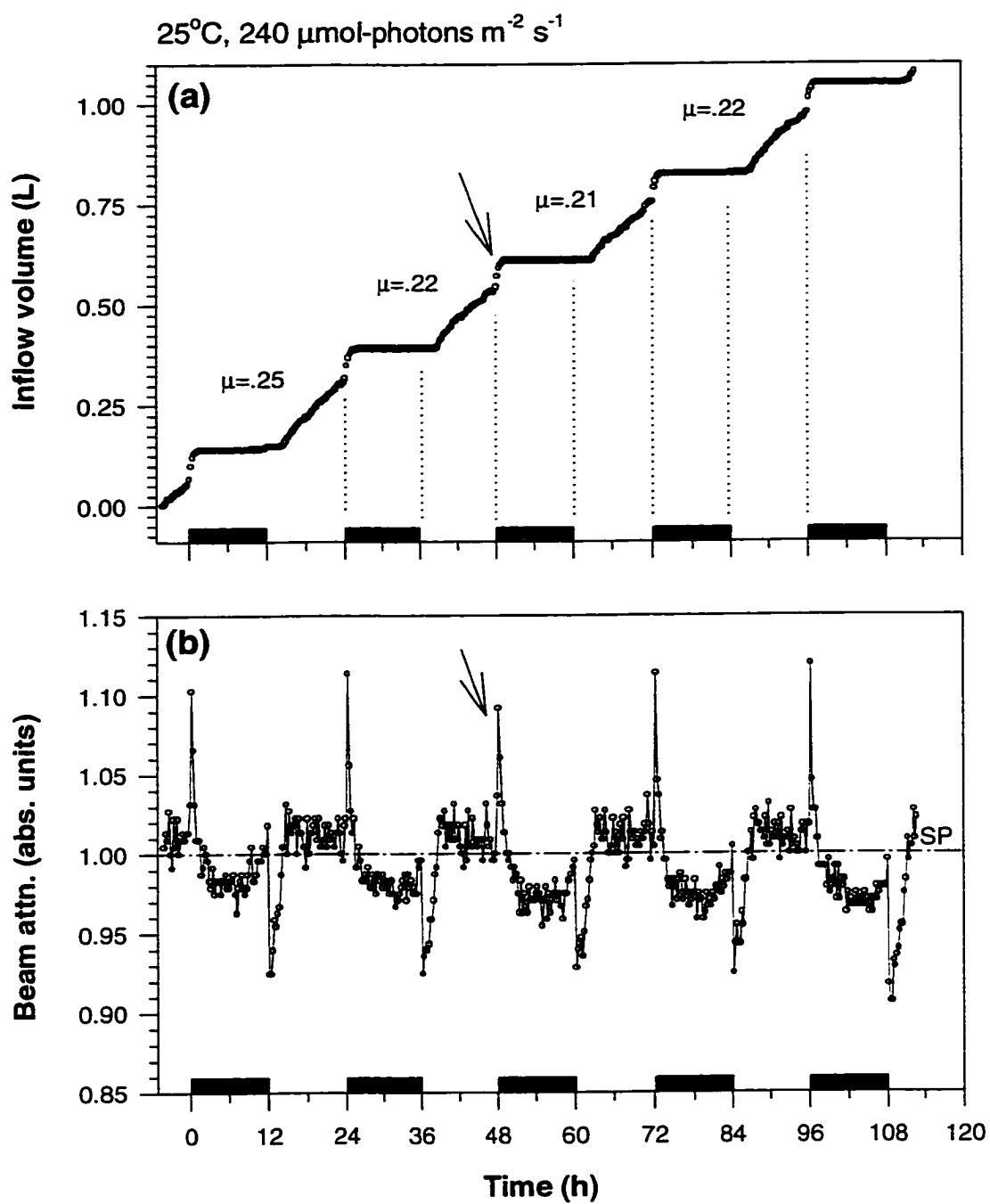


Figure 4.1

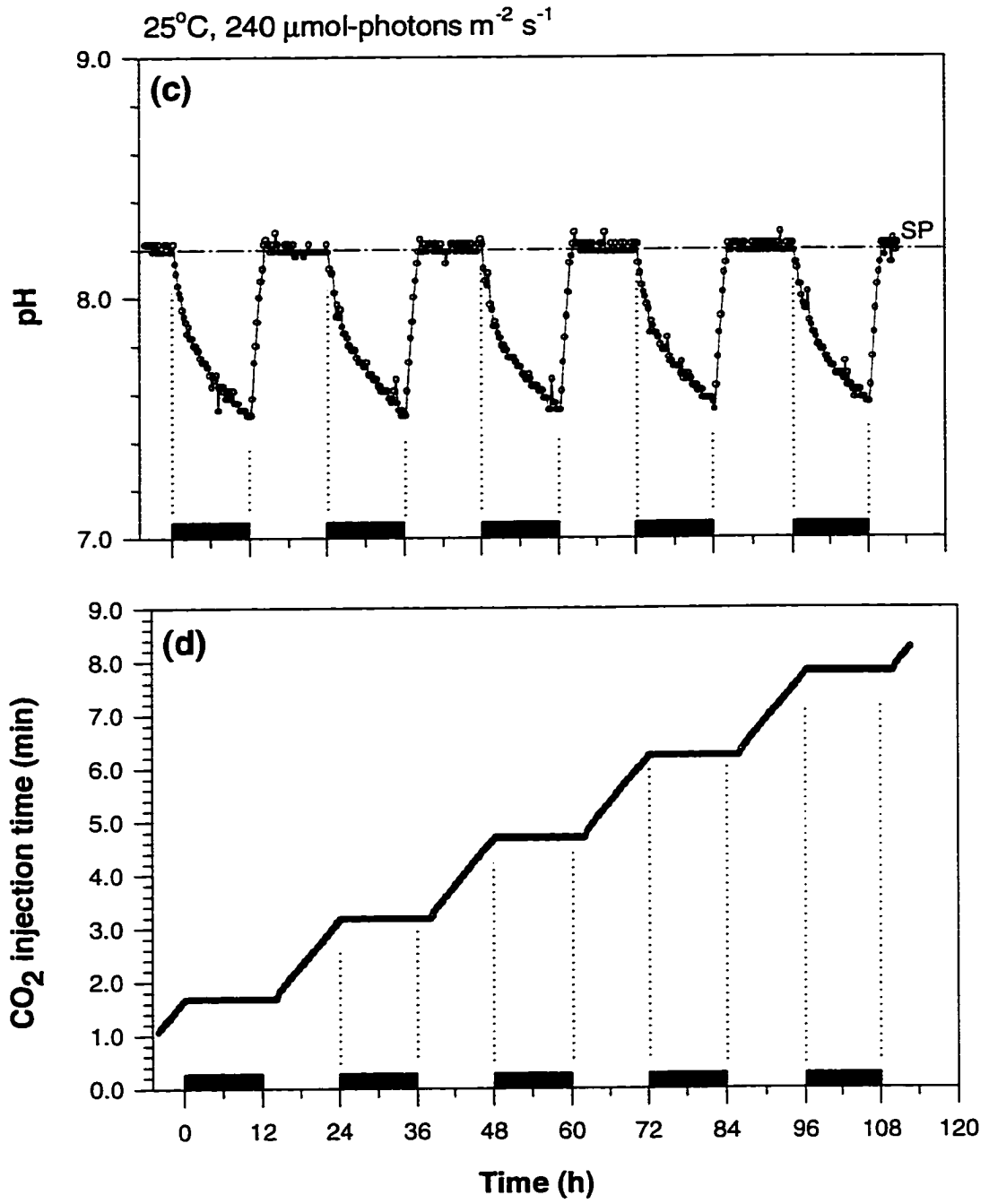


Figure 4.1 (cont.)

This clumping caused a decrease in the attenuation of the light beam (Fig. 4.1b, arrow) even though the cell density had not changed. Thus, while growth was probably taking place as soon as the lights were turned on, the attenuation set point would not be exceeded until the clumps had dispersed and hence the apparent delay in growth according to the recorded dilution rate. The sharp increase in culture attenuation at the end of the light period is probably due to a transitory dispersion of the cell clumps, but this was not confirmed by independent observation and remains speculative. Even so, the short (30 min) increase in attenuation was strong and triggered the medium dilution pumps. This addition of fresh medium would dilute the suspension below the set point and likely contributed to the apparent delay in growth at the start of the next light period.

The light:dark photoperiod also elicited strong diel changes in culture pH (Fig. 4.1c). The decrease in pH of the unbuffered medium during the night was the result of increased CO<sub>2</sub> in the medium from respiration by the cells (in the absence of photosynthesis) and coincident with this is a decrease in beam attenuation (Fig. 4.1b). Carbon fixation during the day caused an increase in culture pH above the setpoint of 8.2 and triggered the addition of CO<sub>2</sub> throughout the light period (Fig. 4.1d). The fact that the culture pH, depressed during the night, rose sharply as soon as the light turned on (Fig.4.1c) supports the observation that growth also started at this time and the delay in apparent growth based on the dilution data was an artifact.

### *DSP production and release rates*

Trace levels of OA diol-ester were detected in the extracts of the cells but not medium. These low levels were probably due to limited hydrolysis of DTX-4 during the cell harvest and extraction process. DTX-4 was the major DSP toxin recovered from the cells at all of the steady states, comprising ~50% of the total DSP recovered from both cells and medium on a molar basis (Table 4.2). On average, OA and DTX-1 in the cell fraction accounted for 24 and 17% of total production respectively. The total production rates of the DSP toxins by *E. lima* varied between 1 and 3 fmol cell<sup>-1</sup> d<sup>-1</sup>. The majority of this variation in production (~ 80%) was explained by differences in the growth rates of the cells (Fig. 4.2). Within the range of growth rates observed, 0.10 to 0.29 d<sup>-1</sup>, the production rates of the DSP toxins could be described as a positive, linear function of the growth rate (Fig. 4.2).

The extraction of known amounts of OA from seawater provided high, although variable, recoveries:  $82 \pm 12\%$  (mean  $\pm$  1 S.E., n = 3). Both OA and DTX-1 were recovered from the medium but OA was clearly the predominant toxin with the concentrations of DTX-1 too low for reliable quantification. The actual concentrations of OA measured in the medium did not exceed 30nM (Table 4.2). The rates of DSP release into the medium were quite variable between the different steady states, averaging ~ 10% of total production. No relationship between release rates and growth rate was evident but the lowest release rates (~1%) were recorded at the highest growth rates and one unusually high rate of release (29%) was found when the growth rate of *E. lima* was reduced at the particularly high temperature of 28 °C (Tables 4.1 and 4.2).

**Table 4.2**

*Exuvialla lima*, DSP toxin production rates in turbidostat culture

Steady State	Toxin production or release rates (fmol toxin cell <sup>-1</sup> day <sup>-1</sup> )										OA in medium (nM)	% of total production released
	$\mu$ (d <sup>-1</sup> )	DTX-4	OA	DTX-1	OA release	DSP (total)	DTX-1	OA release	DSP (total)	OA in medium (nM)		
1	0.17	0.62	0.38	0.25	0.07	1.32	0.25	0.07	1.32	7	8	
2	0.26	1.18	0.52	0.40	0.02	2.12	0.40	0.02	2.12	4	1	
3	0.29	1.54	0.77	0.60	0.05	2.96	0.60	0.05	2.96	1	1	
4	0.22	1.10	0.67	0.47	0.26	2.50	0.47	0.26	2.50	2	11	
5	0.22	1.11	0.35	0.23	0.07	1.76	0.23	0.07	1.76	22	3	
6	0.15	0.82	0.41	0.32	0.06	1.61	0.32	0.06	1.61	6	3	
7	0.12	0.70	0.34	0.27	0.07	1.38	0.27	0.07	1.38	7	5	
8	0.13	0.67	0.34	0.25	0.14	1.40	0.25	0.14	1.40	10	8	
9	0.10	0.50	0.22	0.17	0.13	1.02	0.17	0.13	1.02	24	18	
10	0.14	0.66	0.36	0.22	0.13	1.37	0.22	0.13	1.37	30	10	
11	0.16	0.48	0.20	0.10	0.24	1.03	0.10	0.24	1.03	18	29	

**Figure 4.2** The relationship between DSP toxin production rates by *Exuviaella lima* and growth rate. Squares represent total DSP toxin production rates and circles DTX-4 production rates

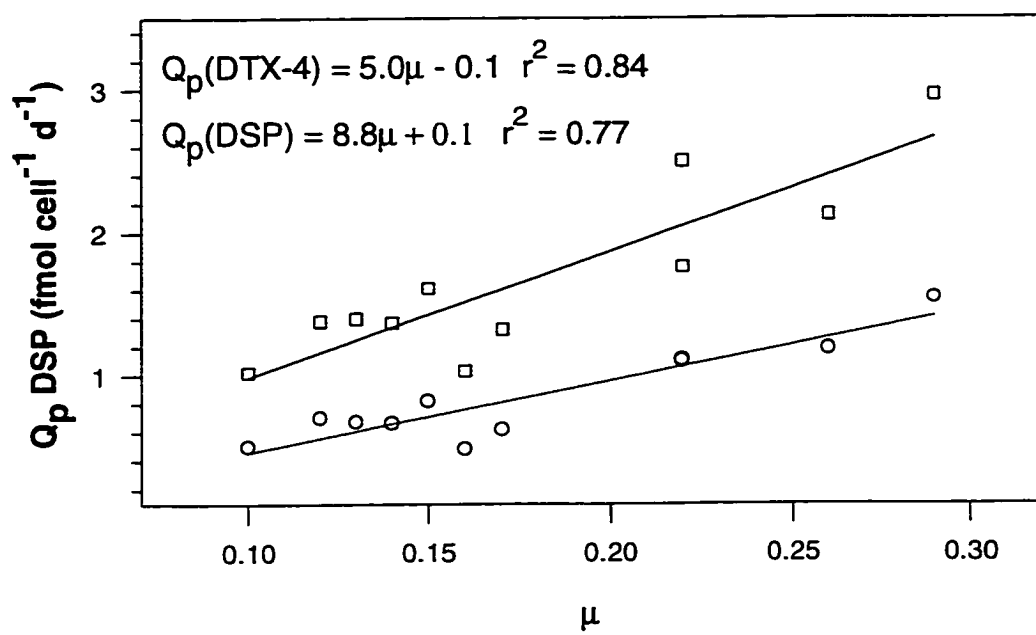


Figure 4.2



## Discussion

### *Growth in turbidostat culture*

*Exuviaella lima* is a cosmopolitan species, occupying a variety of habitats from cold temperate to tropical waters. Vegetative growth through simple binary fission is typical, but multiple mitotic divisions within cysts (Faust, 1993a) and sexual reproduction (Faust, 1993b) have been described; little is known regarding its ecology. *Exuviaella lima* is remarkably resilient in batch culture (McLachlan et al., 1994), a feature which no doubt contributed to its successful continuous culture in this study. Although *E. lima* can tolerate prolonged exposure to 0°C (McLachlan et al., 1994), the optimum growth temperature for both tropical and temperate isolates is between 23 and 28 °C (Faust., 1993a; Jackson et al., 1993) which agrees with the fact that the highest growth rates in this study were found between 22 and 25 °C (Table 4.1).

*Exuviaella lima* is an erratic swimmer, but has been recovered in plankton tows (Marr et al., 1992), although more usually found in the benthos associated with sand, sediment, detritus and macrophytes (Faust, 1993a). Adhesion to these various substrates is facilitated by extensive mucus production (Heil et al., 1993) which appears to be inversely correlated to temperature (Marr et al., 1992) and leads to clumping in culture. The possession of mucocysts is a defining feature of the genus *Exuviaella* and, along with DSP toxin production, is used to separate these benthic dinoflagellates from the planktonic, non DSP-producing proro-centroids which neither possess mucocysts nor produce DSP toxins (McLachlan et al., 1997). The propensity of *E. lima* cells to clump suggests a poor candidate for continuous culture, as a

uniform cell suspension is important. The results of this study indicate that under the appropriate conditions *E. lima* can be successfully cultured at steady state in a turbidostat. The consistent and periodic fluctuations in the beam attenuation data recorded during the culture of *E. lima* are probably responses of the cells to the rapid change of illumination that was under simple on and off control. It is tempting to speculate that the clumping behaviour noted on transition from dark to light and possible dispersal of these clumps following the light to dark transition, could be adaptive as it would be to the advantage of benthic microalgae to remain attached to a substrate in the light, but not the dark. However, the clumping response was also noted to occur in response to other disturbances, such as the cessation of culture stirring for more than a few minutes or the addition of fresh nutrients and is most likely a general response to a disturbance.

#### *Production and release rates of the DSP toxins*

An important finding of this study is the fact that DSP production by *Exuviaella lima* is positively correlated with growth and, therefore, the DSP toxins are constitutive metabolites. In this respect, they are similar to the Paralytic Shellfish Poisoning (PSP) toxins, also of dinoflagellate origin (Anderson et al., 1990), but not the Amnesic shellfish Poisoning (ASP) toxin domoic acid, which is produced by various diatom species of the genus *Pseudo-nitzschia* and is negatively correlated with growth (Bates et al., 1991). The positive linear relationship between the specific growth rate noted above growth rates of  $0.1 \text{ d}^{-1}$  also predicts essentially zero

production at  $\mu = 0 \text{ d}^{-1}$  on the basis of the y-intercepts (Fig. 4.2). However, DSP production is measurable in cells that are no longer dividing under nitrogen limitation (McLachlan et al., 1994) and is also evident in batch cultured cells in the stationary phase (Quilliam et al., 1996). Therefore the linear relationship probably does not hold at low growth rates. The turbidostat is in fact not suited to growing cells at low growth rates and a chemostat would be a more appropriate device (Rhee, 1980). Given the strong positive correlation of DSP toxin production and growth, it becomes evident that any optimization of DSP production by *E. lima* would be best directed by simply optimizing growth rate and the turbidostat is ideally suited to this task (Part 2). The turbidostat could also constitute the basis of an efficient production system for the valuable DSP toxins which are increasingly utilized as tools in studies of protein phosphorylation (Cohen and Cohen, 1989).

At the cell densities of  $\sim 20 \times 10^3 \text{ cells mL}^{-1}$  used in these experiments the actual concentration of OA in the medium ranged from 1 to 30 nM. These concentrations are orders of magnitude less than those found to cause substantial inhibition of diatom growth (Chapter 1). In addition, the above cell densities are much higher than that likely occur in nature; therefore, it is improbable that the DSP toxins released by *E. lima* would have any effect on the growth of other microalgae. High rates of toxin release may occur under extreme conditions as noted in this study at high temperature and also during prolonged exposure to low (5 °C) temperatures (McLachlan et al., 1994). Even so, under more moderate conditions less than 10 percent of production is released which indicates that the DSP toxins are primarily

intracellular. This finding is consistent with batch culture studies which have demonstrated that cellular DSP levels will continue to rise in batch culture well into the stationary phase (Quilliam et al., 1996). As with the timing of toxin production, the retention of DSP toxins within the cell is also seen with the PSP toxins in species of *Alexandrium* which do not release the PSP toxins into the medium (Proctor et al., 1975; Anderson et al., 1990) except following extensive cell lysis (Prakish et al., 1967). In contrast, the ASP toxin domoic acid is apparently released in large amounts to the medium because intracellular levels reach a plateau of between 6 to 10 pg (19 to 32 fmol) cell<sup>-1</sup> (Subba Rao et al., 1990; Bates et al., 1991) in the stationary phase; yet, medium concentrations continue to rise at a linear rate (Bates et al., 1991).

In summary, the DSP toxins produced by *E. lima* are constitutive metabolites which are largely retained intracellularly except under extreme stress. These physiological features of DSP production do not argue for an extracellular role such as the inhibition of competing phytoplankton species. Indeed, even at high cell densities the concentrations of DSP toxins in seawater are orders of magnitude below those shown to inhibit other phytoplankton. Therefore, if the DSP toxins do fulfill a defensive role for *E. lima* it is most likely one directed at predators who ingest the cells.

## Part 2

### Chapter 5

#### **Simplex optimization of the growth rate of two species of *Pseudo-nitzschia*, with respect to irradiance and temperature, using a computer-controlled turbidostat culture system**

##### **Introduction**

An understanding of the environmental factors controlling microalgal growth is important to many diverse areas of research, including primary production and phytoplankton ecology (Harris, 1986), global bio-geochemical cycling in the ocean (Cullen et al., 1993), the production of commercially or ecologically important metabolites (Borowitzka, 1995; Fish and Codd, 1994; Trick et al., 1984) as well as established industrial processes such as wastewater treatment (Talbot et al., 1991). Determining the optimal conditions for phytoplankton growth is not straight-forward, as factors controlling growth do not act independently, and interactions are important in determining the overall response of algal cells (Miller and Kamykowski, 1986; Maddux and Jones, 1964). The classical approach used to determine the response of a species to several environmental variables is factorial. Unfortunately, factorial experiments require a very large number of treatment combinations, and this poses considerable logistical problems. For instance, in studying the responses of *Nannochloris oculata* to light, temperature, salinity and nitrogen source, Terlizzi and Karlander (1980) included 81 different combinations of these variables in their design.

Terlizzi and Karlander used semi-continuous cultures in their study, but most researchers have used batch cultures which, although simple and easily replicated, are often difficult to interpret because they present a variable and poorly defined environment to the algal cells as cell density, growth rates and nutrient levels are continually changing (Rhee, 1980). In contrast, continuous culture methods maintain cells under defined, steady state conditions and are strongly recommended for physiological studies (Madariaga and Joint, 1992). While chemostats are best suited to studying the effects of specific nutrient limitations on cell physiology at low growth rates, turbidostats are employed to examine physiological processes, under non-nutrient limited conditions, at maximum growth rates. An added advantage of the turbidostat is that it also directly measures the specific growth rate of an organism (Skipnes et al., 1980) and thus, is an ideal tool for optimizing the growth rate of phytoplankton. The biggest drawbacks to the use of turbidostats are their cost and complexity. Relatively sophisticated, custom-made electronic controllers are required to ensure stable operation (e.g., Skipnes et al., 1980; Østgaard et al., 1987); this is particularly important when light-dark photoperiods are employed.

Reported here is a method which utilizes turbidostat, continuous culture and a simplex strategy to optimize the growth rates of two diatoms, *Pseudo-nitzschia pungens* Grunow and *P. multiseriata* Hasle (formerly *Nitzschia pungens* f. *multiseriata*), with respect to irradiance and temperature. Also, a turbidostat control mechanism is presented which uses computer data acquisition and control techniques to provide a viable alternative to the complex analog electronics used in the past. Diatoms were

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chosen to test the system because they have relatively short division times and this ensured quick transitions between steady states. An additional advantage of using *P. multiseriis* is that there are numerous, recent, studies on the growth of this diatom, in both batch and chemostat culture under a variety of different environmental conditions, which may be compared with the results of this study. *Pseudo-nitzschia multiseriis* is also of interest because it produces the neurotoxin domoic acid, the causative agent of Amnesic Shellfish Poisoning (Bates et al., 1989; Wright et al., 1989). Samples of *P. multiseriis* grown in the turbidostat were tested for domoic acid production, but this is not a study of toxin production by *P. multiseriis* or a detailed comparative physiology of the two species of *Pseudo-nitzschia*.

## Methods

### *Phytoplankton cultures*

Phytoplankton cultures were acquired from Mr. Kevin Pauley, D.F.O., Moncton, NB. The isolates were obtained from the same location: Stanley Bridge, New London Bay, P.E.I. *Pseudo-nitzschia pungens* (Isolate KP-102) obtained on September 23rd., 1994 and *P. multiseriis* (Isolate KP-104) on October 19th., 1994. The identities of these isolates were confirmed by SEM observations made with the assistance of Mr. David O'Neil (Institute for Marine Biosciences, Halifax). Cultures of both species were maintained at 5°C and 20  $\mu\text{mol-photon m}^{-2} \text{s}^{-1}$  to restrict cell division and prevent large changes in cell size (Admiraal, 1977). Cultures maintained in this manner remained viable for up to one year without transfer.

### *Analyses for domoic acid and silicate*

Analyses for domoic acid production used to confirm S.E.M observations and aid in the identification of the two *Pseudo-nitzschia* species were made by Dr. R. Pocklington, (D.F.O., Bedford Institute of Oceanography, Dartmouth) using HPLC of FMOC-Cl derivatized samples (Pocklington et al., 1990). Analyses of cells grown in the turbidostat were made by HPLC-UV (Quilliam et al., 1989). The HPLC-FLD method is sensitive enough ( $\sim 1 \text{ ng domoic acid mL}^{-1}$ ) that culture material could be analysed without pre-concentration. The HPLC-UV method is less sensitive ( $\sim 500 \text{ ng domoic acid mL}^{-1}$ ) and required a preconcentration step as follows: 100 mL of culture was concentrated on a 10  $\mu\text{m}$  nylon membrane (25 mm diameter) using gentle vacuum. The membrane, with cells, was rolled up and inserted into a micro-centrifuge tube and 500  $\mu\text{L}$  of distilled water added. The cells were extracted by sonication with a fine probe and the resulting homogenate filtered (0.45  $\mu\text{m}$ ) and then analysed by HPLC-UV. This procedure resulted in a 200 fold concentration and provided sufficient sensitivity. Silicate analyses were made according to Strickland and Parsons (1972) with proportional modifications to the volumes of sample and reagents required allowing the use of 1-cm path-length spectrophotometer cuvettes.

### *Medium preparation*

When this study was instigated, medium was prepared by filter sterilizing large quantities of seawater, enriched with nutrients, through sterile 0.22  $\mu\text{m}$  filters. This method was not successful and contamination problems frequently resulted in failed



cultures. Autoclaving 0.22  $\mu\text{m}$  filtered seawater and then adding filter sterilized nutrient stocks once the seawater had cooled (Harrison et al., 1980) solved this problem. Nutrient stock solutions were prepared according to McLachlan (1973) for *f/2* with the following changes: nitrate and phosphate stock solutions were prepared in a 16:1 ratio giving concentrations of 1000 and 62.5  $\mu\text{M}$  respectively. The final concentrations of nutrients added to the seawater were approximately one tenth that described for *f/2*: nitrate 100 $\mu\text{M}$ , phosphate 6.25 $\mu\text{M}$ , iron 1.2 $\mu\text{M}$ , and silicate 10.7 $\mu\text{M}$ . Trace metals were added at one tenth of *f/2* levels and vitamins were added at *f/2* levels. Tris buffer was not used and the culture pH was controlled by the addition of  $\text{CO}_2$  (see below).

#### *General description of the turbidostat*

The Turbidostat system (Fig. 5.1) consisted of two independent units controlled by a single computer. The growth chambers were flanged polycarbonate anaerobic jars (Baxter CanLab) of 2.4 L volume. The anaerobic jars were capped with a stainless steel plate through which stainless steel compression fittings had been fitted to accommodate pH and temperature probes, a sampling port and tubing (glass 6.4 mm O.D 4.8 mm I.D.) to supply  $\text{CO}_2$ , air, medium, and withdraw spent culture. Each jar was enclosed by a transparent acrylic water jacket. An O-ring was used as a seal between the cap and flange. The cap was held in place by two bolts threaded through the cap and water jacket. An overflow tube was situated to maintain the culture volume at 1.0 L, this was set by placing 1.0 L of water in the unit with all tubing and

**Figure 5.1** Diagram of the twin turbidostat system. Note that for clarity fluid, gas and electrical connections are not shown and in addition, the circular light shutter and fluorescent tubes on the right hand unit have been omitted.

Key:

W, waste reservoir

M, medium supply reservoir

WB, water bath

P, peristaltic pump

F, fluorescent light

MS, magnetic stirrer

S, sampling tube

O, outlet tube for spent culture and medium

I, inlet tube for fresh medium

SH, shutter for adjusting irradiance

B, baffle to block stray light from the adjacent unit

OT, outlet for water jacket

IN, inlet for water jacket

E, emitter, infra-red diode

D, detector, phototransistor

S, stirring bar

A, anaerobic jar growth vessel

WJ, water jacket

P, pH sensor

T, temperature sensor

SS, stainless steel head plate

OR, O-ring

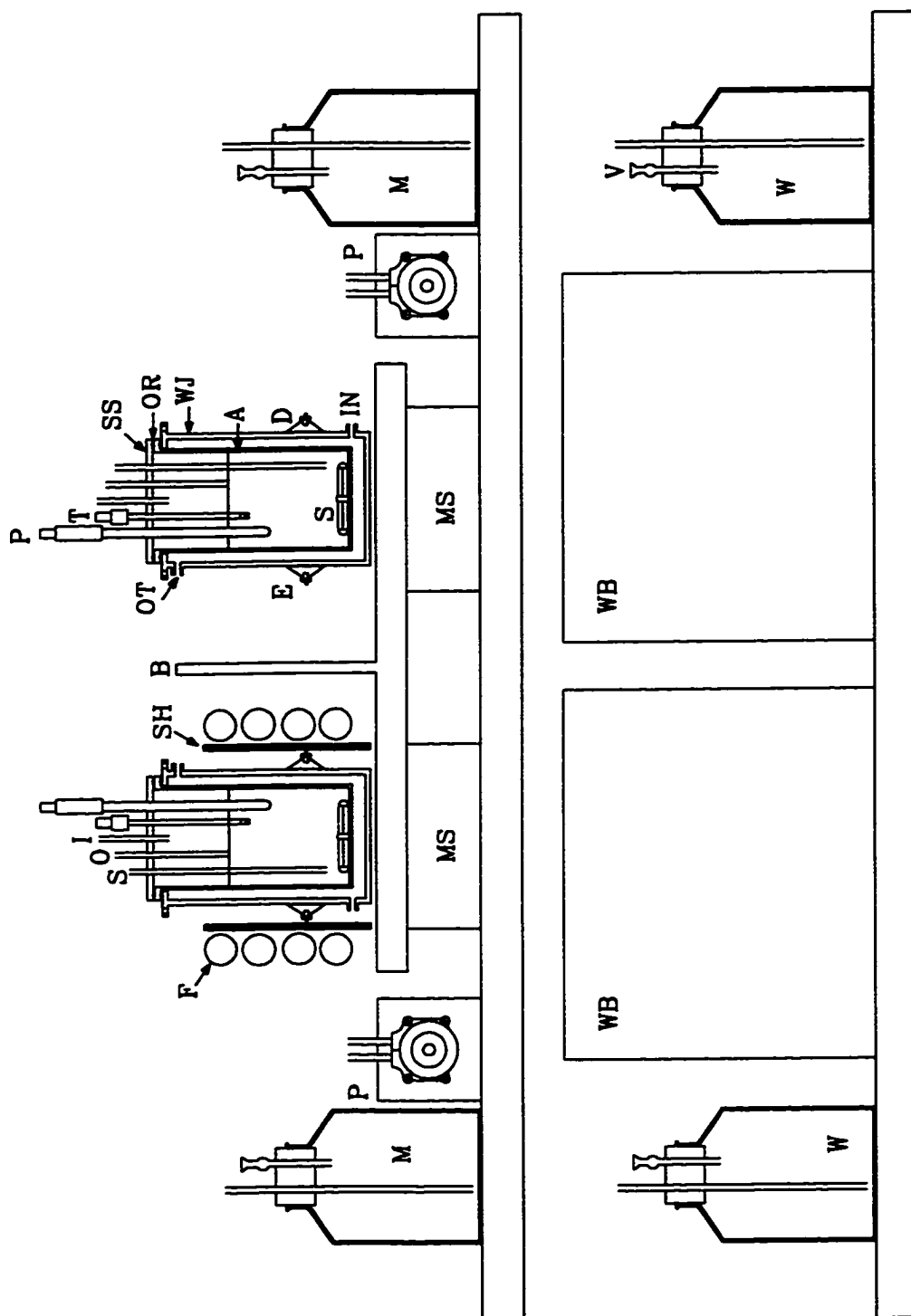


Figure 5.1

probes to be immersed in the vessel in place, loosening the compression fitting holding the overflow tube and adjusting its height so that it just cleared the water surface. In operation, sterile filtered air was periodically injected (typically 1 s every 5 min) into the headspace to maintain a positive pressure and ensuring any culture in excess of 1.0 L would be ejected out of the culture into a receiving vessel. All interconnecting tubing, including that used in the peristaltic pumps, was 9.5 mm O.D. 4.8 mm I.D. silicone. Each water jacket was connected to a separate temperature controlled water bath. Water circulated from the bath through an inlet in the bottom of the water jacket and returned through an overflow tube at the top of the jacket. Temperatures between 3°C and 35°C  $\pm$  1°C were easily maintained by this system and were set manually using the water bath console controls. Stirring was accomplished by a 77 x 12.5 mm stir bar in the bottom of each jar and operated by magnetic stirrers situated beneath the units. Each magnetic stirrer could be turned off or on under computer control and was typically turned on for 5 s min<sup>-1</sup>.

Illumination was supplied by up to 4 circular (20 W, 300 mm diameter, cool-white) fluorescent bulbs surrounding each unit. An adjustable circular shutter between the lights and the unit was used to control the irradiance. A light barrier was placed between both units to prevent stray light from one unit affecting the other (Fig. 5.1). The shutters were composed of four fixed segments and four movable segments on a turntable base, each describing 1/4 of the enclosed circumference. The shutters were periodically calibrated using a 4 $\pi$ -quantum sensor (LI-COR) immersed in the jar with the water jacket and growth chambers filled with water. A pointer on the movable

segments was indexed with marks on the base to mark irradiance values on calibration and set the desired irradiance during operation. Irradiance levels to the nearest  $5 \mu\text{mol-photon m}^{-2} \text{ s}^{-1}$  could be set using this system. The number of bulbs connected and the irradiance were selected manually. The light bank of each unit was turned on and off under computer control. For the studies reported here a 12 h:12 h light:dark photoperiod was employed throughout.

#### *Turbidostat control mechanism*

Turbidostatic control was based on measuring the attenuation of a pulsed (9.1 Hz) infra-red emitting diode (IRED, Radio Shack TIL906-1) light beam ( $\lambda_{\text{max}} = 880 \text{ nm}$ ) transmitted across the culture (light path = 165 mm) and comparing the measured value to a pre-determined set point. The detector was an IR phototransistor (Radio Shack TIL414). The emitter and detector circuits are shown in Figure 5.2a. An optoisolator (Motorola 4N32) was used to interface the logic level from the computer to control the IRED in order to ensure sufficient current handling capacity. Both the IRED and the phototransistor were mounted in aquarium suction cups and attached to opposite sides of the water jacket using a thin layer of transparent silicone adhesive (Fig. 5.2). The attenuation of the IRED beam is due to absorbance, reflection and scattering of the incident light by the cell suspension and components of the culture system. Attenuation (A) was defined as the negative logarithm of the percent transmittance (T) and expressed in absorbance units:

$$A = 2 - \log (T)$$

**Figure 5.2** Circuit diagrams. (a) Circuit diagram and layout of the turbidostat sensor system. Key: DO, digital output line controlling the emitter in the optical isolator (4N32); IRED, infra-red emitting diode; PT, phototransistor; A, aquarium suction cup; R1, 1.0 K $\Omega$ , R2, 100  $\Omega$ ; R3, 1.22 K $\Omega$ . (b) Circuit diagram of the LM35DZ temperature sensor. Key:  $V_{in}$ , 4-30 V;  $V_{out}$ , 10 mV  $^{\circ}\text{C}^{-1}$ ; R4, 10 K $\Omega$ ; C1, 1 $\mu\text{f}$ . (c) Circuit diagram of the a.c power control system. The control bits are derived from a single 8-bit digital output port. The relays are Potter and Brumfield OAC-5, 3A, 120 V a.c. output. Key: All R values are 220  $\Omega$ ; L, a.c. load (e.g., a pump or solenoid).



The percent transmittance of the culture was calculated as the ratio of the power of the beam transmitted across the culture ( $P_o$ ) with respect to that of a reference blank ( $P$ ):

$$T = (P_o / P) \times 100$$

In practical terms,  $P$  and  $P_o$  are measured by the system's analog to digital converter (ADC) as the output of the detector circuit in volts. The reference blank was measured by either recording  $P_o$  of the unit filled with medium before inoculation or by temporarily inserting a blank unit filled with medium into the system, this could be done at any time as sufficient play was provided in all the connections to the growth vessels to allow them to be removed from the water jackets without disconnecting any lines or compromising culture integrity.

To provide immunity from external light sources, including the system's own lights and other electronic noise picked up by the detector, lock-in amplification (synchronous demodulation) was used. This was carried out exclusively in software by an interrupt service routine (ISR). The ISR was triggered by hardware every 55 ms (18.2 Hz). Each time this interrupt was called the detector output would be recorded, then the IRED would be switched on or off as appropriate. Every second interrupt the signal stored when the IRED was off would be subtracted from the signal stored when the IRED was on. Thus, any background signals not in phase with the pulsing IRED would be attenuated. The most recent 100 beam attenuation readings were stored in a circular queue and accessed and averaged by the main program when a reading was required to be recorded or to determine if input medium to dilute the culture was



needed. When the culture density increased beyond the set point the main program determined the error and would turn on the medium input pump for a period of time proportional to that error. Measurements and corrective action were made and recorded every 15 min. Programming the ISR is discussed further below.

The effectiveness of the lock-in amplification scheme to reject external noise was tested by temporarily modifying the software to record two signals, one when the IRED was on ( $IRE D_{on}$ ) the other when the IRED was off ( $IRE D_{off}$ ). A short term recording was then made with the turbidostat set up for normal operation, but with a strong light source (40-W incandescent) positioned 30 cm from the opposite side of the turbidostat and pointing directly at the detector thus acting as an interference. While recording  $IRE D_{on}$  and  $IRE D_{off}$  the light source was turned on and off several times and moved from side to side. If the lock-in amplifier was functioning correctly, the demodulated signal ( $IRE D_{on} - IRE D_{off}$ ) should reject the components of the signal derived from the external light source.

The response of the detector (with the lock-in amplifier operating) to changes in cell density was determined by first recording the detector output with the growth vessel containing filtered seawater (beam attenuation blank) and then replacing the seawater with 895 mL of a batch culture of *Pseudo-nitzschia multiseri* containing  $21.4 \pm 1.8 \times 10^3$  cells mL<sup>-1</sup> (mean  $\pm$  1 SE, as determined by direct microscopic counts of 10  $\mu$ L aliquots, n=5). The detector output was then recorded for multiple dilutions of this culture made by adding 50 or 100 mL portions of sterile medium and mixing well. The seawater, culture and turbidostat were all equilibrated to 15°C for the

calibration. The relationship between beam attenuation and cell density was then determined by linear regression.

#### *Measurement and Control of pH*

The pH of each culture was measured by an autoclavable pH probe connected to a pH meter (Omega PHCN-36). Recorder output from the meter (0 to 1400 mV) directly corresponded to the pH (0 to 14 units) and read by the ADC. Each probe was calibrated before autoclaving using buffer solutions and periodically during operation by withdrawing a sample of the culture and measuring the pH with an independent system and then making the appropriate adjustments. The computer would compare the measured pH to a predetermined set point (typically 8.2) then if the pH exceeded this, calculate the error and trigger a solenoid to inject CO<sub>2</sub> into the culture for a period of time proportional to the error. The pH was not controlled below the set point. Measurements and corrective action were made at 1 min. intervals. Both the pH and the cumulative duration of CO<sub>2</sub> injection were recorded every 15 min.

#### *Measurement of temperature*

Temperature was measured using an integrated sensor (National, LM35DZ). This device is a temperature dependent voltage regulator which accepts an input voltage from 4 to 30 volts and provides an output of 10 mV °C<sup>-1</sup>. The device was inserted into a glass tube with a sealed bottom and kept below the level of the culture. Output from the sensor was filtered by a passive RC filter (Fig. 5.2b) and read by the

ADC. Each sensor was calibrated in a boiling water and melting ice bath to set the 0°C and 100°C reference points. The equation of a straight line fitted to these data was used to relate output voltage to temperature and the calibration factors (slope and intercept from the regression) were incorporated into the software for each sensor.

#### *A.C. Power control*

The control of solenoids, stirring motors, lights and pumps was made by interfacing digital control signals from the computer through a bank of zero-crossing, solid-state a.c. power control relays (Potter and Brumfield, OAC-5) as shown in Figure 5.2c. When the output of the digital line was pulled low (0 V) the input circuit would be completed and a.c. power supplied to the output circuit. On initialization and termination of the control program all digital lines were pulled high (5 V) to ensure the power to all devices was turned off.

#### *Computer control and data logging*

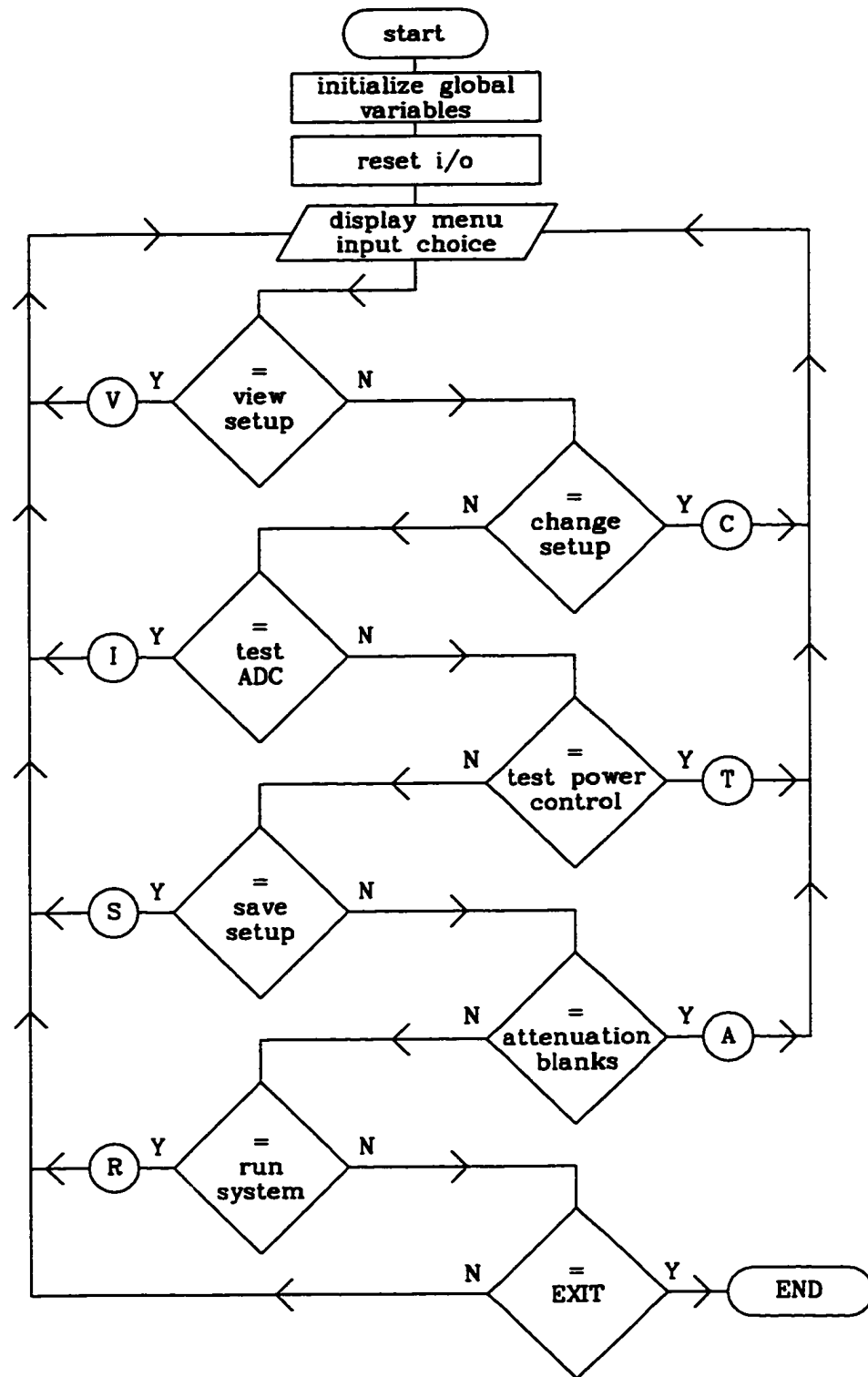
A program written in the C language (Borland Turbo C<sup>®</sup> version 2.0) was used to both control and log data from the turbidostat. An IBM-XT clone fitted with an interface board (Advantech, PCL-711) providing an 8 channel 12-bit analog to digital converter and 2 channel 12-bit digital to analog converter, as well as 16 bits of digital input and output.

A full listing of the C source code is provided in the Appendix. The operation of the program was structured around a main menu that is shown in flow chart form in

Figure 5.3. The operator must specify the relevant operating parameters including, the disk file name to store data, photoperiod, duty cycle for stirring each culture, pH and beam attenuation set points as well as the proportional (in time) gains for the pH and cell density controllers. These operating parameters were then stored in a configuration file for access by the program on initialization. Other routines accessed from the menu include setting the attenuation blanks and various test procedures (Fig. 5.3). Light status (on or off), pH, beam attenuation and CO<sub>2</sub> injection time were stored in an ASCII disk file every 15 min. These values were also displayed on the computers video display.

The most important component of the program is the interrupt service routine (ISR) which controls the turbidostat sensor system and internal program event timing. A flow chart of the ISR is provided in Figure 5.4. The ISR was tied to the user-defined timer interrupt vector present on all IBM and compatible computers (interrupt vector: 1C hex) which is called by internal hardware events every 55 ms. Once initialized, program statements contained within the ISR will be executed every 55 ms, regardless of other events taking place in the program (unless interrupts are disabled). This allows repetitive time based tasks to be done without unnecessary delays in program function. Each call to the ISR (Fig. 5.4) increments the internal program clock while the main program (Fig. 5.5) simply consists of an endless loop which checks when it is time (based on the internal clock) to check the pH, culture density, lights, stirrers, etc., and executes these functions as required. Any function that needs to turn on a device (e.g., a pump or solenoid) for a specified time interval, does so,

**Figure 5.3** Program flowchart for the main menu of the turbidostat control program. The letters referenced by each decision block represent 'cases' acted upon by the switch statement in module turb.C (Appendix). The switch statement then calls the appropriate block of functions to carry out the tasks required.



**Figure 5.3**

**Figure 5.4** Program flow chart for the interrupt service routine (ISR). This routine is called by an internal hardware interrupt every 55 ms.

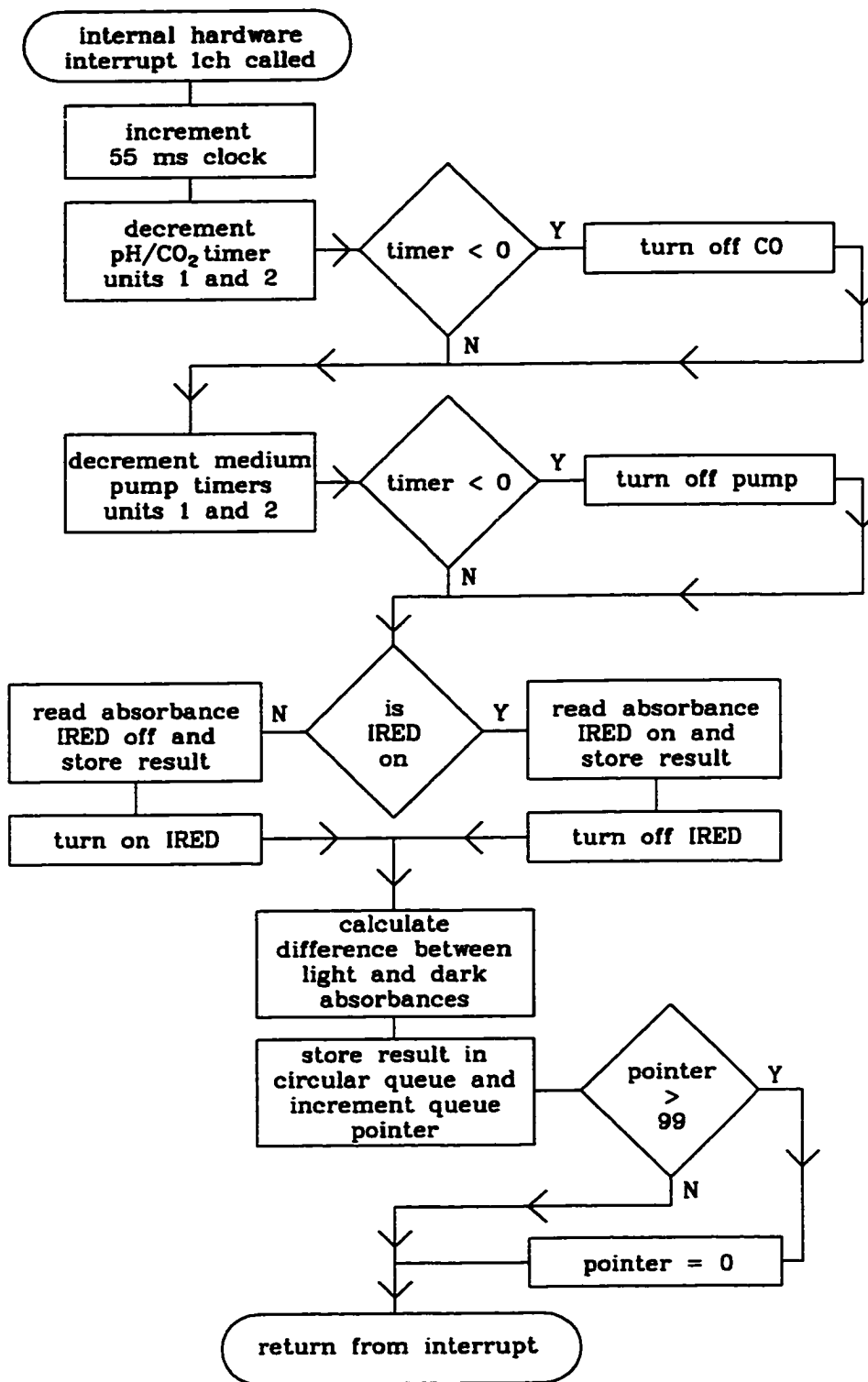


Figure 5.4



**Figure 5.5** Program flow chart illustrating the primary functions used during operation of the turbidostat. The functions are called following selection of the 'run' option from the main menu. Turbidostat operation will continue until a key press is detected at which point the interrupt service routine is disabled, all input and output functions are reset ensuring that all a.c. power circuits are turned off and then control is returned to the main menu.

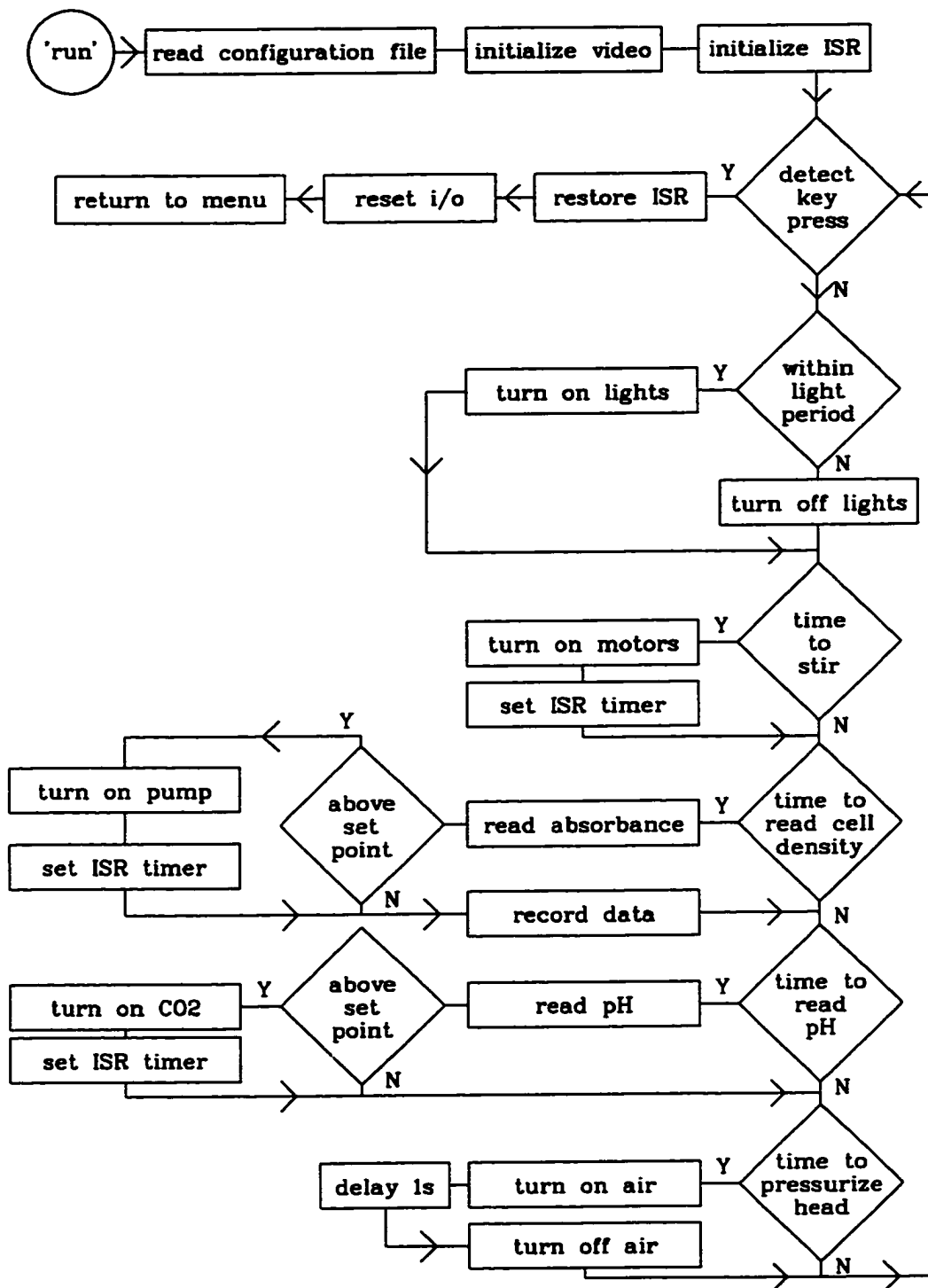


Figure 5.5

then stores the required duration (as an integer representing the number of 55 ms intervals required) in a variable accessed by the ISR. The ISR decrements these variables on each iteration and turns off the device when these values reach zero.

#### *Preparation, inoculation and operation of the turbidostats*

To initiate a run, the growth chamber, cap and all associated probes and tubing were assembled and approximately 1.25 L of distilled water were placed in the unit. Autoclavable filters were attached to the gas inlet ports and covered with aluminum foil. Silicone tubing used to supply and remove medium during operation were autoclaved separately. The vessels were autoclaved while sitting in a shallow pan with approximately 3 cm of water to help prevent checking and cracking of the polycarbonate jar during sterilization. Importantly, the level of electrolyte in the pH and oxygen (if used) probes was lowered and tubes attached to the filling lines to conduct any overflow during the decompression of the autoclave away from the polycarbonate jar. If this was not done the hot electrolyte would severely and permanently etch the plastic it came into contact with. Once removed from the autoclave, the units were placed in a laminar flow hood and the distilled water siphoned off aseptically. After cooling, the units were placed in the water jackets and filled with sterile seawater medium using the peristaltic pumps. All necessary lines were then connected. The water baths were turned on and set to the desired temperatures and the units allowed to equilibrate for several hours or overnight. The control program was then started and blank reference readings were measured for each

unit and stored in the configuration file. The units were inoculated by adding 100 mL of an exponentially growing batch culture through the sampling port via a sterile 50-mL syringe barrel.

The culture was considered in steady state when the dilution rates were constant (within 5%) for three consecutive days. At this point ~90% of the culture would be harvested through a tube attached to the sampling port by clamping shut the overflow tube and pressurizing the headspace of the culture with sterile air. The vessel was then brought to 1.0 L volume with fresh medium and the irradiance and temperature levels reset as directed by the simplex procedure (below).

#### *Calculation of growth rates*

The instantaneous rate of change of cell density ( $dx/dt$ ) in continuous culture is a function of the specific growth rate ( $\mu$ ,  $d^{-1}$ ), cell density ( $x$ ,  $\text{cells mL}^{-1}$ ) and dilution rate ( $D$ ,  $d^{-1}$ ). The dilution rate is defined as the rate of fresh medium added to the vessel ( $V_{in}$ ,  $\text{mL day}^{-1}$ ) with respect to the culture volume ( $V$ ,  $\text{mL}$ ) where:

$$D = V_{in} / V \text{ and}$$

$$dx/dt = (x\mu - Dx) = x(\mu - D)$$

At steady state in a turbidostat cell density is constant and therefore:

$$dx/dt = 0 \text{ therefore, } x(\mu - D) = 0 \text{ and thus } \mu = D$$

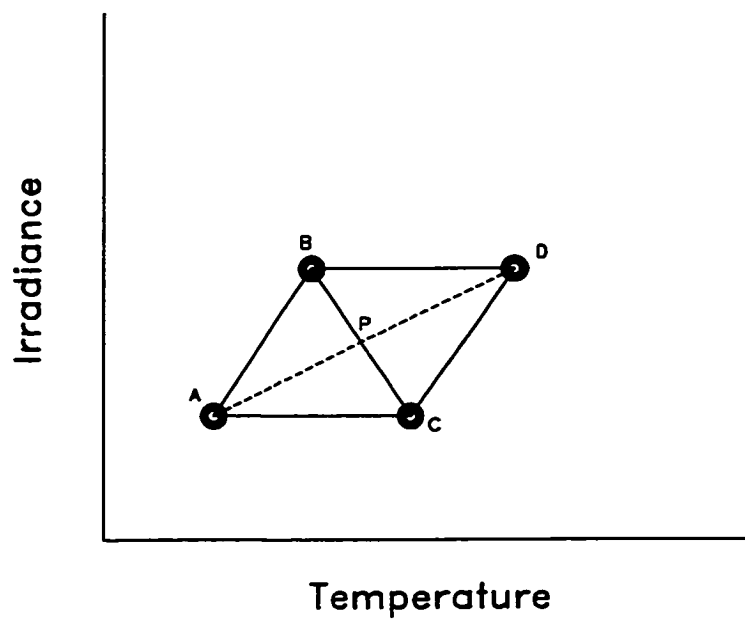
Division rates are calculated as:  $\mu / \log_2$ , where  $\log_2 = 0.693$  (Gulliard, 1973). In practice,  $V_{in}$  was calculated for each turbidostat by recording the total time the supply pump operated over a complete 24 h light:dark cycle and knowing the volume pumped per unit time. Pump calibration was checked and recorded routinely and the segment of silicone tubing running through the pump head replaced frequently.

### *Simplex optimization*

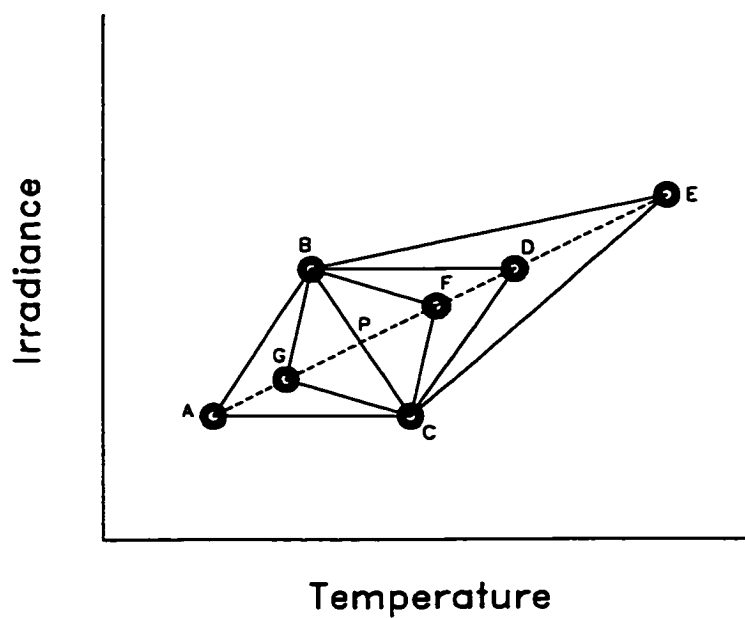
The simplex procedure is used to optimize the response of a system to the variation of more than one parameter. It is a geometric process defined by a simple set of rules (Bratchell et al., 1989). For example, Figure 5.6a illustrates finding the optimal combination of irradiance and temperature for the growth rate of a particular species using a fixed-sized simplex. The process starts by evaluating the response at three initial co-ordinates (combinations of irradiance and temperature) that are the vertices of an equilateral triangle (ABC); this represents simplex number one. A new point (D) is calculated by reflection of the lowest response (A) about the centre point (P) of the line joining the points of best response (B and C) to give simplex number two (BCD). Successive iterations of this process will drive the simplex towards the point of best response. A variable sized simplex may also be used (Fig. 5.6b), which allows it to zero in more rapidly and closely to the optimal point (Ratzlaff, 1987). It starts in the same way as described above, but when the response at the new point D is evaluated the simplex expands or contracts along the axis AD by a factor of 2 according to the magnitude of the response at D with respect to that at A, B, and C.

**Figure 5.6** Simplex optimization. (a) Fixed-sized and (b) Variable-sized optimization procedures. The points A, B and C, represent the three initial combinations of irradiance and temperature chosen to initiate the process. It is assumed for the purposes of the description that the response (i.e. growth rate) at A is the lowest.

(a) fixed-sized simplex



(b) Variable-sized simplex

**Figure 5.6**

There are four possible cases:

Case 1) If D is the best overall response the simplex is expanded to BCE. However, if the expansion proves unsuccessful then case 2 will apply.

Case 2) If The response at D is intermediate between B and C then the point D is retained to give the simplex BCD.

Case 3) If the response is lower than B or C but greater than A then the simplex is retracted to BCF.

Case 4) If the response at D is the worst response the simplex is contracted to BCG.

Once the new point has been evaluated, the simplex is developed once by the fixed-simplex rule and then the new response evaluated according to the rules 1 to 4 (above). This was a modification of the method described by Ratzlaff (1987), intended to prevent overly large changes in the magnitudes of the controlled variables to obviously inappropriate values.

The mathematical derivation of the new points coordinates (X,Y) for each of the above cases is (with reference to Fig. 5.6b) as follows, where point P ( $X_p, Y_p$ ) is calculated as  $[(X_b+X_c)/2, (Y_b+Y_c)/2]$ :

Case 1: point E ( $3X_p-2X_a, 3Y_p-2Y_a$ )

Case 2: point D ( $2X_p-X_a, 2Y_p-Y_a$ )

Case 3: point F ( $1.5X_p-0.5X_a, 1.5Y_p-0.5Y_a$ )

Case 4: point G ( $0.5X_p-0.5X_a, 0.5Y_p-0.5Y_a$ )



Two independent simplex optimization procedures were done using *P. multiseriis*. Fixed-sized simplexes were used and each procedure was initiated from a different starting position. A variable-sized simplex was used to optimize the growth of *P. pungens*.

## Results

### *Phytoplankton cultures*

Observations of 15 separate cells under S.E.M revealed only two rows of poroids in isolate KP-102 and consistently 3 or 4 rows for isolate KP-104. Samples from late-stage batch cultures (14-days) grown in f/2 medium at 20°C and 80  $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$  were checked for domoic acid production. Isolate KP-104 was found to produce moderate levels of domoic acid ( $\sim 1 \text{ pg cell}^{-1}$ ), but no trace of domoic acid was found in the KP-102 culture (detection limit of  $\sim 2 \text{ ng mL}^{-1}$ ), which at a cell density of approximately  $100 \times 10^3 \text{ cells mL}^{-1}$ , means less than  $0.02 \text{ pg cell}^{-1}$  domoic acid). These data support the identification of KP-102 as *Pseudo-nitzschia pungens* and KP-104 as *P. multiseriis*. The cell size of both isolates was similar with widths of 2.5  $\mu\text{m}$  and lengths of 44-50  $\mu\text{m}$  and both species readily formed long chains of 20-30 cells in culture. Isolates KP-102 and KP-104 were both unialgal but not axenic.

### *Turbidostat operation*

When the turbidostats were functioning properly, bacterial contamination was not evident either visually or by microscopic examination. Long term incubation of samples on marine agar would yield colonies of gram negative bacteria. However, given the low phytoplankton cell densities, lack of organic carbon in the medium (e.g., TRIS) and the dynamic nature of the system, the very low levels of bacteria that must have been present were not considered to affect the growth rates measured. Indeed, Fuhs (1969) noted that even if bacteria were present in turbidostat cultures, they were in very low number, did not foul the cell surface of the diatoms and were not considered to influence the growth dynamics of the algae. Once set up the turbidostats were frequently operated for several weeks at a time; ultimately the gradual fouling of the cooling water in the water jacket would necessitate strip down, cleaning and sterilization of the components.

### *Nutrient status and domoic acid production in turbidostat culture*

Growth conditions within the turbidostat were considered to be nutrient replete at cell densities of approximately  $18 \times 10^3$  cells mL<sup>-1</sup> with the medium composition used. The most convincing evidence of this was the fact that cell growth would continue at the same rate until a much higher cell density was reached if the attenuation set point was increased from the normal operating value 0.155 to 0.222 absorbance units, equivalent to  $30 \times 10^3$  cells mL<sup>-1</sup> (Fig. 5.7a).

**Figure 5.7** Turbidostat operation at two different cell densities of *Pseudo-nitzschia multiseries*. (a) Beam attenuation data. (b) Cumulative volume of medium supplied to the turbidostat. The initial beam attenuation set-point of 0.222 corresponded to a cell density of about  $30 \times 10^3$  cells mL<sup>-1</sup>. Although this cell density was attained (a) it could not be maintained and growth ceased after a few days (b). Reducing the beam attenuation set-point to 0.155, equivalent to  $18 \times 10^3$  cells mL<sup>-1</sup>, removed the nutrient limitation and steady state growth was attained (b).

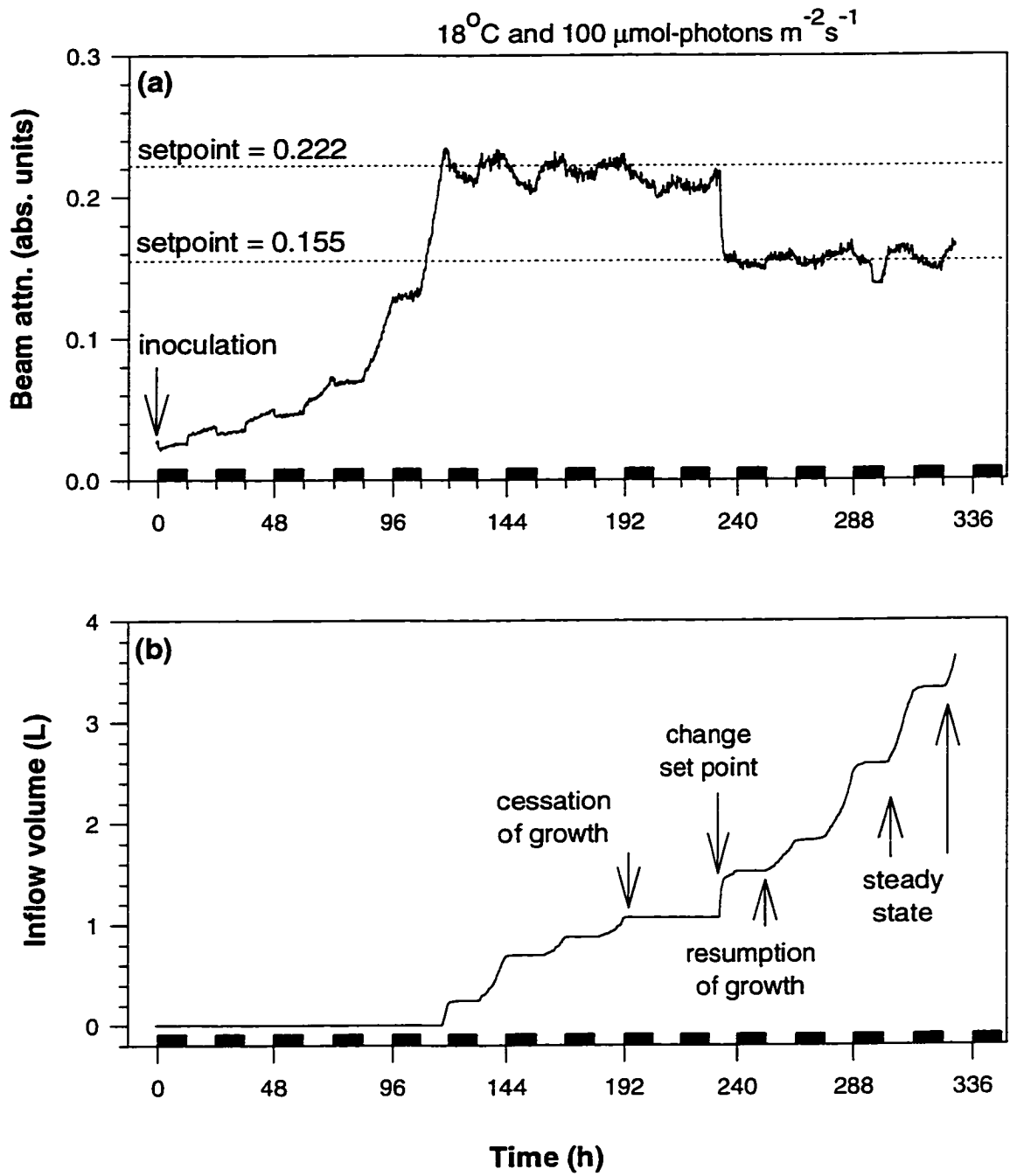


Figure 5.7

Growth eventually ceased at this cell density because of nutrient limitation (Fig. 5.7b), but rapidly resumed on return to a lower cell density to attain steady state (Fig. 5.7a and b).

Domoic acid was detected in *Pseudo-nitzschia multiseriis* cells at steady state in four instances at rates as high as  $2 \text{ pg cell}^{-1} \text{ day}^{-1}$  at specific growth rates between 0.5 and  $0.7 \text{ day}^{-1}$  during an early trial of the simplex method. In each case analyses of the spent culture medium showed very low ( $< 1 \text{ }\mu\text{M}$ ) to undetectable silicate concentrations which correlates with the production of domoic acid. These steady states exhibited lower growth rates than expected based on the interpolation of growth rates obtained in subsequent trials. These cultures were therefore considered to be nutrient limited, and consequently these data were not included in the simplex calculations. All the steady states for *P. multiseriis* used in the simplex procedure yielded cells with no detectable domoic acid. Silicate levels in the effluent from the turbidostats for all the steady states used in the simplex calculations, for both species of *Pseudo-nitzschia*, was never less than  $7 \text{ }\mu\text{M}$ . Seawater used to prepare the medium was also tested on occasion and found to contain between 3 and  $11 \text{ }\mu\text{M}$  silicate and would explain why the effluent from the turbidostat contained levels of silicate at or above that used to enrich the seawater.

#### *Turbidity sensor*

The turbidostat sensor and lock-in amplification system proved to be robust and stable, operating for months at a time, and needed only periodic recalibration. The use

of aquarium suction cups to house the IRED and phototransistor proved to be an effective and versatile means of attaching them to the growth vessel; in fact, they could be easily affixed to virtually any type of transparent vessel with circular or planar cross sections. The suction cups also had the added advantages of sealing the source and detector from condensation and providing an insulating air gap between them and the water jacket. The ability of the lock-in amplifier implemented in software to reject signals out of phase with the emitter is graphically illustrated in Figure 5.8. The signals (a) and (b) in Figure 5.8 are those recorded by the detector with the IRED on and off respectively and the centre trace (c) the demodulated signal i.e., the difference between signals (a) and (b). The effect caused by turning on and off a strong incandescent light source close to the detector is shown by the regions of the graph marked 1, 2 and 3; in regions 2 and 3 the light source was also moved to and away from the detector. Although the external light source created large deviations in the signals in (a) and (b) these are removed from the demodulated signal (c). The demodulated signal shown (Fig. 5.8) is somewhat noisy, but in actual operation this signal is digitally filtered by averaging the most recent 100 readings stored in the circular queue.

The attenuation of the IRED beam was found to be linearly and positively correlated with cell density (Fig. 5.9) and sensitive to changes in cell density of  $\sim 250$  cells  $\text{mL}^{-1}$  which represented about 1% of the working cell density.

The signals acquired during a typical run of the turbidostat are shown in Figure 5.10. At the start of the run, the water bath and light shutter were adjusted to provide

**Figure 5.8** Operation of turbidostat sensor lock-in amplification system. The signals recorded when the IRED was on (a) and off (b) both show strong deflections when the external light source was turned on (points 1, 2 and 3). This 'noise' is effectively attenuated by subtracting (b) from (a) to yield the demodulated signal (c).

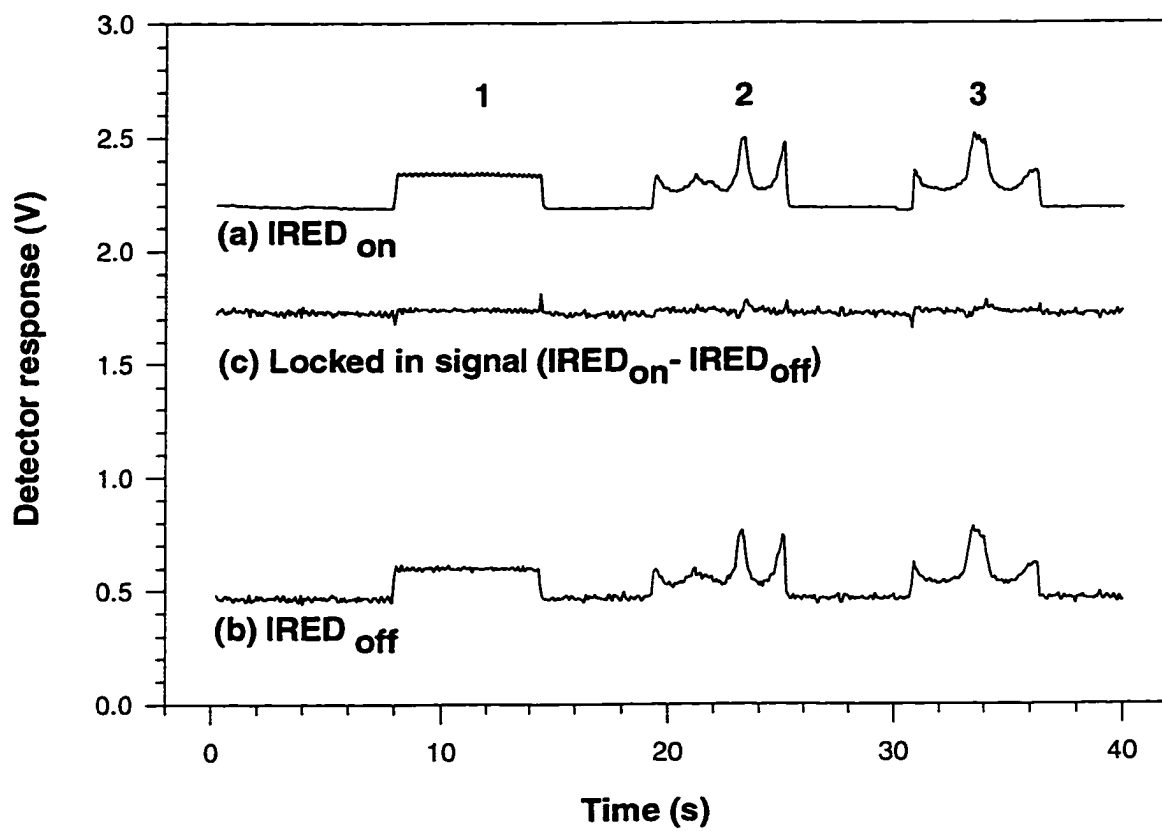


Figure 5.8



**Figure 5.9** Turbidostat detector response as a function of cell density.

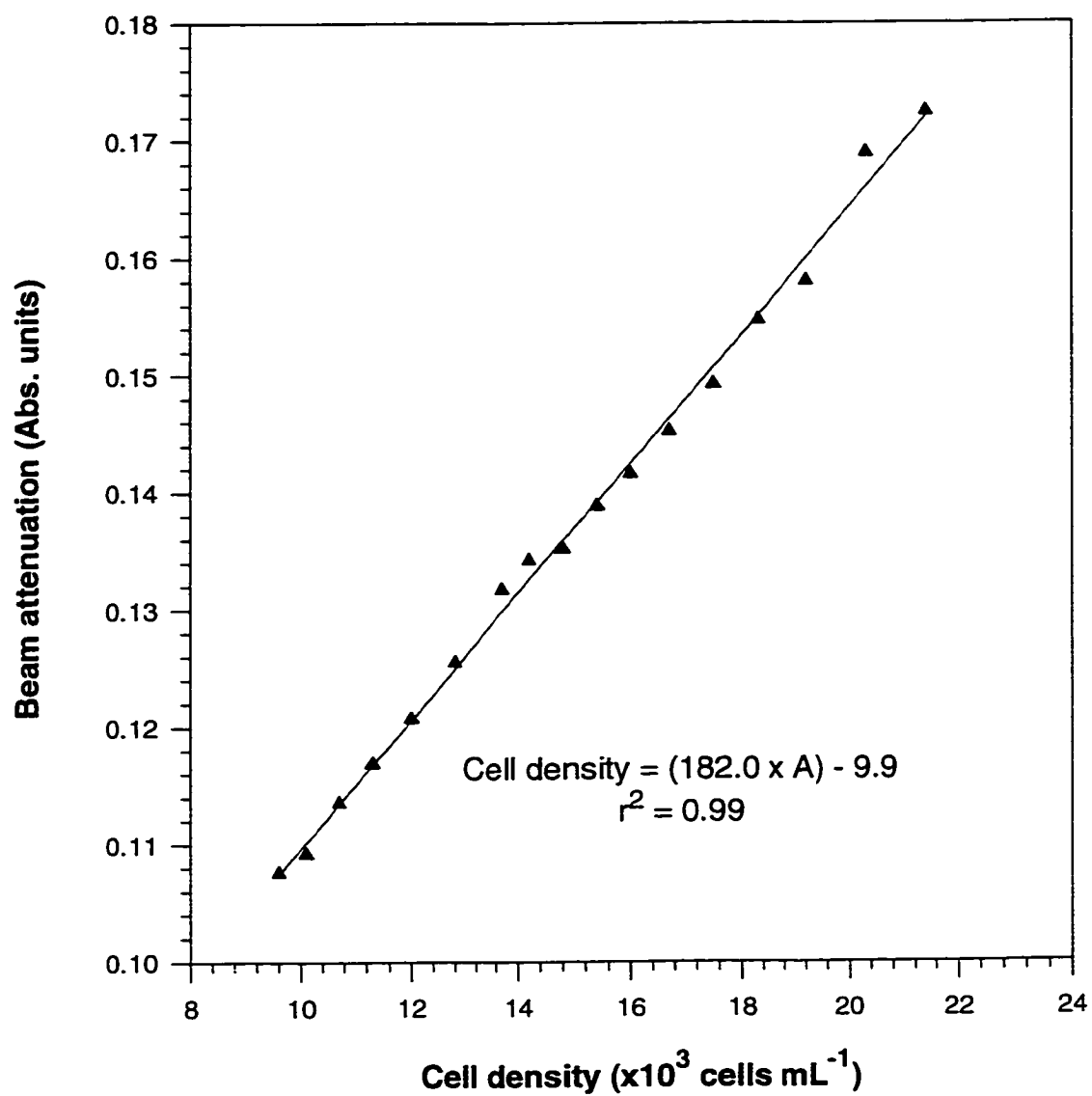


Figure 5.9

**Figure 5.10** A representative sample of the signals recorded during operation of the turbidostat before and at steady state with *Pseudo-nitzschia multiseriata*.

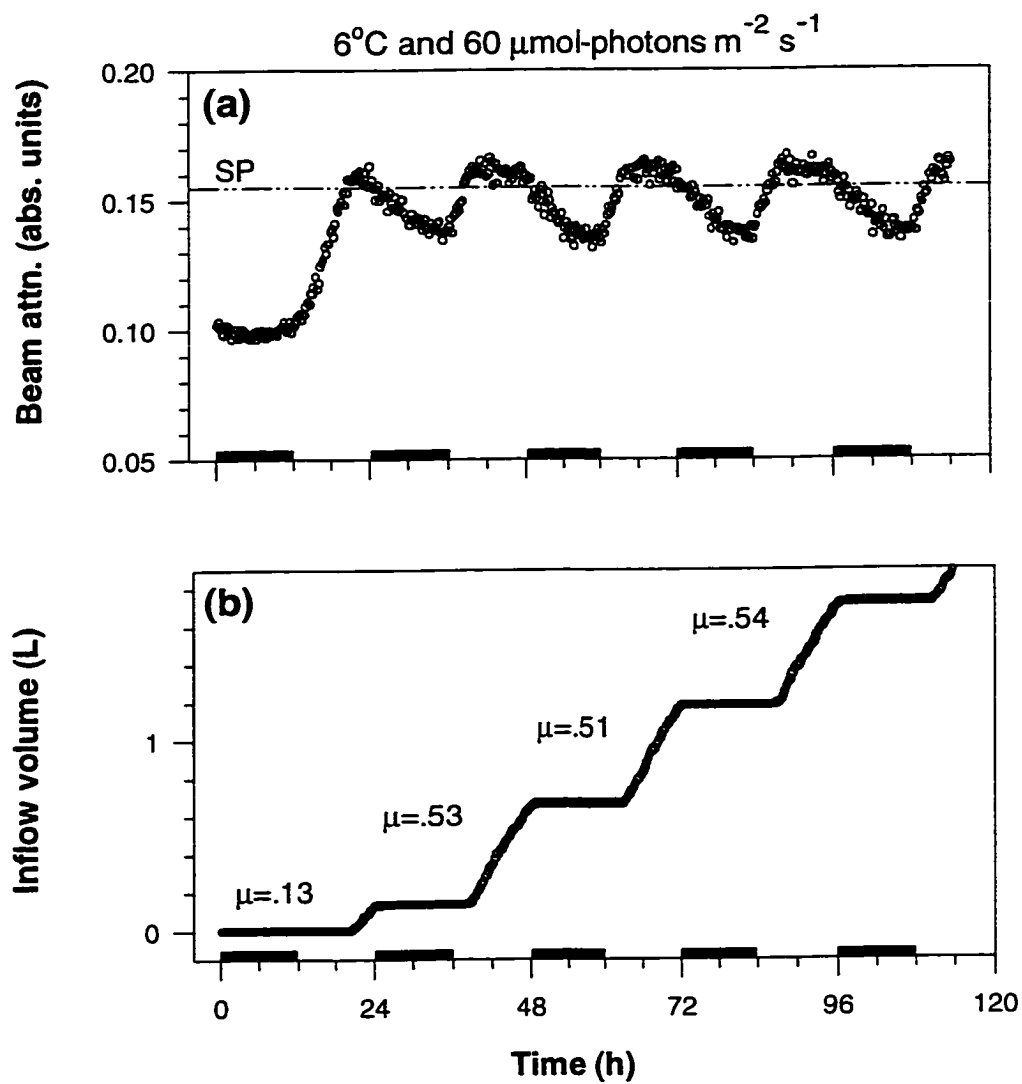


Figure 5.10

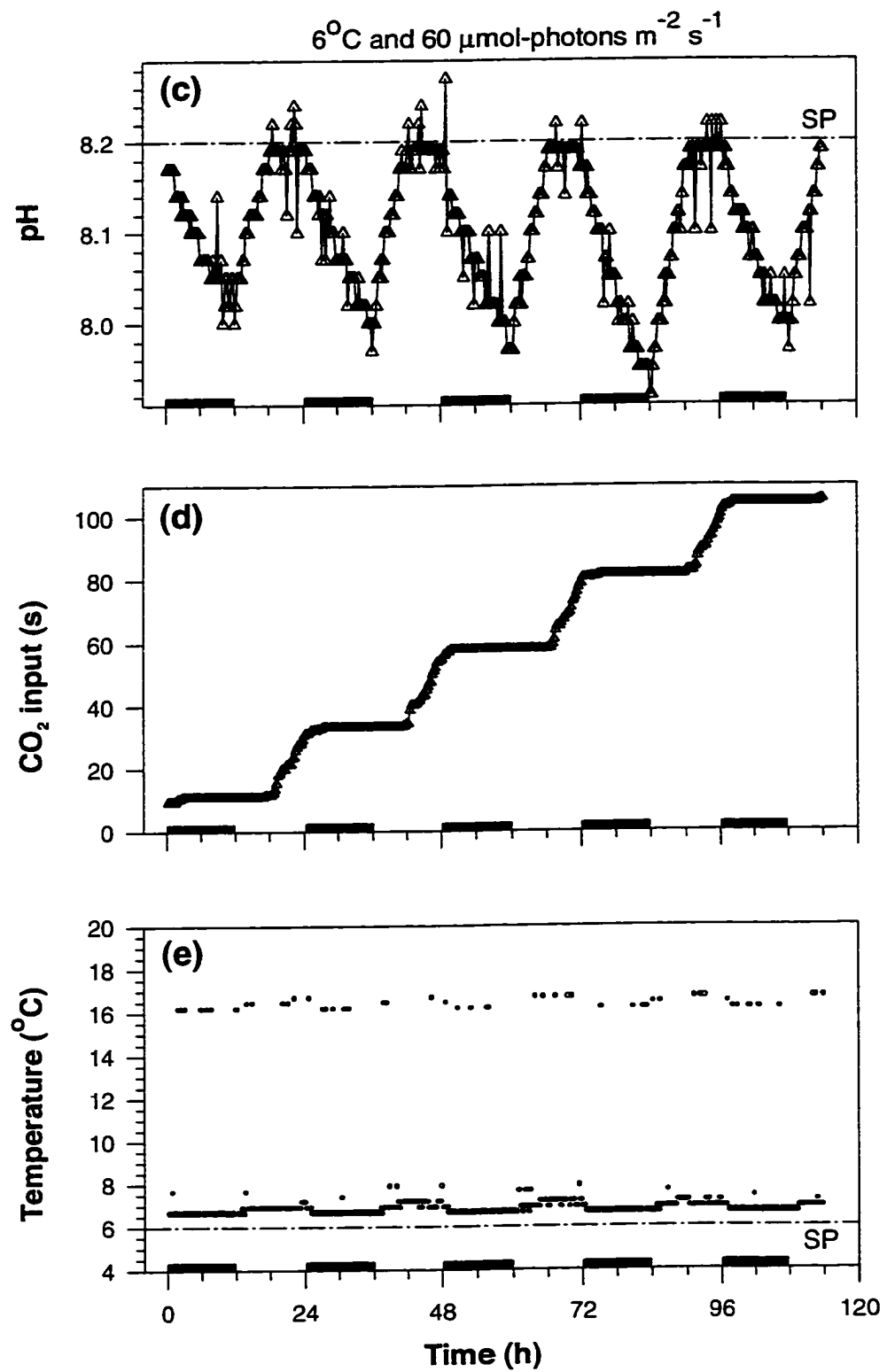


Figure 5.10 (cont.)

a temperature of 6°C and an irradiance of 60  $\mu\text{mol-photons m}^{-2} \text{s}^{-1}$ ; the beam attenuation of 0.10 units indicated a relatively dilute suspension of *Pseudo-nitzschia multiseries* with a cell density of  $\sim 8 \times 10^3$  cells  $\text{mL}^{-1}$  (Fig. 5.10a). There was no significant change in the beam attenuation during the dark part of the photoperiod however, during the first light cycle a steady increase in the beam attenuation occurred. The setpoint value of 0.155, equivalent to about  $18 \times 10^3$  cells  $\text{mL}^{-1}$ , was exceeded during the last few hours of the light period causing the repetitive triggering of the medium supply pump (Fig. 5.10b). The increase in beam attenuation and thus supply of fresh medium, was abruptly stopped at the end of each light period indicating that there was no increase in turbidity and therefore no growth, in the dark. In fact, a gradual decline in the beam attenuation was consistently noted during the dark part of the photoperiod in all the data collected for *P. multiseries* (this was also true for *P. pungens*). Over the next 4 days this pattern of growth was repeated with a mean specific growth rate of  $0.53 \text{ day}^{-1}$  taken as a steady state value (Fig. 5.10b). As with the beam attenuation, the pH of the culture also exhibited diel periodicity (Fig. 5.10c). The pH increased during the day, exceeded the set point, and triggered the injection of  $\text{CO}_2$  (Fig. 5.10d). As soon as the lights were shut off the pH declined steadily throughout the night (Fig. 5.10c). The stepped nature of the pH signal (Fig. 5.10c) is owing to the fixed resolution of the ADC. The temperature data (Fig. 5.10e) was noisy during both light and dark periods often recording sporadic spikes. This effect was possibly due to the operation of the magnetic stirrers while the temperature readings were being made. A slight  $\sim 0.5^\circ\text{C}$  increase was observed when the lights were

turned on. This stepped increase was both rapid and consistent during the light period and may have been caused by a direct radiant effect on the sensor (which was black and housed in clear tubing) rather than representing an actual increase in the water temperature.

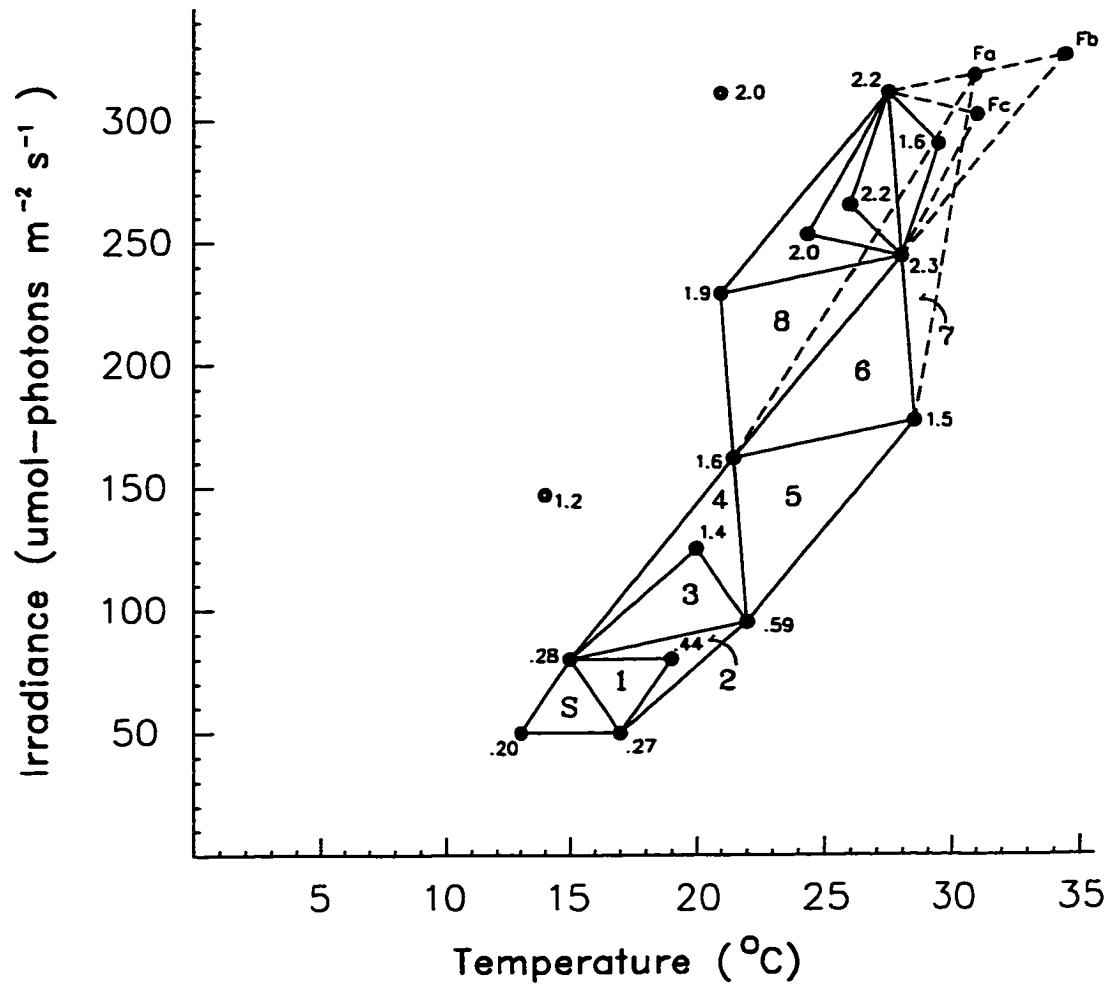
*Simplex Optimization of the growth rate of Pseudo-nitzschia multiseriis and P. pungens*

Three simplex optimization experiments were run; two with *Pseudo-nitzschia multiseriis* that utilized a fixed sized simplex with different starting points and one with *P. pungens* that used a variable sized simplex procedure. Growth rates were measured for 36 steady states for *P. multiseriis* and 16 for *P. pungens*. In all cases the simplex procedures were successful at directing new choices for light and temperature combinations that resulted in increased growth rates.

The variable sized procedure (Fig. 5.11) used with *P. pungens*, increased the growth rate from a high value of  $0.28 \text{ day}^{-1}$  on the starting simplex to  $2.3 \text{ day}^{-1}$ , the highest rate achieved, in 6 iterations. Two expansions of the initial simplex took place at iterations 2 and 4 resulting in much wider changes in light and temperature between steady states. Ultimately, an additional expansion was attempted on iteration 7 ( $31^\circ\text{C}$  and  $320 \mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ ) and resulted in the failure of the culture (point Fa, Fig. 5.11). The experiment was continued following re-preparation and inoculation of the turbidostat set at  $21^\circ\text{C}$  and  $230 \mu\text{mol-photon m}^{-2} \text{ s}^{-1}$  permitting the successful completion of two additional simplexes; however, following iteration 8 a new point

**Figure 5.11** Simplex optimization of the growth rate of *Pseudo-nitzschia pungens* with respect to irradiance and temperature using a variable-sized simplex. The specific growth rate ( $\mu$ ,  $\text{d}^{-1}$ ) achieved at steady state is indicated beside each point. A maximum rate of  $2.3 \text{ day}^{-1}$  was attained at  $28 \text{ }^{\circ}\text{C}$  and  $244 \text{ } \mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ . The letter 'S' indicates the initial simplex. The point Fa indicates values of temperature and irradiance selected by the simplex procedure that caused the culture to fail. Points Fb and Fc were not tested, but were assumed to cause culture failure.





**Figure 5.11**

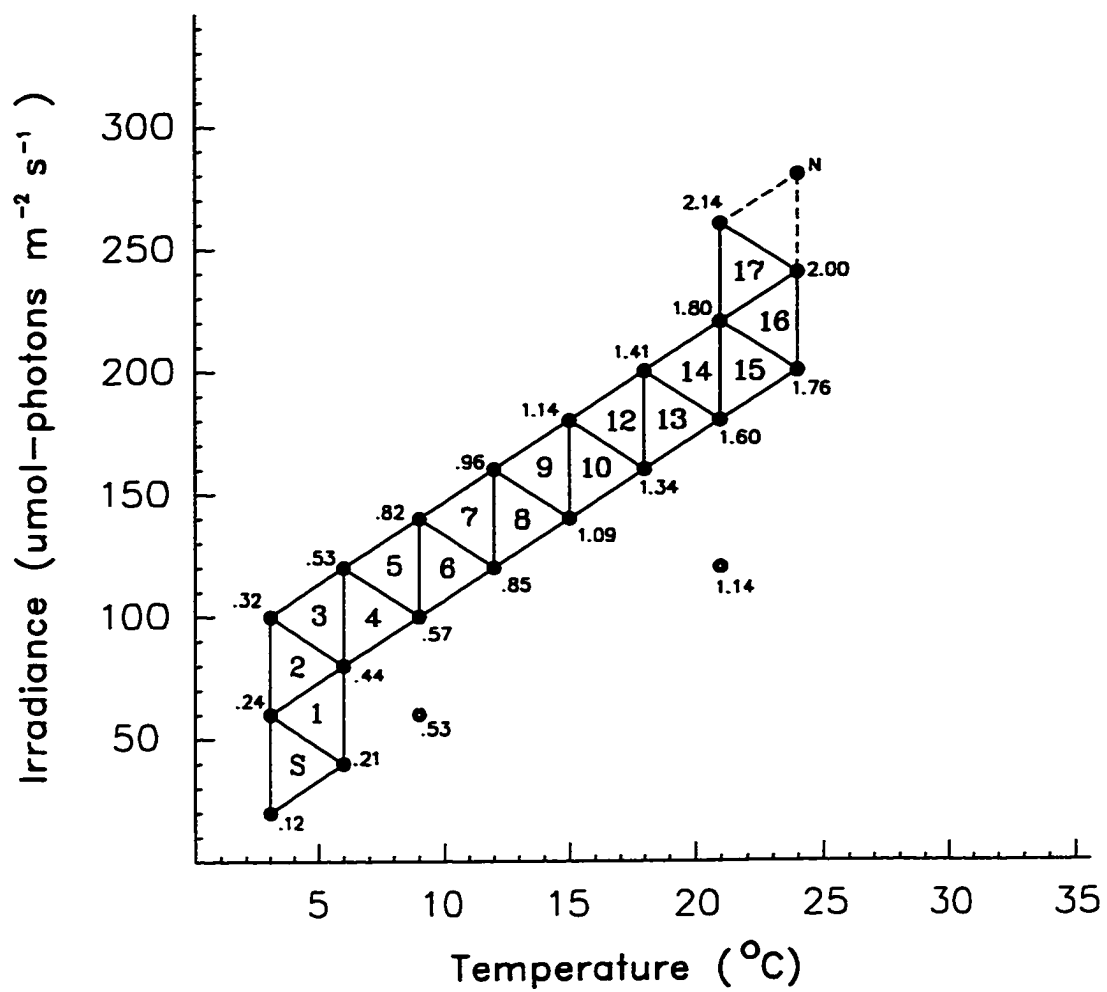
was calculated at 35°C and 325  $\mu\text{mol-photon m}^{-2} \text{s}^{-1}$  (point Fb, Fig. 5.11) which would clearly have resulted in a failed culture. Failure at these values was assumed and a contraction of the simplex to give a new point at 24 °C and 250  $\mu\text{mol-photon m}^{-2} \text{s}^{-1}$  was made and steady state achieved. Reflection of this point to 31 °C and 300  $\mu\text{mol-photon m}^{-2} \text{s}^{-1}$  (point Fc, Fig. 5.11) would have again resulted in failure and the simplex was contracted to 26 °C and 265  $\mu\text{mol-photon m}^{-2} \text{s}^{-1}$ . Although this point, and a subsequent reflection, achieved steady states the growth rates did not exceed the value of 2.3  $\text{day}^{-1}$  obtained at 28°C and 245  $\mu\text{mol-photon m}^{-2} \text{s}^{-1}$  and therefore the optimization procedure was considered complete.

Two independent optimizations of the growth of *P. multiseriis*, using fixed sized simplexes, are shown in Figures 5.12 and 5.13. Each simplex was initiated from a different set of starting points and run in independent turbidostat units. The paths of the two simplex procedures converged with iteration 12 of the first series (Fig. 5.12) and 8 of the second series (Fig. 5.13). In neither series was an ultimate optimum confirmed due to considerations of time. In both cases it is unlikely that significantly higher growth rates would have been attained with further iterations given the already high temperatures, irradiances and growth rates for both trials. Where the simplexes from both series converged the specific growth rates obtained were comparable with the exception of that of 2.5  $\text{day}^{-1}$  obtained at 24°C and 240  $\mu\text{mol-photon m}^{-2} \text{s}^{-1}$  in the second series (Fig. 5.13) which was 0.5  $\text{day}^{-1}$  higher than that obtained in the first series (Fig. 5.12). Also it is noted that the next points chosen at the end of each series indicated that the paths of the two simplexes were about to diverge.

**Figure 5.12** The first simplex optimization of the growth rate of *Pseudo-nitzschia multiseriis* with respect to irradiance and temperature using a fixed-sized simplex.

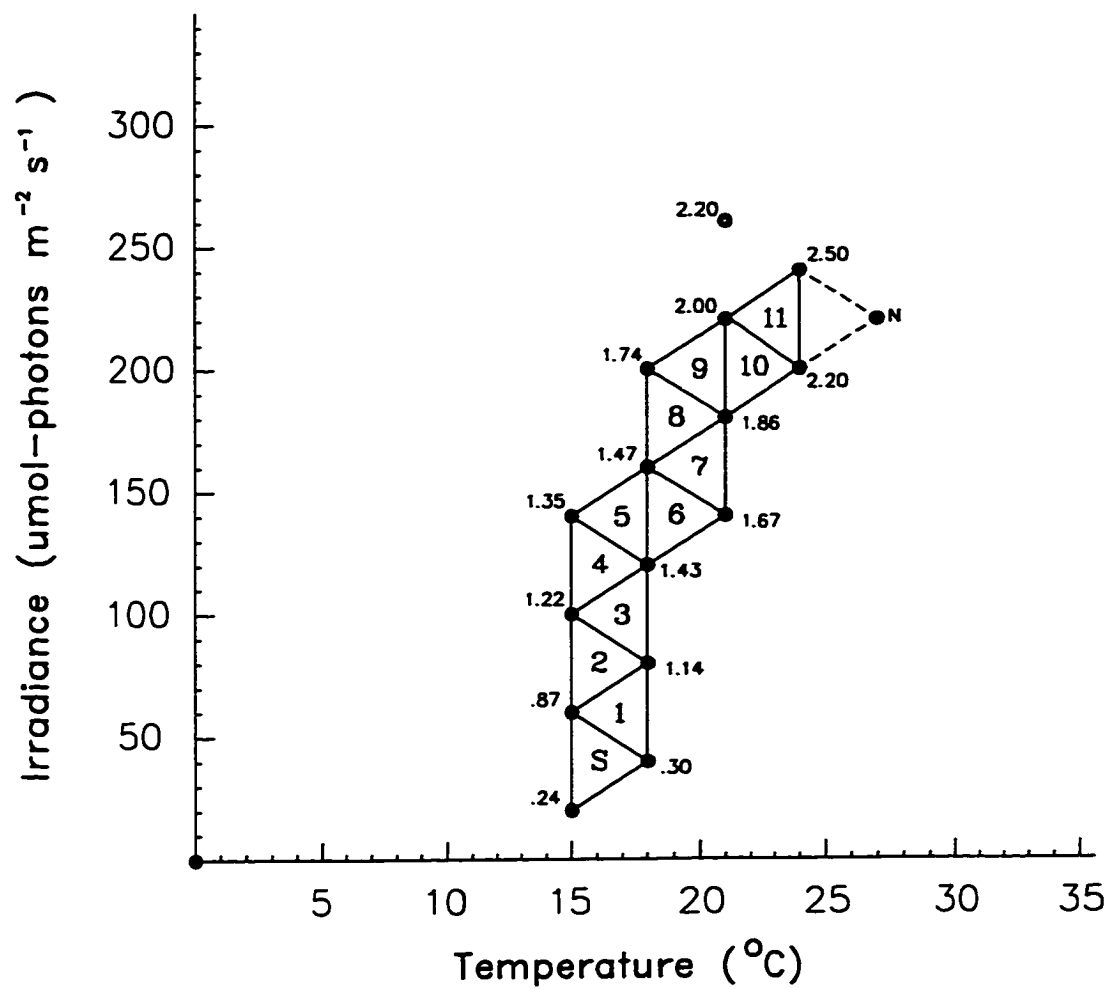
The specific growth rate ( $\mu$ ,  $\text{d}^{-1}$ ) achieved at steady state is indicated beside each point. A maximum rate of  $2.14 \text{ d}^{-1}$  was attained at  $21 \text{ }^{\circ}\text{C}$  and  $260 \mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ .

A true optimum was not reached in this trial. The letter 'S' indicates the initial simplex the letter 'N' the next predicted point.



**Figure 5.12**

**Figure 5.13** The second simplex optimization of the growth rate of *Pseudo-nitzschia multiseries* with respect to irradiance and temperature using a fixed-sized simplex. The specific growth rate ( $\mu$ ,  $d^{-1}$ ) achieved at steady state is indicated beside each point. A maximum rate of  $2.5 d^{-1}$  was attained at  $24\text{ }^{\circ}\text{C}$  and  $240\text{ }\mu\text{mol-photon m}^{-2}\text{ s}^{-1}$ . A true optimum was not reached in this trial. The letter 'S' indicates the initial simplex the letter 'N' the next predicted point.

**Figure 5.13**

The specific growth rates measured for both species of *Pseudo-nitzschia* at high levels of irradiance and temperature (above 20°C and 100  $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ ) were quite similar (Figs. 5.11, 5.12 and 5.13); however, the specific growth rates of *P. pungens* below these values was markedly lower than that for *P. multiseriata*.

## Discussion

### *Turbidostat culture*

Turbidostat cultures of microalgae offer a means to overcome many of the disadvantages inherent in batch culture as cells are maintained in a time-independent steady state under defined physiological conditions with cell density, nutrient levels and growth rates held constant (Rhee, 1980). Importantly, turbidostat cultures are open systems with continuous input of fresh medium and removal of spent medium and cells, this means that ambient nutrient levels can be kept low and extracellular products will not accumulate. With a turbidostat the specific growth rate can be measured continuously (Skipnes et al., 1980) and so the effect of changing culture conditions can be directly assessed. It could be argued that phytoplankton grown under light:dark photoperiods are by definition not at steady state; however, the definition of a steady state used here is that growth rates were constant between successive 24-h intervals.

The central component of a turbidostat system is a closed-loop control system which serves to continually monitor culture turbidity, compare the measured turbidity to a set-point and operate a medium supply pump to dilute the culture when the set

point is exceeded. To avoid measurement errors owing to fluctuating ambient light, previous designs have used lock-in amplifiers implemented in hardware (Østgaard et al., 1987; Skipnes et al. 1980). These circuits are relatively complex and require many components, including signal sources, active filters, and integrators. Software techniques are considered preferable when integrating relatively low frequency signals (i.e., those significantly less than the ADC conversion rate) because calibration and tuning problems are easily solved and unnecessary hardware is eliminated (Ratzlaff, 1987). The system presented here is very simple with minimal hardware requirements costing only a few dollars per unit. A computer with data acquisition hardware is required, but as these are standard components in modern systems, regardless of the control technique, the only additional overhead required is in the software. The controller described here proved to be sensitive, versatile and reliable and thus provides a viable and cheap alternative to controllers realised entirely in hardware.

Many factors that influence the optical properties of a plankton suspension are species specific (Østgaard et al., 1987) and many species of phytoplankton have been used. It is thus difficult to directly compare the performance of the current design with that of others. In addition, few researchers have provided calibration data in their reports. Maddux and Jones (1964) successfully operated their turbidostat with *Nitzschia closterium* at cell densities between 10 to 100 x 10<sup>3</sup> cells mL<sup>-1</sup>. Skipnes et al. (1980) reported that their sensor system was sensitive to changes in absorbance of ~ 1.2 % when growing various diatom species at densities of 50 x 10<sup>3</sup> cells mL<sup>-1</sup>. The performance of the sensor system used in this study is certainly comparable, the



turbidostat was operated at a cell density of approximately  $20 \times 10^3$  cells  $\text{mL}^{-1}$  and based on the calibration data (Fig. 5.9) changes in cell density of  $\sim 250$  cells  $\text{mL}^{-1}$  ( $\sim 1\%$ ) were detectable. When the turbidostat was operating during the light period the cell density was maintained at  $19 \times 10^3 \pm 1 \times 10^3$  cells  $\text{mL}^{-1}$  (Fig. 5.10a). This value exceeded the set point by about  $1 \times 10^3$  cells  $\text{mL}^{-1}$  and represents the steady state error typical of proportional control.

The 12 h light: 12 h dark photocycle clearly imposed a strong underlying rhythm on the physiology of the diatoms. Increases in culture absorbance only took place during the light and abruptly halted as soon as the lights were extinguished; this is consistent with light-dependent carbon assimilation. The decline in culture absorbance noted during each dark period for *Pseudo-nitzschia* is interesting (Fig. 5.10a); similar observations were recorded for the dinoflagellate *Exuviaella lima* (Chapter 4). Østgaard and Jensen (1982) noted that the turbidity of *Skeletonema costatum* grown in batch culture and monitored turbidometrically varied between the end of the light and dark periods, but the results presented here are much more striking. Several factors may account for the diel variation in culture turbidity. It is well known that photosynthesis in microalgae is time dependent (Harding et al., 1981) and cellular concentrations of chlorophyll-*a* undergo diel variation, increasing during the light and declining in the dark (Sournia, 1974), but because the turbidity or attenuation in these experiments was measured at 880 nm it is unlikely that changing concentrations of chlorophyll-*a* ( $\lambda_{\text{max}} = 420$  and  $660$  nm) would be responsible for the

observed decline in absorbance. However, Østgaard and Jensen (1982) measured turbidity at 660 nm and this undoubtedly influenced their results. Turbidity is believed to correlate with biomass or cell carbon rather than cell number (Østgaard and Jensen, 1982), therefore the decline in the dark could simply be due to respiration. This interpretation of a reduction in cell carbon (up to 33%!) during the dark was made by Weiler and Karl (1979) for the dinoflagellate *Ceratium furca*. This interpretation is supported here by a concomitant decline in culture pH during each dark period (Fig. 5.10c), presumably a result of the release of CO<sub>2</sub> from the cells to the medium. It is difficult to assess how common this phenomenon is given the limited use of turbidostats and the fact that most are operated using continuous irradiance (Fuhs, 1969; Skipnes et al., 1980; Østgaard et al., 1987; Wangersky et al., 1989).

#### *Nutrient status and domoic acid production in turbidostat culture*

Early studies with *Pseudonitzschia multiseries* (Subba Rao et al., 1990; Bates et al., 1991; Lewis et al., 1993), concluded that domoic acid production is coincident with the cessation of cell division. An available source of nitrogen is required (Bates et al., 1991); therefore growth must be limited by a non-nitrogenous nutrient. Silicate was identified as the nutrient in *f/2* media most likely to be limiting during the stationary phase and doubling the silicate from 55 µM to 105 µM increased the final cell yield, delayed the onset of stationary phase and hence domoic acid production (Bates et al., 1991). The assumption that only non-dividing *Pseudo-nitzschia* cells under silicate limitation produce toxin is contradicted by field observations of domoic

acid production by rapidly dividing cells of *P. multiseriis* (Smith et al., 1990b). Recent studies report production of domoic acid by exponentially growing cells in chemostats under either silicate or phosphate limitation. Therefore the limitation, not cessation, of cell division, under nutrient-limiting conditions is a prerequisite for toxin production (Pan et al 1996a, 1996b). This conclusion is in accord with the observations of domoic acid production here. The cause of the low levels of silicate that caused domoic acid production in this study, despite the fact that cell densities did not increase, remains unknown. The low levels may have been caused by silicate precipitation in the unbuffered medium (Smith-Palmer et al., 1985).

### *Simplex Optimization*

Simplex optimization procedures have been advocated as a means of increasing the performance of bacterial (Bull et al., 1990) and microalgal (Harker et al., 1995) cultures, but to my knowledge have never actually been applied. The results of this investigation demonstrate that simplex strategies are effective in directing the adjustment of two variables, irradiance and temperature, simultaneously to optimize the growth rates of the diatoms *Pseudo-nitzschia pungens* and *P. multiseriis*.

The actual direction taken by the simplex development is interesting. The initial movement along the irradiance axis at the start of the optimization procedure for *P. multiseriis* (Figs. 5.4 and 5.5) indicates that, at first, stepwise changes in irradiance were more important for increasing growth than those for temperature. At some point, in all three simplex procedures the path of the simplex was such that new points were

chosen to increase temperature and irradiance simultaneously. The variable-sized simplex procedure was able to reach an optimum much more rapidly than the even-sized method, but was more likely to result in picking lethal combinations of variables to test because of the large changes in the controlled variables. This would constitute a serious problem in a fully automated system and pre-determined limits to the controlled variables would have to be specified from the start to avoid unnecessary culture failures.

The simplex optimization approach used in this study represents an alternate, operational approach to mechanistic (e.g., Cullen et al., 1993) and descriptive (Miller and Kamykowski, 1986) models seeking to understand the controls of phytoplankton growth. Although the simplex technique is empirical in nature, the response data generated during the course of optimization could be modelled to gain insight into the underlying processes (Harker et al., 1995). Probably the most useful application for an automated simplex system would be in the commercial production of biomass and valuable secondary metabolites. Importantly, simplex procedures are not limited to two variables or simply growth responses. For instance, in the case of *P. multiseriis* it would be particularly relevant to also control the concentration of silicate in the medium and monitor domoic acid production. Domoic acid is a valuable metabolite and the potential of *P. multiseriis* as a commercial source of this toxin has been pointed out (Laycock et al., 1989). Given that domoic acid production can occur during exponential growth and the obvious kinetic advantages of continuous culture (Trilli, 1990), the use of optimized turbidostats could well constitute an efficient means

of production. This is underscored by the stable operation of the turbidostat in this study at specific growth rates of  $2.2 \text{ d}^{-1}$  ( $3.2 \text{ divisions d}^{-1}$ ) for *P. multiseriis* exceeding the previously reported maximum rate for *P. multiseriis* ( $1.8 \text{ divisions d}^{-1}$ : Pan 1993) by a factor of nearly 2.

*Growth rates of Pseudo-nitzschia multiseriis and P. pungens.*

Batch culture studies with *Pseudo-nitzschia multiseriis* report an optimal growth temperature of  $20^{\circ}\text{C}$  (Lewis et al., 1993; Pan et al., 1993). In this study, under continuous culture  $20^{\circ}\text{C}$  was clearly sub-optimal. The reasons for this difference may be owing to the use of continuous culture which in general will support higher growth rates than batch culture (Epply, 1972) and the fact that, at the optimum temperatures determined for growth, other studies used different irradiances and photoperiods than here. For instance, Pan et al. (1993) used high levels of continuous irradiance ( $350\text{--}450 \mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ ), which is known to depress the growth rates of some species (Paasche, 1968). The maximum growth rates for both species of *Pseudo-nitzschia* were found between  $24$  and  $28^{\circ}\text{C}$  which is in accord with the fact that many temperate diatoms have optima in this range (Eppley, 1972). The growth of *P. multiseriis* at very low temperatures is also in agreement with reports of domoic acid contamination of mussels on the Gulf coast of New Brunswick in December of 1988 during sub-zero water temperatures, under ice cover, and subsequent demonstration of growth and domoic acid production in culture at  $0^{\circ}\text{C}$  (Smith et al., 1993).

In this study, irradiance had a very important effect on the growth rate of *P. multiseriis*: i.e., at 18°C the specific growth rate increased from 0.30 at 40  $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$  to 1.74  $\text{day}^{-1}$  at 200  $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$  (Fig. 5.5). Bates et al. (1991) also found an increase in growth rate ( $\mu$ ) from 0.35 to 0.64  $\text{day}^{-1}$  on increasing irradiance from 45 to 145  $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$  with *P. multiseriis* grown at 10°C on a 10:14 light:dark photoperiod. These effects of irradiance on growth rate contradict Lewis et al., (1993) who found no difference in growth rates of *P. multiseriis* cultured between 5 and 25°C at 80 or 180  $\mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ . Finally, it is noted that at low irradiance ( $< 100 \mu\text{mol-photon m}^{-2} \text{ s}^{-1}$ ) and moderate to low temperatures ( $< 20^\circ\text{C}$ ), the growth rates exhibited by *P. multiseriis* were 2 to 3 times higher than those of *P. pungens*. This is consistent with the field data reported by Smith et al. (1990a) who found a transition between a bloom dominated by *P. pungens* to one dominated by *P. multiseriis* in the Cardigan River, P.E.I. in 1988 to coincide with declining irradiance and temperature.

## General Discussion

The DSP toxins produced by *Exuviaella lima* are positively correlated with growth and are therefore constitutive metabolites. They follow the same general physiological patterns of production and accumulation as the PSP toxins and also, like the PSP toxins, are predominantly intracellular (Chapter 4). Although some of the DSP toxins are released from the cell, the concentrations of these toxins in seawater, even at the unnaturally high cell densities employed in laboratory culture, are orders of magnitude below that indicated by dose-response data to influence significantly the growth of other phytoplankton species (Chapter 1). These physiological characteristics suggest, if indeed these toxins are allelochemicals, that they are most probably directed at potential predators rather than competing phytoplankton (Paerl, 1988). The fact that some DSP toxins are found in the medium is not surprising. It has been long recognised that even healthy populations of natural phytoplankton release up to 16% of their photoassimilated carbon as a wide variety of low molecular weight compounds (Hellebust, 1965). Release of organic matter by phytoplankton cultures is even higher, up to 50% of photoassimilated carbon, particularly in stressed cells and following exponential growth (Hellebust, 1965; Sharp, 1977). The physiological mechanisms responsible for the release of organic matter are unknown although models suggest that passive diffusion is largely responsible (Bjørnsen, 1988) hence, the concept of the phytoplankton cell as a "leaky bag". The fact that PSP toxins have not been detected in the medium of phytoplankton cultures (Anderson et al., 1990) is probably more a

result of difficulties in detecting these small, charged, and highly water soluble molecules in seawater at trace levels rather than any true physiological distinction between PSP and DSP producing cells with respect to toxin release.

Suggestions have been made (Hu et al., 1995b) that one possible function of the water soluble DSP toxin DTX-4 is to facilitate DSP excretion. This may be true in the sense that enhanced water solubility might increase the passive release of this DSP compound from the cell into the aqueous medium; but, the fact that the continuous culture data (Chapter 4) demonstrate that ~90% of production is retained within the cell during exponential growth and that batch culture studies have found continual elevation of intracellular levels even after weeks in post-exponential growth (Quilliam et al., 1996) argue against active excretion of the DSP toxins. Further, the physiological characteristics of DSP toxin production and release contrasts sharply with algal metabolites with established extracellular roles. Particularly good examples are the metal-binding phytochelatins and siderophores. These compounds serve to either ameliorate metal toxicity or act as carriers in high-affinity uptake systems for metal micronutrients (McKnight and Morel, 1979). Importantly, these metabolites are not constitutive, but are induced in direct response to metal ion toxicity or limitation (Wilhelm and Trick, 1995; Ahner et al., 1994) and in the case of schizokinen production by species of *Anabaena* (Clark et al., 1987), clearly excreted, as only 10-20% of production remains within the cells and medium concentrations reach high micromolar concentrations while the cells are still in exponential growth.



It is evident that the suggestions raised in Chapter 1 regarding the allelopathic potential of OA towards competing phytoplankton are not consistent with the physiological patterns of toxin production and release by *E. lima*. Even so, the use of the diatom *Thalassiosira weissflogii* as a bioassay organism has provided interesting data regarding the relative toxicity, transformation and metabolism of the DSP toxin family. Firstly, it was found that OA diol-ester elicits approximately equal toxicity towards *T. weissflogii* as OA when added to the seawater medium (Chapter 2) despite the fact that the diol-ester is not an active phosphatase inhibitor. Secondly, *T. weissflogii*, when challenged with OA diol-ester, would oxidatively metabolise the majority of the toxin to a variety of more water soluble derivatives (Chapter 3). The rapid and extensive metabolism of the ester inferred that it was entering the diatom cell with apparent ease, which is particularly interesting because cell walls and membranes are known to pose a significant barrier to the diffusion of OA itself (Klump et al., 1990). The toxicity of the ester could then be explained to be the result of even minor hydrolysis to OA once within the cell (Chapters 2 and 3); thus, the diol-ester facilitates the transfer of OA across cell membranes. It may then be speculated that the major DSP endotoxin, DTX-4, is a defensive agent and its hydrolysis to the ester following cell lysis (Quilliam and Ross, 1996) provides a means of transporting OA into the cells of the predator. The efficacy of this putative defense would depend on the ability of the target cells to metabolise and excrete the diol-ester before sufficient hydrolysis to OA occurred, either spontaneously, or with assistance of the target cells own esterases, to cause irreparable damage.

From the point of view of assessing the risk of DSP posed by *E. lima*, the physiological patterns of toxin production and retention suggest that the single most important consideration is a thorough understanding of the factors that influence the growth and standing stock of this species. A similar conclusion could also be drawn for the PSP toxin producing dinoflagellates where toxin production and cell content can largely be explained in terms of growth rate (Anderson et al., 1990). Searching for specific conditions which may "trigger" toxin production independently of growth, as has been suggested for ASP production by *Pseudo-nitzschia multiseriata*, where toxin production is negatively correlated with growth in response to specific nutrient limitations (Bates et al., 1991; Hallegraeff, 1993), would be less rewarding.

The turbidostat culture system described in Part 2 is suited to the characterization of the physiology of toxin production, and the effect of environmental conditions on the growth of microalgae. Given that there are multiple interacting environmental factors which control microalgal growth the adoption of efficient automated strategies would be desirable (Bull et al., 1990). The successful performance of the simplex procedure in optimizing the growth rate of species of *Pseudo-nitzschia* to two environmental variables simultaneously (Part 2) demonstrates the potential utility of such an approach. Unfortunately, there are several drawbacks to the use of continuous culture, these are cost, complexity and time (Rhee, 1980). However, such limitations are largely technical: compact, low volume and multi-channel turbidostat systems are feasible (Østgaard et al., 1987) as are cheap and robust sensor systems (Part 2). Increasingly, microalgae are regarded as an untapped source

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of pharmaceutical and bioactive compounds (Borowitzka, 1995). Computer-controlled turbidostat culture could constitute an efficient means of optimizing culture conditions and ultimately be utilized in industrial production.

### **Concluding remarks**

Differences, not similarities, typify many aspects of the ecology and physiology of toxigenic microalgae, but some generalizations may be made. ASP, DSP and PSP toxins, although chemically dissimilar, have the potential to inhibit predators or competitors of microalgae and a defensive role for such compounds is possible (Carmichael, 1986). In DSP and PSP producing algae, cellular levels of toxin, not toxin production rates, will tend to increase whenever growth is limited by light, temperature, salinity or nutrients provided the limiting nutrients do not restrict substrate for toxin synthesis. ASP toxin production and cell content increases with growth limitation. An increase in toxin content in response to declining growth rate is therefore common to all three type of algae. Similar patterns of growth and secondary metabolite accumulation are found in terrestrial plants and have been correlated with resistance to herbivory (Byrant et al., 1983). Growth and secondary metabolism are considered to be competing sinks for fixed carbon (Herms and Mattson, 1992) and it is speculated that the plant is responding to environmental limitation by apportioning finite resources to both growth and secondary metabolism (e.g. defence) such that fitness is optimised (Tuomi, 1992). The above observations do not on their own constitute proof of function. However, for the DSP toxins, the demonstration of a

potential biochemical mechanism that serves to transport OA into a target cell *via* an inactive, lipophilic intermediate, adds weight to the argument. Other roles for the DSP toxins are certainly possible. The excessively high ratio of DSP toxins to intracellular phosphatases certainly contradicts a regulatory function, but this does not mean these compounds do not or were not evolved to fulfil such a role. Further, a particularly interesting attribute of the DSP toxins is that they are ionophores; yet, a possible role in ion transport by the DSP compounds remains unexplored.

Assigning a defensive function to secondary metabolites from marine microalgae is particularly problematic (Naylor, 1982); by definition it must be demonstrated that the presence of the metabolite confers an adaptive advantage to the producer *in situ* (Whittiker and Feeny, 1971), and suitable experimental designs have not been forthcoming (Smayda, 1980). The problem of singling out key features to study from the complex web of microflora-meiofauna interactions has been termed "tropical rainforest syndrome" (Reise, 1992), and aptly illustrates the problem of determining a specific function for a compound such as OA because of its potentially broad spectrum of activity - multiple biochemical processes in all eukaryotes. Therefore, although the hypothesis that certain secondary metabolites produced by terrestrial plants (Harborne, 1993) and marine macroalgae (Hay and Fenical, 1988) can function as defensive agents is accepted, the paucity of basic knowledge of microalgal ecology, due to the inherent difficulties of scale and environment remains a major obstacle in studying the chemical ecology of microalgae.

## Appendix

This appendix provides a source code listing of the turbidostat control program. The program is broken down into 4 separate modules that are concerned with different aspects of the program. Module `turb.c` contains the main menu and functions that control the interrupt service routine. Module `turb_io.c` provides functions that either control or read data from external hardware. Module `turbcyc.c` provides a variety of functions that are polled in a continuous loop and controls the actual operation of the turbidostat. Module `config_2.c` provides the functions required to record and modify data provided by the operator and define some basic graphical functions for the video display. All the modules require the inclusion of a header file (`turb.h`) which defines variables used by the program and prototypes of all the functions used by each module. The prototype functions are used by the compiler for error checking by describing the type of variable returned by or passed to a function, e.g., "unsigned int `analog_in(int)`" indicates that the function "analog\_in" must be passed an integer (the analog channel to be read) and returns an unsigned integer (the result of the conversion) to the calling routine. Numbers preceded by 0x are in hexadecimal. Items delimited with the symbols `/* */` are comments and ignored by the compiler. Compilation of the modules is guided by the project file (below) which describes the modules and associated header files required, other header files are also used for functions required to access the operating system (MS-DOS), and various mathematical functions. These header files are defined by Borland Turbo C<sup>®</sup> 2.1 in their standard library and are marked in the program code with the compiler directive `"#include"`.



```

/* -----set up and control of DAC on Control Board -----*/
/*-----*/
#define dac_1_high 0x175
#define dac_1_low  0x174
#define dac_2_high 0x177
#define dac_2_low  0x176

/* -----setup and control of ADC system-----*/
/*-----*/
#define ch1 0x0      /* ph_1 */
#define ch2 0x1      /* ph_2 */
#define ch3 0x2      /* temp_1 */
#define ch4 0x3      /* temp_2 */
#define ch5 0x4      /* od_880_1 */
#define ch6 0x5      /* od_880_2 */
#define ch7 0x6      /* level_1 not used current system */
#define ch8 0x7      /* level_2 not used current system*/
#define ch9 0x8      /* oxygen_1 not used current system*/
#define ch10 0x9     /* oxygen_2 not used current system*/

/* -----function prototypes -----*/
/*-----*/

/* module turb */
void get_info();
void view_info();
void write_info();
char file_exist();
void read_config();
void save_existing_info();
void write_screen();
void rectan_1(int,int,int,int);
void h_line(int,int,int);
void v_line(int,int,int);

/* module turb */
void set_timer();           /*put addr of new ISR in interrupt vector table */
void reset_timer();        /* restore old ISR */
void interrupt new_timer(); /* new int service rtn */
void display_menu();

```

```

/* module turb_io.c */
    void ddelay();
    unsigned int analog_in(int);
    void c_turn_on(int);
    void c_turn_off(int);
    void turn_on(int);
    void turn_off(int);
    void set_io();
    void reset_dac(int);
    void take_sample();
    void read_abs();
    float read_diode();
    float read_ph()
    float read_temp();
    float bits_to_volts(int);

/* module turb_cyl */
    void write_data();
    void cycle_od_stat();
    void cycle_chemo_stat();
    void cycle_sample();
    void cycle_light();
    void cycle_ph_stat();
    void cycle_stir();
    void cycle_blow();

/* -----structure and union definitions-----*/
/*-----*/
struct s_nibble{
unsigned low:4;
unsigned high:4;
};

struct s_byte{
    unsigned _0:1;
    unsigned _1:1;
    unsigned _2:1;
    unsigned _3:1;
    unsigned _4:1;
    unsigned _5:1;
    unsigned _6:1;
    unsigned _7:1;
};

```



```

union u_bits{
    char byte;
    struct s_byte bit;
    struct s_nibble nibble;
};

```

*The main program module turb.c*

```

/*-----*/
/*-----main program-----*/
/*-----*/

```

```

#include "turb.h"
#include "dos.h"
#include "stdio.h"
#include "conio.h"

```

```

struct time system_time;
struct date system_date;

```

```

struct system_var{
    char file_str[13];      /* file name to store data */
    int mode;              /* 0 = chemostat, 1 = turbidostat */
    int pump_1_sec;       /* pump 1 on times per 15 min for chemostat operation */
    int pump_2_sec;       /* pump 2 on times per 15 min for chemostat operation */
    int stir_ontime;      /* duration of stirring */
    int stir_interval;    /* interval when stirring motors are off */
    int lights_on;        /* lights on time 24 hr clock, hrs only */
    int lights_off;       /* lights off time 24 hr clock, hrs only */
    int light_override;   /* for operation under constant illumination */
    float ph_gain;        /* gain value for time-proportional ph controller */
    float ph_set_1;       /* unit 1 pH set point */
    float ph_set_2;       /* unit 2 pH set point */
    float od_gain;        /* gain value for time-proportional turbidostat controller */
    float od_set_1;       /* optical density set points */
    float od_set_2;
    float blank_880_1;    /* optical densities of seawater blanks from calibration */
    float blank_880_2;
} turbo_var;

```

```

void interrupt (*old)();          /* 32 bit far pointer global */
unsigned long int tick=0;        /* raw 55 ms resolution timer*/

```

```

/* define global variables */
float ph_1, ph_2, co2_ontime_1, co2_ontime_2;
float od_1, od_2, pump_1_ontime, pump_2_ontime;
float lockin_1[99], lockin_2[99];
float abs_880_1=0;
float abs_880_2=0;
float pt_1,pt_2;
long int ph_command_1, ph_command_2, od_command_1, od_command_2;
int light_status;

main()
{
    set_io(); /* all digital power controls off position */
    read_config(); /* get existing configuration data from file */
    while(1) display_menu1(); /* run menu */
}

/* -----function display menu-----*/
/*-----*/
/* This function displays the menu and calls operating functions as required. On
termination all i/o functions are reset and the old ISR replaced */

void display_menu1()
{
    int i;
    char response;
    clrscr();

    gotoxy(10,1);cputs("Chemostat System Variables");
    gotoxy(20,3);cputs("(V)iew configuration");
    gotoxy(20,4);cputs("(C)hange or create");
    gotoxy(20,5);cputs("(T)est power control");
    gotoxy(20,6);cputs("(I)nput testsfor ADC");
    gotoxy(20,7);cputs("(S)ave setup");
    gotoxy(20,8);cputs("(A)bsorbance blanks");
    gotoxy(20,9);cputs("(R)un system");
    gotoxy(20,10);cputs("(E)xit");
    response=getch();

```

```
switch(response){

case 'v':
    if(file_exist()=='y'){
        view_info();
        break;}
    else {
        gotoxy(20,9);cputs("file not found");
        getch();
        break;}

case 'c':
    get_info();
    write_info();
    break;

case 's':
    write_info();
    break;

case 't':
    clrscr();
    printf("hit key to turn on co2_1\n");getch();turn_on(co2_1);
    printf("hit key to turn off co2_1\n");getch();turn_off(co2_1);
    printf("hit key to turn on co2_2\n");getch();turn_on(co2_2);
    printf("hit key to turn off co2_2\n");getch();turn_off(co2_2);
    printf("hit key to turn on stir motors\n");getch();turn_on(stir);
    printf("hit key to turn off stir motors\n");getch();turn_off(stir);
    printf("hit key to turn on air\n");getch();turn_on(air);
    printf("hit key to turn off air\n");getch();turn_off(air);
    printf("hit key to turn on pump_1\n");getch();turn_on(pump_1);
    printf("hit key to turn off pump_1\n");getch();turn_off(pump_1);
    printf("hit key to turn on pump_2\n");getch();turn_on(pump_2);
    printf("hit key to turn off pump_2\n");getch();turn_off(pump_2);
    set_io();
    break;

case 'e':
    set_io();
    reset_dac(1);
    reset_dac(2);
    exit(0);
```

```

case 'I':
    read_config();                /* read config data */
    set_timer();                  /* start ISR */
    clrscr();                     /* clear screen */
    while(1){
        if(kbhit()) break;       /* repeat until key pressed */
        read_abs();              /* get abs_880's as volts */
        gotoxy(20,10);printf("pH_1 = %2.3f",read_ph(1));
        gotoxy(20,11);printf("pH_2 = %2.3f",read_ph(2));
        gotoxy(20,12);printf("unit_1 detector output = %3.2f volts",abs_880_1);
        gotoxy(20,13);printf("unit_2 detector output = %3.2f volts",abs_880_2);
        gotoxy(20,14);printf("Temperature_1 = %2.3f volts",read_temp(1));
        gotoxy(20,15);printf("Temperature_2 = %2.3f volts",read_temp(2));
        delay(5000);             /* delay 5 sec */
    }
    reset_timer();
    set_io();
    break;

case 'a':
    read_config();                /* read in old data then modify */
    set_timer();                  /* start ISR */
    clrscr();
    printf("\n wait 10 seconds");
    delay(8000);                  /* ISR is operating to fill circular queue */
    read_abs();                   /* get abs_880's as volts */
    gotoxy(20,20);printf("unit_1 %3.2f",abs_880_1);
    gotoxy(20,22);printf("unit_2 %3.2f",abs_880_2);
    turbo_var.blank_880_1=abs_880_1; /*mean blank voltages */
    turbo_var.blank_880_2=abs_880_2;
    set_io();                     /* reset all i/o */
    reset_timer();                /* restore old ISR */
    write_info();
    printf("\n .... hit key to return to menu");
    getch();
    break;

```

```

case 'r':
  read_config();
  write_screen();
  set_timer();
  while(1){
    if(kbhit()) break;
    cycle_sample();
    cycle_light();
    cycle_stir();
    if(turbo_var.mode==1) cycle_od_stat();
    if(turbo_var.mode==0) cycle_chemo_stat();
    cycle_ph_stat();
    cycle_blow();
  }
  set_io();
  reset_dac(1);
  reset_dac(2);
  reset_timer();
  break;

default:
  set_io();
  break;
}
}

/*-----function set_timer-----*/
/* This function uses the far pointer "old", defined above, to store the address of the
existing ISR routine and places the address of the new timer service routine in the
interrupt vector table. The setvect and getvect functions are defined in dos.h */

void set_timer()
{
  old=getvect(0x1c);
  setvect(0x1c,new_timer);
}
/* pointer to existing bios handler */
/* replace with pointer to function new_timer */

```

```

/*-----function new_timer-----*/
/* This function replaces the existing timer interrupt handler and will be called every
55 ms. The internal software timer "tick" is incremented on each call and the timers
controlling both the CO2 solenoids and medium input pumps are decremented. On time
out these are turned off. The routine also functions as the lock-in amplifier by
determining the difference between the phototransistor signal with the IR diode off and
on and storing the result in a circular queue */

```

```

void interrupt new_timer()
{
    static int flash=0;          /*stores the status of the diodes, 1 is on 0 is off */
    static int lock_index=0;    /* pointer for the circular queues storing absorbance data */
    static float lockin_1_hi,lockin_1_lo,lockin_2_hi,lockin_2_lo; /* temporary storage */

    tick ++;                    /* increment 55ms. clock */

    if(--ph_command_1 <= 0) turn_off(co2_1);          /*turn off on time out */
    if(--ph_command_2 <= 0) turn_off(co2_2);

    if(--od_command_1 <= 0) turn_off(pump_1);        /*turn off on time out */
    if(--od_command_2 <= 0) turn_off(pump_2);

    if(flash==1) {
        lockin_1_hi=bits_to_volts(analog_in(ch5));
        lockin_2_hi=bits_to_volts(analog_in(ch6));
        outportb(io_latch_c,(inportb(io_latch_c)&0xfe)); /* ir diodes off */
        flash=0;
    }

    else {
        lockin_1_lo=bits_to_volts(analog_in(ch5));
        lockin_2_lo=bits_to_volts(analog_in(ch6));
        outportb(io_latch_c,(inportb(io_latch_c)|0x01)); /* ir diodes on */
        flash=1;
    }

    /* calculate and store difference */
    lockin_1[lock_index]=lockin_1_hi-lockin_1_lo;
    lockin_2[lock_index]=lockin_2_hi-lockin_2_lo;
    if(++lock_index==99) lock_index=0; /* reset if end of array */
}

```

```

/*-----function reset_timer-----*/
/* This function restores old ISR address in vector table using setvect defined in dos.h
*/
void reset_timer()
{
    setvect(0x1c,old);
}

/*-----end module turb.c-----*/

```

*The module turb\_io.c*

```

/*-----*/
/*-----module turb i/o-----*/
/*-----*/
#include "stdio.h"
#include "turb.h"
#include "math.h"

extern struct system_var{
    char file_str[13];
    int mode;
    int pump_1_sec;
    int pump_2_sec;
    int stir_ontime;
    int stir_interval;
    int lights_on;
    int lights_off;
    int light_override;
    float ph_gain;
    float ph_set_1;
    float ph_set_2;
    float od_gain;
    float od_set_1;
    float od_set_2;
    float blank_880_1;
    float blank_880_2;
} turbo_var;

extern float lockin_1[];
extern float lockin_2[];
extern float abs_880_1;
extern float abs_880_2;
extern unsigned long int tick;

```

```

/*-----function ddelay-----*/
/* user delay rtn for a to d converter settle time */
void ddelay()
{
int i;
for(i=0;i<=30;i++);
}

/*-----function set_io -----*/
/* Setup configuration of the digital i/o ports: smartlab board portl b0->b7 connected.
l is on 0 is off . Note independent relays on port c-low are gnd/0 = on */

void set_io()
{
    outportb(io_control,port_mode);    /* a,b and c low out, c high in */
    outportb(io_latch_b,0x00);        /* all off 0 = off pcmd bd */
    outportb(io_latch_c,0xff);        /* l = off */
}

/*-----function turn_off for port b -----*/
void turn_off(int plug)
{
    outportb(io_latch_b,(inportb(io_latch_b)&(~plug)));
}

/*-----function turn_on for port b -----*/
void turn_on(int plug)
{
    outportb(io_latch_b,(inportb(io_latch_b)|plug));
}

/*-----function turn_off for port c -----*/
void c_turn_off(int plug)
{
    outportb(io_latch_c,(inportb(io_latch_c)|plug));
}

/*-----function turn_on for port c-----*/
void c_turn_on(int plug)
{
    outportb(io_latch_c,(inportb(io_latch_c)&(~plug)));
}

```



```

/*-----function reset dac -----*/
void reset_dac(int unit) /* zero output voltages */
{
    if(unit==1){
        outportb(dac_1_low,0x00);
        outportb(dac_1_high,0x00);}
    if(unit==2){
        outportb(dac_2_low,0x00);
        outportb(dac_2_high,0x00);}
}

/*-----function read ph-----*/
float read_ph(int unit) /* 1 volt = ph 10 */
{
    if (unit==1) return(bits_to_volts(analog_in(ch1)))*10;
    if (unit==2) return(bits_to_volts(analog_in(ch2)))*10;}

/*-----function bits_to_volts -----*/
float bits_to_volts(int bits)
{
    return(bits*.00122); /* range is 0 to +5v = 4096 */
}

/*-----function read_temp -----*/
float read_temp(int unit)
{
    int i;
    unsigned long int value=0;
    if(unit==1){
        for(i=1;i<=100;i++) value+=analog_in(ch3);
        return((100.2*bits_to_volts(value/100))-1.1); /* calibration for sensor 1 */
    }
    if(unit==2){
        for(i=1;i<=100;i++) value+=analog_in(ch4);
        return((99.7*bits_to_volts(value/100))-0.9); /* calibration for sensor 2 */
    }
}

```

```

/*-----function analog_in -----*/
/* this function returns an unsigned integer as a result of the conversion of the
channel number passed by the calling routine to the ADC. The input range is fixed at
-5 -> +5 volts = 0 -> 4095 */

unsigned int analog_in(channel)
int channel;
{
int i,j;                               /* conversion loop control */
unsigned int outword;                  /* returns valid 12 bits data */
    outportb(0x170,channel);           /* select channel */
    outportb(0x171,0);                 /* clear register */

    for(i=1;i<=6;i++){
        inportb(0x17c);ddelay(); }     /* hi_convert */
    for(j=1;j<=8;j++){
        inportb(0x17a);ddelay(); }     /* lo_convert */

    outword=inportb(0x173);             /* read high byte */
    outword=outword << 8;              /* shift into position */

    outword=outword+inportb(0x172);    /* append in low byte */
    return(outword);                   /* unsigned interger return */
}

/*-----function read_abs -----*/
/* This routine calculates the absorbance for each unit by averaging the circular queues
and setting the global variables abs_880_1&2*/

read_abs()
{
int j;
    disable();
    abs_880_1=0;abs_880_2=0;
    for(j=0;j<99;j++) abs_880_1+=lockin_1[j];abs_880_1/=99;
    for(j=0;j<99;j++) abs_880_2+=lockin_2[j];abs_880_2/=99;
    enable();
}

```

```

/*-----function take_sample-----*/
void take_sample()
{
    turn_on(sampling_pump);          /* peristaltic to sampler */
    delay(4000);                    /* flush system lines */
    c_turn_on(pin_3);delay(100);c_turn_off(pin_3); /*pulse pin_3 remote port */
    delay(4000);                    /* allow sample to be taken */
    turn_off(sampling_pump);
}

/* -----end module turb i/o-----*/
/*-----*/

```

*The module turb\_cyl.c*

```

/*-----*/
/* -----module of turbidostat cycling functions-----*/
/*-----*/

```

```

#include "turb.h"
#include "dos.h"
#include "conio.h"
#include "stdio.h"

```

```

extern unsigned long int tick;          /* raw 55 ms. count global */
extern int light_status;
extern long int ph_command_1, ph_command_2, od_command_1, od_command_2;
extern float co2_ontime_1, co2_ontime_2, ph_1, ph_2;
extern float abs_880_1, abs_880_2, pump_1_ontime, pump_2_ontime;
extern float pt_1,pt_2;

```

```

extern struct time system_time;
extern struct date system_date;

```

```

extern struct system_var{
    char file_str[13];
    int mode;
    int pump_1_sec;
    int pump_2_sec;
    int stir_ontime;
    int stir_interval;
    int lights_on;
    int lights_off;
}

```

```

int light_override;
float ph_gain;
float ph_set_1;
float ph_set_2;
float od_gain;
float od_set_1;
float od_set_2;
float blank_880_1;
float blank_880_2;
} turbo_var;

```

```

/* -----function cycle_ph_stat-----*/
/* This function checks to see if more than 60 s have elapsed since the last pH
reading. If so, the ph of each unit will be read as stored in the global variables ph_1
and ph_2. If either pH is greater than the set point an error term is generated. A ph
lower than the set point is ignored. The CO2 solenoid will be turned on when the error
exceeds 0.1 units. An error is limited to a maximum of 0.8 units (to prevent excessive
reaction if there is a bad reading) and multiplied by the proportional controller gain.
This value is given to the variable "pH_command" and constitutes the number of
55ms. intervals that the CO2 solenoid will be turned on. Each iteration of the ISR will
decrement "ph_command" and the CO2 solenoid is turned off by the ISR when "pH
command" is decremented to zero */

```

```

void cycle_ph_stat()
{
    static unsigned long int last_time;
    int cycle_time=60;                /* every minute */
    float elapsed_seconds, error_1, error_2;

    elapsed_seconds = tick/18.2;
    if ((elapsed_seconds-(float)last_time)>=(float)cycle_time){
        last_time += cycle_time;

        ph_1=read_ph(1);                /* global extern float */
        ph_2=read_ph(2);                /* global extern float */

        error_1=ph_1-(turbo_var.ph_set_1);if(error_1<0) error_1=0;
        if(error_1==0) turn_off(co2_1);    /* double check, ensure solenoid is off */
        if(error_1>0.8) error_1=0.8;      /* limit extent of action */
        error_2=ph_2-(turbo_var.ph_set_2);if(error_2<0) error_2=0;
        if(error_2==0) turn_off(co2_2);
        if(error_2>0.8) error_2=0.8;
    }
}

```

```

disable();                                /* changing a variable used in isr !!! */
ph_command_1=(int)(error_1*turbo_var.ph_gain); /* 55 ms. counts */
ph_command_2=(int)(error_2*turbo_var.ph_gain); /* global extern int */
enable();

if(error_1>=0.1){
    turn_on(co2_1);                        /* open valve if rqd turned off by ISR */
    co2_ontime_1+=error_1*turbo_var.ph_gain/18.2; /* record time */
}
if(error_2>=0.1){
    turn_on(co2_2);
    /* open valve, turned off by ISR when command expires */
    co2_ontime_2+=error_2*turbo_var.ph_gain/18.2;
}
}
}

```

```

/* -----function cycle_od_stat-----*/
/* This function checks to see if the transmittance of the cultures has been measured in
the last 15 minutes and if not calls read_abs() to do so. If the cell density exceeds the
setpoint as measured by % transmittance an error term is generated and the medium
input pump turned on. The length of time it will operate is determined by the error
multiplied by the proportional controller gain. This value represents the number of 55
ms periods the pump will be on. The pumps are turned off after this by the ISR.
Finally this function calls write_data(), which writes all the current data to disk and
updates the video display. */

```

```

void cycle_od_stat()
{
    static unsigned long int last_time;
    int cycle_time=900;                    /* every 15 minutes */
    float elapsed_seconds, error_1, error_2;

    elapsed_seconds = tick/18.2;
    if ((elapsed_seconds-(float)last_time)>=(float)cycle_time){
        last_time += cycle_time;

        read_abs();                        /* update abs_880's as volts*/
        pt_1=abs_880_1/turbo_var.blank_880_1*100; /* calc % transmittances */
        pt_2=abs_880_2/turbo_var.blank_880_2*100;
    }
}

```

```

error_1=(turbo_var.od_set_1)-pt_1;if(error_1<0) error_1=0;
error_2=(turbo_var.od_set_2)-pt_2;if(error_2<0) error_2=0;

disable();          /* changing a variable used in isr !!! */
od_command_1=(int)(error_1*turbo_var.od_gain); /* 55 ms. counts */
od_command_2=(int)(error_2*turbo_var.od_gain); /* global extern int */
enable();

if(error_1>0){
    turn_on(pump_1); /* open valve if rqd turned off */
    pump_1_ontime+=error_1*turbo_var.od_gain/18.2;
}
if(error_2>0){
    turn_on(pump_2); /* by ISR when command expires */
    pump_2_ontime+=error_2*turbo_var.od_gain/18.2;
}
write_data();
}

}

/* -----function write_data-----*/
/* This function appends all current data to a disk file and updates the video display */

void write_data()
{
    FILE *data_out;
    float elapsed_seconds, temp_1,temp_2,mins;
    elapsed_seconds = tick/18.2;
    mins=elapsed_seconds/60;

    temp_1 = read_temp(1);
    temp_2 = read_temp(2);
    gettime(&system_time);
    getdate(&system_date);

    if((data_out=fopen(turbo_var.file_str,"a"))==NULL){
        puts("file error cannot open file\n");
        exit(1);
    }
}

```

```

fprintf(data_out,"%6.1f %d %2.2f %3.1f %7.2f %3.1f %2.2f %2.2f %3.1f %7.2f %3.1f
%2.2f\n",
        mins,light_status,ph_1,pt_1, pump_1_ontime, co2_ontime_1,temp_1,
        ph_2,pt_2, pump_2_ontime, co2_ontime_2,temp_2);
fclose(data_out);
/* write data to video
display */
gotoxy(8,3);printf("%d:",system_time.ti_hour);
gotoxy(11,3);printf("%d",system_time.ti_min);
gotoxy(68,3);printf("%d",system_date.da_year);
gotoxy(65,3);printf("%d:",system_date.da_mon);
gotoxy(62,3);printf("%d:",system_date.da_day);

gotoxy(18,10);printf("%6.1f",elapsed_seconds/60);
gotoxy(21,12);printf("%2.2f",ph_1);
gotoxy(21,16);printf("%3.1f",co2_ontime_1);
gotoxy(21,18);printf("%2.2f",temp_1);
gotoxy(18,20);printf("%3.1f",pt_1);
gotoxy(18,22);printf("%7.2f",pump_1_ontime);

gotoxy(58,10);printf("%6.1f",elapsed_seconds/60);
gotoxy(61,12);printf("%2.2f",ph_2);
gotoxy(61,16);printf("%3.1f",co2_ontime_2);
gotoxy(61,18);printf("%2.2f",temp_2);
gotoxy(58,20);printf("%3.1f",pt_2);
gotoxy(58,22);printf("%7.2f",pump_2_ontime);
}

/* -----function cycle_stir-----*/
/*This function permits intermittent stirring of the two cultures based on the duty cycle
entered during the system configuration. The local static variable "status" is used to
indicate when the motors are turned on or off. Unlike either the pH or OD control
functions, which use the ISR to keep track of the running time, this function will also
turn off the motors after the on time interval is exceeded. This is because the stirring
interval does not require accurate timing.*/

void cycle_stir()
{
    static unsigned long int last_time;
    long int cycle_time;
    static int status;
    float elapsed_seconds;

```

```

elapsed_seconds = tick/18.2;
cycle_time = turbo_var.stir_interval;
if ((elapsed_seconds-(float)last_time)>=(float)cycle_time){
    last_time += cycle_time;
    turn_on(stir);
    status=1;
}

if ((status==1)&&(elapsed_seconds-(float)last_time)>=turbo_var.stir_ontime){
    turn_off(stir);
    status=0;
}
}

/* -----function cycle_light-----*/
/* This function controls the photoperiod using the function gettimeofday() defined in dos.h
and therefore requires the correct time to be entered using the MS-DOS "time"
command. The logic used is identical to that in the function cycle_stir(). */

void cycle_light()
{
    if(turbo_var.light_override==1){
        turn_on(light);
        light_status=1;
        return;
    }

    gettimeofday(&system_time);
    if((int)system_time.ti_hour>=turbo_var.lights_on &&
        (int)system_time.ti_hour<turbo_var.lights_off){
        turn_on(light);
        light_status=1;}

    else {
        turn_off(light);
        light_status=-1;}
}

```



```

/* -----function cycle_blow----- */
/* This function controls a solenoid allowing sterile compressed air to be injected into
the headspace of the culture (5 sec. every 30 min.) to ensure a constant level is
maintained by forcing out culture through the overflow tube.*/

```

```

void cycle_blow()
{
    static unsigned long int last_time;
    long int cycle_time=1800;
    static int status;
    float elapsed_seconds;

    elapsed_seconds = tick/18.2;
    if ((elapsed_seconds-(float)last_time)>=(float)cycle_time){
        last_time += cycle_time;
        turn_on(air);
        status=1;
    }

    if ((status==1)&&(elapsed_seconds-(float)last_time)>=5){
        turn_off(air);
        status=0;
    }
}

```

```

/* -----function cycle_chemo_stat----- */
/* This function provides an alternate for cycle_turbostat to run the unit as a
chemostat. The pumping times are entered during configuration. Optical data is
recorded, but not used to change the pumping times. */

```

```

void cycle_chemo_stat()
{
    static unsigned long int last_time;
    int cycle_time=900;                /* every 15 minutes */
    float elapsed_seconds;

    elapsed_seconds = tick/18.2;
    if ((elapsed_seconds-(float)last_time)>=(float)cycle_time){
        last_time += cycle_time;

        read_abs();                    /* update abs_880's as volts*/
        pt_1=abs_880_1/turbo_var.blank_880_1*100; /* calc % transmittances */
        pt_2=abs_880_2/turbo_var.blank_880_2*100;
    }
}

```

```

    if(light_status==1){
        turn_on(pump_1);
        turn_on(pump_2);
        disable();          /* changing a variable used in isr !!! */
        od_command_1=(int)(turbo_var.pump_1_sec*18.2); /* 55 ms. counts */
        od_command_2=(int)(turbo_var.pump_2_sec*18.2); /* global extern int */
        enable();
    }

    write_data();
}
}

/* -----function cycle_sample-----*/
/* This function is used to control an external Brinkman autosampler which requires
an electrical pulse to initiate operation, this is achieved by calling the function
take_sample(). Six samples are taken over a 24-hour period. */
void cycle_sample()
{
    int i;
    static int sample[6]={0,4,8,12,16,20}; /* set sample hours */
    static int status[6]={0,0,0,0,0,0}; /* status 0 not taken */

    gettimeofday(&system_time); /* get system time in hours 0-23 are valid */

    for(i=0;i<=5;i++){
        if(sample[i]==system_time.ti_hour && status[i]==0){
            take_sample(); /* rtn to operate pump and sampler */
            status[i]=1; /* set status to signify sample acquired */
        }
    }
}

/* -----end turb cycling functions-----*/
/*-----*/

```

*The module config\_t.c*

```

/*-----*/
/* -----turboconfig module-----*/
/*-----*/
#include "dos.h"
#include "stdio.h"
#include "conio.h"
#include "turb.h"

extern struct system_var{
char file_str[13];
int mode;
int pump_1_sec;
int pump_2_sec;
int stir_ontime;
int stir_interval;
int lights_on;
int lights_off;
int light_override;
float ph_gain;
float ph_set_1;
float ph_set_2;
float od_gain;
float od_set_1;
float od_set_2;
float blank_880_1;
float blank_880_2;
} turbo_var;

/* -----function view_info-----*/
/* Function to read and display the contents of the configuration file "turbo.fig" on the
video screen */

void view_info()
{
read_config();
clrscr();
gotoxy(1,1);

printf("\ndata file name is %s\n\n",turbo_var.file_str);

printf("stir interval is %d sec\n",turbo_var.stir_interval);
printf("stir duration is %d sec\n\n",turbo_var.stir_ontime);

```

```
printf("lights on at %d:00 hours\n",turbo_var.lights_on);
printf("lights off at %d:00 hours\n\n",turbo_var.lights_off);
printf("light override logic status is %d\n",turbo_var.light_override);
```

```
printf("ph proportional controller gain is %3.1f\n",turbo_var.ph_gain);
printf("ph set point_1 is %1.2f\n",turbo_var.ph_set_1);
printf("ph set point_2 is %1.2f\n",turbo_var.ph_set_2);
printf("od proportional controller gain is %4.1f\n",turbo_var.od_gain);
printf("od set point 1 is %1.3f\n",turbo_var.od_set_1);
printf("od set point 2 is %1.3f\n",turbo_var.od_set_2);
printf("blank 880 1 is %3.2f\n",turbo_var.blank_880_1);
printf("blank 880 2 is %3.2f\n",turbo_var.blank_880_2);
printf("pump_1_time/900s is %d\n",turbo_var.pump_1_sec);
printf("pump_2_time/900s is %d\n",turbo_var.pump_2_sec);
printf("mode is %d\n",turbo_var.mode);
printf(".. hit key to return to main menu");
getch();
}
```

```
/* -----function get_info-----*/
/* Function to allow the operator to enter the configuration information into the data
structure turbo_var */
```

```
void get_info()
{
    int answer;
    /* first read in existing information & calibration so it is not lost */

    read_config();

    clrscr();gotoxy(1,1);
    printf("enter DOS file name to accept data ");
    gets(turbo_var.file_str);

    printf("input interval for stirring (sec) ");
    scanf("%ld",&turbo_var.stir_interval);

    printf("input duration of stirring (sec) ");
    scanf("%d",&turbo_var.stir_ontime);

    printf("input lights on time (24 hour clock-hrs only) ");
    scanf("%d",&turbo_var.lights_on);
```

```

printf("input lights off time (24 hour clock-hrs only) ");
scanf("%d",&turbo_var.lights_off);

printf("to override photoperiod for continuous light enter 1 else 0 ");
scanf("%d",&turbo_var.light_override);

printf("enter ph proportional control gain ");
scanf("%f",&turbo_var.ph_gain);

printf("input pH set point_1 ");
scanf("%f",&turbo_var.ph_set_1);

printf("input pH set point_2 ");
scanf("%f",&turbo_var.ph_set_2);

printf("enter od proportional control gain");
scanf("%f",&turbo_var.od_gain);

printf("input od set point unit 1");
scanf("%f",&turbo_var.od_set_1);

printf("input od set point unit 2");
scanf("%f",&turbo_var.od_set_2);

printf("input pump_1 on time/900 (sec) ");
scanf("%d",&turbo_var.pump_1_sec);

printf("input pump_2 on time/900 (sec) ");
scanf("%d",&turbo_var.pump_2_sec);

printf("input mode 0=chemo, 1=turbo");
scanf("%d",&turbo_var.mode);

printf("\nhit key to return to main menu");
getch();
}
/* -----function write_info-----*/
/* This function is used to write the contents of the structure turbo_var to disk */

void write_info()
{
FILE *config_file;

```

```

    if((config_file=fopen("turbo.fig","wb"))==NULL){
        puts("file error cannot open file\n");
        exit(1);
    }
    fwrite(&turbo_var,sizeof(turbo_var),1,config_file);
    fclose(config_file);
}

/* -----function file_exist-----*/
/* Standard protocol to check if a file exists before commencing any read/write
operations. The result of the inquiry is returned as type char, either a "y" or "n" */

char file_exist()
{
    FILE *check_file;
    char answer;
    clrscr();
    if((check_file=fopen("turbo.fig","rb"))==NULL){ /* check if exist */
        fclose(check_file);
        return 'n';
    }
    else {
        fclose(check_file);
        return 'y';
    }
}

/* -----function read_config-----*/
/* This function reads the contents of the configuration file into the data structure
turbo_var */

void read_config()
{
    FILE *config_file;

    if(file_exist()=='y'){
        if((config_file=fopen("turbo.fig","rb"))==NULL){
            puts("file error cannot open file\n");
            exit(1);
        }
    }
}

```

```

    fread(&turbo_var,sizeof(turbo_var),1,config_file);
    fclose(config_file);
}
}

/* -----function write_screen-----*/
/* This function provides graphics and text for the video display */
void write_screen()
{
clrscr();
gotoxy(15,5);printf("Chemostat_1");
gotoxy(50,5);printf("Chemostat_2");
h_line(1,4,79);
h_line(1,6,79);
v_line(39,9,13);

gotoxy(5,10);printf("Time (min)");
gotoxy(5,12);printf("pH (units)");
gotoxy(5,14);printf("Oxygen (uM)");
gotoxy(5,16);printf("Co2 (sec)");
gotoxy(5,18);printf("Temp (C)");
gotoxy(5,20);printf("Abs (units)");
gotoxy(5,22);printf("pump_1 (sec)");

gotoxy(45,10);printf("Time (min)");
gotoxy(45,12);printf("pH (units)");
gotoxy(45,14);printf("Oxygen (uM)");
gotoxy(45,16);printf("Co2 (sec)");
gotoxy(45,18);printf("Temp (C)");
gotoxy(45,20);printf("Abs (units)");
gotoxy(45,22);printf("pump_2 (sec)");

}

/* -----function rectan_1-----*/
/* Draws a rectangle using ASCII character codes to screen coordinate points passed
*/

void rectan_1(int x1,int y1,int x2,int y2)
{
int i;
gotoxy(x1,y1);putch(218);
gotoxy(x2,y2);putch(217);

```

```

gotoxy(x2,y1);putch(191);
gotoxy(x1,y2);putch(192);
gotoxy(x1+1,y1);for(i=1;i<=x2-x1-1;i++) putch(196);
gotoxy(x1+1,y2);for(i=1;i<=x2-x1-1;i++) putch(196);
for(i=1;i<=y2-y1-1;i++) {
    gotoxy(x1,y1+i);
    putch(179);
    gotoxy(x2,y1+i);
    putch(179);    }
}

/*-----function h_line-----*/
void h_line(int x1,int y1,int len)
{
int i;
gotoxy(x1,y1);
for(i=1;i<=len;i++) putch(205);
}

/*-----function v_line-----*/
void v_line(int x1,int y1,int len)
{
int i;
for(i=1;i<=len;i++){
    gotoxy(x1,y1+i);
    putch(179);  }
}

/*-----end turbostat configuration module-----*/
/*-----*/

```



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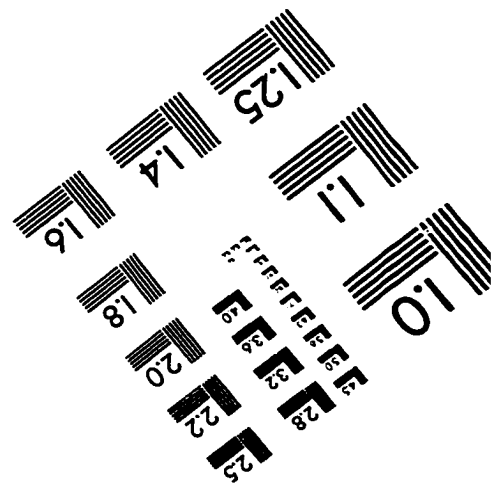
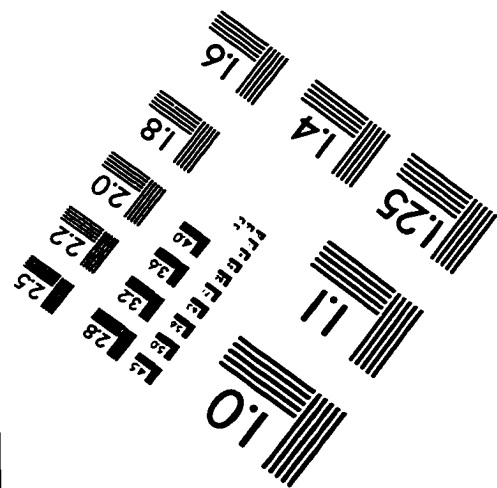
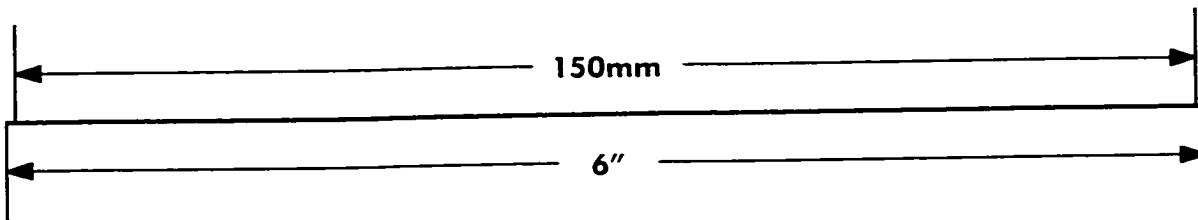
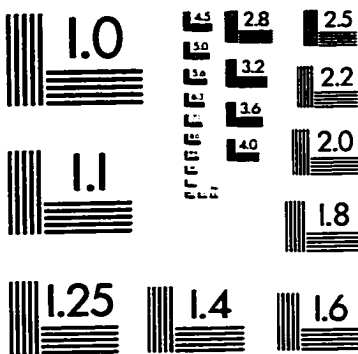
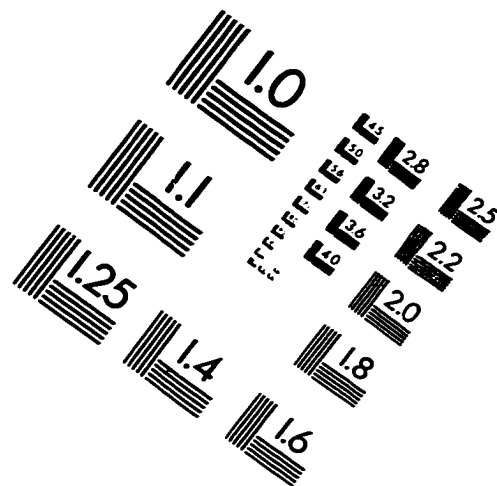
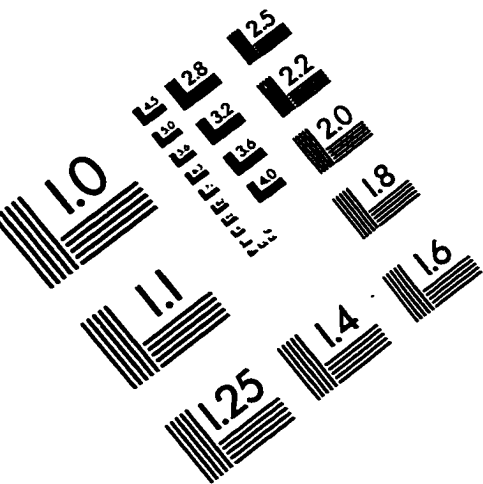
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