DYNAMICS OF DIARRHETIC SHELLFISH TOXINS FROM THE DINOFLAGELLATE *PROROCENTRUM LIMA* IN THE BAY SCALLOP *ARGOPECTEN IRRADIANS*

by

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For my parents,

Nita and Don

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Abstract

Bivalve molluscs can acquire diarrhetic shellfish poisoning (DSP) toxins via ingestion of toxigenic dinoflagellates. The dynamics and metabolic fate of DSP toxins were investigated in the bay scallop, *Argopecten irradians*, when exposed to cells of the epibenthic dinoflagellate *Prorocentrum lima*, a known producer of DSP toxins, in controlled laboratory microcosms. Toxin parameters determined were uptake and detoxification rates, and anatomical compartmentalization of toxin components. Tissue and algal extracts were analyzed by liquid chromatography-mass spectrometry (LC-MS) and by liquid chromatography with UV detection (LC-UV) for okadaic acid (OA), dinophysistoxin-1 (DTX1) and recently identified OA-esters.

No mortalities occurred and no feeding inhibition was observed for juvenile and adult bay scallops during the two week exposure to *P. lima* cells. Clearance rates were similar for scallops exposed to equivalent biovolume cell concentrations of toxigenic *P. lima* and the non-toxic diatom *Thalassiosira weissflogii*; however, absorption efficiency of organic matter was significantly lower for *P. lima* as a diet relative to *T. weissflogii*. Examination of scallop fecal ribbons revealed that a significant portion of ingested *P. lima* cells were capable of survival and cell division following passage through the scallop gut. Although DSP toxin concentrations in bay scallop viscera tissue exceeded commonly accepted regulatory levels (2 $\mu g\ g^{-1}$) within 24 hours of exposure to *P. lima*, after 2 weeks of exposure, total DSP toxin retained in scallop tissues was < 1% of the total toxin ingested over the same period. Most of the total toxin body burden was associated with the viscera (76%) in adult scallops, however a significant portion was associated with gonadal tissue (12%). Toxin levels were relatively low in gill, mantle and adductor tissue (<12% of total toxin body burden). During the depuration period, rapid release of DSP toxins from scallops indicated that toxins were poorly bound to the tissues. Detoxification of viscera tissue was biphasic, comprised of a loss of labile toxin components (30% of the total toxin load) within the initial 16 hours of depuration, followed by a much slower release of the remaining toxin at a rate of 8.4% d^{-1}. Metabolic conversion pathways of DSP toxins were examined *in vitro* by incubating purified DSP toxins with scallop tissue homogenates. Dinophysistoxin-4 (DTX4), a sulfated ester derivative of OA, was hydrolyzed within minutes to form OA-diol ester and OA in tissue homogenates containing ruptured *P. lima* cells. However, in the absence of *P. lima* esterases, both DTX4 and OA-diol ester remained stable in scallop tissue homogenates. This suggests that *in vivo* conversions of DSP toxins within scallops occur exclusively as a result of endogenous esterases liberated from *P. lima* cells during digestion within the scallop gut.

Abbreviations and Symbols

ADAM	9-anthryldiazomethane
AE	absorption efficiency
ANOVA	analysis of variance
b	allometric size exponent
C	concentration
°C	degrees Celsius
$CHCl_3$	methanol
cm	centimetre
CR	clearance rate
CR_S	weight-standardized clearance rate
CTC	time-specific cellular toxin content
CTI	cumulative toxin ingested
d	day
DCA	deoxycholic acid
DF	dry weight of feces
df	degrees of freedom
DSP	diarrhetic shellfish poisoning
DTX1	dinophysistoxin-1
DTX2	dinophysistoxin-2
DTX3	dinophysistoxin-3
DTX4	dinophysistoxin-4
D8OA	okadaic acid-diol ester
E	ash-free dry weight proportion of feces
F	ash-free dry weight proportion of algae
F	flow rate
FDR	fecal deposition rate
fmol	femtomol
g	gram
HPLC-FD	high performance liquid chromatography-fluorescence detection
ht.	height
HCl	hydrochloric acid
I	number of cells ingested
i.d.	internal diameter
IMB	Institute for Marine Biosciences
IR	ingestion rate
ISP-MS	ion-spray mass spectrometry
k	division rate
K_e	growth constant
Kpsi	kilo pounds per square inch
L	litre
λ	exponential decay coefficient
LC-MS	liquid chromatography-mass spectrometry
LC-UV	liquid chromatography-ultraviolet light detection
L:D	light:dark
ln	natural log
m	metre
Me-OA	methyl-okadaic acid ester
Me-DTX1	methyl-dinophysistoxin-1 ester
mg	milligram
min	minute
mL	milliltre
mm	millimetre

mM	millimolar
MU	mouse units
µg	microgram
µm	micrometre
µmol	micromol
µM	micromolar
N	cell concentration
n	number
NRC	National Research Council (Canada)
ng	nanogram
nm	nanometre
NS	Nova Scotia
OA	okadaic acid
OACS-1	okadaic acid calibration solution-1
P	probability value
pg	picogram
PSP	paralytic shellfish poisoning
PTX	pectenotoxin
r^2	correlation coefficient
RP	reverse phase
s	second
SD	standard deviation
SE	standard error
SNK test	Student-Newman-Keuls test
SPE	silica solid-phase-extraction
spp.	species
STXeq	saxitoxin equivalents
TAE	toxin accumulation efficiency
TTBB	total toxin body burden
TCI	total number of cells ingested
t	time
T	toxin concentration
UV	ultraviolet light
V	volume
wt.	weight
YTX	yessotoxin

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Chapter 1. General introduction and objectives

Background

Diarrhetic shellfish poisoning (DSP) poses a serious public health risk and constitutes an economic threat to harvesters of cultured and wild shellfish (Shumway, 1990). Bivalve molluscs acquire DSP toxins by ingesting dinoflagellates from the water column and perhaps via feeding on resuspended benthic material. The known dinoflagellate producers of DSP toxins include several species of the genus *Dinophysis* and a few benthic *Prorocentrum* species (Yasumoto et al., 1990; Andersen, 1996).

Consumption of shellfish contaminated by DSP toxins results in symptoms similar to severe gastroenteritis, including diarrhea, nausea, abdominal cramps and vomiting. Diarrhetic shellfish toxins induce diarrheic symptoms by acting as potent protein phosphatase inhibitors in metabolic processes, resulting in the passive loss of fluids (Aune and Yndestad, 1993). Although at present, Canada does not have official regulations concerning DSP toxins in shellfish, the regulatory level in Japan and some European countries is 0.2 μg OA g^{-1} total soft tissue (Quilliam, 1995) or 2 μg OA/g digestive gland tissue (Hallegraeff, 1993). The diarrheagenic effects of DSP toxins are not known to be lethal to humans, and victims usually recover within a few days. However, it has recently been proposed that OA and DTX1 are powerful cancer promoters and that chronic exposure to these toxins could stimulate the growth of gastrointestinal tumours (Dickey *et al.*, 1993; Aune and Yndestad, 1993). The tumorgenic properties of DSP toxins have only been demonstrated in mice (Fujiki et al, 1989) and have not yet been linked to such activity in humans.

Global Distribution

Diarrhetic shellfish poisoning has been an expensive problem for the shellfish industry worldwide during the past two decades (Fig. 1.1), especially in Japan and Europe, where it is the most significant phycotoxin problem (Cembella and Stabell, 1990; Andersen, 1996)). One DSP incident alone affected 5000 mussel consumers in Spain in 1981 and resulted in costly shellfish harvesting closures (Reguera *et al.*, 1993). The French (Belin, 1993), Chilean (Lembeye *et al.*, 1993), Swedish (Underdahl *et al.*, 1985), Norwegian and Danish (Andersen,

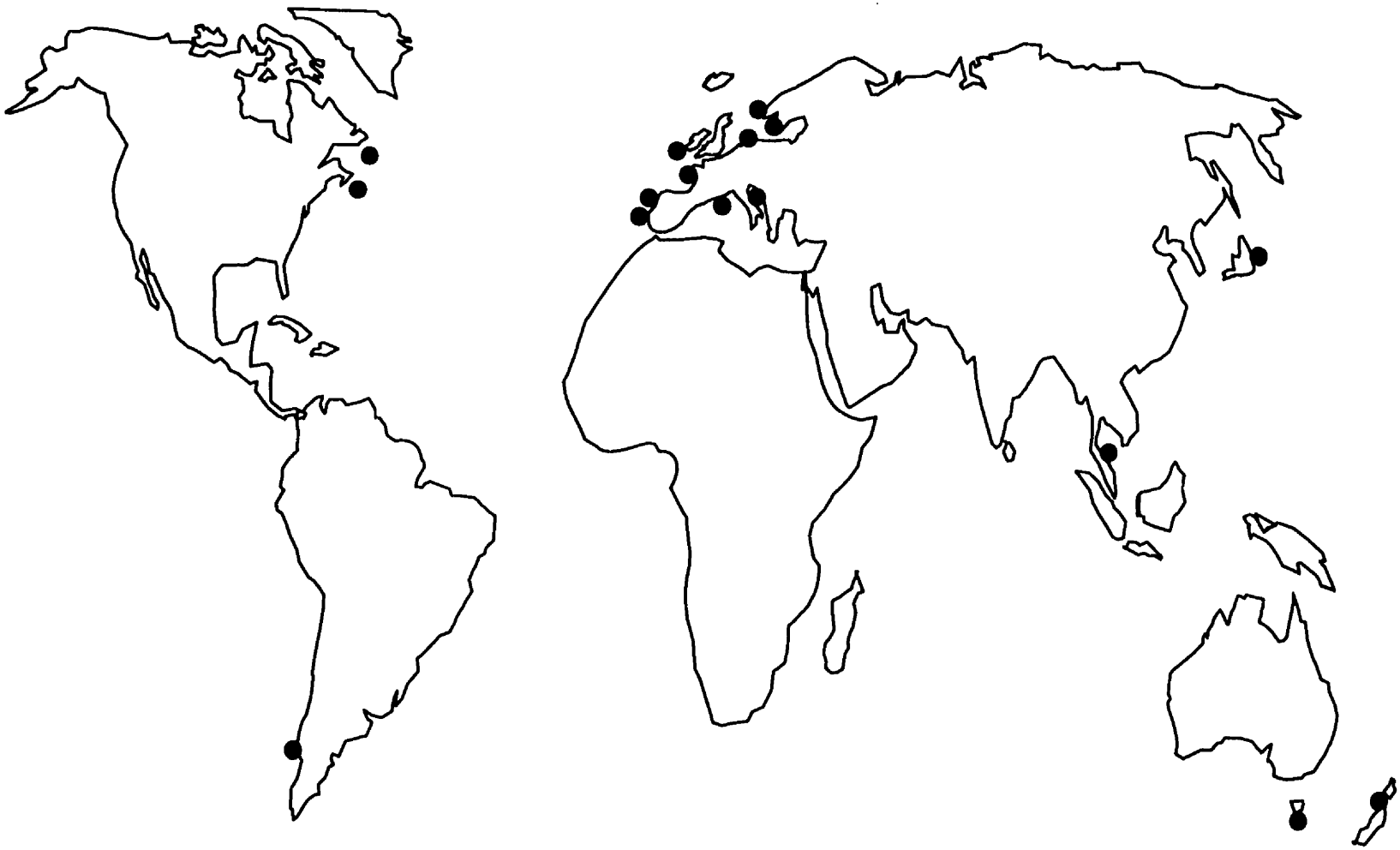


Fig. 1.1. Global distribution of diarrhetic shellfish poisoning incidents (●).

1996) mussel industries have also been significantly by DSP events. Cases of DSP have been confirmed following the consumption of toxic scallops (Yasumoto et al., 1978; 1989), Pacific oysters (Yasumoto et al., 1978; Kawabata, 1989), cockles and clams (Andersen, 1996; Lembeye et al., 1993). Most of the reported DSP events have implicated *Dinophysis* spp. as the causative dinoflagellates (Kat, 1985; Gago et al., 1991; Hageltorn, 1989); however, a few incidents have been linked to the presence of *Prorocentrum lima* (Yasumoto et al., 1989; Van Egmond et al., 1993; ICES, 1992).

North America has been relatively free of confirmed DSP cases until recently. Retrospective examination of documented shellfish-related illnesses has led researchers to suggest that previous DSP cases in Canada and the United States may have been misdiagnosed as bacterial gastroenteritis. Cembella and Todd (1993) suggested that one of the first DSP episodes may have occurred in New Brunswick in 1972 when dozens of people became ill after consuming oysters, whereas Freudenthal and Jijina (1998) estimated that 12 unconfirmed DSP cases probably occurred in New York during the 1980's due to contaminated mussels, clams and scallops. Cembella (1989) confirmed the presence of DSP toxin (OA) in natural phytoplankton populations dominated by *Dinophysis norvegica* and *D. acuminata* in the Gaspé region of Quebec, but this was not linked to shellfish toxicity in this region. In 1990, 16 people experienced DSP related symptoms after consuming cultured mussels from Mahone Bay, Nova Scotia (Quilliam *et al.*, 1993). High levels of DTX1 was detected in the suspect mussels, as well as the presence of thecal fragments attributable to *D. norvegica* in the mussel gut contents, leading investigators to suggest that the DSP causative organism was *D. norvegica* (Quilliam et al., 1993). However, phytoplankton samples collected in the vicinity two weeks after the incident contained a relatively high abundance of *Dinophysis* species but DSP toxins were not detected when these samples were analyzed (Jackson *et al.*, 1993). Repeated attempts to confirm the presence of either OA or DTX1 in net-haul material rich in *Dinophysis* spp. from Nova Scotia waters, using both high-performance liquid chromatography with fluorescence detection (HPLC-FD) and mass spectrometry (ISP-MS) have been consistently unsuccessful (A. Cembella, pers. comm.). This is in spite of the fact that episodic low levels (< 0.1 μg OA eq. g^{-1}) of DTX1 are frequently detected in mussels and scallops from local aquaculture sites.

A strain of the benthic dinoflagellate *Prorocentrum lima* (Fig. 1.2) was isolated from the same locality as the 1990 DSP incident in Mahone Bay and was shown to produce OA and DTX1 in culture (Jackson et al., 1993). The occurrence of *P. lima* was relatively rare in the original plankton-tow samples subsequent to the 1990 DSP incident, however this could be explained by its predominately benthic existence. It has been suggested that *P. lima* was the source of the DSP toxins in the Mahone Bay mussels (Quilliam et al., 1993) and this has been further supported by the presence of *P. lima* on the byssal threads and shells of DSP contaminated mussels from the same site during a later DSP episode (Jackson et al., 1993). The DSP incident in Mahone Bay in 1990 occurred in the aftermath of a major storm with strong onshore winds and a rapid increase in surface temperatures (Cembella, 1992). It is possible that this meteorological event may have caused vertical mixing of the water column. This could have resulted in the resuspension of *P. lima* from the benthos, or dislodging of epizootic colonies of *P. lima* cells attached to mussels and their substrate (mesh "socks"), into the water column where it would be available for filtration by mussels hanging on longlines.

Toxin Chemistry

The lipophilic polyether compounds associated with the DSP toxin complex include okadaic acid (OA) and its dinophysistoxin derivatives (DTX 1-3) (Fig. 1.3), pectenotoxins (PTX 1-3), and yessotoxins (YTX) (Hu et al., 1993). Although all of these compounds have been shown to be acutely toxic to mice following intraperitoneal injection, only OA, DTX1 and DTX3 are associated with diarrhea in humans (Aune and Yndestad, 1993).

Recently, within the timeframe of this thesis research, liquid chromatography combined with ion-spray mass spectrometry (LC-MS) has been used to identify new naturally-occurring ester derivatives of OA from an eastern Canadian strain of *Prorocentrum lima*, including okadaic acid diol ester (D8OA) and DTX4, a polar compound, in which the primary hydroxyl group of D8OA is esterified with a trisulfated end group (Hu et al., 1995; Quilliam and Ross, 1996). Quilliam et al. (1996) demonstrated that DTX4 is the dominant intracellular DSP component in a strain of *P. lima* (PA) isolated from Nova Scotoa.. However, when cells were disrupted during simple extraction procedures, a rapid enzymatically-catalyzed reaction occurred in which DTX4 was hydrolyzed to D8OA, which in turn, was more slowly hydrolyzed to OA

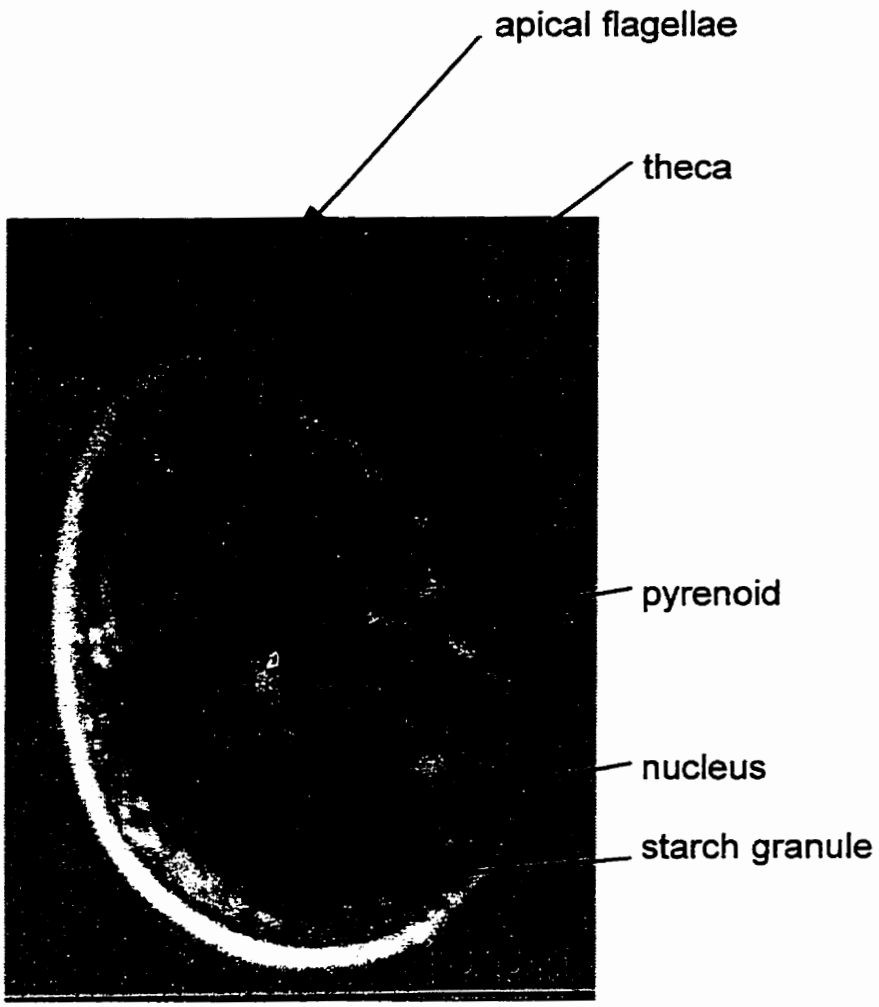
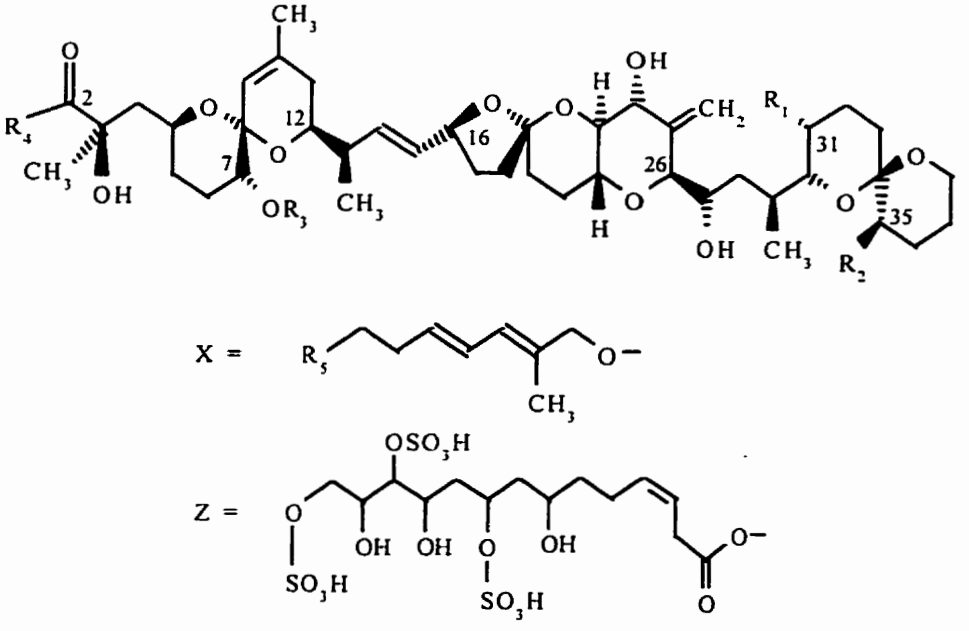


Fig. 1.2. Nomarsky interference micrograph of *Prorocentrum lima* (strain PA) cell.



R1	R2	R3	R4	R5	Mol. Wt.	
CH_3	H	H	H	–	804.5	Okadaic acid (OA)
CH_3	CH_3	H	H	–	818.5	DTX1
H	CH_3	H	H	–	804.5	DTX2
(H or CH_3)		Acyl	H	–	1014-1082	DTX3
CH_3	H	H	X	H	928.5	OA diol ester (D8OA)
CH_3	H	H	X	Z	1472.6	DTX4

Fig. 1.3. Structures of okadaic acid and its naturally occurring derivatives.

(Fig. 1.4). Furthermore, in the presence of aqueous methanol, a portion of the D8OA component underwent methanolysis to form methylated okadaic acid (Me-OA), an artifact of the extraction procedure. Quilliam et al. (1996) hypothesized that digestion of *P. lima* cells by shellfish would result in similar hydrolysis reactions due to esterases derived from disrupted cells and from the shellfish digestive gland. Although DTX4 and OA both act as phosphatase inhibitors, D8OA does not display this property. Thus, processes influencing the biotransformation of DSP toxins in shellfish can affect the total toxicity of the tissues and thereby have serious implications for regulatory authorities.

Motivation

In recent years, several researchers have expressed concern regarding the apparent increase in both the global distribution and frequency of occurrence of harmful algal blooms (eg. Smayda, 1990). Whether this phenomenon is due to a shift in oceanographic conditions that favour the growth of flagellates, or simply, a result of an increase in the use of coastal water for aquaculture leading to increased detection of toxic events (Hallegraeff, 1993), it is inevitable that the risk DSP is a problem that will continue to hamper the growing aquaculture industry in future years (ICES, 1992). Regulatory authorities and shellfish harvesters must have an understanding of how exposure to DSP toxins will affect commercial shellfish products. Thus, it is critical to understand species-specific kinetics and metabolism of DSP toxins in shellfish (Van Egmond, 1993), including rates of uptake and depuration, anatomical compartmentalization and pathways of biotransformation.

Controlled feeding studies of paralytic shellfish poisoning (PSP) toxins in bivalve molluscs have provided much needed information concerning the kinetics and metabolism of such toxins in a number of shellfish species (Bricelj et al., 1990, 1991; Bricelj and Cembella, 1995). Although several reports have related the incidence of DSP toxins in shellfish to phytoplankton blooms (Rodriguez et al., 1989; Zhao et al., 1993; Gilgan et al., 1994), few studies have attempted to describe the actual kinetics of DSP toxins in bivalve molluscs either under laboratory controlled conditions (Pillet et al., 1995) or in field populations (Haamer et al., 1990; Marcaillou Le-Baut et al., 1993a; Reguera et al., 1993). Furthermore, previous studies of DSP toxin kinetics have been unable to examine biotransformation processes due to limitations

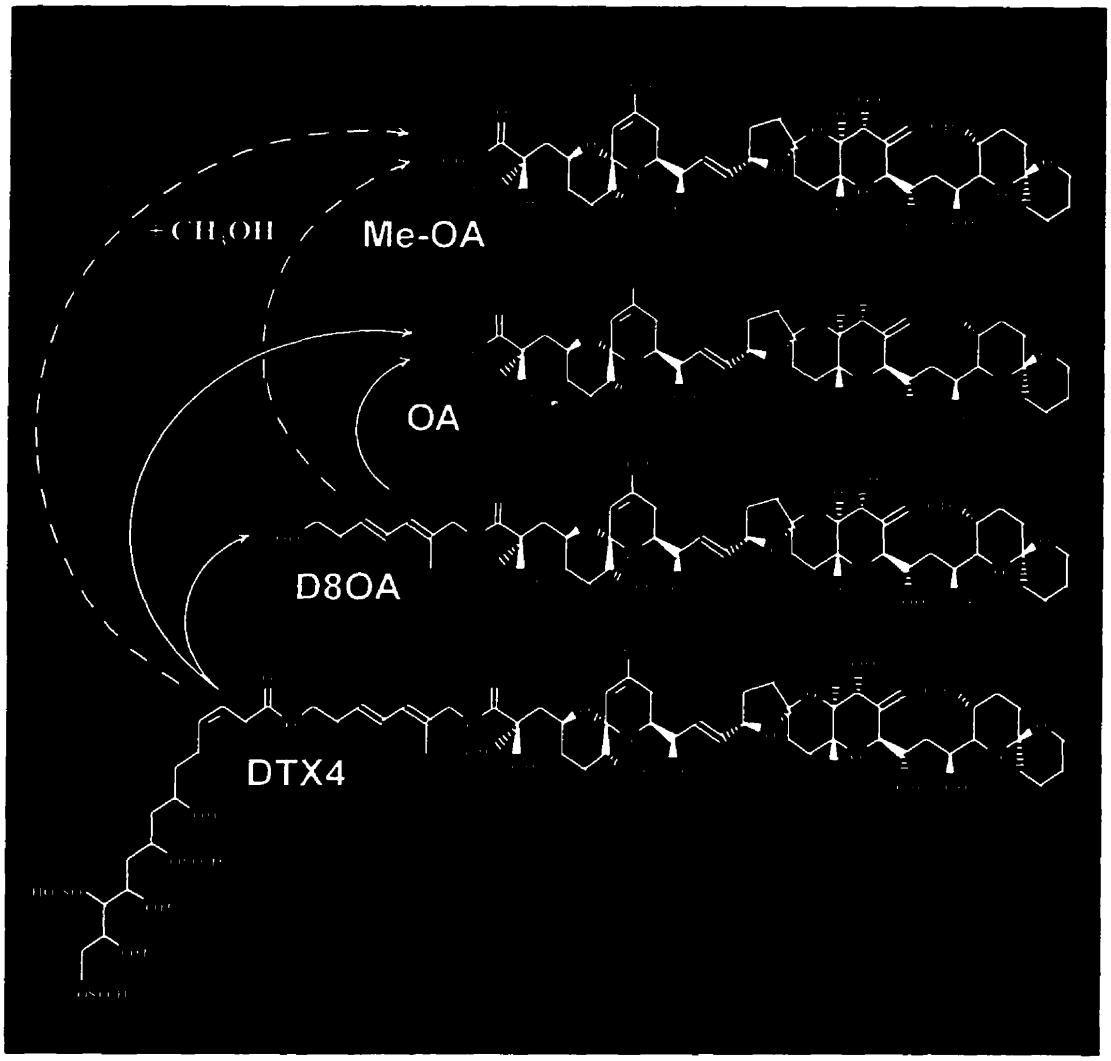


Fig. 1.4. Hydrolytic conversion of DTX4 to okadaic acid diol ester (D8OA) and OA . Dashed line indicates methanolysis of OA-esters to methylated OA (Me-OA).

imposed by the employed methods of detection. The most widely used DSP toxin detection method, the mouse bioassay, is unable to discriminate between the various toxins and only provides a value of total toxicity; moreover, false positives are prone to occur following intraperitoneal injection into mice due to the lethal effect of shellfish-derived free fatty acids (Hamano et al., 1985). Detection of DSP toxins by high performance liquid chromatography followed by fluorescence detection (HPLC-FD) is a much more sensitive and reliable method. However, only DSP toxins with a free carboxylic acid group (OA, DTX1, DTX3) are detected by derivation with the fluorescent reagent (Lee et al., 1987; Quilliam, 1995). Liquid chromatography combined with ion-spray mass spectrometry (LC-MS) has proven to be an extremely valuable analytical method for the identification and quantification of novel DSP toxin analogues, such as D8OA and DTX4 (Pleasance et al., 1990; 1992; Hu et al., 1995; Quilliam, 1995). The application of LC-MS to the analysis of DSP toxins in shellfish is highly advantageous since toxin kinetic parameters can be determined for individual toxin compounds. Thus, resolving biotransformation pathways, along with rates of toxin uptake and release, would provide insight into how DSP toxins are affected following ingestion of toxigenic dinoflagellates by shellfish.

An investigation of phycotoxin kinetics in shellfish must also consider physiological feeding parameters of the species, and whether these processes are altered by exposure to toxigenic dinoflagellates. Species-specific selection against PSP toxin-producing dinoflagellates has been demonstrated in feeding studies performed by Shumway and co-workers (Shumway et al., 1985; Shumway and Cucci, 1987; Gainey and Shumway, 1988), in which bivalve responses included shell valve closure, reduced clearance rates and increased pseudofeces production. Shumway et al. (1985) also argued that bivalves can use post-ingestive sorting mechanisms, such as decreased digestion and absorption rates, to limit assimilation of toxins into tissues. Although it has been hypothesized that similar protective feeding mechanisms are employed by shellfish to prevent accumulation of DSP toxins (Haamer et al., 1995), few studies have attempted to test this hypothesis (Pillet and Houvenaghel, 1995). If toxigenic dinoflagellate cells are selectively egested by bivalves in fecal ribbons, it is of interest to determine whether undigested cells remain viable and are capable of continued growth following release into the water column and/or the benthos. Previous studies, demonstrating the survival of PSP-

toxigenic *Alexandrium tamarense* (Scarratt et al., 1993) and *A. fundyense* (Bricelj et al., 1993) in mussel feces, have warned that such a process could serve as a potential seed source for subsequent dinoflagellate blooms, particularly when live shellfish stocks are transferred to new locations.

In the present study, DSP toxin kinetics and related physiological feeding processes in the northern bay scallop (*Argopecten irradians irradians*) were investigated when exposed to DSP toxigenic *Prorocentrum lima* cells as a food source. The bay scallop was selected for this study due to the abundance of literature on feeding physiology available for this species (Davis and Marshall, 1961; Palmer, 1980; Bricelj and Kuenstner, 1989) and its potential as a prime aquaculture species. Although at present, blue mussels (*Mytilus edulis*) are the most important cultured bivalve species in Nova Scotia, there is considerable local interest in the commercial culture of the bay scallop (*Argopecten irradians*) in Nova Scotia (Scarrattt, 1992; Couturier, 1990). This species has traditionally been an important bivalve product for shellfish harvesters in the northeastern United States (Rhodes, 1991). Recently, the bay scallop has been successfully established in culture in China (Chew, 1990) where it has become a 200,000 tonne per year fishery since 1980. Field grow-out studies at selected sites along the eastern shore of Nova Scotia, including Mahone Bay, (Mallet and Carver, 1987; 1988) have suggested that the bay scallop is a feasible candidate for culture in this region. The extremely fast growth rate of the bay scallop makes it the only bivalve species that can be grown to marketable size in Nova Scotian waters in a single grow-out season. However, due to the relatively small size of the adductor muscle, marketability for the bay scallop depends largely on the growers' ability to sell the product on the half-shell as either whole or "roe-on" (gonads attached) scallops. Thus, a clear understanding of compartmentalization of DSP toxins in the various tissues of the bay scallop (e.g.- adductor vs. gonad vs. viscera) is required.

OBJECTIVES

The main goal of this research project was to investigate the dynamics of diarrhetic shellfish toxins from the dinoflagellate *Prorocentrum lima* in the bay scallop *Argopecten irradians*. Specific objectives were to determine:

1) The fate of *Prorocentrum lima* cells ingested by bay scallops.

2) How feeding physiology parameters of the bay scallop are affected by exposure to DSP-toxigenic *Prorocentrum lima*.

3) The rate of DSP toxin uptake into scallop tissues from a homogeneous suspension of *Prorocentrum lima* supplied at a constant rate in a closed system.

4) Tissue compartmentalization of DSP toxins in juvenile and adult scallops.

5) Processes affecting biotransformation of DSP toxins within scallop tissues, *in vivo* and *in vitro*.

6) The detoxification rate of DSP toxins from various scallop tissues.

Chapter 2. Uptake of DSP toxins by juvenile bay scallops

2.1. Introduction

The relation of DSP incidence in bivalve molluscs to blooms of planktonic *Dinophysis* spp. has been frequently documented over the past two decades (Yasumoto et al., 1980; Van Egmond et al., 1993); however very few studies have attempted to describe the physiological feeding parameters resulting in the uptake and accumulation of DSP toxins by bivalves. Although field studies have provided useful information concerning *in situ* toxin concentrations in shellfish (Haamer et al., 1990; Marcaillou-Le Baut et al, 1993a), the kinetics of DSP toxins in bivalves can only be approximated in the field due to lack of control of environmental variables such as water temperature, phytoplankton concentration and cellular toxin content. By exposing bivalves to simulated "blooms" of toxigenic dinoflagellates in controlled laboratory microcosms, a more precise determination of physiological feeding parameters leading to the accumulation of toxins in shellfish tissue can be achieved (Bricelj et al., 1990). An additional advantage of laboratory feeding studies is that bivalves can be more closely monitored for detrimental effects caused by exposure to toxic dinoflagellates (Shumway et al., 1987; Bardouil et al., 1993).

The focus of the present chapter is to examine rates of feeding and DSP toxin uptake in juvenile bay scallops when exposed to DSP-toxigenic cells of *Prorocentrum lima* as a food source in a controlled laboratory environment. The effect of toxic microalgal exposure on the feeding physiology and survival of juvenile bivalves is of special interest due to their high weight-specific metabolic rates and specific nutritional needs to meet growth requirements (Lesser and Shumway, 1993). Bay scallop populations are especially susceptible to harmful effects caused by microalgal blooms. Since these animals usually spawn only once in their lifetime, the loss of a single juvenile year-class could well result in severe recruitment failure for the population (Rhodes, 1991; Bricelj and Kuenster, 1989).

Scallop tissues were analyzed for DSP toxin content using high performance liquid chromatography with fluorescence detection (HPLC-FD) techniques used in previous DSP toxin studies (Lee et al., 1987; Marcaillou-Le Baut and Masselin, 1990), as well as by

liquid chromatography combined with ion-spray mass spectrometry (LC-MS). The latter technique, a powerful analytical tool for the detection and identification of DSP toxins (Quilliam and Ross, 1996), was used to dtermine the concentration of DSP toxin derivatives, including okadaic acid (OA), DTX1, as well as ester derivatives of OA recently discovered in *Prorocentrum* cultures (Hu et al., 1992; 1993), but never reported before in shellfish tissue.

2.2. Materials and Methods

2.2.1. Algal culture

The epibenthic marine dinoflagellate *Prorocentrum lima* (strain PA), isolated from Mahone Bay, Nova Scotia, was cultured in modified K-medium (Keller et al., 1985) prepared from filtered UV-sterilized (1 μm) natural seawater from the IMB Aquaculture Station at Sandy Cove, Nova Scotia. Dinoflagellates were grown in non-axenic, unialgal batch cultures contained in 2.8 L polycarbonate Fernbach flasks on a 14:10 L:D photocycle, at an incident irradiance of 90 μmol m^{-2} s^{-1} at 17 °C. Relatively low light irradiance was selected to provide optimum growth conditions for *P. lima*, a species known to be shade adapted (Morton and Norris, 1990; Faust, 1995). Polycarbonate Fernbach flasks were selected to minimize cell adhesion and to provide adequate surface area for gas exchange. Flasks were swirled frequently to prevent algal cells from further aggregating on the bottom or adhering to the sides of the flasks.

The growth cycle of *P. lima* under these conditions was monitored to determine the rate of cell division, duration of culture cycle phases and the maximum cell concentrations in the cultures. Cell concentrations were determined by taking 5 mL aliquots from swirled Fernbach flasks, fixing the homogenized sample in Lugol's iodine solution and enumerating cells in a 0.1 mL Palmer-Maloney chamber under a phase contrast microscope (100 X magnification). Division rates were calculated according to Guillard (1973) using the equations: $k\ (\text{div}\ d^{-1}) = K_e/\ln 2$ and $K_e = \ln(N_1/N_0)/(t_1-t_0)$, where k = division rate, K_e = growth constant, t = time and N = cell concentration. The mean division rate of *P. lima* cultures (n = 5) during exponential growth was calculated to be 0.17 d^{-1}.

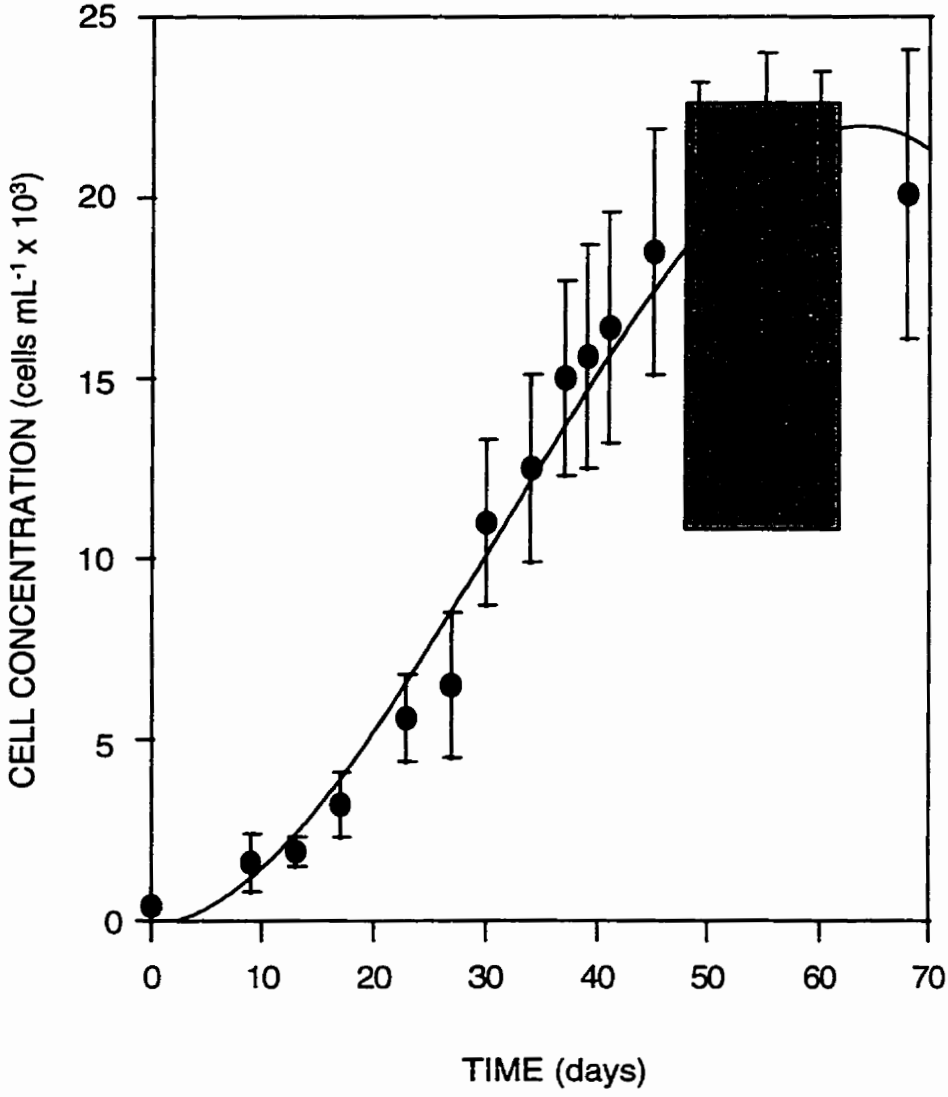
For feeding experiments, cultures of *P. lima* were inoculated in staggered sequence and harvested at the same point of late exponential growth (cell density *ca.* 2 x 10^7 cells L^{-1}) to ensure that each culture fed to scallops was in the same growth phase (Fig. 2.1). The diatom *Thalassiosira weissflogii* (isolated from Long Island, N.Y.), a species known to be nutritionally suitable for bay scallop growth (Bricelj and Kuenstner, 1989), was used as a non-toxic food source during acclimation of scallops and as a control experimental diet for feeding study comparisons with toxic *P. lima* cells. *Thalassiosira weissflogii* was cultured on Fritz's f/2 growth medium supplemented with silicate in 300 L columns at 17 °C and at an incident irradiance of 600 μmol m^{-2} s^{-1}.

2.2.2. Scallop maintenance

Several hundred juvenile bay scallops were obtained from the provincial shellfish hatchery in Ship Harbour, Nova Scotia and transferred to the National Research Council Aquaculture Station at Sandy Cove in May, 1993. Scallops were held in upwellers in flow-through raceways (*ca.* 9 L min^{-1} unfiltered seawater) and maintained at 18 °C. During pre-conditioning, scallops were fed a cultured diet of the diatom *Thalassiosira weissflogii* delivered via a peristaltic pump from a 200 L feeding column and dripped into the upwellers at a rate adjusted to provide an ambient concentration of diatom cells of approximately 2000 cells mL^{-1} in the upwellers.

2.2.3. Determination of clearance rates

Short-term measurements of clearance rates (volume of water swept clear of particles per unit time) (Bayne and Newell, 1983) were conducted to determine the feeding activity of juvenile bay scallops exposed to DSP toxin-producing *Prorocentrum lima* cells. Clearance rates were also determined for juvenile scallops exposed to equivalent biovolume concentrations of *T. weissflogii*, a species used in previous studies of bay scallop feeding activity (Bricelj and Kuenstner, 1989; Bricelj and Shumway, 1991). Using cell dimension measurements determined using an optical micrometer with phase contrast microscopy (400 X magnification), the cellular biovolume for *P. lima* (spheroid volume)



Division rate, k = = 0.17 d^{-1} Temperature = 17°C

Division Time = 5.75 d Light Intensity = 90 µmol m^{-2} s^{-1}

Fig. 2.1. Growth cycle of *Procentrum lima* cultures used for toxin uptake experiments. Shaded area marks period when cells were harvested for feeding experiments.

and *T. weissflogii* (cylinder volume) were calculated as 2.5 x 10^4 μm^3 and 1.5 x 10^3 μm^3, respectively. Thus, the cell biovolume of *P. lima* was approximately 16.7 times larger than that of *T. weissflogii*.

Twenty-five scallops of similar size (mean shell ht. = 12.71 mm, SE = 0.48; mean wet tissue weight = 166.75 mg, SE = 11.65) were divided into five groups of five animals and placed in 500 mL glass beakers containing algal suspensions at an initial cell concentration of 5000 cells mL^{-1} (*T. weissflogii*) or 300 cells mL^{-1} (*P. lima*). Two other beakers containing the algal suspension without scallops were used as controls for each experiment. The algae were kept in suspension by an air stone placed in each beaker. No pseudofeces were produced at these cell concentrations and no feces were produced within the short feeding period of this study. Samples of the suspension (5 mL) were taken from each beaker at the beginning of the exposure period and after 20 minutes for determination of cell concentration by microscopic counts. Following each feeding study, scallops were sacrificed for determination of total wet tissue weight.

Clearance rates were estimated according to the equation: $CR = \ln(C_0/C_1) \times V/t \times n^{-1}$ (Bricelj et al., 1990), where C_0 and C_1 are the initial and final cell density, respectively, V is the volume of suspension, t is the time interval and n is the number of scallops. Clearance rates were converted to weight-standardized rates for a scallop of 1g total wet body weight according to the equation: $CR_S = CR_e/W_e^b$, where CR_e and W_e are the clearance rate and total wet tissue weight of the experimental animal, respectively and *b* is the exponent of the allometric equation relating clearance rate and body size (= 0.75; Bayne and Newell, 1983).

2.2.4. Long-term toxin exposure

Juvenile bay scallops (mean shell ht. = 19.65 mm, SD = 1.55 ; mean total wet tissue weight = 0.498 g, SD = 0.129, n = 99) were held for 14 days in a recirculating aquarium containing 30 L of filtered (1 μm) UV-treated seawater at a constant temperature of 17 °C (Fig. 2.2). Cultures of *P. lima* were harvested daily and continuously metered from a stock tank into the aquarium with a peristaltic pump to yield an approximately constant cell density of 10^5 cells L^{-1} throughout the toxification period. Water was also pumped from the aquarium into a waste tank at the same rate as the inflow to maintain a constant

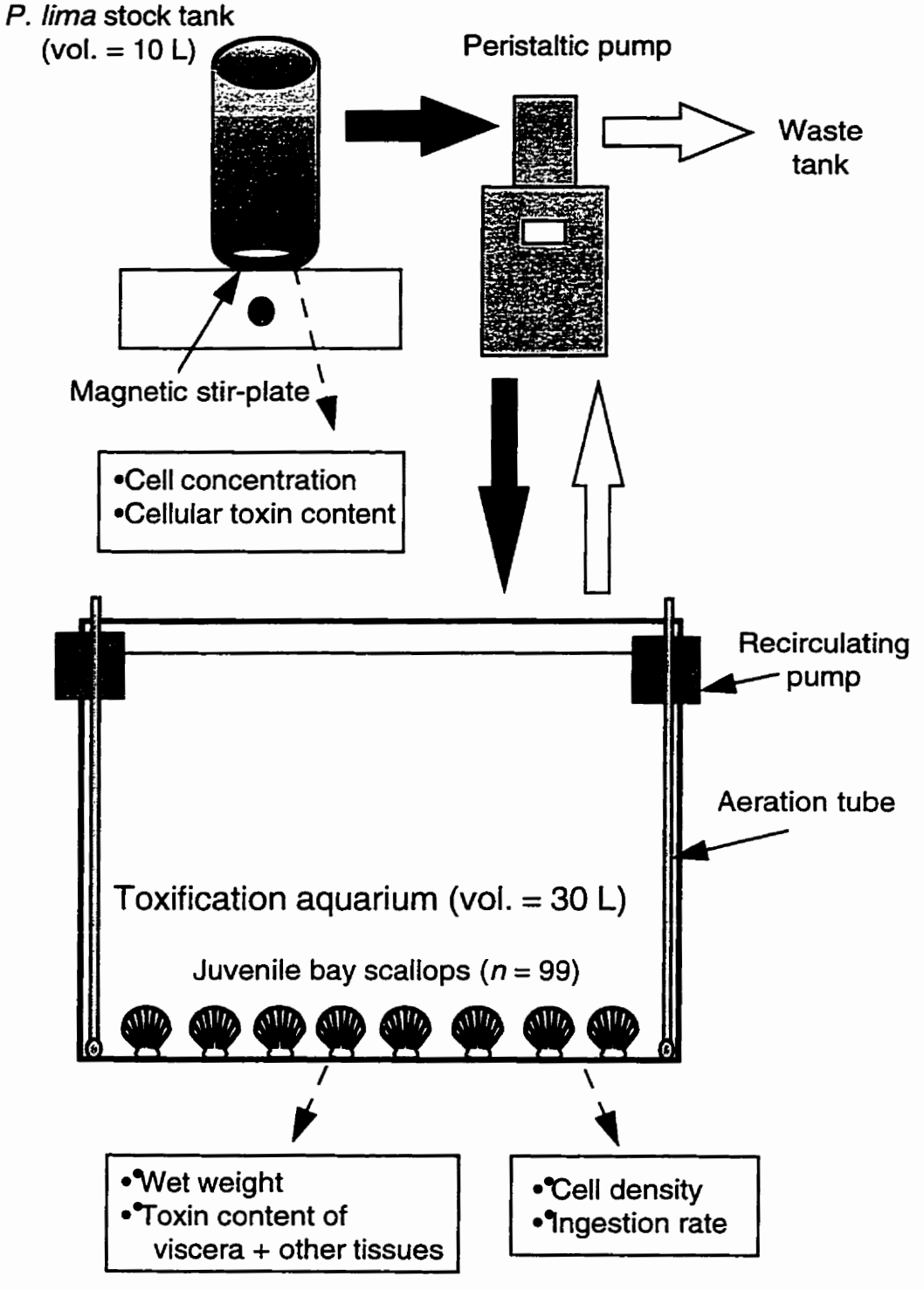


Fig. 2.2. Experimental apparatus used for the toxin exposure experiment. Measured parameters are indicated below.

volume of water in the aquarium. *Prorocentrum lima* cells were maintained in suspension in the stock tank by a magnetic stir-bar. Cells were kept in suspension in the aquarium by the action of two recirculating pumps mounted on each end and two aeration tubes mounted along the bottom of the aquarium. The apparatus was tested over a 24 h period prior to the experiment to ensure that *P. lima* cells were not lost by mechanisms other than ingestion by scallops (e.g. - sedimentation, attachment to tank surfaces, disruption by pumps). The flow created by the pumps did not appear to inhibit the feeding activity of the scallops (i.e. - normal "shell gape" of scallops was observed). The entire volume of water in the aquarium was exchanged every two days to prevent fouling and re-ingestion of fecal material by the scallops.

Algal cell concentrations in the aquarium were determined microscopically (100 X magnification) using a 1.0 mL Sedwick-Rafter counting chamber. The number of cells ingested by all of the scallops in the aquarium was determined each day by calculating the number of cells delivered from the stock tank, the number of cells removed by the exit (waste) flow and the change in cell concentration in the aquarium. Weight-specific ingestion rates were standardized according to the mean wet weight of all scallops used in this study (0.50 g, SD = 0.13, n = 99). Weight-specific values of cumulative toxin ingested (CTI) were determined according to the equation: CTI = TCI x CTC, where TCI is the weight-specific total number of cells ingested, CTC is the time-specific cellular toxin content. Toxin accumulation efficiency (TAE) was determined by the equation: TAE = TTBB/CTI x 100, where TTBB is the total toxin body burden (as determined by LC-MS) and CTI is the cumulative toxin ingested.

Replicate (n = 3) samples of three pooled juvenile scallops were removed at regular intervals from the aquarium for toxin analysis and replaced with scallops not previously exposed to *P. lima*, which were sampled at the end of the experiment. Scallops were pooled to ensure that adequate quantities of tissues were extracted for toxin detection. The replacement scallops maintained a constant number of scallops in the aquarium and provided a duplicate time series for toxin analysis (Series II). For each pool, the tissues were removed and divided into two groups: viscera (digestive gland-stomach complex) and all other tissues (including gills, mantle and adductor muscle). Tissues were weighed,

quick-frozen in isopropanol dry ice and kept on dry ice until transfer to the main laboratory (*ca.* 2 - 4 h) where they were stored at -80 °C.

2.2.5. Toxin extraction

Tissues were extracted in 8.0 mL of 80% aqueous methanol by homogenization for 3 min using a Brinkman tissue homogenizer. Following centrifugation for 20 min at 4000 x g (4°C), an accurate 5.0 mL aliquot of the supernatant was transferred to a glass centrifuge tube, and subjected to a liquid-liquid partitioning clean-up procedure in which the sample was extracted twice with 5 mL aliquots of hexane (Quilliam, 1995). After discarding the hexane layers, the sample was extracted with 1 mL of water and 6 mL of chloroform. The chloroform layer was transferred to another glass centrifuge tube following which the aqueous layer was extracted again with 6 mL of chloroform. The combined chloroform layers were evaporated to dryness under a stream of nitrogen. The residues were re-dissolved in 0.25 mL methanol and filtered by centrifugation (2000 x g) through a 0.45 µm cartridge-filter (Millipore Ultrafree-MC) for analysis by HPLC-FD and LC-MS.

Toxin content of *P. lima* cells was determined by removing 400 mL of algae from the stock tank at two day intervals. The cell concentration in the sample was determined by microscopic counts and the cells were concentrated by centrifugation at 4000 x g for 20 minutes (4 °C). The wet weight of the pellet was determined, after which the algal cells were disrupted by 10 s bursts of probe sonication for 3 minutes in 10 volumes of methanol. Sonicated samples were centrifuged for 10 minutes at 4000 x g (4 °C) and the supernatant was centrifuged through a 0.45 µm cartridge-filter for analysis by HPLC-FD and LC-MS.

2.2.6. Toxin analysis and quantitation

High-performance liquid chromatography with fluorescence detection (HPLC-FD)

Following methods developed by Lee et al. (1987) and modified by Quilliam (1995), fluorometric detection of OA and related compounds containing a carboxyl group (OA and DTX1) was enabled by derivatization with 9-anthryldiazomethane (ADAM), a fluorescent reagent.

Aliquots (10 µL) of algal and scallop tissue extracts were transferred to 1.5 mL amber vials along with 100 µL of ADAM solution (0.2% w/v in methanol) and 10 µL of an internal standard, deoxycholic acid (DCA) (7 µg/mL). Solutions were vortexed for 0.5 min. and heated in the dark for 1 hour at 37°C to allow the derivatization reaction to reach completion.

A silica solid-phase extraction (SPE) clean-up procedure was used to prepare ADAM-derivatized samples for analysis by HPLC. Supelco SPE LC-Si cartridges were placed on an SPE vacuum manifold and conditioned twice with 5 mL of ACS-grade chloroform followed by 5 mL of chloroform/hexane (1:1). The derivatized residues were redissolved and transferred to the cartridges using 3 x 300 µL aliquots of chloroform/hexane and drained slowly through to waste. The cartridges were washed slowly with 5 mL of chloroform/hexane and 5 mL of chloroform. ADAM derivatives were eluted directly into clean glass centrifuge tubes with 5 mL of 10% methanol in chloroform. The elutates were evaporated to dryness under a stream of nitrogen, following which the dried residues were redissolved in 500 µL of methanol and transferred to a 1.5 mL amber vial for storage.

Toxin peaks were resolved on a Lichrospher 100 RP-18 column (25 cm x 4 mm i.d., 5 µm particle size) maintained at 25 °C with a flow rate of 1 mL min^{-1} and an injection volume of 10 µL. Isocratic elution was performed with 80% acetonitrile in deionized water. The fluorescence detector was set for excitation at 254 nm and emission at 412 nm. Toxin peaks were quantified directly by comparison of relative chromatogram peak areas with OACS-1, a standard okadaic acid calibration solution [National Research Council Marine Analytical Chemistry Standards Program (MACSP)]. Quantitation of DTX1 relative to OACS-1 was based on the valid assumption that the molar response for DTX1 and OA are equal (Quilliam, 1995). Toxin concentrations were converted to µg g^{-1} wet wt. tissue according to the molar masses of the toxins (Fig. 1.3).

Liquid chromatography-mass spectrometry (LC-MS)

As an atmospheric-pressure ionization method for mass spectrometry (MS), ion-spray has been proven to be highly sensitive for detection and quantitation of DSP toxins in algal and shellfish tissue extracts (Hu et al., 1992; Pleasance et al., 1990, 1992; Quilliam 1995). Positive ion-spray liquid chromatography (ISP-LC) was used to analyze scallop tissue and

algal extracts for DTX1, OA and OA-esters: Me-OA, Me-DTX1 and OA-diol ester (D8OA). Samples were analyzed using an API-III LC-MS system (SCIEX, Thornhill, Ont.) equipped with an ion-spray interface (Quilliam, 1995). Toxin peaks were quantified by direct comparison of relative chromatogram peak areas with OACS-1. It was assumed that each of the toxins has a molar response equal to that of OA.

2.2.6. Statistical analysis

Statistical analysis of data were performed using SigmaStat statistical software (Version 1.0, 1992, Jandel Scientific, San Rafael, CA). Data sets were tested for normality (Kolmogorov-Smirnov test) and equal variance (Levene median test) prior to use of parametric tests (e.g., t-test, ANOVA, linear regression). Non-parametric tests (e.g., Kruskal-Wallis one-way ANOVA, Wilcoxon signed-rank test) were employed for data that failed the assumptions of normality and equal variance. An analysis of variance (ANOVA) was used to determine if the % molar toxin content varied significantly ($P < 0.05$) between tissue compartments and *P. lima* cells. Following the ANOVA, pairwise multiple comparisons of arc-sine transformed levels of individual toxins were made beween each of the tissue compartments and the *P. lima* cells.

2.3. Results

2.3.1. Clearance rates

Mean weight-standardized (1 g wet wt.) clearance rates of juvenile bay scallops exposed to volume equivalent cell densities of *Thalassiosira weissflogii* or *Prorocentrum lima* were 634 mL h^{-1} (SD = 185) and 767 mL h^{-1} (SD = 149), respectively. There was no significant difference between the clearance rates of the scallops when exposed to either alga (t-test, t = -1.25, df = 8, $P > .05$). Scallops did not exhibit any unusual feeding behaviour when exposed to *P. lima*, such as shell valve closure or violent clapping/ swimming activity. Cell concentrations in the control beakers remained constant during the feeding period for both of the algal suspensions, indicating that cell depletion in the beakers containing scallops was due to scallop ingestion and not settlement of cells.

2.3.2. Long-term toxin ingestion and uptake patterns

The time-averaged *P. lima* cell concentration in the feeding aquarium during the two week-exposure period was 131 cells mL^{-1} (SD = 90) (Fig. 2.3). Weight-specific ingestion rates (1 g wet wt.) of scallops remained relatively constant around a mean of 1.0×10^6 cells $d^{-1}g^{-1}$ wet wt. (SD = 2.5×10^5), thus the cumulative number of cells ingested increased linearly over the exposure period according to the equation: $I = 0.936 \times 10^6 t$, $r^2 = 0.99$, where I = number of cells ingested and t = exposure time (days), equation forced through zero (Fig. 2.4).

There were no apparent detrimental physiological responses in juvenile scallops caused by long-term exposure to toxigenic *P. lima*. Scallops appeared to be actively feeding at all times (i.e. - wide shell gape). Prolonged shell closure, shell clapping and violent swimming behaviour were not observed during the exposure period. Most of the scallops were byssally attached to the tank walls or bottom surface during the experiment. During periods when water in the tank was exchanged, the animals were often gently removed from the tank surface to facilitate the exchange process. Within a day most of the animals were observed to re-attach to the tank surface, indicating that byssus production and climbing behaviour were probably unaffected by exposure to *P. lima*. No mortalities occurred during the entire exposure period.

Concentrations of DSP toxins in *Prorocentrum lima* cells and scallop tissues reported in this section are results from LC-MS analysis. Total cellular toxin (DTX1 + OA + D8OA) content in *Prorocentrum lima* cells varied considerably during the exposure period (Fig. 2.5), ranging from 31.9 pg $cell^{-1}$ at the beginning of the exposure period to 2.4 pg $cell^{-1}$ on day 5. The mean total cellular toxin content was 9.8 pg $cell^{-1}$ (SD = 11.2), however the coefficient of variation for this value was 110%.

Series I scallops attained toxin saturation levels within 24 h of exposure, followed by relatively constant toxin levels for the remainder of the experiment (ca. 4-5 $\mu g\ g^{-1}$). Series II scallops (reverse time-series sampled at the end of the experiment) exhibited a similar temporal toxin pattern except that a peak in visceral toxin content (7.3 $\mu g\ g^{-1}$) occurred in scallops exposed to *P. lima* for 13 days. Mean total toxin content in Series I and II visceral tissues, averaged for all samples after the first day of exposure, was 4.39 $\mu g\ g^{-1}$

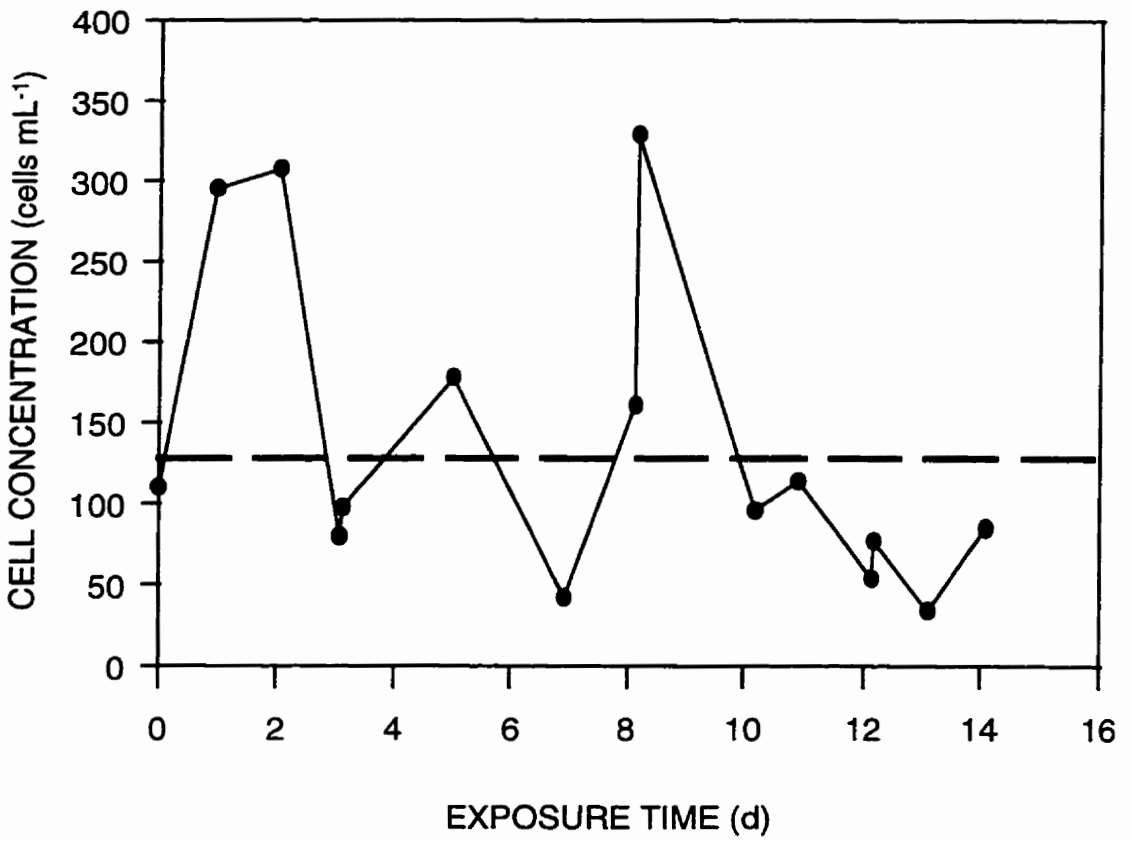


Fig. 2.3. Concentration of *Procentrum lima* cells in aquarium during exposure period. Dashed line indicates time-averaged cell density.

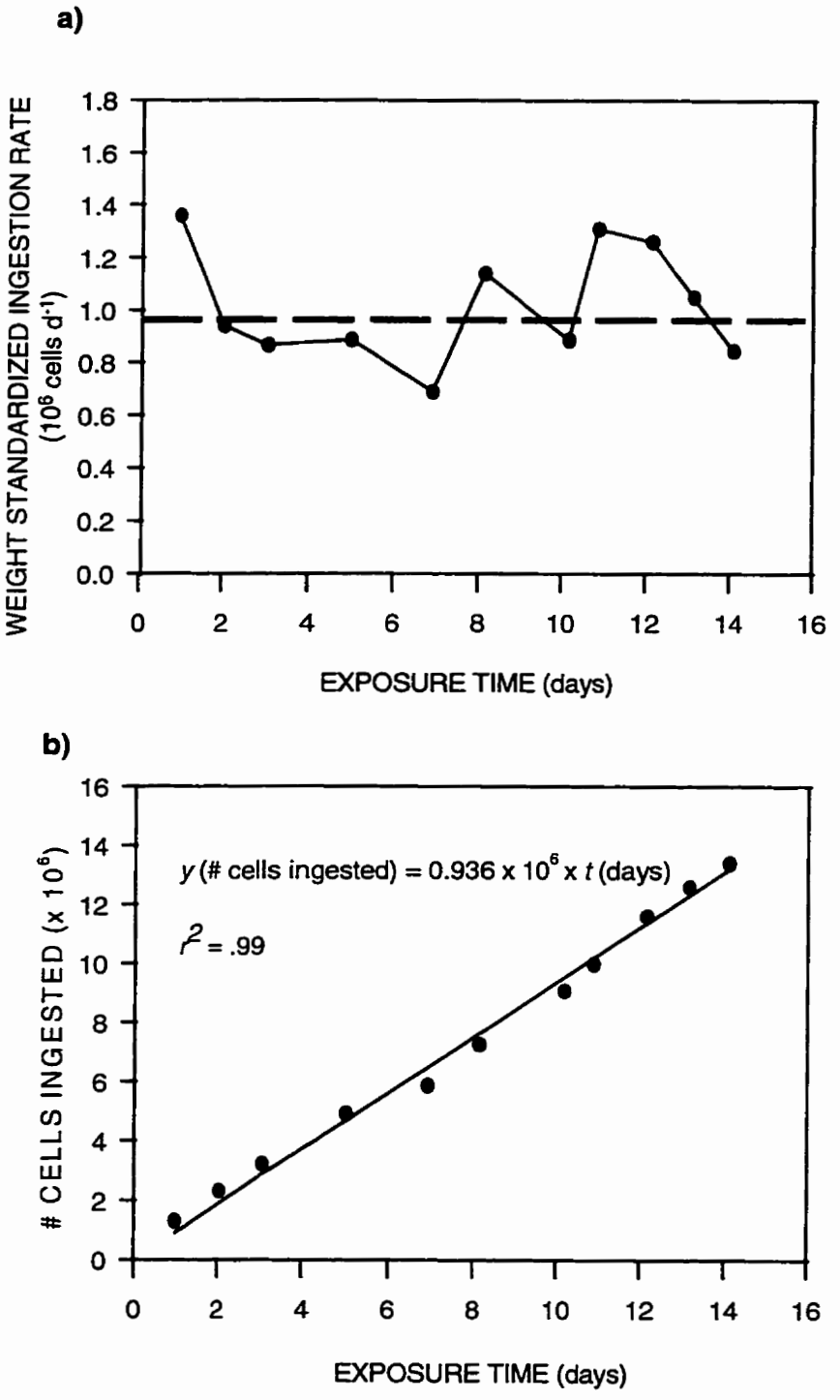


Fig. 2.4. a) Ingestion rates of *P. lima* cells by *Argopecten irradians* during exposure period. Dashed line indicates time-weighted mean. b) Cumulative number of cells ingested.

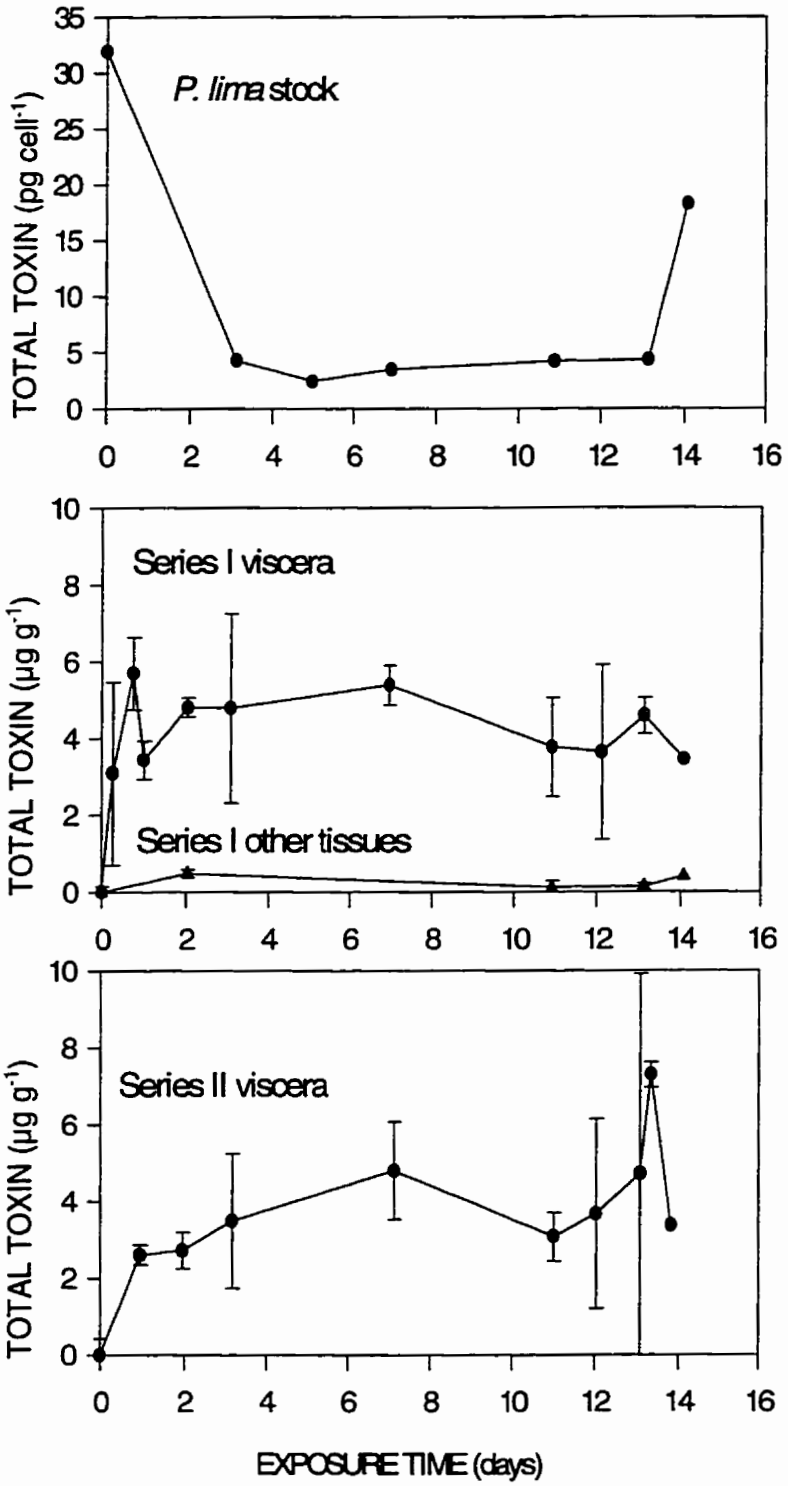


Fig. 2.5. Temporal pattern of DSP toxin content in *Prorocentrum lima* cells and scallop tissues during exposure period.

(SD = 0.86) and 3.97 μg g^{-1} (SD = 1.47), respectively. There was no significant difference between the toxin content of the two sets of visceral tissues (t-test, t = 0.734, df = 16, p > .05). In both series, toxin levels in viscera exceeded accepted regulatory limits (2 μg OA g^{-1} tissue) within one day of exposure. Remarkably, this limit was exceeded in Series I visceral tissue after only six hours of exposure to *P. lima*.

Toxin concentrations were far greater in scallop visceral tissue than in other tissues (gills, mantle, adductor) (Fig. 2.5). For this reason, the "other tissues" pool was analyzed less frequently than viscera for Series I scallops and not analyzed at all for Series II scallops. Similar to visceral tissues, other tissues attained maximum toxin content within one day of exposure, after which toxin content remained relatively constant. The mean total toxin content in Series I other tissues, averaged over the exposure period after one day of exposure to *P. lima*, was only 0.28 μg g^{-1} (SD = 0.19).

The mean tissue contribution to total wet weight in the scallops for viscera and all other tissues was 26.7% and 73.3% (SD = 4.5), respectively (Fig. 2.6). However, the contribution of these tissues to total toxin body burden was essentially reversed, with viscera and all other tissues comprising 85% and 15% (SD = 8.7), respectively.

Unlike the relationship describing the cumulative number of *P. lima* cells ingested, cumulative toxin ingested (CTI) was non-linear over the two week exposure period (Fig. 2.7). Due to the extremely high toxin content of *P. lima* cells at the beginning of the exposure period, CTI increased very rapidly during this period, followed by a relatively slower rate of toxin ingestion when the toxin content of *P. lima* cells decreased to lower levels. The temporal pattern of total toxin body burden was quite different from that of cumulative toxin ingested. As described previously for visceral tissues, total toxin body burden was saturated after one day of toxin exposure and remained relatively constant thereafter. This pattern is reflected in the calculated toxin accumulation efficiency (TAE) in scallops over the exposure period (Fig. 2.7). After six hours of exposure, only 10% of the ingested toxin was accounted for in scallop tissues. This value decreased rapidly to 2 to 3% after one day of exposure, followed by a gradual decrease to < 1% after two weeks of exposure.

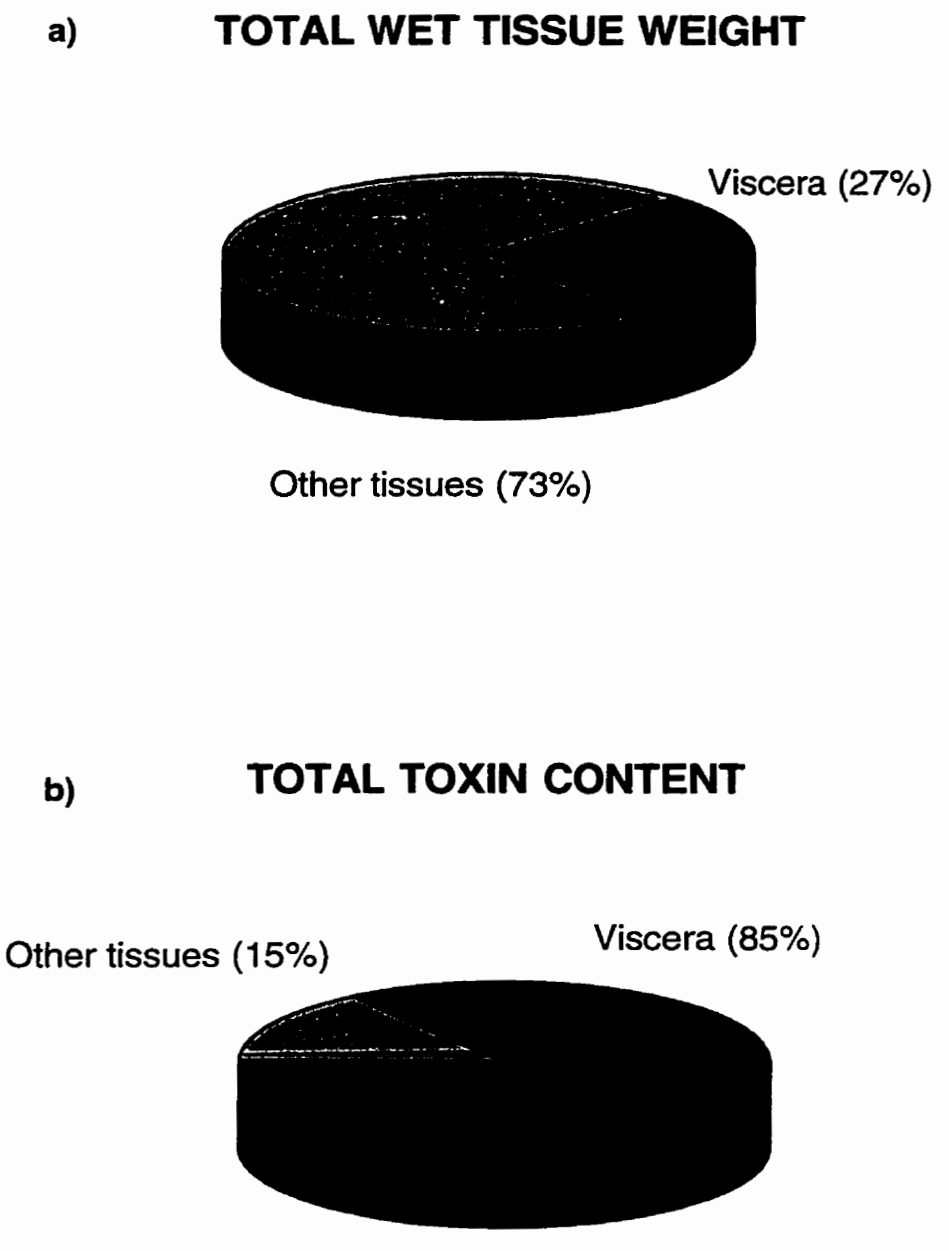


Fig. 2.6. Contributions (%) of juvenile scallop tissues to: a) total wet tissue weight and b) total toxin body burden. Values averaged for all samples after the first day of exposure. Viscera composed of digestive gland-stomach complex.

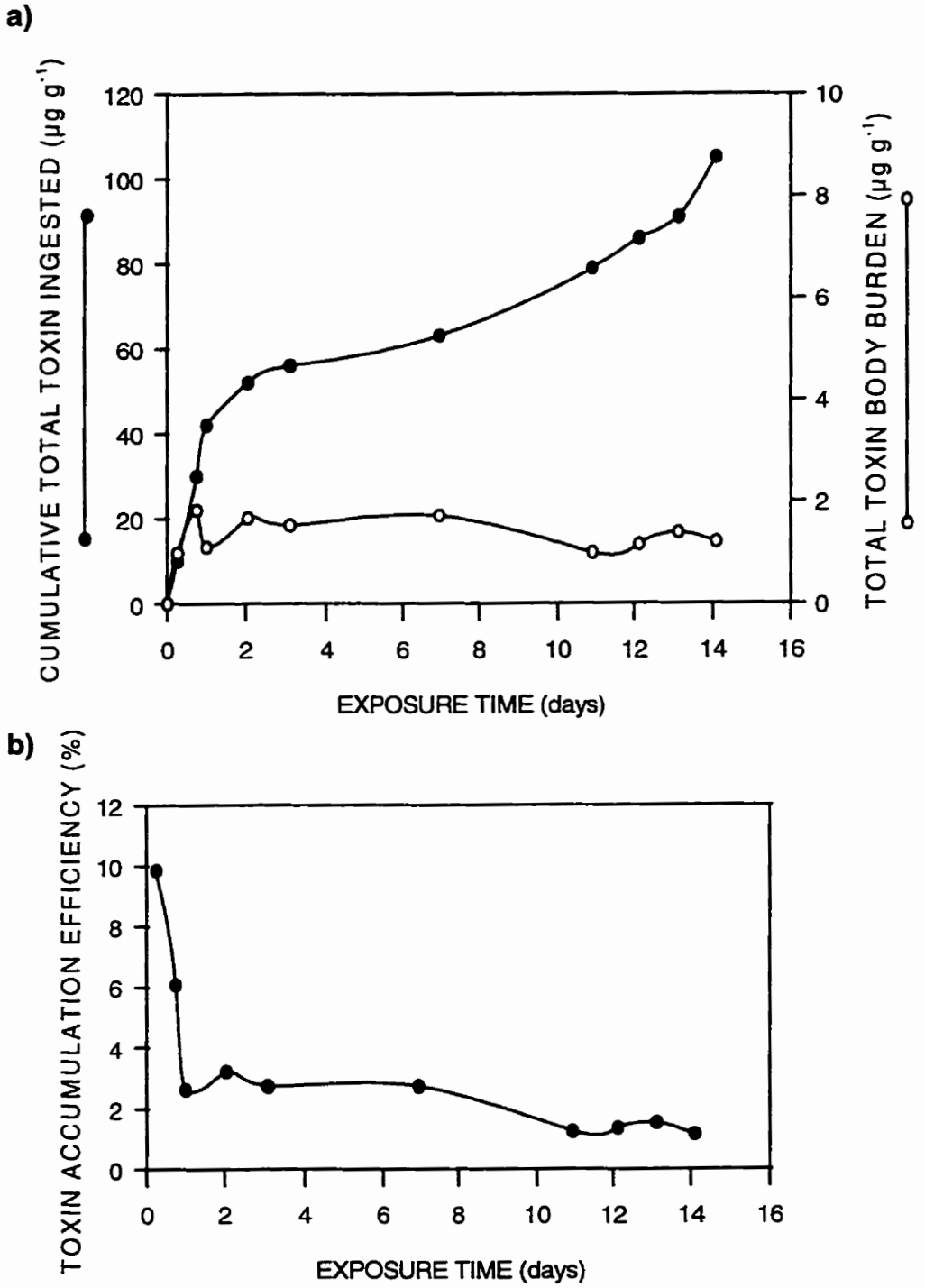


Fig. 2.7. a) Total DSP toxin ingested and accumulated in whole scallop tissues. b) Efficiency (%) of DSP toxin accumulation in whole scallop tissues. Data shown for Series I scallops only.

2.3.3. Toxin composition

Examination of the individual toxin composition in *P. lima* and scallop extracts was enabled by LC-MS analysis. Concentrations of the following compounds were quantified in extracts: Dinophysistoxin-1 (DTX1), okadaic acid (OA), okadaic acid diol-ester (D8OA), and the methyl esters of OA and DTX1 (Me-OA and Me-DTX1) (Fig. 1.3). Me-OA and Me-DTX1 have been found to be artifacts formed from D8OA and DTX1 via a methanolysis reaction during extraction of these toxins in methanol (N. Ross, pers. com., IMB, National Research Council, Halifax, NS), therefore Me-OA and Me-DTX1 concentrations were combined with the toxins from which they were derived, D8OA and DTX1, respectively. On a mean % molar basis, methyl esters comprised 4.9% (SD = 7.8) of total toxin content in *P. lima* extracts and 15.1% (SD = 6.7) in scallop tissue extracts. The higher proportion of methyl esters in tissue extracts is probably due to the fact that the tissues were extracted in 80% aqueous methanol, while *P. lima* cells were extracted in 100% methanol. This pattern is consistent with findings by Quilliam and Ross (1996) that methyl esters formation is more prevalent in the presence of aqueous methanol than in 100% methanol.

Concentrations of the individual toxins following conversion of methyl ester values back to D8OA and DTX1 in *P. lima* and scallop tissues are depicted in Fig. 2.8. All of the toxin concentrations determined in *P. lima* cell extracts were well above the detection limit of 0.01 pg $cell^{-1}$. The detection limit for the scallop tissue extracts was 30 ng g^{-1} tissue, which was greatly exceeded by all of the toxins, with the exception of DTX1 in Series I non-visceral tissues. In general, individual toxin temporal variations in scallop tissues seemed to parallel each other. A characteristic rank order of toxin concentration in scallop tissues became apparent: D8OA > OA > DTX1. However, this pattern was not consistent in *P. lima* cells.

Prorocentrum lima and scallop tissues were examined for temporal trends in individual toxin composition on a % molar basis (Fig. 2.9). Linear regression was used to determine whether the % molar contribution of each toxin increased or decreased over the two week exposure period (H_a: slope differs significantly from zero, $P < 0.01$). No significant trends were observed for toxin composition in *P. lima* cells. As was the case with total toxin

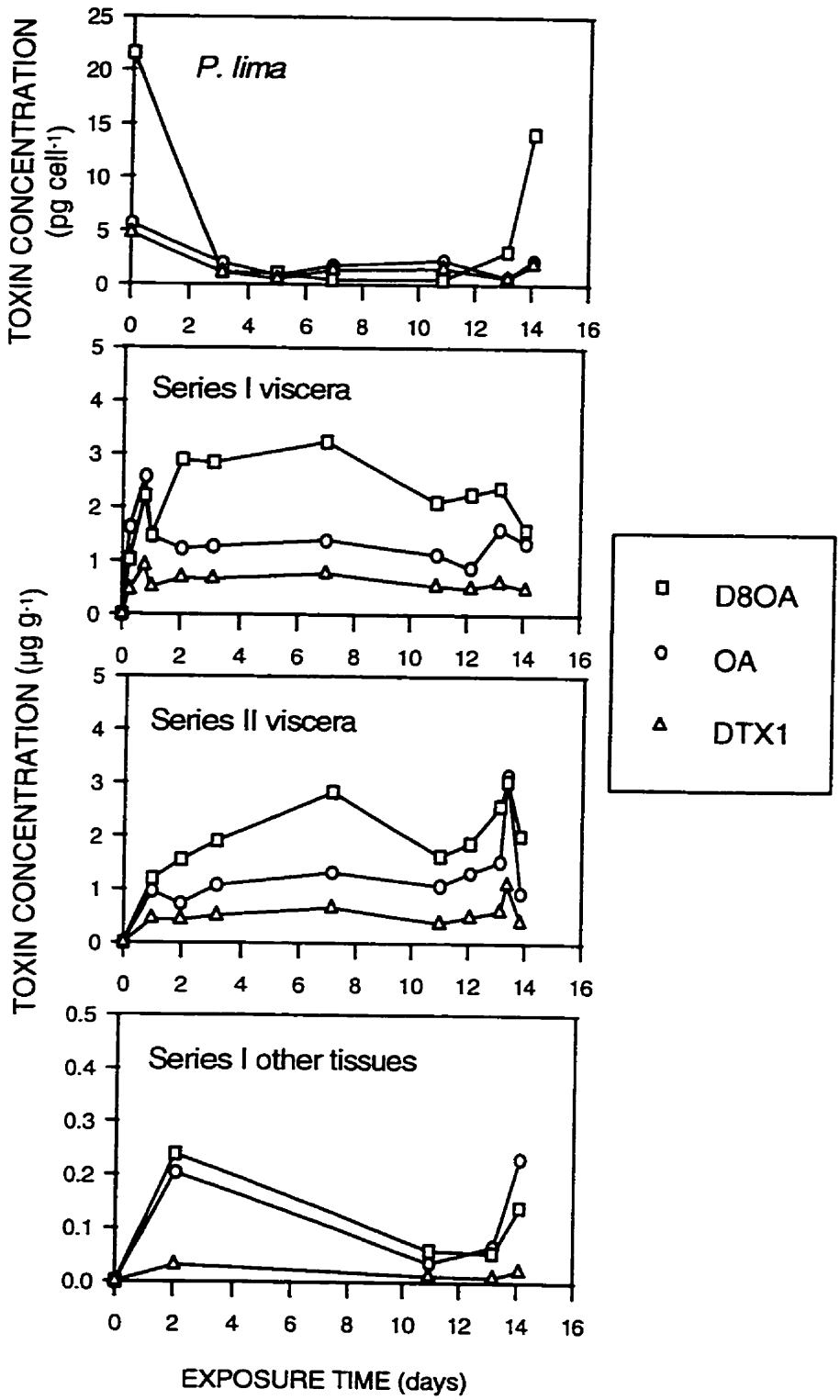


Fig. 2.8. Concentration of individual DSP toxins (see Fig. 1.1) in *P. lima* cells and scallop tissues.

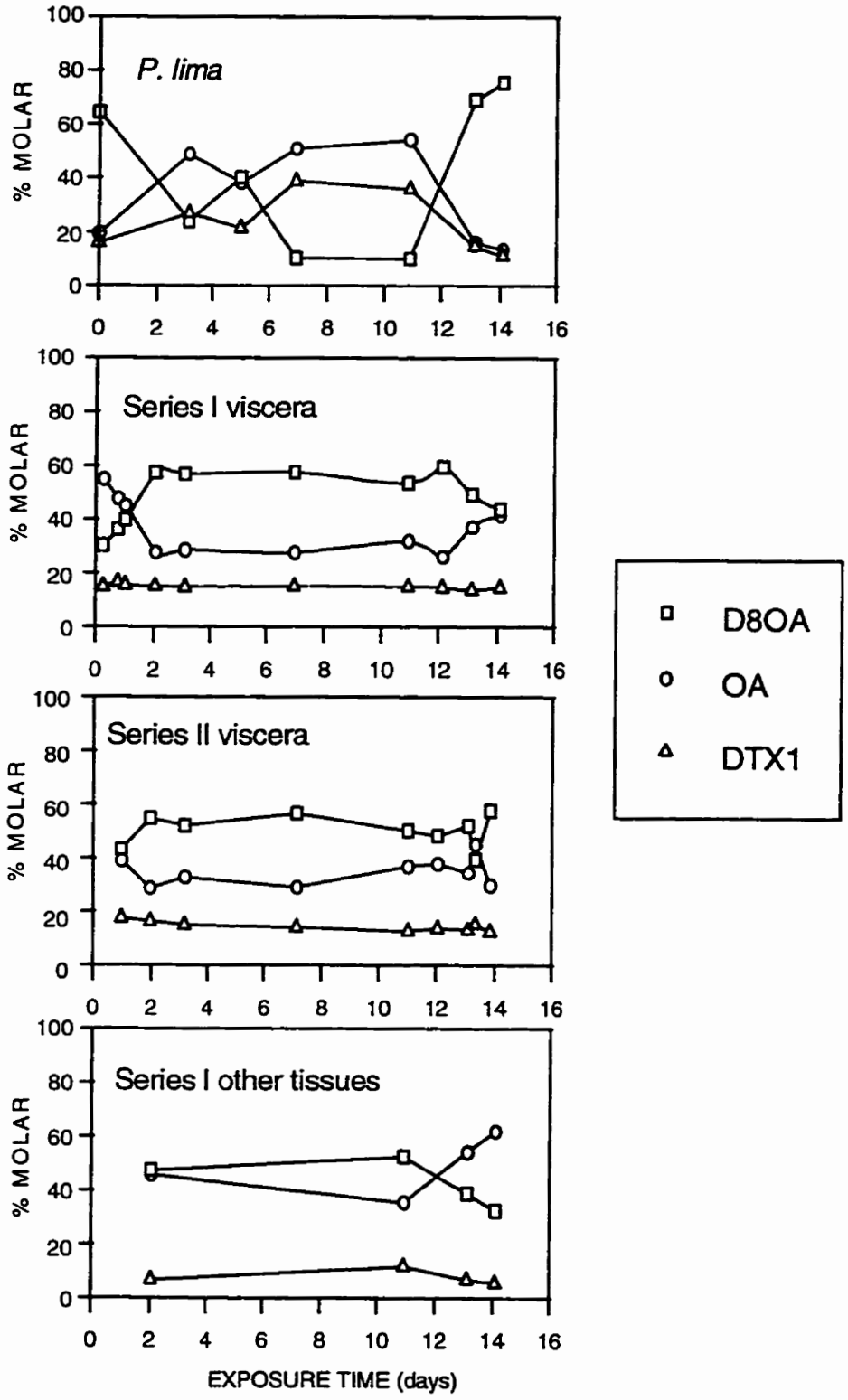


Fig. 2.9. Relative (% molar) toxin composition in *P. lima* cells and scallop tissues.

content, toxin composition fluctuated significantly over the experiment. In *P. lima* cells, the % molar contribution of DTX1 remained relatively constant (mean = 23.6%, SD = 10.8), however D8OA and its hydrolysis end-product, OA, cycled inversely relative to each other throughout the time-course (mean D8OA = 41.9%, SD = 28.1; mean OA = 34.5 %, SD = 17.8). The toxin composition in scallop tissues was much more consistent over the exposure period. There were no significant temporal trends in % molar content for any of the toxins in the scallop tissues. The % molar contribution of DTX1 in scallop tissues was constant over time, whereas slight fluctuations in D8OA and OA content occurred at the beginning and end of the exposure period, coinciding with peaks in the D8OA and total toxin content in ingested *P. lima* cells (Fig. 2.8).

The relative % molar content of each toxin, averaged over the experimental period, was compared between ingested *P. lima* cells and scallop tissues using Kruskal-Wallis one-way ANOVA for sample sets with unequal variances coupled with SNK multiple pairwise comparisins ($P < 0.05$) (Fig. 2.10, Table 2.1). There were no significant differences between the % molar toxin content of *P. lima* and scallop tissues for D8OA or OA, however DTX1 content was determined to be significantly higher in *P. lima* cells than in non-visceral tissues (Dunn's multiple comparison, $H = 12.7$, $df = 3$, $P = 0.005$). Mean arc-sine transformed levels of individual toxin levels in scallop tissues were tested for significant differences using a one-way ANOVA coupled with Student-Newman-Keul's method for pairwise multiple comparisons ($P < 0.05$) (Fig. 2.10, Table 2.1). DTX1 was significantly higher in both series of visceral tissues than in other tissues ($F = 32.6$, $df = 2$, $P < 0.0001$). Although D8OA content was slightly greater in visceral tissue than in other tissues, this difference was not significant; however OA, the hydrolytic end-product of D8OA, was significantly higher in non-visceral tissues ($F = 3.99$, $df = 2$, $P = 0.035$).

Visceral tissue and *P. lima* cells share a very similar toxin profile, which may be attributed to toxin derived from undigested cells in the scallop digestive system. However, non-visceral tissues have relatively less DTX1 and more OA than visceral tissue. It is interesting to note that both series of visceral tissue had extremely similar toxin profiles.

To test whether D8OA was being converted to OA in scallop tissues, the ratio of OA to D8OA was examined in ingested cells and in each of the tissue compartments. An

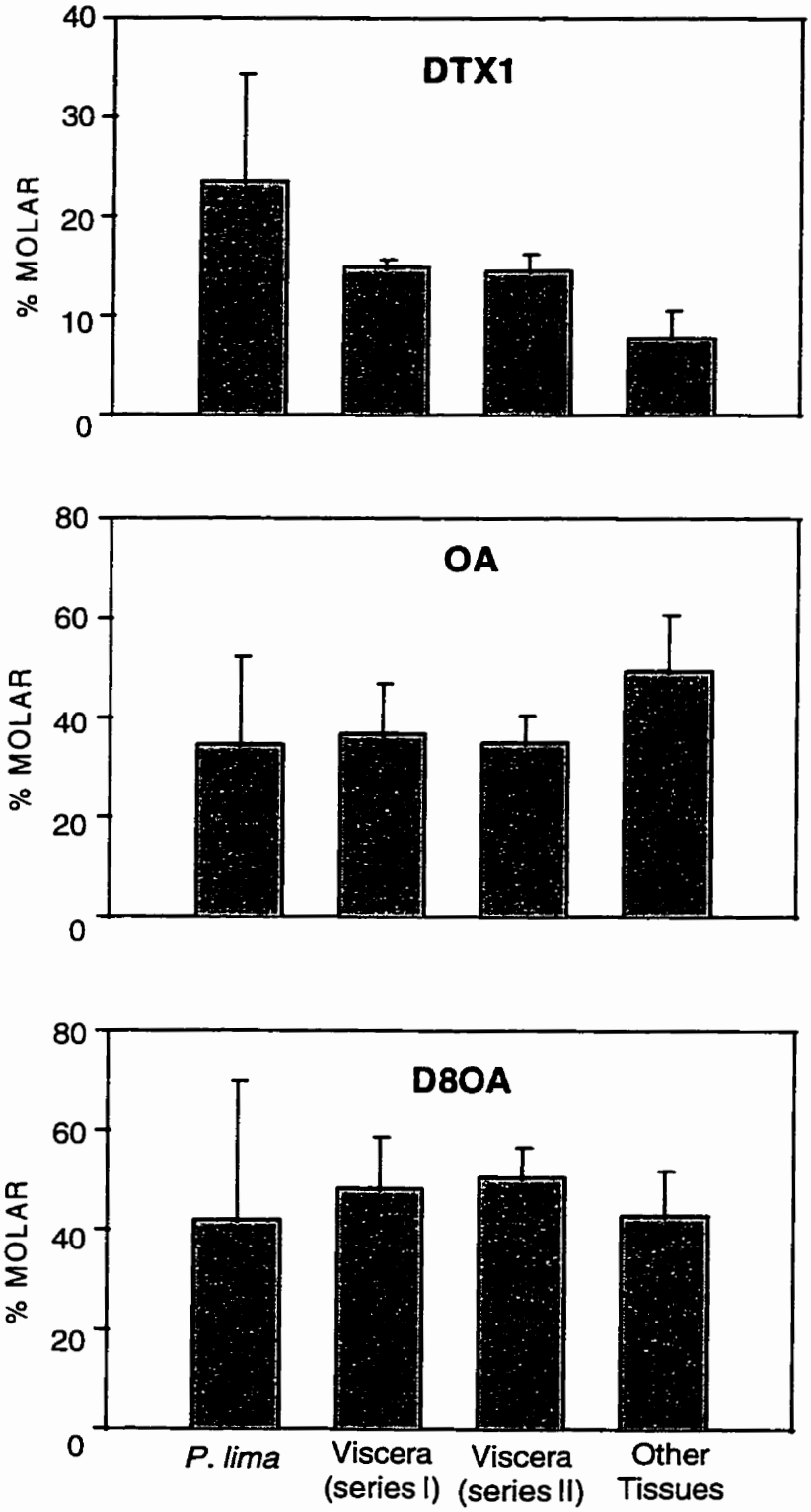


Fig. 2.10. Mean (% molar) levels of individual DSP toxins in *P. lima* cells and scallop tissues during exposure period. Error bars = 1 standard deviation.

Table 2.1. Mean % molar toxin content (SD) of DSP toxins in ingested *Prorocentrum lima* cells and in scallop tissues. Means not connected by a line are significantly different at $P < 0.05$ (one-way ANOVA, SNK multiple pairwise comparisons).

Toxin	*P. lima*	Series I Viscera	Series II Viscera	Other Tissues
DTX1	23.6 (10.7)	14. 9 (0.7)	14.6 (1.7)	7.8 (2.7)
OA	34.5 (17.8)	36.7 (10.0)	35.0 (5.4)	49.3 (11.3)
D8OA	41.9 (28.1)	48.4 (10.3)	50.5 (6.0)	42.9 (8.9)

increase in the OA/D8OA ratio, over time or between compartments, would indicate that either D8OA was converted to OA or possibly, that OA was selectively retained over D8OA in scallop tissues. Linear regression analysis was used to determine whether the OA/D8OA ratio changed over time in either *P. lima* cells or in scallop tissues. No significant temporal trends in this ratio were detected ($P > 0.05$) in any of the compartments, although variations in OA/D8OA were apparent between compartments (Fig. 2.11). There was no consistency in the OA/D8OA ratio in *P. lima* cells over time. During the beginning and end of the experimental period when total toxin content was very high, the ratio was low (<1), however when the total toxin content was low, the ratio was relatively high. Overall, the time-averaged OA/D8OA ratio in *P. lima* cells was 2.0 (SD = 2.3), however the coefficient of variation (CV) was > 100%.

In scallop tissues, the ratio of OA to D8OA was more constant. In visceral tissue, the ratio dropped within the first two days of exposure to *P. lima* and remained relatively constant thereafter, however, the OA/D8OA ratio increased during the last few days of the experiment in the remaining tissues. The mean OA/D8OA ratio, averaged over the entire exposure period, was 0.79 (SD = 0.30) and 1.24 (SD = 0.53) for viscera and non-viscera tissues, respectively. There was no significant difference in the average OA/D8OA ratio between ingested *P. lima* cells and scallop visceral tissues (ANOVA on arc-sine transformed values, $P > 0.05$).

Linear regression analysis was used to examine the relationship between okadaic acid derivatives (OA, D8OA) and DTX1 in *P. lima* cells and in scallop tissues (viscera and other tissues combined) (Fig. 2.12). The linear relationship of DTX1 to OA and D8OA was significant for both algae and tissues ($P < 0.05$). In *P. lima* cells the slope was greater for D8OA than OA, while in scallop tissues the slopes were roughly equal.

2.3.4. Comparison of toxin analysis by HPLC-FD and LC-MS

Prorocentrum lima samples and Series I visceral tissue were analyzed by HPLC-FD (Fig. 2.13), as well as LC-MS (Fig. 2.14), enabling a comparison of results obtained by the two methods (paired t-test, $P < 0.05$). Mean OA and DTX1 time-series values for the two methods (Fig. 2.15), indicated that OA values obtained by HPLC-FD were significantly greater than those obtained by LC-MS, in both *P. lima* cells (df = 6, $P = 0.019$) and

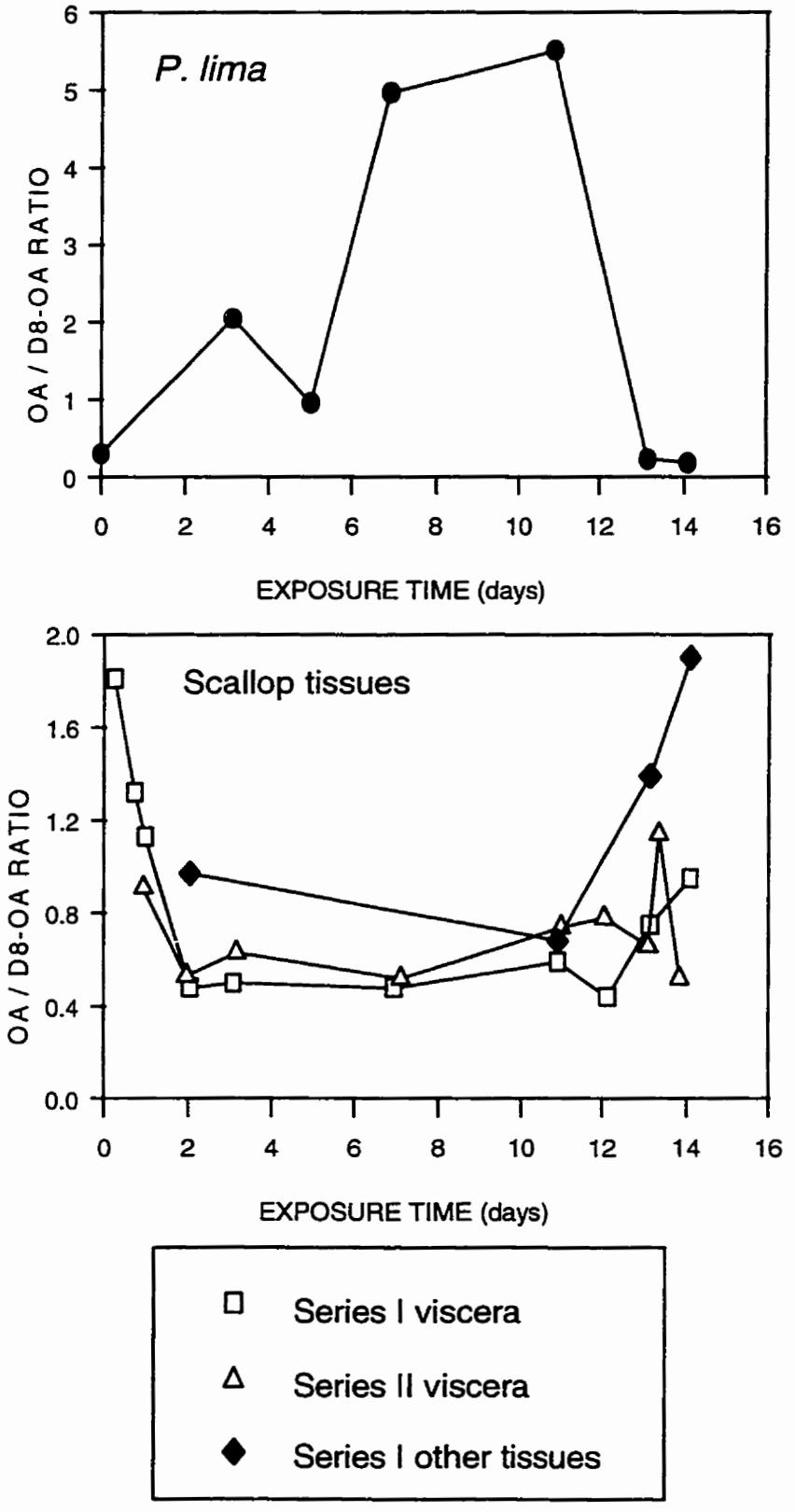


Fig. 2.11. Temporal pattern of OA/D8-OA ratio in *P. lima* cells and scallop tissues.

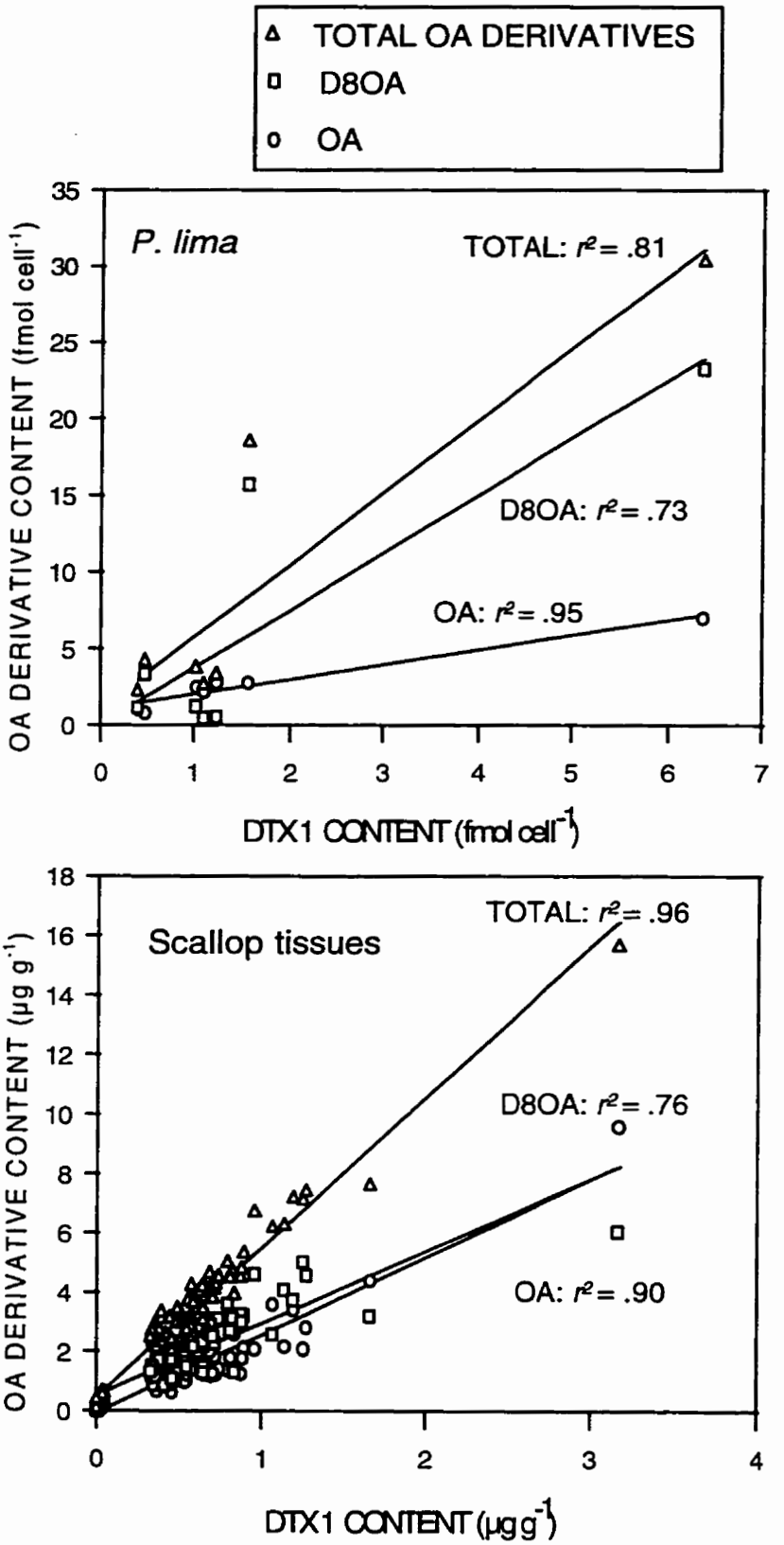


Fig. 2.12. Relationship between the concentration of DTX1 and okadaic acid derivatives in a) *P. lima* cells and b) scallop tissues.

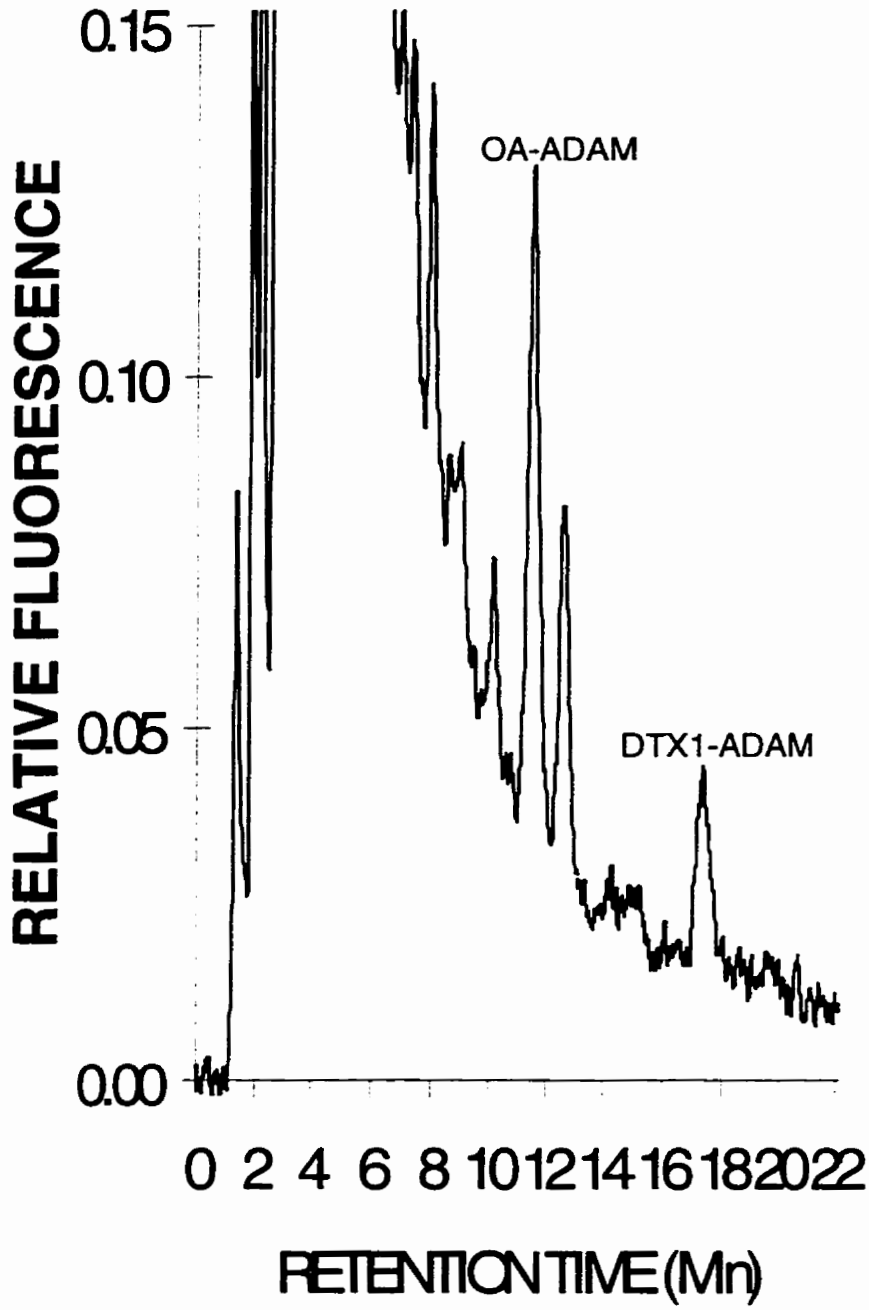


Fig. 2.13. HPLC-FD analysis of bay scallop viscera extract derivatized with ADAM.

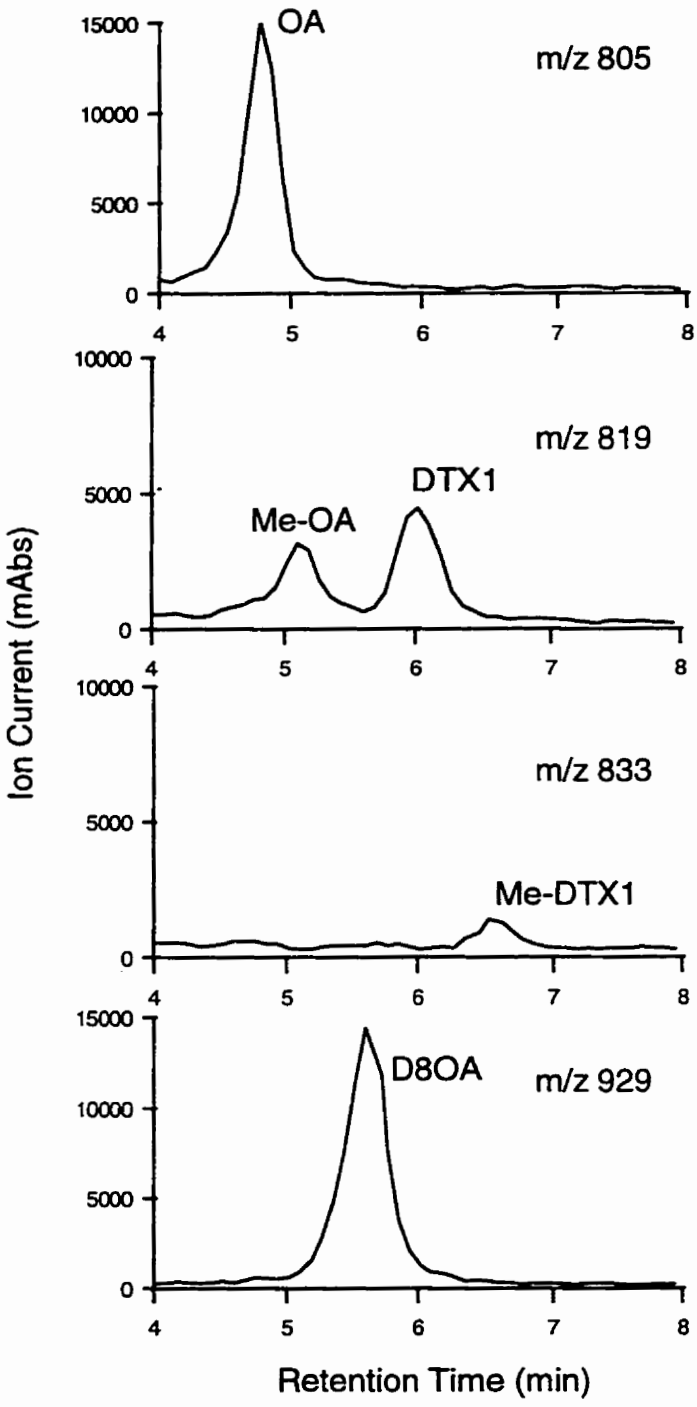


Fig. 2.14 Positive ion-spray LC-MS analysis of bay scallop viscera extract.

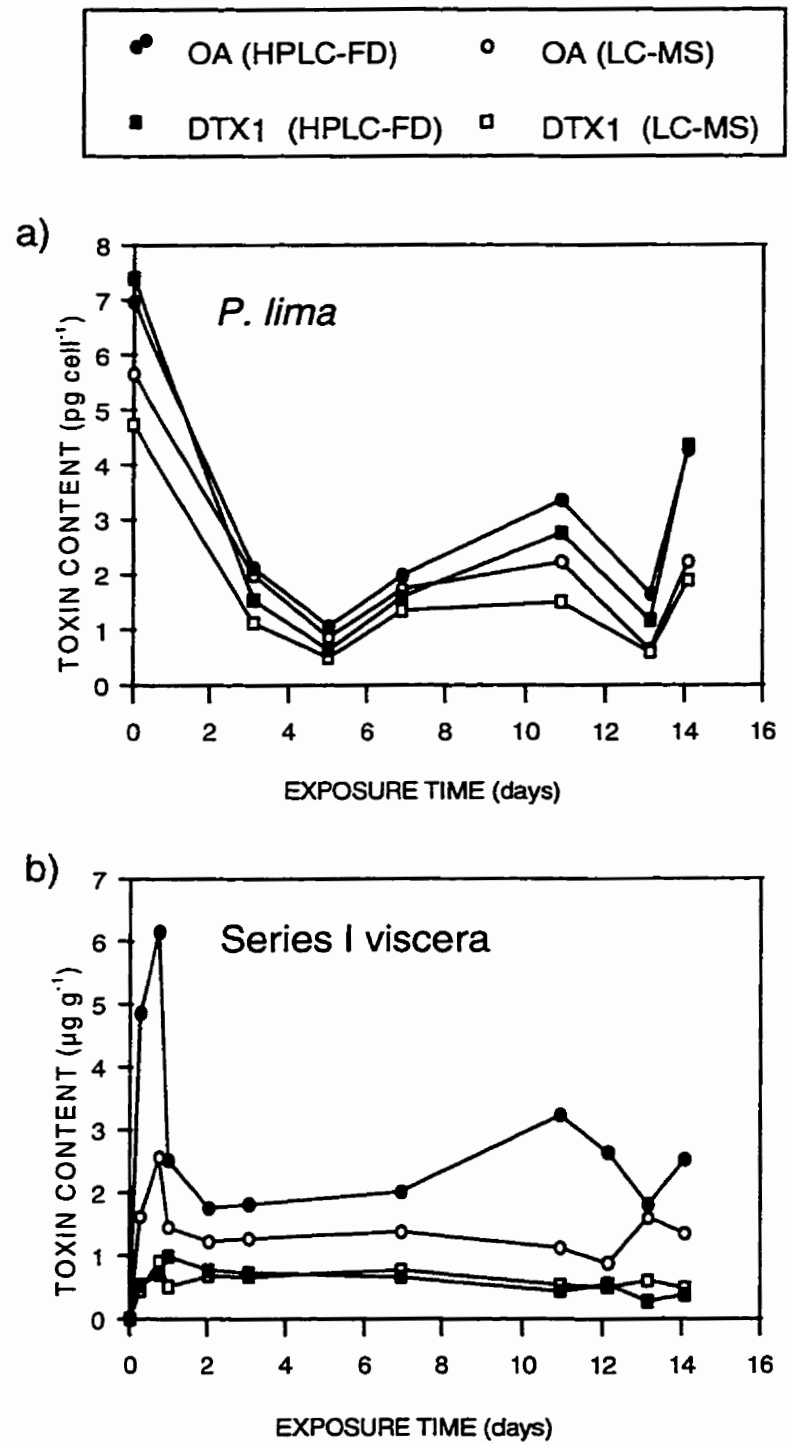


Fig. 2.15. Mean OA and DTX1 content in a) *P. lima* cells and b) scallop visceral tissue, as determined by HPLC-FD and LC-MS analyses.

scallop viscera (df = 31, $P < 0.0001$). Values for DTX1 determined by HPLC-FD were significantly greater in *P. lima* samples ($P = 0.032$), however there was no significant difference for viscera samples. When total DSP toxin results were compared for HPLC-FD (OA + DTX1) and LC-MS (D8OA + OA + DTX1) samples (Fig. 2.16), there was no significant difference in *P. lima* samples and only a slightly significant difference in viscera samples ($P = 0.045$). The reduction in differences among the two methods when D8OA was added to LC-MS values suggests that the high OA values in the HPLC-FD analysis may be partly due to hydrolytic conversion of D8OA to OA during ADAM derivatization. Linear regression analysis indicated that HPLC-FD and LC-MS values for *P. lima* samples were highly correlated for DTX1, OA and total toxin ($P < 0.01$), however a linear correlation was only significant for OA in scallop viscera (Fig. 2.17).

2.4. Discussion

Feeding and survival

When exposed to high concentrations (10^5 cells L^{-1}) of DSP-toxigenic *Prorocentrum lima*, juvenile bay scallops were able to ingest the cells without any apparent adverse physiological effects. Unlike previous studies which reported mortalities of juvenile *Argopecten irradians* when exposed to the dinoflagellates *Gyrodinium aureolum* (Lesser and Shumway, 1993) and *Prorocentrum mininum* (Wikfors and Smolowitz, 1993), no mortalities were experienced when bay scallops were exposed to *P. lima* in the present study. The high survival rate over the two-week study without reduction in tissue weight suggests that the scallops were able to meet their maintenance requirements by feeding on *P. lima* cells. This point was further strengthened by the similar clearance rates of scallops exposed to either *P. lima* or the diatom *Thalassiosira weissflogii*, a proven source of suitable nutrition for *A. irradians* (Bricelj and Kuenster, 1989). An indirect, yet excellent indication from this study that juvenile bay scallops may be capable of surviving prolonged exposure to DSP-toxigenic *P. lima* was the persistent climbing behaviour that scallops exhibited in the aquarium, similar to that of natural populations in seagrass beds. Climbing behaviour and the continued production of byssal threads require metabolic energy; this

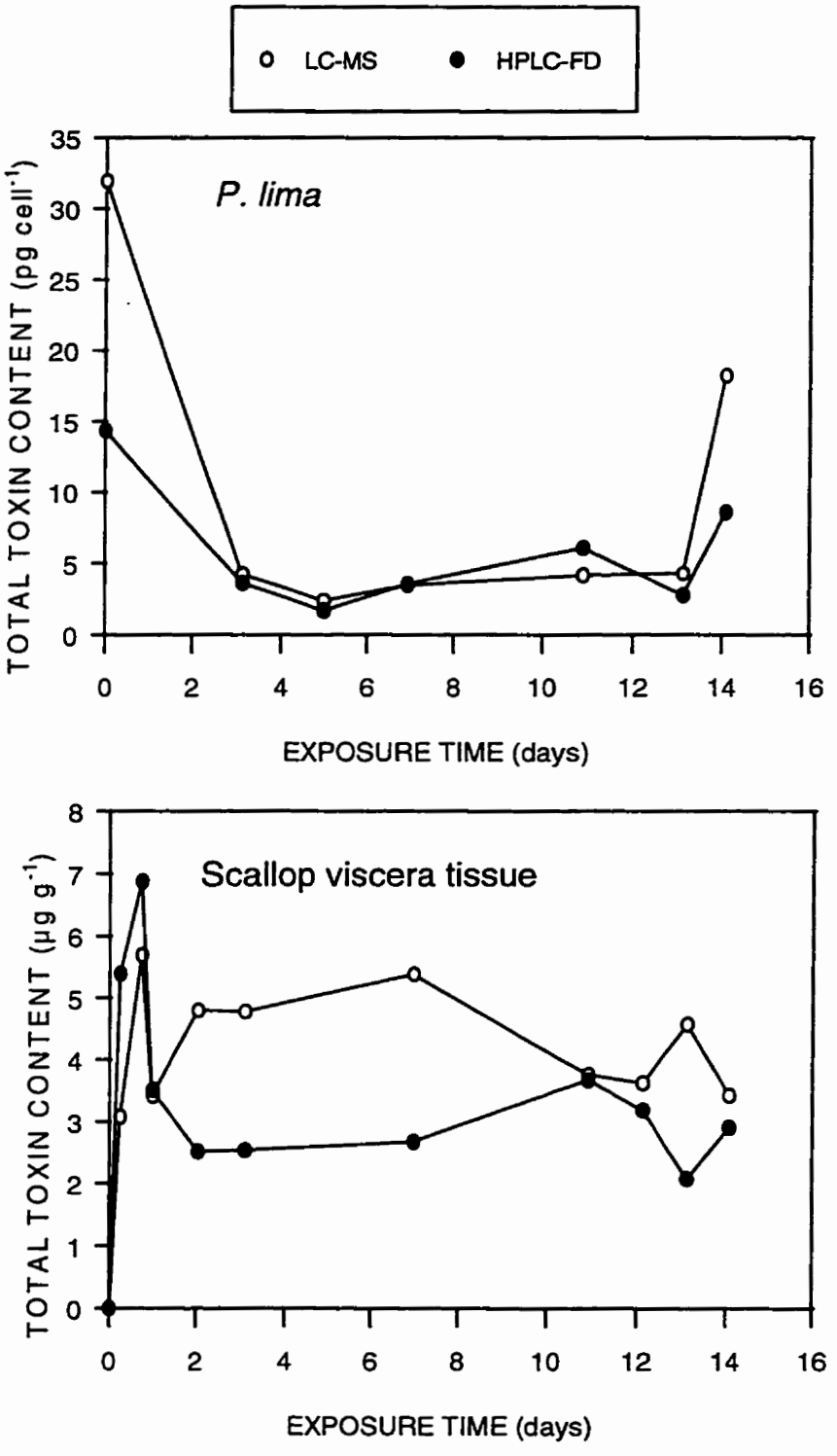


Fig. 2.16. Total DSP toxin content in a) *P. lima* cells and b) scallop visceral tissue as determined by HPLC-FD (OA + DTX1) and LC-MS (D8OA + OA +DTX1).

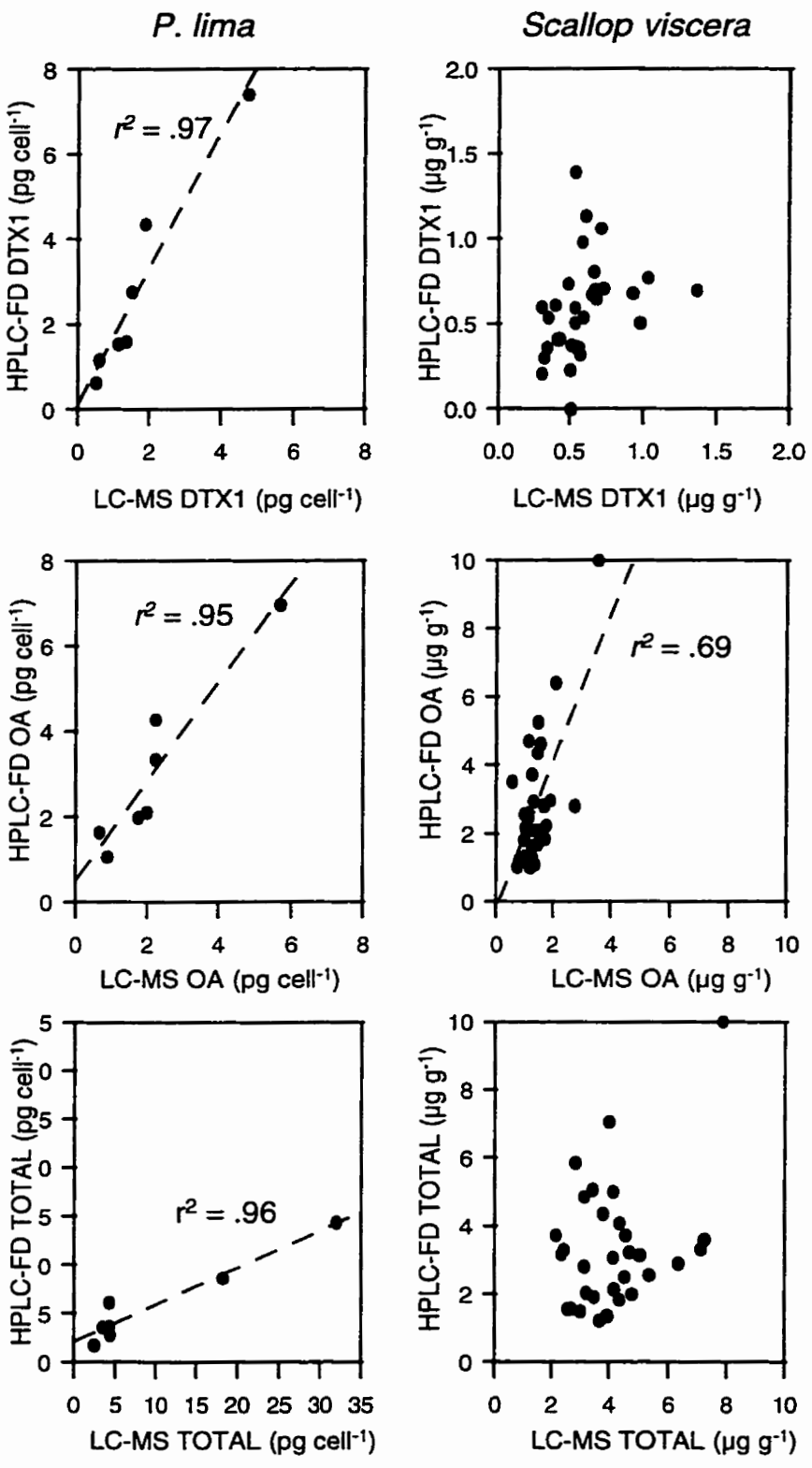


Fig. 2.17. Comparison between values HPLC-FD and LC-MS values for DSP toxin content in *P. lima* cells and scallop tissues. Regression lines are only shown for significant correlations ($P < 0.05$, Pearson product moment correlation, r).

demand would be fulfilled only if basic maintenance requirements were first being met by a food source or the utilization of endogenous reserves.

Toxin uptake and accumulation

The faster rate of toxin uptake in Series I scallops was a result of exposure to a very highly toxic culture of *P. lima* during the first three days of the experiment. Attempts were made to avoid such variations in the cellular toxin content of *P. lima* by growing batch cultures in staggered sequence under identical growth conditions. Extreme variations in the toxin content of *P. lima* have also been reported in ecophysiological studies of this dinoflagellate by McLachlan et al. (1994) and by Morton et al. (1994). Both of these studies attributed high toxin levels to cells which have been forced into stationary growth phase by growth limited conditions such as low temperature or depleted nutrients. Following the same argument, the highly toxic batch of *P. lima* fed to scallops at the beginning of the experiment may have entered stationary growth phase much earlier than the other batches due to an unusually high growth rate and early depletion of nutrients.

Although unintended, the extreme variation in *P. lima* toxin content during the feeding study provided an indication of how bay scallops respond when exposed to varying levels of DSP toxin content in dinoflagellates. Relatively constant ingestion rates throughout the study and similar temporal profiles for Series I and II scallops demonstrated that cellular toxin content has very little effect on the feeding parameters of bay scallops. The temporal profile of Series I scallops indicated that peak toxin loads were attained within the first day of exposure to *P. lima*, after which no more toxin was retained, suggesting that tissues had become saturated with DSP toxins or that scallops had optimized their digestive processes to maximize toxin elimination. In Series II animals, peak toxin loads were recorded in scallops exposed to *P. lima* for 14 days, however it must be remembered that the Series II profile represents a reverse time-series. Thus, scallops with the highest toxin loads were initially exposed to the highly toxic batch of *P. lima* fed to scallops at the beginning of the experiment. This evidence demonstrates that bay scallops only retained DSP toxins from *P. lima* cells ingested at the beginning of the exposure period, after which ingested DSP toxins were efficiently eliminated from the animals, preventing further accumulation of toxins in the tissues.

The efficiency of DSP toxin accumulation in bay scallop tissues was less than 1% after two weeks of exposure. Thus, juvenile bay scallops readily ingested *P. lima* cells for two weeks but retained very little of the DSP toxins associated with the cells after the first day of exposure. Furthermore, since most of the toxin load was associated with visceral tissue, it appears that the great majority of the toxin load associated with the scallop tissues remains a labile (unbound) component within the gut, rather than being transferred and sequestered from the viscera to other tissues.

Toxin composition

The extreme variations in the toxin composition of *Prorocentrum lima* cells depicted in this study were consistent with similar observations made by Quilliam and Ross (1996), who attributed the fluctuations to enzyme-mediated toxin conversion processes that operate when the cells are disrupted during extraction procedures. These researchers reported that following extraction of cell pellets in methanol, the main cellular DSP toxin component, DTX4, is rapidly converted to D8OA, which in turn is converted to OA and Me-OA at a much slower rate. To alleviate this problem, Quilliam et al. (1996) found that immersion of the pellet in boiling water prior to extraction in methanol prevented the enzymatic conversion reactions from occurring. This information did not become available until after the present study was completed. Apparent fluctuations in the OA and D8OA content of *P. lima* cells fed to scallops were likely due to enzymatic conversions of D8OA to OA during extraction of frozen tissues in methanol. The degree to which the conversions were completed would depend on the length of time that the frozen pellets were allowed to thaw prior to extraction in methanol. The relatively low level of variation in cellular DTX1 content during the feeding study agrees with the findings of Quilliam and Ross (1996) and suggests that this toxin component is not directly linked to the DTX4 - D8OA - OA enzymatic pathway.

In scallop tissues, the relative amounts of each toxin component were much more consistent than in the dinoflagellate cells. This may be due to both physical and chemical digestive processes within the scallop gut which would disrupt *P. lima* cells sufficiently to facilitate the same enzymatic reactions that occurred during the extraction of cell pellets. Since the bulk of the conversion reactions had probably already taken place after ingestion

of the cells by the scallops, extraction procedures would have comparably less effect on the determined toxin composition.

The gradual decrease in the relative OA content and concomittent increase in D8OA depicted in visceral tissue (especially Series I) during the first two days of the study suggested that the amount of cell disruption was decreasing during this period, resulting in less D8OA being converted to OA. This evidence may indicate feeding acclimation by the scallop resulting in digestion of *P. lima* cells following initial exposure. As was the case with *P. lima* extracts, the relative DTX1 content in scallop tissues remained remarkably stable during the feeding study, indicating that toxin conversion pathways between DTX1 and OA-derivatives are unlikely to exist within scallop tissues.

Toxin profile comparisons showed a progressive drop in the % molar composition of DTX1 from *P. lima* cells (24%) to viscera (15%), and subsequently to non-visceral tissues (8%). This evidence suggests that DTX1 may be eliminated faster than OA-derivatives from scallops following ingestion of *P. lima* cells. Since OA and DTX1 have very similar chemical structures, it seems unlikely that OA would be more efficiently bound to scallop tissues than DTX1. However, it is possible that the scallops may have selectively eliminated DTX1 over OA in order to reduce exposure to DTX1, which has been shown to have a slightly greater cytotoxic potency than OA (Hamano et al., 1985).

Analytical methodology

Comparisons of toxin analysis results obtained by HPLC-FD and LC-MS revealed that significant discrepancies existed, especially when individual toxin components were examined. In both *P. lima* cell extracts and scallop viscera, OA levels were consistently higher when analyzed by HPLC-FD. However, when total toxin was compared, including D8OA, LC-MS results were much closer to those of HPLC-FD. Since the fluorescent reagent (ADAM) used to detect toxin in the HPLC-FD technique cannot bind to OA-esters due to the absence of a free carboxylic acid group, this evidence suggests that some of the D8OA component may have been hydrolyzed to OA during the ADAM derivatization procedure. Since DTX1 does not appear to be undergo such hydrolytic pathways, this would explain the relatively similar results obtained by the two techniques for this toxin.

Overall, HPLC-FD and LC-MS results were fairly well correlated for *P. lima* extracts, whereas correlations for scallop viscera samples were poor. Failure to fully eliminate lipids from tissue samples during the clean-up step of the extraction procedure may have resulted in the presence of compounds which interfered with ADAM derivatization and thus, contributed to the high variability in the viscera HPLC-FD analysis. Such inconsistencies with the relatively tedious and labour intensive HPLC-FD procedure agreed with observations made by Cembella and Stabell (1990) who reported that comparisons of results obtained by this method were discouraging, mainly due to complications associated with the ADAM-derivatization step. Although Pleasance et al. (1990) found good correlations between HPLC-FD and LC-MS results, they also noted complications associated with impurities and ADAM derivatives in samples analyzed by HPLC-FD.

For research purposes it is obvious that the shorter extraction procedure, higher sensitivity, better accuracy and ability to analyze OA-ester components prove LC-MS to be a superior technique for the analysis of DSP toxins. However, since ion-spray mass spectrometry is a luxury which few regulatory authorities can afford, HPLC-FD will continue to be used as a more accurate detection method than mouse bioassays which have been notoriously inconsistent for DSP toxin detection (Lee et al., 1987; Andersen, 1996; Quilliam, 1995). The results of the present study suggest that HPLC-FD results for DSP toxin analysis should be interpreted carefully, especially with regard to OA-esters that are not detected by this method. Esters derivatives of OA represent a hidden, yet potential group of toxins which can be readily hydrolyzed to yield the active toxin OA. A suggested way to alleviate this problem would be to hydrolyze the OA-esters in shellfish tissue to OA prior to HPLC-FD analysis. This could be accomplished by subjecting the sample to a freeze-thaw process as Quilliam et al. (1996) suggest, or perhaps, by simply adding a *P. lima* esterase solution to shellfish homogenates.

Chapter 3. DSP toxin uptake, compartmentalization and detoxification in adult bay scallops.

3.1. Introduction

The results of the previous chapter demonstrated that juvenile bay scallops will ingest DSP-toxigenic *Prorocentrum lima* cells and accumulate low levels of toxins without any apparent physiological impairments. However, to gain a more complete understanding of the fate of ingested DSP toxins, post-ingestive feeding processes must be investigated in addition to clearance rates. Several feeding studies have revealed that bivalve molluscs will compensate for changes in food quality and quantity by adjusting digestive processes, thus influencing the food ration ultimately absorbed (Bayne, 1985; Willows, 1992; Bayne et al., 1984). Shumway et al. (1985) showed that although the oyster, *Ostrea edulis*, ingested *Prorocentrum minimum* (non-toxic) cells at relatively high rates, an abundance of intact cells in fecal ribbons indicated that the cells were rejected from the digestive system via post-ingestive sorting mechanisms. A focal point of the present chapter is the fate of *P. lima* cells and associated DSP toxin components following ingestion by *Argopecten irradians*. Physiological parameters including clearance rates (Hildreth and Crisp, 1976) and absorption efficiencies (Conover, 1966) were determined using individual continuous-flow feeding chambers (Bricelj and Malouf, 1984) which enable a more precise measurement of feeding rates than the static chambers used in the previous chapter

The possibility of toxigenic dinoflagellate cells surviving passage through bivalve digestive systems is an area of special concern for shellfish growers. Shellfish stocks may be transferred from areas where toxic dinoflagellates occur to "clean" waters to depurate (Silvert and Cembella, 1995). If live dinoflagellate cells are released into clean waters via fecal deposition from newly transferred toxic shellfish, they may divide and form blooms in the new region. Studies have indicated that such a scenario is quite possible for PSP-toxin producing *Alexandrium tamarense* cells ingested by *Mytilus edulis* (Bricelj et al., 1993; Scarratt et al., 1993), however no studies have examined whether DSP-toxin producing dinoflagellates can survive gut passage. An objective of the work presented in the present

chapter was to investigate the likelihood of survival of *P. lima* cells following ingestion by *A. irradians*.

Toxin kinetics were investigated using techniques similar to those indicated in the previous chapter, however pre-reproductive (gonads not fully mature) adult scallops were used rather than juveniles. New markets for scallops are encouraging the sale of "half-shell" (whole animal) and "roe-on" (gonad attached to adductor) products rather than just the adductor muscle (Cembella et al., 1994), which is traditionally the only part of the scallop consumed in North America. Due to the relatively small adductor muscle, interest in marketing gonads and other tissues of bay scallops is especially strong (Couturier, 1990; Scarratt, 1992). Thus, knowledge of phycotoxin partitioning amongst bay scallop tissue is important for industry consideration. Another important issue of concern to industry is the rate at which phycotoxins are depurated from contaminated shellfish stocks. Data from previous studies suggest that sea scallops (*Placopecten magellanicus*) are prone to retain PSP toxins for extended periods of time (Shumway and Cembella, 1993), however very little information is available concerning DSP toxin depuration rates from scallops (Kikuchi et al., 1992; Tazawa et al., 1989).

3.2. Materials and Methods

3.2.1. Algal culture and scallop maintenance

Prorocentrum lima and *Thalassiosira weissflogii* were cultured as previously described in 2.2.1. Pre-reproductive adult bay scallops (shell ht. = 30 - 40 mm), from the same stock as the juvenile scallops used in the experiments described in Chapter 2, were acquired from a bottom grow-out site managed by Little Harbour Fisheries (Little Harbour, NS) in November, 1994. Scallops were transferred to the National Research Council Aquaculture Research Station in Sandy Cove, NS, where they were held in flow-through raceways maintained at 16 - 18 °C with a flow rate of approximately 9 L $min.^{-1}$ of unfiltered natural seawater. Scallops were maintained on a diet of the non-toxic diatom *Thalassiosira weissflogii* as described previously in 2.2.2.

3.2.2. Determination of clearance rates

Experiments were conducted to determine the effect of cell concentration on clearance and ingestion rates of adult bay scallops using equivalent cell biovolume concentrations (2.2.3.) of *Prorocentrum lima* or a control diet, the non-toxic diatom *Thalassiosira weissflogii*. Individual adult bay scallops (n = 7) (mean shell ht. = 40.34 mm, SD = 2.94) were randomly placed in parallel flow-through feeding chambers (Fig. 3.1). Empty scallop shells were placed in two of the chambers during each feeding study, serving as control chambers. Feeding chambers were constructed from transparent plexiglass to enable visual observation of the scallops during feeding studies. Each feeding chamber measured 75 mm wide x 110 mm long x 75 mm deep. An inflow valve was present 35 mm above the chamber bottom and a plexiglass baffle was inserted 20 mm from the inflow end of the chamber. An exit valve, 15 mm above the bottom was fitted with an elbow shaped segment of glass tubing, which served to control the height of water in each chamber (50 mm) and prevented fecal ribbons produced by the scallops from escaping the chamber through the outflow.

Algal suspensions were mixed to appropriate concentrations with filtered (1 μm) UV-treated natural seawater in a 100 L stock tank and kept in suspension by an air-stone. A Masterflex high-capacity peristaltic pump was used to pump the algal suspensions from the stock tank into a 30 L aerated header tank fitted with an overflow valve that maintained a constant volume in the tank. Suspensions were gravity-fed from the header tank to the individual feeding chambers via Tygon tubing (i.d. = 20 mm) at a controlled flow rate of 180 mL $min.^{-1}$. The action of the pump was gentle enough not to disrupt the *P. lima* or *T. weissflogii* cells and wide shell gapes exhibited by scallops indicated that the flow did not inhibit feeding activity.

Scallops were exposed to six different cell concentrations of *T. weissflogii* (860, 1200, 3730, 6480, 14530, 20700 cells mL^{-1}) and *P. lima* (36, 83, 135, 177, 243, 426 cells mL^{-1}). Scallops were allowed to acclimate to each cell concentration for 90 minutes, at which time samples of algal suspension (100 mL) were taken simultaneously from the outflow of each of the seven feeding chambers and two control chambers. Samples were preserved using Lugol's iodine solution and stored in glass scintillation vials. Cell concentrations

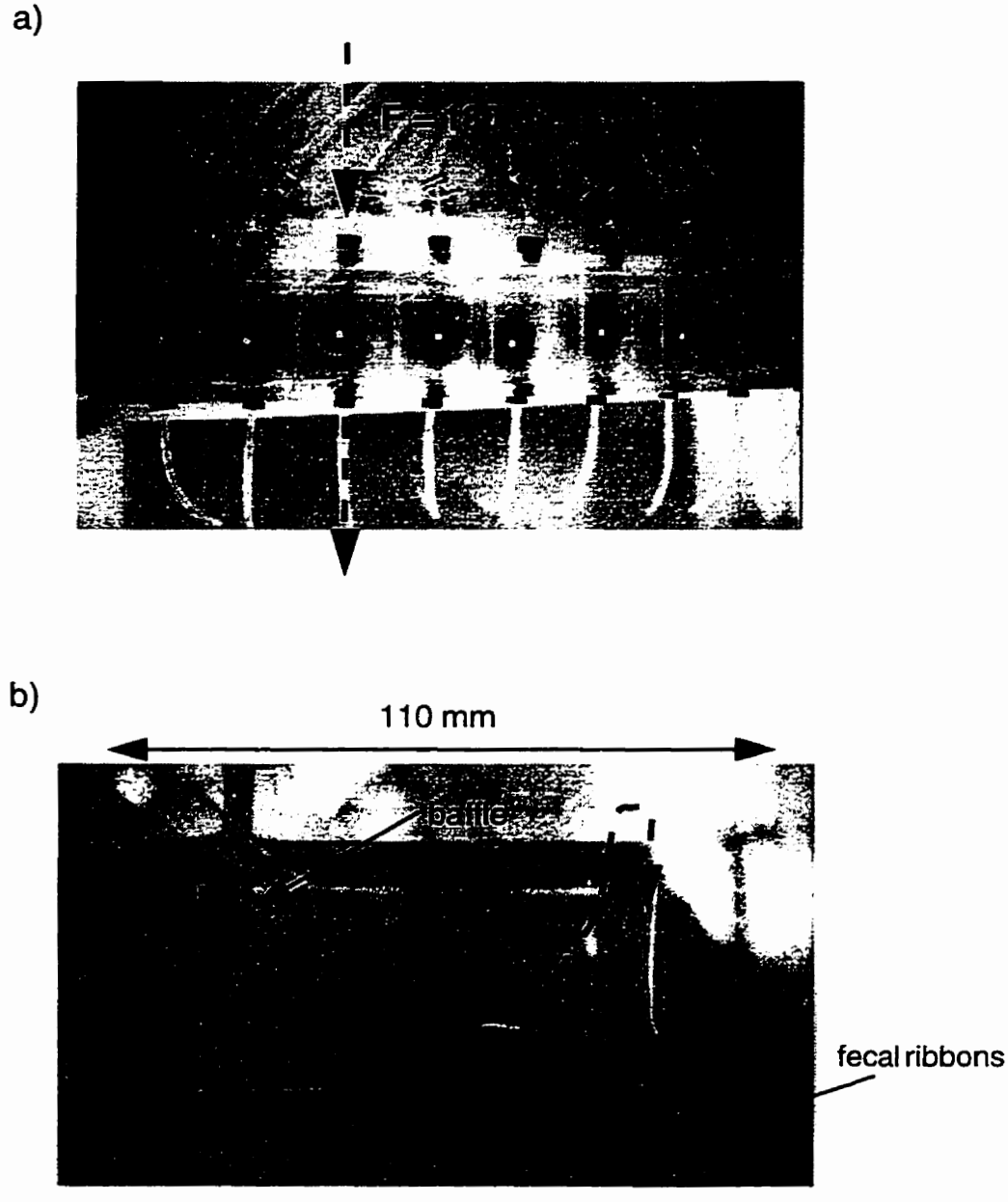


Fig. 3.1. Top (a) and side (b) views of flow-through feeding chambers. Dashed lines indicate direction of water flow.

were determined by microscopic enumeration in a 0.1 mL Palmer-Maloney chamber (*T. weissflogii*) and a 1.0 mL Sedwick-Rafter chamber (*P. lima*). As a confirmation of optical cell counts, cell concentrations were also measured using an electronic particle counter (Coulter Multisizer) equipped with a 100 μm aperture.

Clearance rates (CR) were calculated according to the equation: $F \times (C_1 - C_2)/C_1$ (Hildreth and Crisp, 1976), where F is the flow rate in the feeding chambers, C_1 is the cell concentration in the inflow (mean outflow of two control chambers) and C_2 is the cell concentration in the outflow of each feeding chamber. Clearance rates were converted to weight-standardized rates for a scallop of 1g total wet body weight as described previously.

Any feces and pseudofeces produced were removed with a Pasteur pipette five minutes prior to collection of outflow suspension samples. Following clearance rate experiments, scallops were sacrificed for determination of total wet tissue weight and total dry tissue weight after lyophilization for 24 hours in a freeze-dryer.

3.2.3. Short-term toxin loading

A short-term (2 d) feeding study was conducted to determine how DSP toxins are compartmentalized within adult scallop tissues following ingestion of *Prorocentrum lima* cells.

Twenty-four pre-reproductive adult bay scallops (mean shell ht. = 38.63 mm, SD = 0.50) were exposed to DSP-toxigenic *P. lima* cells (mean cell density = 80 cells mL^{-1}) in an 80 L recirculating aquarium using methods described previously in 2.2.4. Triplicate 100 mL samples of the *P. lima* stock were taken over the course of the exposure period (0h, 24h, 48h) to determine whether the total toxin content and/or relative toxin profile of *P. lima* cells in the stock tank varied over the two day period.

All of the scallops were removed and divided into three pools of eight scallops at the end of the two day exposure. The following tissues were removed from each scallop: viscera (stomach-digestive gland complex), gonad (including intestinal loop section), mantle, gills and adductor muscle. Metanephridium and heart tissue were included with the viscera. Within each pool, tissues were combined, resulting in triplicate samples of each tissue. The combined tissue samples, each pooled from eight scallops, provided highly

concentrated extracts for the analysis of toxins in non-visceral tissues, which were hypothesized to be significantly lower in toxin content than visceral tissue. Combined tissue pools were weighed, immediately immersed in 4:1 (v:w) 80 % aqueous methanol and stored at -20 °C for later toxin extraction and analysis. Tissues were immersed in methanol prior to freezing to avoid the possibility of toxin hydrolysis occurring during freezing and thawing of samples (Quilliam and Ross, 1996) which may have contributed to the toxin profile variations noted in Chapter 2.

3.2.4. Long-term feeding study

A long-term toxin exposure and detoxification study was conducted to determine the kinetics of DSP toxin uptake, compartmentalization and detoxification in adult bay scallop tissues. Another objective of this study was to measure physiological indices of scallop feeding activity (clearance rate, absorption efficiency, fecal deposition) during the long-term toxin exposure period.

Toxin uptake and detoxification

Pre-reproductive adult bay scallops (n = 61, mean shell ht. = 38.21 mm, SD = 3.89; mean total wet tissue wt. = 4.68 g, SD = 1.22) were exposed to DSP-toxigenic *Prorocentrum lima* for 13 days in an 80 L aquarium at 17 °C, using methods described previously in 2.2.4. Three scallops were removed at frequent intervals for toxin analysis and replaced with scallops of similar size to maintain a constant number of scallops in the aquarium. Scallops were immediately dissected into three tissue groups: viscera, gonad (including intestinal loop) and all other tissues (including adductor muscle, mantle and gills). Wet tissue weights were determined, following which the tissues were placed in plastic centrifuge tubes containing 9:1 (v:w) 80 % aqueous methanol and temporarily stored on dry ice (< 6h). Samples were ultimately stored at -20 °C to await subsequent toxin extraction.

Following the toxin exposure period, scallops were detoxified for one week by immediate transfer to another 80 L aquarium containing the non-toxic diatom *Thalassiosira weissflogii*, which was continuously metered from a column into the aquarium with a peristaltic pump to yield an approximately constant cell concentration of

2500 cells mL^{-1}. Scallops were removed at frequent intervals for toxin analysis, as described previously. To reduce the chance of scallops re-ingesting toxic *P. lima* cells from fecal deposits, the entire volume of water in the tank was exchanged each day. Fecal ribbons were collected from the aquarium each day and examined microscopically for intact *P. lima* cells. After one week of detoxification in an enclosed aquarium, the remaining scallops were transferred to a flow through seawater raceway supplemented with *T. weissflogii*, from which scallops were sampled for toxin analysis for two more weeks. The remaining scallops (n = 6) were removed after eight weeks of detoxification to determine if any toxin remained in the tissues after a prolonged depuration period.

Physiological indices

Clearance rates, fecal deposition rates and absorption efficiencies of scallops were determined after one hour, three days, and eight days of exposure to *Prorocentrum lima* cells. The same measurements were also determined for scallops exposed to equivalent biovolume cell concentrations of *Thalassiosira weissflogii*, one day prior to exposure to *P. lima* and after five days of detoxification. Seven labeled scallops were removed from the aquarium and placed in the flow-through feeding chambers previously described (Fig. 3.1). Algal suspensions (*P. lima* = *ca.* 100 cells mL^{-1}, *T. weissflogii* = *ca.* 1600 cells mL^{-1}) were pumped from a stock tank into the feeding chambers using methods described in 3.2.2. Cell concentrations in the feeding chambers were approximately the same as that which the scallops were exposed to in the aquarium.

After allowing scallops to acclimate for 90 min. in the feeding chambers, clearance rates were measured as described in 3.3.2. Fecal ribbons produced in each of the feeding chambers were gently removed using a Pasteur pipette and filtered onto preweighed, precombusted 25 mm Whatman GF/F filters. Samples of concentrated algal stocks (15 mL) (*P. lima* and *T. weissflogii*) were also collected by vacuum filtration onto GF/F filters. Salt was removed from the samples by washing filters under vacuum with 10 mL 4% ammonium formate. Filters were temporarily stored on dry ice to prevent degradation of organic material, following which they were dried at 80 °C for 48 h, weighed, combusted at 450 °C for 6 h, and weighed again after cooling in a dessicator. All weights were determined to ± 10 μg.

The absorption efficiency (% AE) of organic matter ingested by the scallops was calculated according to Conover (1966): AE = (F-E)/[(1-E) x F] x 100, where F is the ash-free dry weight proportion of the algae and E is the ash-free dry weight proportion of the feces. Ash-free dry weight was determined as the difference between the dry and combusted weights of the filters. Fecal deposition rate (FDR) was determined as: FDR = DF/t, where DF is the dry weight of feces produced and t = time in feeding chambers. This value was standardized for a scallop of 1 g wet weight according to: $FDR_S = FDR_e/W_e^b$, where W = the wet weight of the scallop and b = the allometric weight exponent (0.75) (Bayne and Newell, 1983).

Fecal samples: Toxin composition and cell viability

Fecal ribbons produced by scallops after twelve days of exposure to *Prorocentrum lima* cells were gently removed using a Pasteur pipette and rinsed by allowing to the feces to settle in a scintillation vial containing filtered seawater (1 µm). Feces were transferred by Pasteur pipette to another vial containing filtered seawater and kept cool on dry ice. Fecal samples were further subsampled to determine the toxin profile, and to establish viability of *P. lima* cells.

Fecal samples were prepared for toxin analysis by probe-sonicating (10 s bursts at 50% pulse-duty cycle) fecal ribbons in 2 mL of 80% aqueous methanol for 3 min followed by centrifugation for 10 min at 4000 x g. The supernatant was filtered through a 0.45 µm cartridge-filter for analysis by LC-MS.

Viability of *P. lima* cells within fecal ribbons was determined by inoculating triplicate samples of intact or disrupted (vortexed for 30 s) fecal ribbons in flasks containing 200 mL of K-medium. A 5 mL sample of *P. lima* stock culture was also inoculated in triplicate as a control for comparison of cell division rates. Cultures were exposed to a 14:10 L:D photocycle at an incident irradiance of 90 µmol m^{-2} s^{-1} at 17 °C. Cell concentrations in each of the flasks were determined every seven days over a four week period by enumerating 5 mL subsamples in a 0.1 mL Palmer-Maloney chamber under phase contrast microscopy (100x magnification). Division rates were calculated according to Guillard (1973) as described previously in 2.2.1.

Fecal samples taken from scallops after two and twelve days of exposure to *P. lima* cells were photographed at 1000x magnification using Nomarsky interference microscopy. *P. lima* cells in the feces were examined for obvious visual characteristics such as thecal integrity and cell motility.

3.2.5. Toxin extraction and analysis

Toxins were extracted from scallop tissues according to methods described in 2.2.5. Extract residues were re-dissolved in 100 µL methanol and prepared for LC-MS analysis by centrifugation through a 0.45 µm cartridge-filter.

Prorocentrum lima samples (100 mL) taken from the stock tank during the toxin uptake period were concentrated to form a pellet by centrifugation at 4000 x g for 20 min (4 °C). Immediately following centrifugation, 80 % aqueous methanol was added to each pellet to a make up a volume of exactly 2.0 mL. Cells were disrupted by 10 s bursts of probe sonication for 3 min and centrifuged for 10 min at 4000 x g (4 °C). The supernatants were centrifuged through a 0.45 µm cartridge-filter for LC-MS analysis.

Algal and scallop tissue extracts were analyzed for DTX1, OA and OA esters by positive ion-spray mass spectrometry following liquid chromatography as described in 2.2.6. Selected scallop tissue extracts were also analyzed for DTX4, a water soluble toxin, using the negative LC-MS ion-mode (Quilliam and Ross, 1996). To ensure that DTX4 was not lost to the water-soluble phase during the liquid-liquid partitioning clean-up of scallop tissue extracts (see 2.2.6.), both the water and lipid-soluble phases, as well as the initial crude methanol extract, were subjected to LC-MS analysis. LC-UV detection (Quilliam et al., 1996) was used to analyze for D8OA and DTX4 in algal and scallop tissue extracts. Both of these compounds contain conjugated double bonds included in the diol-ester chain, which fluoresce at 238 nm.

3.3. Results

3.3.1. Cell density dependent clearance and ingestion rates

Clearance rates of scallops exposed to *Prorocentrum lima* cells declined exponentially over a concentration range of 36 to 426 cells mL^{-1} (Fig. 3.2a). Pseudofeces production

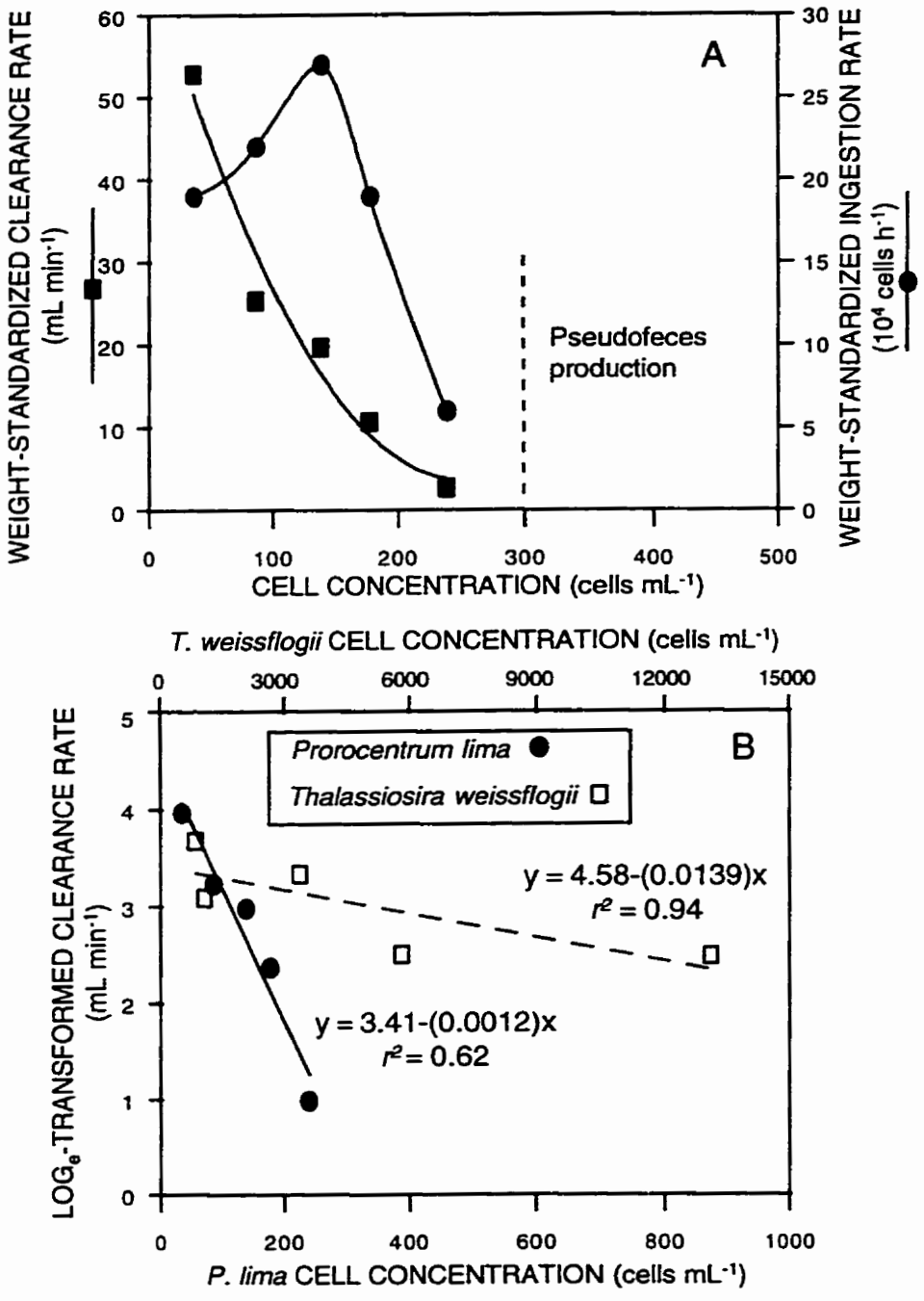


Fig. 3.2. A) Weight-standardized clearance and ingestion rates of *Argopecten irradians* exposed to *Prorocentrum lima*. B) Linear regressions of $\log_e$-transformed weight-standardized clearance rates of *A. irradians* exposed to equivalent biovolume cell concentrations of *P. lima* and *Thalassiosira weissflogii*.

was only observed at the highest *P. lima* cell concentration tested (426 cells mL^{-1}), therefore clearance rates could not be calculated at this concentration since the number of cells rejected in the pseudofeces would have to be determined. Maximum ingestion rates (CR x cell concentration) of *P. lima* cells occurred at cell concentrations of approximately 130 cells mL^{-1}.

Clearance rates of scallops fed equivalent biovolume concentrations of the non-toxic diatom *Thalassiosira weissflogii* also declined exponentially with increasing cell concentrations, however $\log_e$-transformed regression equations indicate that the rate of decline this relationship was much less than for *P. lima* cells (Fig. 3.2b). Although there was no significant difference in clearance rates of scallops exposed to the two algal diets at *P. lima* equivalent biovolume cell concentrations below 150 cells mL^{-1} (*t*-test, $P > 0.05$, $n = 7$), clearance rates were significantly lower for scallops exposed to *P. lima* at cell concentrations greater than 200 cells mL^{-1} (*t*-test, $P < 0.0001$, $n = 7$). The feeding behaviour of scallops appeared the same for both diets: scallops fed actively on *P. lima*, except at the highest cell concentrations, when scallop valves were observed to shut frequently.

3.3.2. Anatomical compartmentalization of toxins

Mean total (DTX1 + OA + D8OA) toxin content of *P. lima* cells in the stock tank was 3.42 pg $cell^{-1}$ (SD = 0.49) over the course of the two day feeding study. There were no significant differences in either the total toxin content or the relative % molar toxin profile of *P. lima* cells sampled from the stock tank after 0, 24 and 48 h (repeated measures ANOVA, $P > 0.05$) (Fig. 3.3).

After two days of exposure to *P. lima* cells, total DSP toxin concentrations in visceral and gonadal tissues were approximately 1 $\mu g\ g^{-1}$ while in the gills, mantle and adductor muscle toxin concentrations were less than 0.1 $\mu g\ g^{-1}$ (Fig. 3.4). Total toxin concentrations were slightly greater in visceral than in gonadal tissues, however this difference was not significant (*t*-test, $P > 0.05$). Although gills, mantle and viscera comprised most of the wet weight of the scallops (Fig. 3.4), most of the total toxin body burden was confined to the viscera (Fig. 3.5b). Gonadal tissue accounted for only 4 % of

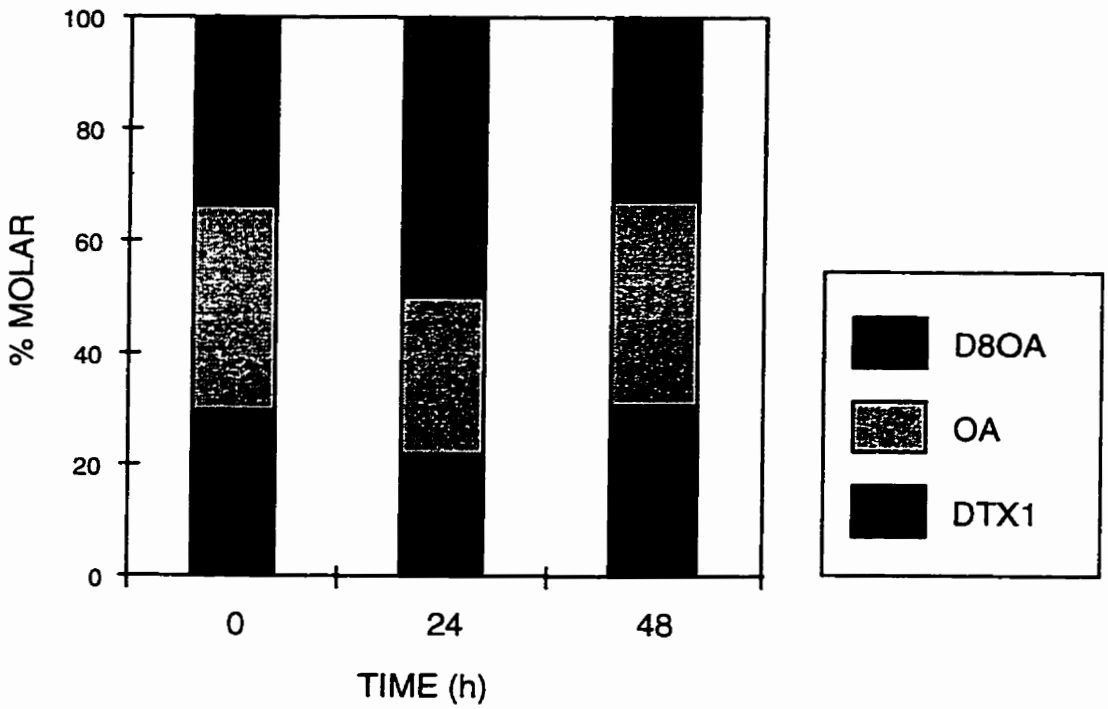


Fig. 3.3. Relative % molar DSP toxin content of *Prorocentrum lima* cells in the stock tank over 48 h feeding experiment.

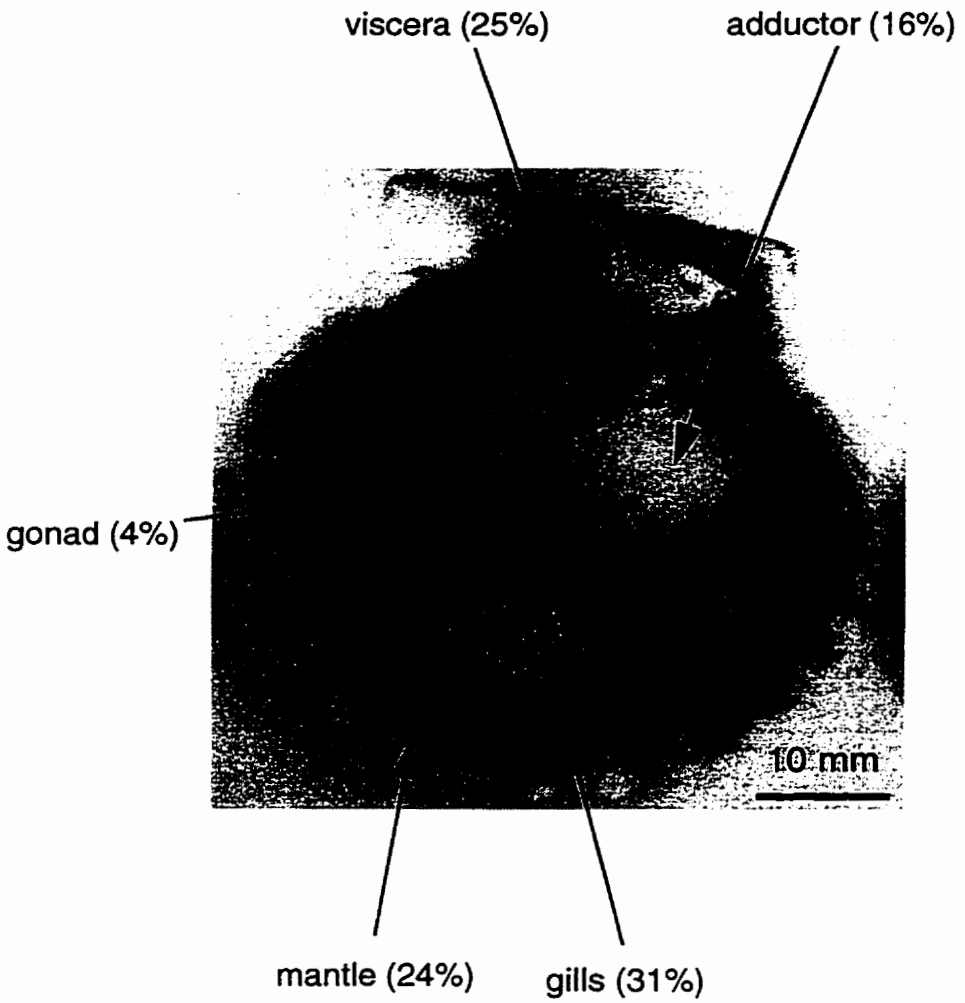
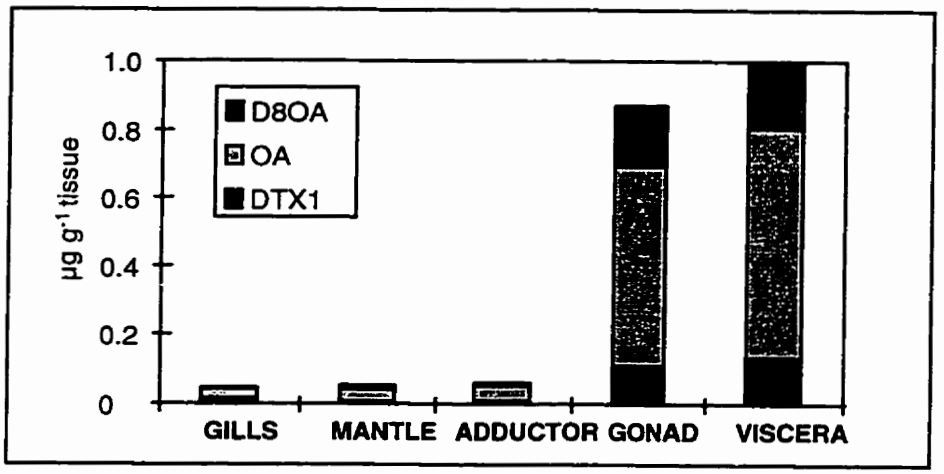


Fig. 3.4. Contribution of tissues to total wet body weight in bay scallops.

a)



b)

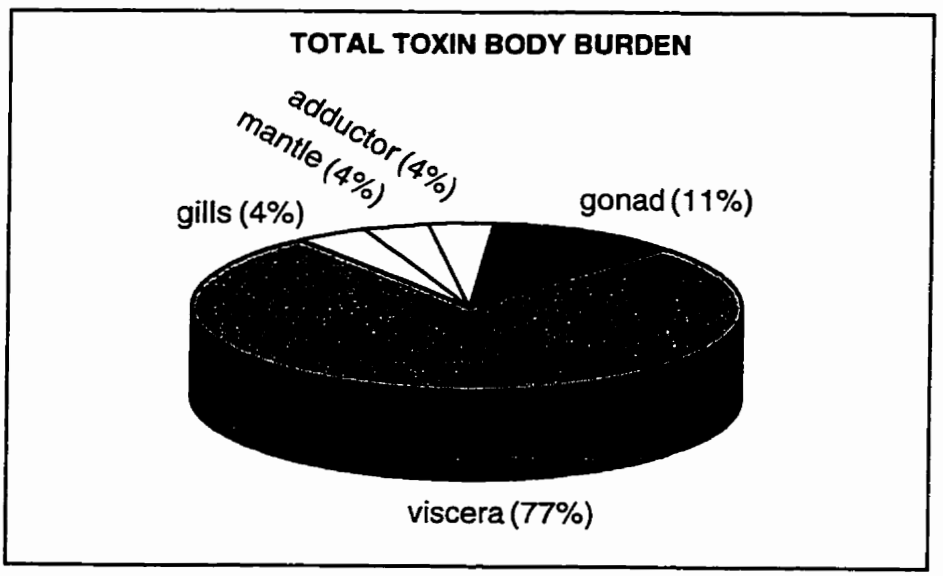


Fig. 3.5. DSP toxin concentrations (a) and % contribution to total toxin body burden (µg) (b) in bay scallop tissues following 2 day exposure to *P. lima* cells. Viscera composed of digestive gland-stomach complex.

the wet tissue weight in the reproductively immature scallops, however 11 % of the total toxin body burden was present in this tissue.

An examination of the relative % molar toxin composition of *P. lima* cells and scallop tissues indicated that the dinoflagellate cells contained a lower percentage of OA and a higher percentage of D8OA and DTX1 than the tissues (Fig. 3.6a). This is also evident in the fact that both the ratios of OA/D8OA and OA-derivatives/DTX1 were markedly lower in *P. lima* cells than in any of the tissues except for the gills (Fig. 3.6b). Among the tissues, mantle, adductor muscle, gonads and viscera all had similar toxin profiles, characterized by a dominance of OA, especially in the adductor muscle. The toxin composition of gills was intermediate between that of ingested *P. lima* cells and the other tissues. The ratio of OA-derivatives/DTX1 in the gills, which resembled that of *P. lima* cells, was significantly less than in the other tissues (ANOVA, $P = 0.0023$).

3.3.3. Effects of toxins on feeding physiology

Prorocentrum lima cell concentrations in the aquarium fluctuated around a time-weighted mean of 173 cells mL^{-1}, during which time scallops ingested the cells at a mean rate of 1.57×10^6 cells d^{-1} g^{-1} (SD = 9.9×10^5) (Fig. 3.7a). Linear regression analysis was used to calculate the cumulative weight-specific ingestion rate: IR = 2.0×10^6 cells d^{-1} g^{-1} (Fig. 3.7b). No mortalities occurred over the 13 d exposure period, nor did scallops exhibit unusual feeding behaviour. In general, scallops appeared to be unaffected by exposure to toxigenic *P. lima* cells.

Weight-standardized clearance rates of scallops varied significantly over the pre-exposure, exposure and detoxification period (one-way repeated measures ANOVA, $P = 0.0148$, $n = 7$) (Fig. 3.8a). Pairwise multiple comparisons indicated that clearance rates were significantly higher during the detoxification period than prior to exposure to *P. lima* or during the first three days of the exposure period (SNK, $P < 0.05$). There was no significant shift in clearance rates when scallops were changed from a diet of *Thalassiosira weissflogii* (pre-exposure) to *P. lima*. Clearance rates increased during the second week of exposure and continued to climb during detoxification.

There was no significant difference in the % organic matter content of *Prorocentrum lima* (mean = 80.4 %, SD = 4.6) or *Thalassiosira weissflogii* cells (mean 79.0 %, SD =

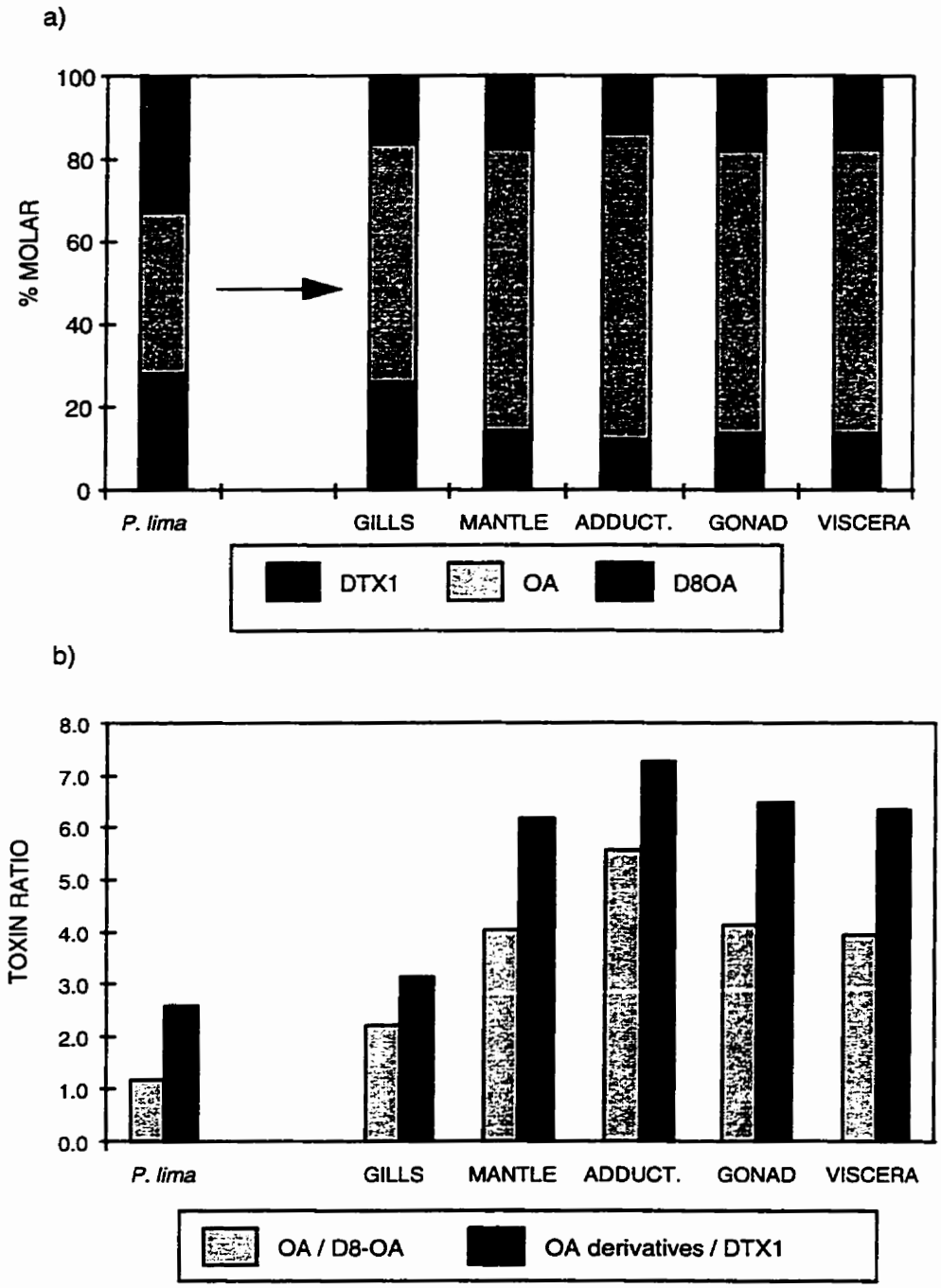


Fig. 3.6. a) Relative % molar DSP toxin content in *Argopecten irradians* tissues after two days exposure to *Prorocentrum lima* cells. b) DSP toxin ratios in *P. lima* cells and *A. irradians* tissues.

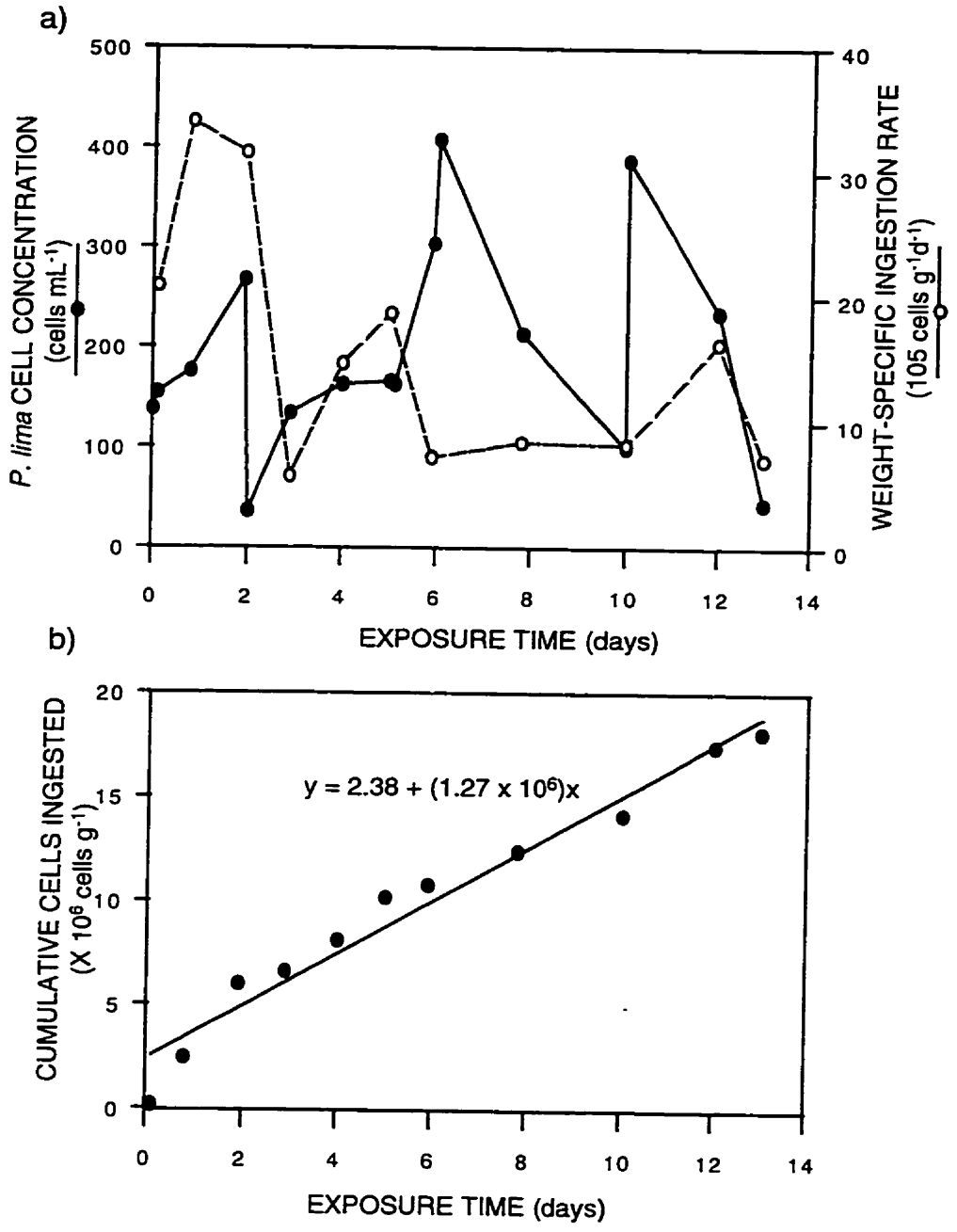


Fig. 3.7. a) *Prorocentrum lima* cell concentration and weight-specific cellular ingestion rates of *Argopecten irradians*. b) Linear regression of cumulative cells ingested during 13 d exposure period.

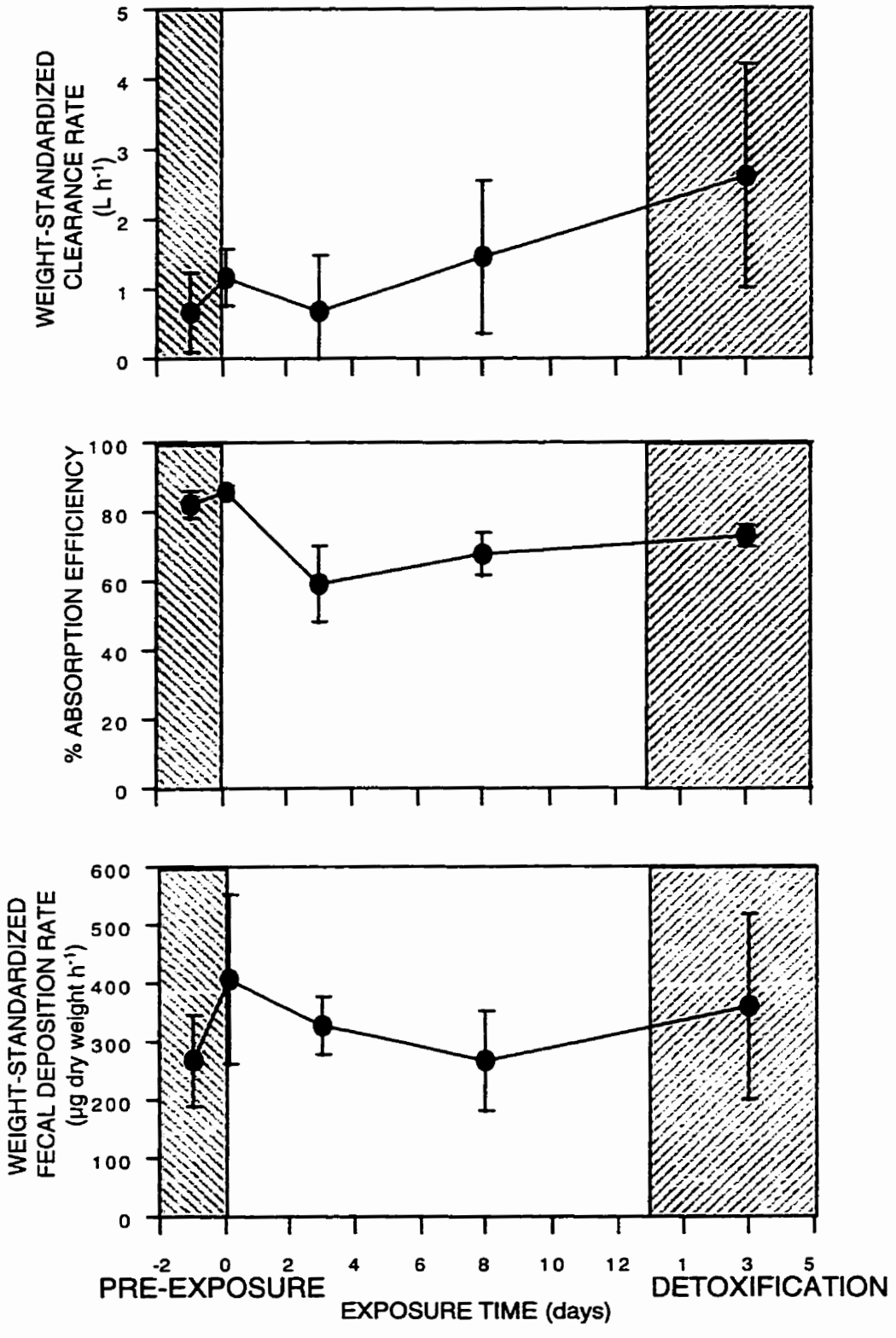


Fig. 3.8. Physiological feeding indices of *Argopecten irradians* exposed to *Prorocentrum lima*. Hatched areas indicate pre-exposure and detoxification periods, during which scallops were exposed to *Thalassiosira weissflogii*. Error bars represent ± 1 standard deviation, $n = 7$.

9.2) (t-test, $P > 0.05$, $n = 5$). The absorption efficiency (AE) of organic matter by scallops varied significantly over the course of the feeding study (one-way repeated measures ANOVA on arc-sine transformed values, $P = 0.0003$, $n = 7$) (Fig. 3.8b). Absorption efficiencies were significantly lower after three days of exposure to *P. lima* than at the beginning of the exposure period (SNK, $P < 0.05$, $n = 7$). Although AE values appeared to increase during detoxification when scallops were returned to a diet of *T. weissfloggi*, this increase was not significant.

Fecal deposition rates by scallops were not significantly different over the exposure period (one-way repeated measures ANOVA, $P = 0.0996$, $n = 7$), fluctuating around a weight-standardized (1 g wet weight tissue) mean of 325 µg dry weight h^{-1} (SD = 60) (Fig. 3.8b).

3.3.4. Effects of gut passage on *Prorocentrum lima* cells

Microscopic examination of fecal samples (1000x magnification) revealed that intact and motile *Prorocentrum lima* cells were prevalent in scallop fecal ribbons throughout the exposure period. They were frequently observed swimming within the fecal ribbons, as well as along the exterior margins of the ribbons, suggesting that these cells had recently migrated from the feces into the surrounding medium. Evidence that some of the ingested *P. lima* cells were digested during passage through the scallop gut was provided by the presence of *P. lima* thecal fragments and free starch granule bodies within the feces. A greater proportion of digested *P. lima* cells within fecal ribbons was observed at the beginning of the exposure period than during the latter portion (Fig. 3.9), coinciding with the observed decrease in absorption efficiency.

Prorocentrum lima cells contained in fecal ribbons were able to divide at rates comparable to those of *P. lima* cultures when inoculated into growth media (Fig. 3.10). Growth curves were similar for cells inoculated from intact and dispersed fecal ribbons, indicating that *P. lima* cells were capable of freeing themselves from feces.

3.3.4. Toxin dynamics: uptake and detoxification

Total DSP toxin content (DTX4 + D8OA + OA + DTX1) of *Prorocentrum lima* cells fluctuated between 22.7 and 6.8 fmol $cell^{-1}$ over the exposure period, averaging 12.4 fmol

a)

b)

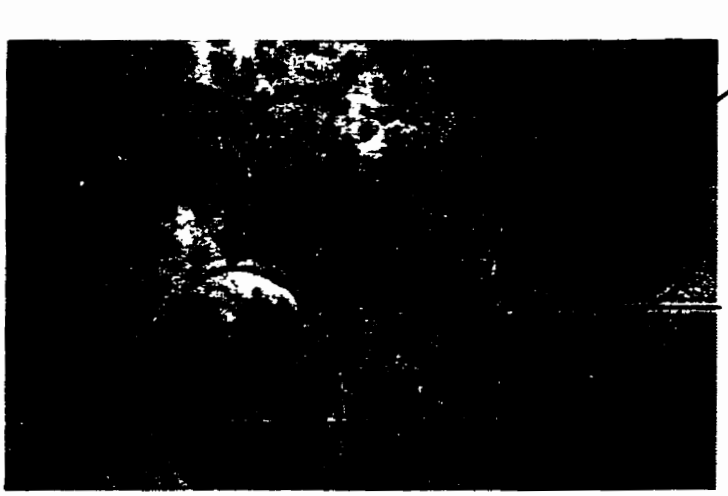


Fig. 3.9. Nomarsky interference photo micrographs of fecal samples produced by bay scallops after 2 days (a) and 12 days (b) of exposure to *Prorocentrum lima* cells at 10^5 cells L^{-1}

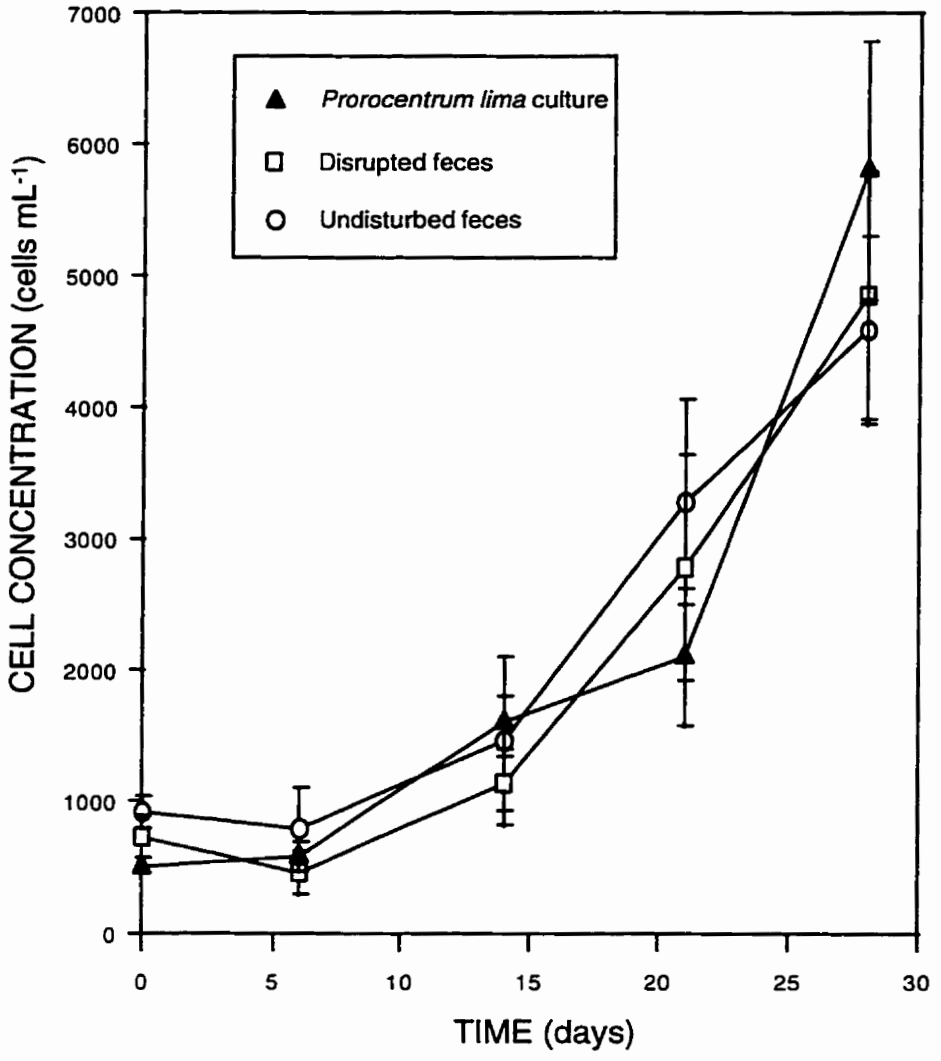


Fig. 3.10. Growth of *Prorocentrum lima* cells in K-medium after inoculation from either *P. lima* culture or fecal ribbons of *Argopecten irradians* following ingestion of *P. lima* cells (undisturbed and disrupted fecal ribbons). Error bars = 1 standard deviation, $n = 3$.

cell^{-1} (SD = 5.2) (Fig. 3.11a). These values were expressed in units of fmol cell^{-1} rather than pg cell^{-1} due to the considerably larger molecular weight of DTX4 (1472.6) relative to OA (805.5). Mean total toxin content of *P. lima* cells was 10.0 (SD = 4.2) if expressed in terms of pg OA eq. cell^{-1}. Total toxin content of scallop tissues as determined by LC-MS was < 1% of the cumulative toxin ingested by the animals over the exposure period (Fig. 3.11b). After 13 days of exposure to *P. lima* cells, scallops had ingested an estimated 197 μg total DSP toxin g wet wt.$^{-1}$, however total toxin concentration in whole scallop tissues peaked at only 1.00 μg g^{-1} after 8 days of exposure (Fig. 3.10c). Thus, toxin accumulation efficiency (% TAE) in scallop tissues decreased exponentially from 8.1 % after only 2 hours of exposure to approximately 0.4 % for the remainder of the study (Fig. 3.11c). Total toxin concentration in whole scallop tissue exceeded DSP toxin regulatory levels of 0.2 μg g wet wt.$^{-1}$ accepted in Europe and Japan (Quilliam, 1995) after only 18 hours of exposure to *P. lima*. A decrease in total toxin concentration in scallop tissues observed midway through the study (8 d) coincided with the presence of *P. lima* cells containing low levels of toxin.

Temporal patterns of total DSP toxin content in viscera, gonad and other tissues (mantle, gills and adductor muscle) were similar during the toxin exposure period although toxin uptake rates were greatest in visceral and gonadal tissue (Fig. 3.12). Pearson product-moment correlations indicated that total toxin concentrations in each of the tissue compartments were significantly correlated during the toxin uptake period ($P < 0.05$). In all tissues toxin content peaked after 8 days, following which a decline in toxin levels for the remaining 5 days of exposure coincided with a substantial decrease in the toxin content of ingested *P. lima* cells. Over the course of the uptake period, toxin concentrations in each of the tissue compartments followed the same rank order: viscera > gonads >> other tissues.

Tissue wet weights, expressed as percentage of total body weight, remained constant throughout the uptake and detoxification periods. Viscera, gonad and other tissues averaged *ca.* 21%, 5% and 74% of the total body weight, respectively (Fig. 3.13a). During the uptake period, visceral tissue comprised most (76%) of the total toxin body burden (TTBB), while gonadal and other tissues accounted for 11% and 13%, respectively

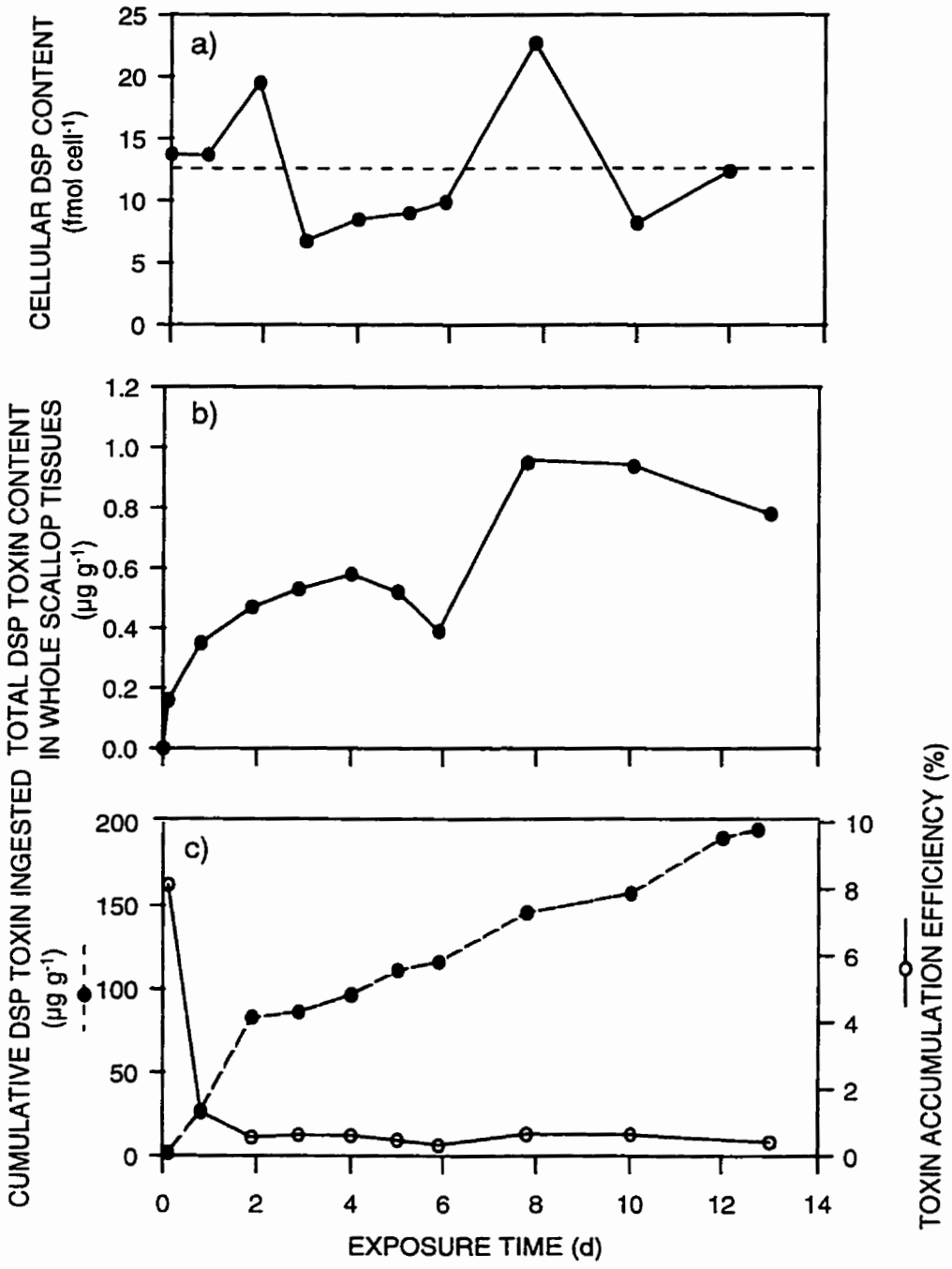


Fig. 3.11. a) Total DSP toxin content in *Prorocentrum lima* cells. Horizontal dashed line represents time-weighted mean toxin content. b) Total DSP toxin concentration in whole scallop tissues during 13 day exposure. c) Cumulative DSP toxin ingested by scallops during exposure period. Expressed as µg toxin per g whole scallop tissue.

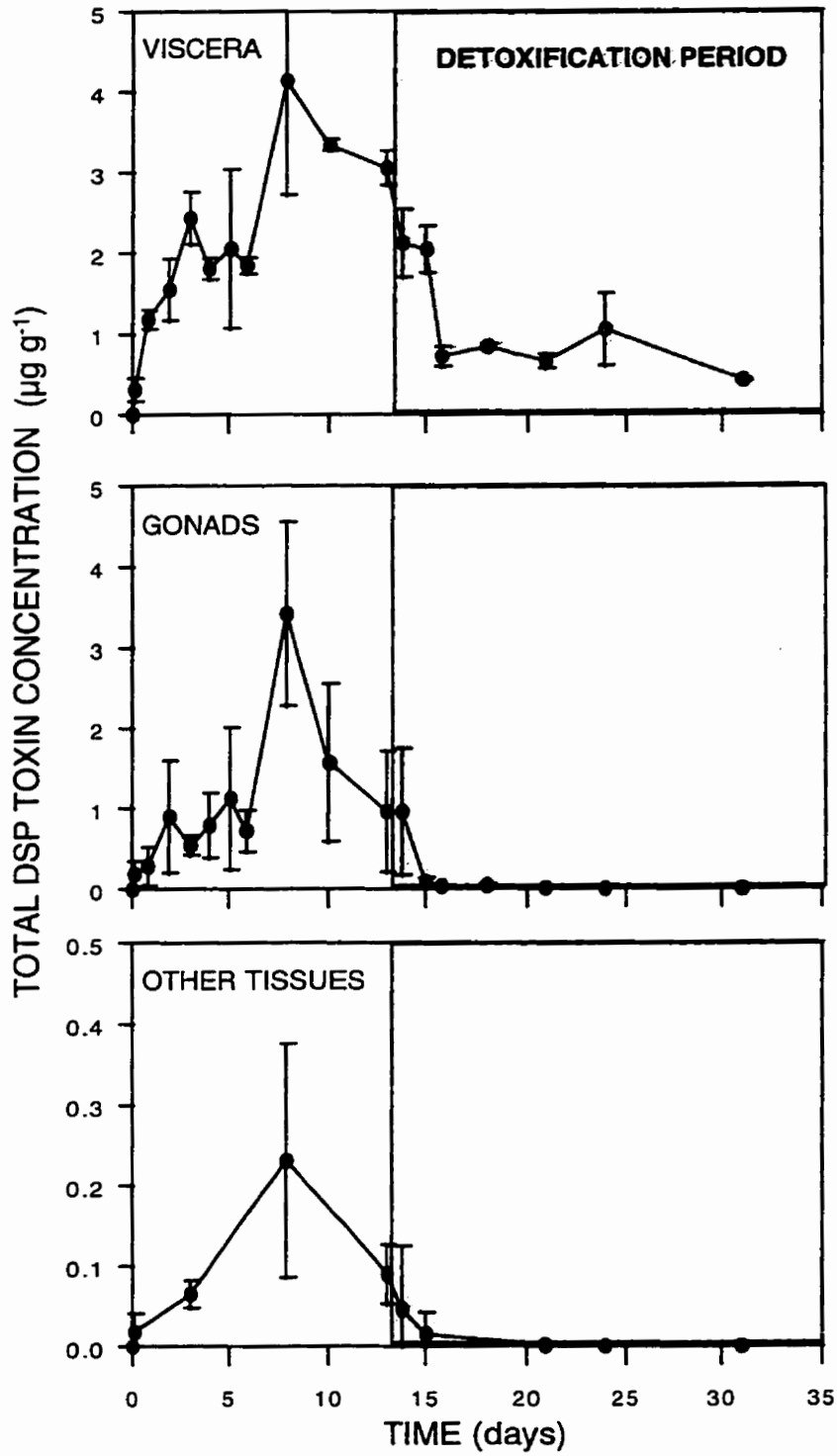


Fig. 3.12. Temporal pattern of DSP toxin uptake and loss (hatched area) in *Argopecten irradians* tissues during a 13 day exposure to *Prorocentrum lima*, followed by a 3 week detoxification period. Error bars represent ± 1 standard deviation ($n = 3$).

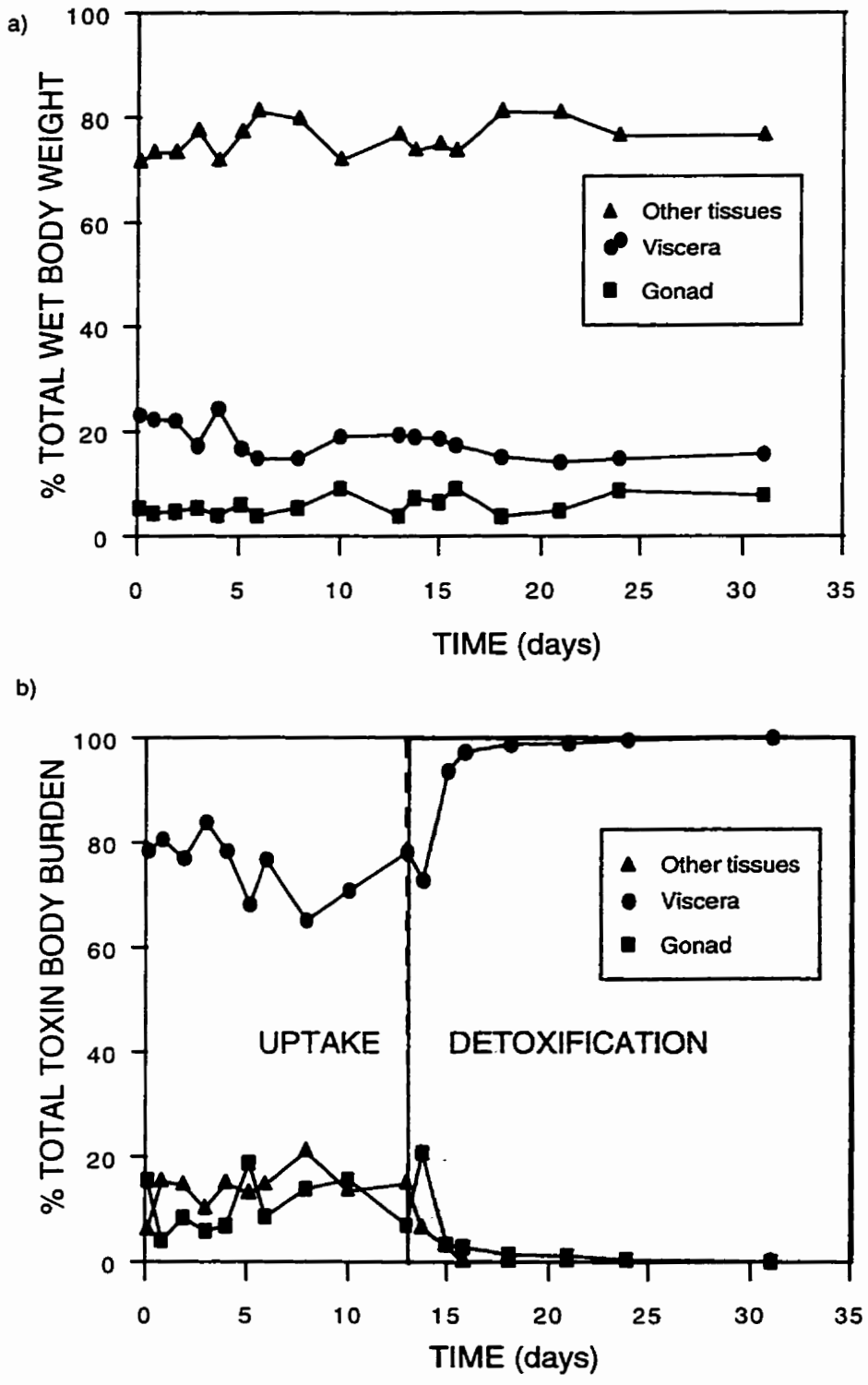


Fig. 3.13. Contribution (%) of *Argopecten irradians* tissues to: a) total wet body weight; b) total DSP toxin body burden.

(Fig. 3.13b). During the first two days of detoxification, visceral tissue represented 96% of TTBB, after which virtually all of the remaining toxin load was confined to this tissue.

Total toxin concentration in gonads was compared to the condition index (gonad wet weight/total body wet weight x 100) to determine whether the reproductive condition of gonads had an effect on DSP toxin content in this tissue, however no significant correlation existed between the two variables (Pearson product-moment correlation, P = 0.15).

Detoxification in all tissues followed an exponential pattern, however toxin release rates were far more rapid in gonads and other tissues than in viscera (Fig. 3.14). Detoxification data was fitted to the general exponential loss equation: $T_t = T_0 e^{-\lambda t}$, where T_t = % of toxin concentration at start of detoxification, λ = exponential decay coefficient and t = time (d). Toxin loss from the viscera appeared to follow a biphasic pattern, characterized by a rapid release of toxins during the initial 16 hours of detoxification (30 % of the original toxin load), followed by a much more gradual detoxification process over the ensuing weeks, although a higher sampling frequency would be required yo confirm this pattern. Exponential loss curves were fitted to viscera data after the first day of detoxification. While toxin loss from visceral tissue was calculated to be 8.4 % d^{-1}, gonads and other tissues detoxified far more rapidly at rates of 50 % d^{-1} and 68 % d^{-1}, respectively. Toxin levels in gonads and other tissues were undetectable within 5 days of detoxification, however toxin concentrations in visceral tissues still remained at levels above regulatory limits (1-2 µg g viscera^{-1}) after 11 days of detoxification. No DSP toxins were detected in visceral tissues after two months of detoxification.

Live *Prorocentrum lima* cells were observed by microscopy (400 X magnification) in fecal ribbons produced by scallops up until the fourth day of detoxification. Pigments and thecal fragments derived from digested *Thalassiosira weissflogii* cells were abundant in fecal samples, however, unlike *P. lima* cells, very few of these diatom cells were intact.

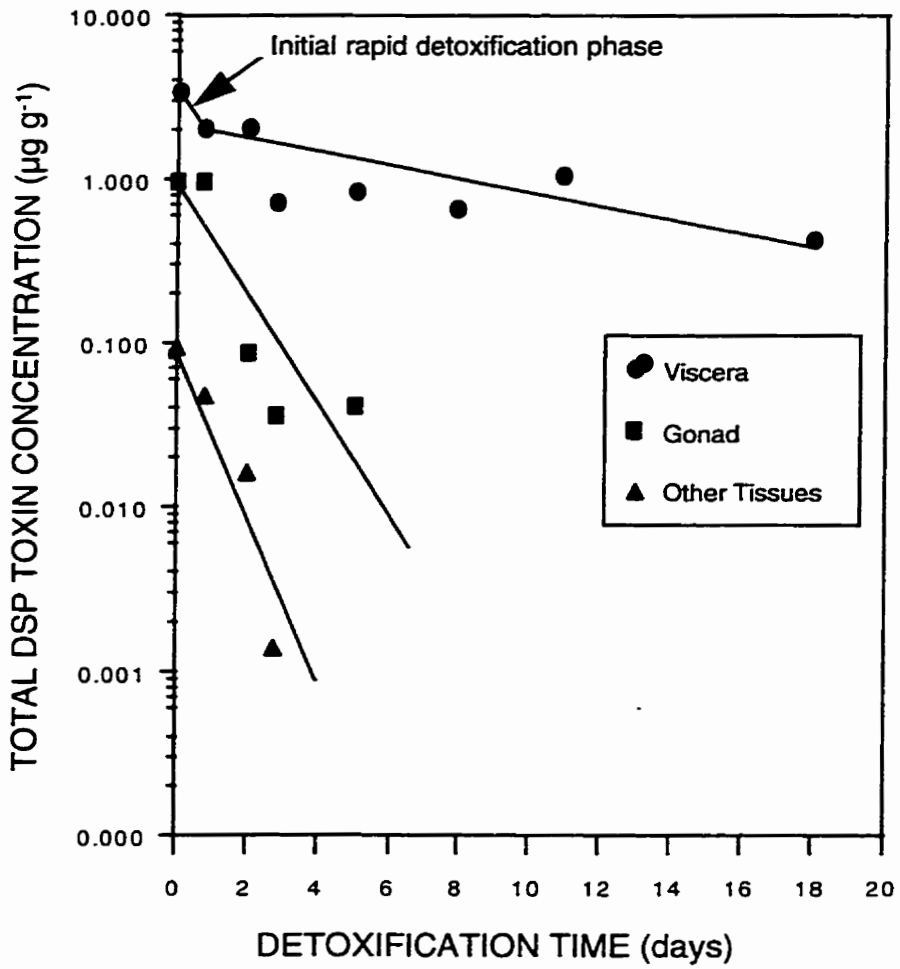


Fig. 3.14. Loss of DSP toxins from *Argopecten irradians* tissues following exposure to DSP-toxigenic *Prorocentrum lima*. Detoxification rates fitted to the general exponential loss equation: $T_t = T_0 e^{-\lambda t}$, where T_t = toxin concentration (µg g^{-1}) T_0 = toxin concentration at beginning of detoxification (T_0 = 16 hours for viscera), λ = exponential decay coefficient (% d^{-1}) and t = time (days).

Viscera: $T_t = 1.87e^{-0.088}$, $r^2 = 0.62$
Gonad: $T_t = 0.96e^{-0.684}$, $r^2 = 0.80$
Other tissues: $T_t = 0.09e^{-1.137}$, $r^2 = 0.90$

3.3.5. Toxin composition

Temporal patterns

Relative toxin composition (% molar) of *Prorocentrum lima* cells and scallop tissues over the periods of toxin uptake and detoxification is depicted in Fig. 3.15. As determined by LC-UV analysis, DTX4 was detected in *P. lima* cell extracts in extremely variable amounts (0-5.5 fmol cell^{-1}). While the relative proportions of OA and DTX1 in *P. lima* samples remained fairly constant over the exposure period, D8OA levels fluctuated inversely with its sulfated parent toxin, DTX4.

Analyses by both LC-UV and negative ion-spray LC-MS failed to detect DTX4 in any of the extract phases taken from visceral tissues on day 8 of the exposure period, at which time toxin concentrations had reached maximum levels in the scallops. Since DTX4 was not detected in these samples, no other scallop tissues extracts were analyzed for DTX4. In visceral tissues, D8OA was the principal toxin present, fluctuating inversely with OA throughout the toxin uptake and detoxification periods (Fig. 3.15). Temporal changes in the relative concentrations of D8OA and OA in visceral tissues were not significant when tested by linear regression analysis (H_0: slope differs from zero), however a significant ($P = 0.0015$), yet slow, decrease in the relative concentration of DTX1 occurred during the toxin uptake period. Levels of DTX1 (% molar) were constant in visceral tissues during detoxification.

Levels of D8OA and OA also varied inversely in gonadal tissues throughout the feeding study, however unlike in visceral tissue, the two toxins were present in relatively equivalent proportions. There was no temporal trend detected for any of the toxins in the gonads. Although mantle/gill/adductor tissues were sampled less frequently during the exposure period, a significant enrichment in OA (linear regression, $P = 0.0076$) and a decrease in D8OA ($P = 0.0265$) occurred in this tissue compartment over the time-frame of the uptake/detoxification study.

A trend common to each of the tissue compartments, as well as ingested *P. lima* cells, was the relatively stable levels of DTX1 compared to the large fluctuations of other OA derivatives. In general, for each of the compartments studied, DTX1 accounted for 10 - 20

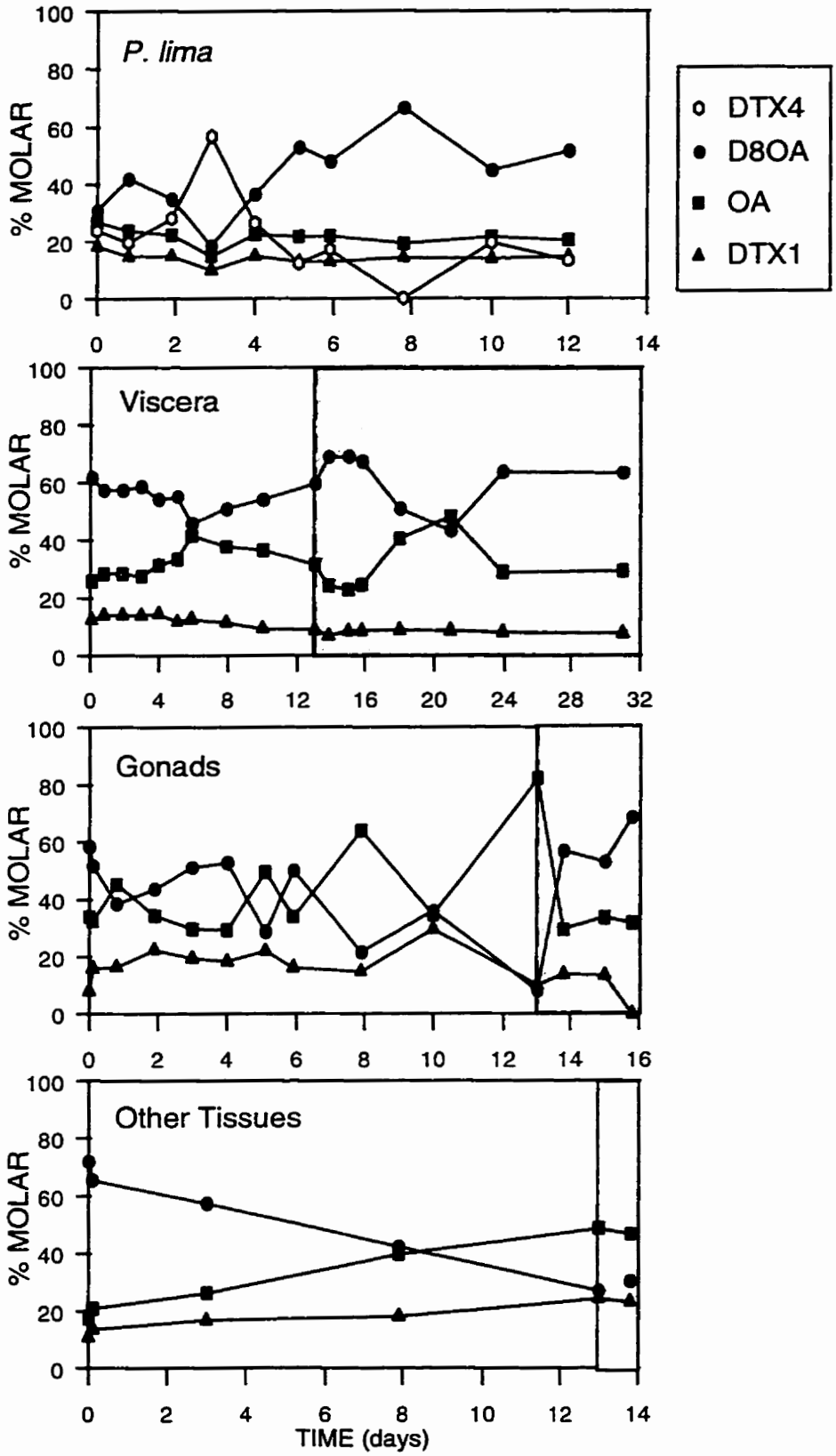


Fig. 3.15. Relative (% molar) composition of DSP toxins in *Argopecten irradians* tissues and ingested *Prorocentrum lima* cells during toxin exposure and detoxification periods (beginning of detoxification indicated by dashed line).

% (molar) of the total toxin present. The remainder varied widely among OA, D8OA and DTX4.

Relative toxin composition

The toxin composition of scallop feces produced on day 12 of the uptake study was almost identical to that of *P. lima* cells and was characterized by the presence of DTX4 (Fig. 3.16a), indicating that a large proportion of the feces was composed of intact *P. lima* cells. Mean (averaged over the whole uptake period) arcsine transformed levels of individual DSP toxins in scallop tissues were compared to *P. lima* cells by pairwise multiple comparisons (Dunnet's test, $P < 0.05$). D8OA levels were significantly higher in visceral and other tissues than in *P. lima* cells, while OA levels were higher in visceral and gonadal tissues. There was no significant difference in DTX1 levels between tissues and dinoflagellate cells. Comparisons between tissue compartments indicated that gonadal tissues contained significantly more OA and less D8OA than either visceral or other tissues. OA enrichment in gonads was also apparent when the OA/D8OA molar ratios were examined (Fig. 3.16b). The ratio of all OA derivatives (DTX4 + D8OA + OA) to DTX1 was similar for *P. lima* cells and non-visceral tissues, however visceral tissues were enriched in OA derivatives compared to DTX1, especially during the detoxification phase, when the viscera contained significantly lower proportions of DTX1 than during the uptake period (*t*-test, $P < 0.0001$) (Fig. 3.16c).

3.4. Discussion

Short-term feeding responses

At *Prorocentrum lima* concentrations of 100 cells mL (10^5 cells L^{-1}), adult bay scallops ingested the toxigenic cells as readily as equivalent biovolume concentrations of the non-toxic diatom *Thalassiosira weissflogii*. However, scallop clearance rates were much more inhibited by increases in *P. lima* cell concentrations than of *T. weissflogii*. This evidence indicates that although bay scallops appear unaffected by exposure to DSP-toxigenic *P. lima* at concentrations of 10^5 cells L^{-1}, higher cell concentrations cause scallops to rapidly reduce feeding rates, presumably to reduce exposure to the harmful effects of DSP toxins.

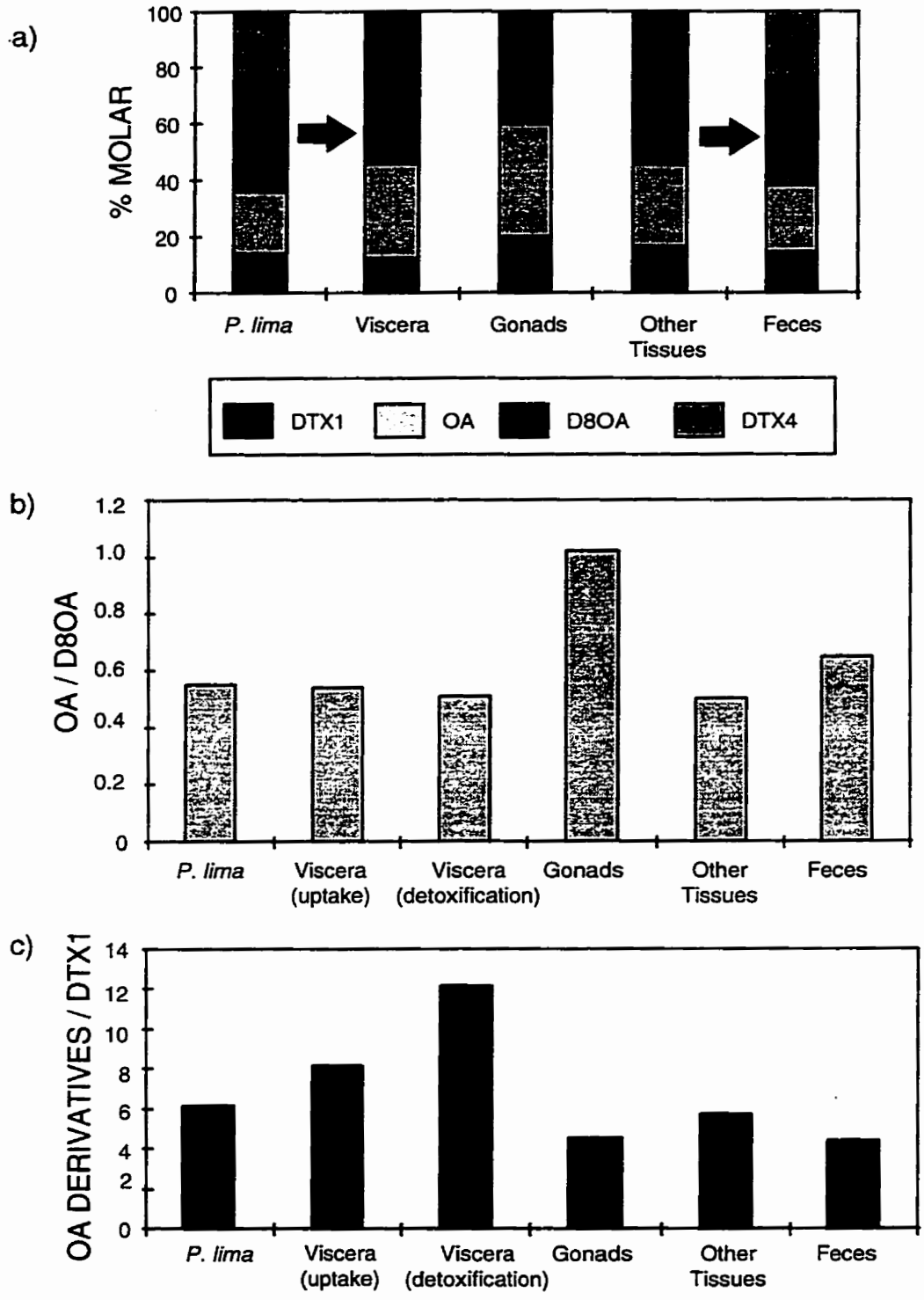


Fig. 3.16. Mean DSP toxin profiles in ingested *Prorocentrum lima* cells, *Argopecten irradians* tissues and fecal ribbons: a) relative % molar toxin content; b) ratio of OA to D8OA; c) ratio of OA derivatives to DTX1. Arrows indicate direction of toxin flow. Dashed lines refer to toxin ratios in *P. lima* cells.

It is possible that scallops have post-ingestive mechanisms to protect themselves from DSP toxins which may become overwhelmed at high cell concentrations. This would force scallops to cope with the toxins using pre-ingestive mechanisms, including reduced clearance rates, pseudofeces production and intermittent valve closure, all of which were observed at very high concentrations of *P. lima*. Peak ingestion rates determined at 130 *P. lima* cells mL^{-1} indicated that scallops were probably ingesting *P. lima* cells at maximal rates throughout both the juvenile and adult long-term feeding studies, during which cell concentrations were maintained at approximately 100 - 200 cells mL^{-1}.

Anatomical compartmentalization of toxins

Short-term loading of scallops with DSP toxins indicated that most of the total toxin body burden was confined to the viscera (77%), whereas gonadal tissue comprised a smaller, yet significant portion (11%). Although the subsequent depuration study indicated that high DSP toxin levels in gonadal tissues were derived from labile toxin components in the intestinal loop section of this tissue, shellfish marketing authorities should nonetheless be warned that bay scallop roe can become quite toxic during exposure to DSP toxigenic dinoflagellates. Gills, mantle and adductor tissue each contained only 4% of the total toxin load and can be considered low risk tissues for DSP toxin accumulation. Due to the very low toxin concentrations in each of these tissues (< 0.1 $\mu g\ g^{-1}$), they were combined and treated as one compartment for the subsequent long-term toxin uptake and depuration study.

Examination of DSP toxin profiles revealed that all of the scallop tissue compartments were enriched in OA relative to *P. lima* cells, presumably due to hydrolysis of the D8OA component following ingestion of cells, as suggested in the previous chapter. Since D8OA is not considered to be a phosphatase inhibitor (Hu et al., 1995), the D8OA to OA shift in scallop tissues represents an increase in total toxicity. Also consistent with the results of the juvenile toxin uptake study, was the decrease in relative DTX1 content in tissues compared to *P. lima* cells, supporting the hypothesis that DTX1 may be selectively eliminated from scallop tissues at a slightly faster rate than OA-derivatives. An interesting result of this study was the unique toxin profile exhibited in gills, which contained a higher percentage of DTX1 and a lower OA/D8OA ratio than other tissues. The resemblance of

the gill toxin composition to that of *P. lima* cells suggests that a significant portion of the toxin component may have been derived from intact *P. lima* cells trapped amongst the gill filaments. Although scallops were lightly rinsed with filtered seawater prior to dissection, it is quite plausible that mucilage aggregates of newly filtered *P. lima* cells may have remained amongst the gill filaments following excision of the tissue. Although total toxin content in adductor muscle was low, it should be noted that this compartment contained the highest and lowest proportions of OA and DTX1, respectively. This evidence lends support to the hypothesis that D8OA is hydrolyzed to OA, and DTX1 is selectively eliminated as DSP toxins are subjected to scallop digestive processes, since toxin located in the adductor muscle represents a component which has already been digested and transferred from the viscera.

Feeding physiology

As was the case with juvenile bay scallops, when exposed to toxigenic *Prorocentrum lima* cells, adults ingested the cells over a two week duration without suffering any mortalities. Feeding parameter measurements suggested that bay scallops may use post-ingestive selection to cope with DSP toxins ingested with *P. lima* cells. Absorption of organic matter from ingested food decreased by approximately 25% when scallops were changed from a diet of non-toxic diatoms (*Thalassiosira weissflogii*) to toxigenic *P. lima*. Since both species of microalgae were composed of 80% organic matter, it cannot be argued that *P. lima* is assimilated less efficiently due to poorer organic content. One explanation for the lower AE for *P. lima* cells is that the cells may not have been as easily digested as those of *T. weissflogii*. The rapid increase in clearance rates exhibited when adult scallops were depurated on a diet of non-toxic diatoms may have been an attempt by the scallops to compensate for nutritional losses incurred during exposure to *P. lima* cells. This suggests that although scallops were able to survive two-weeks of exposure to toxigenic *P. lima*, longer toxin exposure may have resulted in nutritional deficiencies and poor growth.

Fecal deposition rates of scallops were unaffected by exposure to *P. lima*, demonstrating that scallops did not enhance elimination of DSP toxins via decreasing gut residence times. However, an apparent increase in the incidence of intact *P. lima* cells in

fecal ribbons during the latter portion of the exposure period suggested that the proportion of intestinal feces (ingested material that bypasses the digestive gland) may increase after extended *P. lima* exposure. An important finding of this study was that *P. lima* cells were able to survive gut passage and subsequently divide in culture media. This evidence demonstrates that a substantial risk is incurred when shellfish stock are transferred from areas where DSP-toxigenic strains of *P. lima* exist, since release of live cells in fecal pellets can seed new sites with this dinoflagellate. Aquaculturists should be advised to allow stock to clear gut contents for at least three days in contained tanks prior to introduction to a new site.

Toxin uptake and depuration

Adult bay scallops accumulated DSP toxins at rates rapid enough to exceed regulatory levels (0.2 $\mu g\ g^{-1}$ whole $tissue^{-1}$) in less than 18 hours. However, since overall toxin assimilation efficiency in scallop tissue was less than 1%, the initial toxin load was likely a result of newly ingested *P. lima* cells in the gut (including intestinal loop passing through the gonad). This point is further supported by the similarity between fluctuations in *P. lima* cellular toxin content and tissue toxin content. The observed decrease in DSP toxin concentration in tissues midway and again at the end of the toxin exposure, was clearly a result of decreases in the cellular toxin content of ingested *P. lima* cells. In each of the tissue compartments, toxin loads peaked on the eighth day of exposure, coinciding with a peak in cellular DSP toxin content of 22.7 $fmol\ cell^{-1}$. The close coupling of tissue toxin load with cellular toxin content strongly suggests that DSP toxicity in scallop tissue was mainly derived from labile toxin components which experience a short residence time (< 1 day) in scallop tissues. The labile toxin load would have been a result of both intact and recently digested *P. lima* cells, primarily confined to the scallop gut. This would account for both the relatively high toxin level in gonadal tissue and low levels in mantle, gills and adductor, since the gonad includes part of the intestinal tract while the other tissues have little direct contact with the gut. Evidence that gonadal toxin content was independent of its condition index supported the hypothesis that DSP toxins in gonads were composed of a labile intestinal component rather than a sequestered fraction in the actual tissue. These findings are favourable for shellfish growers, since toxin accumulation in gonadal tissue

would present a long-term marketing problem for tissue consumption, as well as pose a threat to the reproductive capacity of maturing gonadal tissue.

Depuration of DSP toxins from bay scallop tissues reiterated findings that the majority of the toxin load in scallops was composed of a labile component. Rapid detoxification of gonadal tissue and the gill/mantle/adductor compartment in less than 5 days demonstrated that DSP toxins were poorly, if at all, bound to these tissues. Moreover, this evidence suggests that non-visceral tissues should be easily rendered fit for human consumption via depuration following DSP toxin contamination. An apparent biphasic detoxification patterns exhibited in visceral tissue suggests that DSP toxins were present as both a labile and bound fraction in this tissue. Rapid release of approximately 30% of visceral toxin during the first 16 hours of depuration was likely a result of evacuation of intact and partially digested *P. lima* cell via fecal deposition. However, depuration of the remaining toxin load in the viscera occurred at a considerably slower rate (8.4% d^{-1}), and represents toxins that were bound to gut tissue. Persistent presence of DSP toxins in the viscera even after two weeks of detoxification indicates that scallop visceral tissue is not likely to be depurated easily in the event a DSP incident. However, rapid loss of toxins from non-visceral tissues suggests that regulatory authorities should test both sets of tissues for DSP toxicity since adductor and gonadal tissues appear to be fit for consumption long before visceral tissue is free of toxins.

Toxin composition

Analytical methods for the detection of DTX4, a slightly water-soluble derivative of OA, were developed at the time the present study was conducted. Evidence of DTX4 in Prorocentrum lima cells fed to scallops added another dimension in the examination of DSP toxin transformation in scallops following ingestion of P. lima cells. Although DTX4 in cell extracts was detected at levels slightly less than D8OA and OA, it is assumed, according to the results of Quilliam and Ross (1996), that DTX4 was the principle cellular DSP toxin component prior to rupture of cells via the extraction process. Failure to detect DTX4 in scallop tissue indicates that this compound was hydrolyzed to D8OA and OA upon digestion of P. lima cells by scallops. This process may have been facilitated either by scallops digestive enzymes or by esterases endogenous to P. lima cells. Detection of

DTX4 in fecal pellets confirmed the presence of live, toxin-producing cells in the feces and suggests that DTX4 in live P. lima cells present in the scallop viscera was hydrolyzed during homogenization of the tissue for toxin extraction.

Amongst scallop tissues, relative toxin composition was similar with the exception of evidence of OA enrichment in gonadal tissues. Results of this study suggest that toxin in this tissue is confined to the intestinal loop, therefore it is possible that digestive processes influencing hydrolysis of DTX4 to D8OA are more complete in this section of the digestive tract than in the viscera which would contain recently ingested cells as well as digested material. Further support for the hypothesis that DTX1 is selectively eliminated from scallop tissues was provided by detoxification data, which revealed a lower proportion of DTX1 in the viscera during the detoxification stage than during toxin uptake.

Chapter 4. Biotransformation processes

4.1. Introduction

As well as understanding pathways of phycotoxin uptake and loss in shellfish, knowledge of metabolic conversions of toxins following ingestion of toxigenic microalgae by bivalve molluscs is also of critical importance. Such biotransformations can lead to dramatic shifts in net tissue toxicity (Bricelj and Cembella, 1995; Bricelj et al., 1990), and possibly result in the appearance of *de novo* toxins not previously detected in ingested microalgal cells (Cembella et al., 1993). Such conversion processes can be facilitated by enzymes derived from either shellfish digestive glands or ingested microalgal cells.

A combination of physical (Beninger and Le Pennec, 1991) and chemical (Seiderer et al., 1982) digestive processes that operate within the gut of bivalve molluscs presents a multitude of complicating factors for attempts to distinguish toxin conversion processes linked to shellfish digestive enzymes from catabolic processes endogenous to ingested cells. To alleviate this problem, researchers examining paralytic shellfish toxin conversions have incubated purified PSP toxins with shellfish tissue homogenates (Shimuzu and Yoshioka, 1981; Sullivan et al., 1983), thereby eliminating any physical processes induced by live animals. Although Quilliam and Ross (1996) demonstrated that DTX4 in *P. lima* cells can be hydrolyzed to D8OA and OA following cell disruption, questions remained concerning the fate of this metabolic pathway in shellfish tissue. Therefore, an *in vitro* toxin incubation experiment, similar in design to those described above, was employed in the present study to determine the main factors responsible for the conversion of DSP observed in bay scallop tissue in the previous two chapters.

4.2. Materials and Methods

4.2.1. *In vitro* incubation of DSP toxins with visceral and gonadal tissue

Visceral and gonadal (including intestinal loop) tissues were excised separately from adult bay scallops (n = 7) (mean shell height = 38.6 mm), weighed and immediately homogenized with 4:1 (v:w) 50 mM Tris HCl buffer (pH = 7.5), while cooling in an ice bath. Enzymatically inactive homogenates were obtained by placing 1.5 mL aliquots of

each homogenate in 2 mL plastic microcentrifuge tubes, followed by immersion in boiling water for 2 min.

Suspensions of ruptured *Prorocentrum lima* cells were obtained by passing cell pellets (50 mL of culture centrifuged at 4000 x g for 10 min.) resuspended in 0.2 mL 50mM Tris HCl buffer through a chilled French press. Pressures produced by the press exceed 10 Kpsi and are sufficient to rupture *P. lima* cells without harming the activity of endogenous enzymes liberated from disrupted cells (Quilliam and Ross, 1996).

Purified DSP toxins produced by *P. lima* cultures were obtained from Dr. J. Wright, Institute for Marine Biosciences, Halifax, NS. Although a toxin solution containing only DTX4 was preferred for this study, this was not possible due to the degradative nature of DTX4. Therefore, the toxin solution contained DTX4 accompanied by slightly lower concentrations of D8OA, OA and DTX1. Aliquots (350 µL) of purified toxin solution were incubated in 1.5 mL plastic microcentrifuge tubes with tissue homogenates according to Table 4.1 Each tube contained one of several different homogenates chosen to determine biotransformation processes of DSP toxins in the presence of scallop and/or *P. lima* endogenous enzymes.

Incubations were performed in duplicate at room temperature (*ca.* 22 °C) and sampled at intervals of 0, 0.5, 4 and 22 h. At each interval, incubation tubes were vortexed for 30 s following which, 100 µL aliquots were removed from each tube and combined with 400 µL of methanol in a 1.5 mL centrifuge tube, thereby arresting enzymatic activity. It is important to note that the initial "time 0" sample actually occurred after 30 s of vortexing, during which time the toxins were briefly exposed to enzymatic activity. Toxins were extracted by centrifuging samples at 6600 x g for 3 min., following which the supernatants were decanted. Pellets were rinsed with 100 µL of 80% aqueous methanol, centrifuged, and the combined supernatants were evaporated to dryness in a vacuum centrifuge. Following resuspension in 100 µL of 80% aqueous methanol, extracts were passed through a 0.45 µm cartridge-filter (Millipore Ultrafree-MC) and transferred to crimp-topped autosampler vials for toxin analysis and quantitation by LC-UV (D8OA, DTX4) and positive ionspray LC-MS (OA, DTX1) as previously described in sections 3.2.5 and 2.2.6, respectively.

Table 4.1. Solution volumes (mL) used for *in vitro* incubation of purified DSP toxins with scallop tissue homogenates and/or *P. lima* cell homogenates. Incubation solutions are as follows: C = control; BV = boiled viscera; BG = boiled gonad; FV = "fresh" viscera; FG = "fresh" gonad; PL = ruptured *P. lima* cell homogenate; FV + PL = fresh viscera combined with *P. lima* homogenate.

	C	BV	BG	FV	FG	PL	FV+PL
DSP toxin solution	350	350	350	350	350	350	350
Tris Buffer	350	150	150	150	150	200	-
Viscera homogenate	-	-	-	200	-	-	200
Boiled viscera	-	200	-	-	-	-	-
Gonad homogenate	-	-	-	-	200	-	-
Boiled gonad	-	-	200	-	-	-	-
P. lima homogenate	-	-	-	-	-	150	150
Total volume (μL)	700	700	700	700	700	700	700

4.2.2. *In vitro* incubation of *Prorocentrum lima* cells with visceral tissue

A 50 mL culture of *Prorocentrum lima* (age = 90 days, stationary phase growth) was centrifuged at 4000 x g for 10 min. The supernatant was decanted to waste and the pellet was resuspended in 5 mL of Tris HCl buffer (pH = 7.5). Cells suspended in the buffer solution were observed to be intact when examined under phase contrast microscopy. Aliquots (700 µL) of the *P. lima* suspension were incubated with 700 µL of either Tris HCl buffer, scallop viscera homogenate or boiled (2 min.) viscera homogenate in 1.5 mL plastic microcentrifuge tubes. Incubations were performed at room temperature and sampled (100 µL) at intervals of 0, 0.5, 4 and 22 h. Toxins were extracted as described in 4.2.1. and analyzed for DTX4 and D8OA by LC-UV.

4.3. Results

4.3.1. Toxin incubations

Analysis by LC-UV indicated that concentrations of DTX4 decreased over the 22 h incubation period in all of the samples (Fig. 4.1a), however this trend was much more rapid and pronounced in incubations that included ruptured *P. lima* cell homogenate. In these samples, DTX4 concentrations decreased to approximately 50% of the initial concentrations in the control incubations after only 30 s exposure to *P. lima* homogenate. After an incubation period of 30 min., DTX4 was not detected in either of the samples containing *P. lima* homogenate. Thus, hydrolysis of DTX4 was rapid in the presence *of P. lima* homogenate and the inclusion of scallop viscera homogenate did not appear to have any additional effects. In the incubations that did not contain *P. lima* homogenate, DTX4 concentrations remained relatively constant during the first 4 hours of incubation. After 22 h of incubation, DTX4 levels had decreased to 30% of initial concentrations in the control and 60% and 75% in fresh viscera and gonad incubations.

A rapid increase in D8OA concentration during the first 30 min. of incubation provided evidence that DTX4 hydrolysis to D8OA had occurred in both of the incubations containing *P. lima* homogenate (Fig. 4.1b). Net increases in D8OA were not apparent in

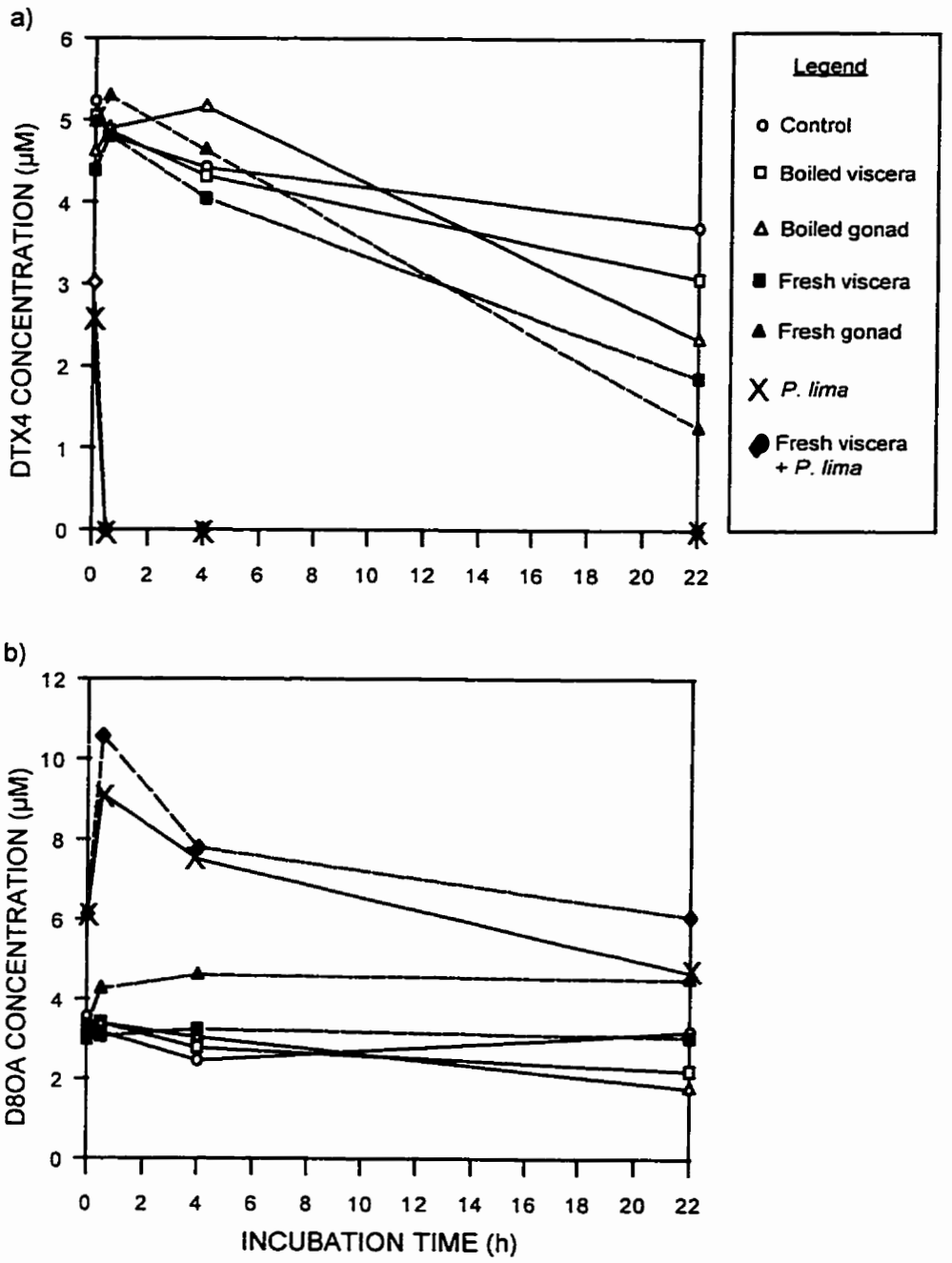


Fig. 4.1. Concentrations of DTX4 (a) and D8-OA(b) in homogenates incubated with purified DSP toxins, as analyzed by LC-UV.

the incubations that did not contain *P. lima* homogenate, with the exception of a slight D8OA increase in the fresh gonad incubation (38%).

Positive ion-mode LC-MS analysis was only performed on the control, fresh viscera and *P. lima* homogenate + fresh viscera incubations. DTX1 results were used to normalize concentrations of the okadaic acid derivatives (DTX4, D8OA and OA). Normalized results indicated that all of the toxins remained at relatively constant levels in the control, contrasting with the incubation containing *P. lima* homogenate combined with scallop viscera, in which DTX4 was rapidly hydrolyzed to D8OA during the first 30 min. of incubation followed by a more gradual hydrolysis to OA over the following 22 h (Fig. 4.2). In the incubation containing only viscera homogenate, a slight decrease in DTX4 levels over the 22 h incubation period was accompanied by a small increase in D8OA, while OA levels appeared to remain constant.

4.3.2. *P. lima* cell incubations

As analyzed by LC-UV, DTX4 concentrations in the control homogenate remained at levels near 0.9 μM during the first 4 hours of incubation, however after 22 hours, the DTX4 concentration had decreased by approximately 80 % (Fig. 4.3a). Initial DTX4 concentrations in boiled viscera homogenate were substantially lower than the control and remained at this level for the duration of the incubation period. In fresh viscera homogenate, initial DTX4 concentration was 90% lower than the control, after which the concentration of this toxin decreased to negligible levels by the end of the incubation period.

Concentrations of D8OA in the control homogenate followed a similar temporal pattern as DTX4, characterized by a marked decrease after 22 hours of incubation (Fig. 4.3b). In both of the viscera homogenates, D8OA concentrations increased during the first 30 min. of incubation, followed by a substantial decrease in this toxin over the rest of the period. Although D8OA patterns were similar in the two viscera homogenates, the sharp

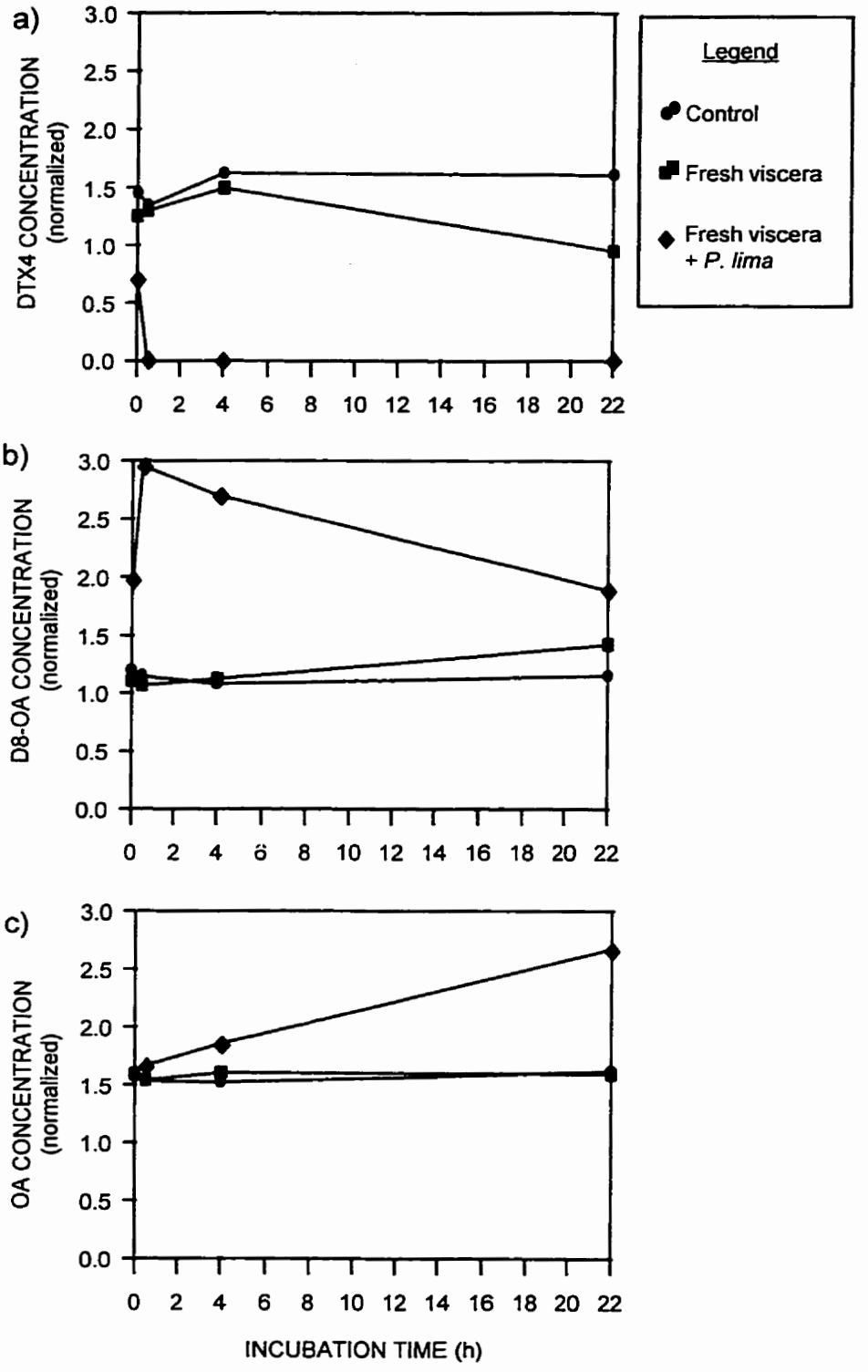


Fig. 4.2. Concentrations of DTX4 (a), D8-OA (b) and OA (c) in homogenates incubated with purified DSP toxins. DTX4 and D8-OA analyzed by LC-UV. OA analyzed by LC-MS. Toxin concentrations normalized to DTX1, as analyzed by LC-MS.

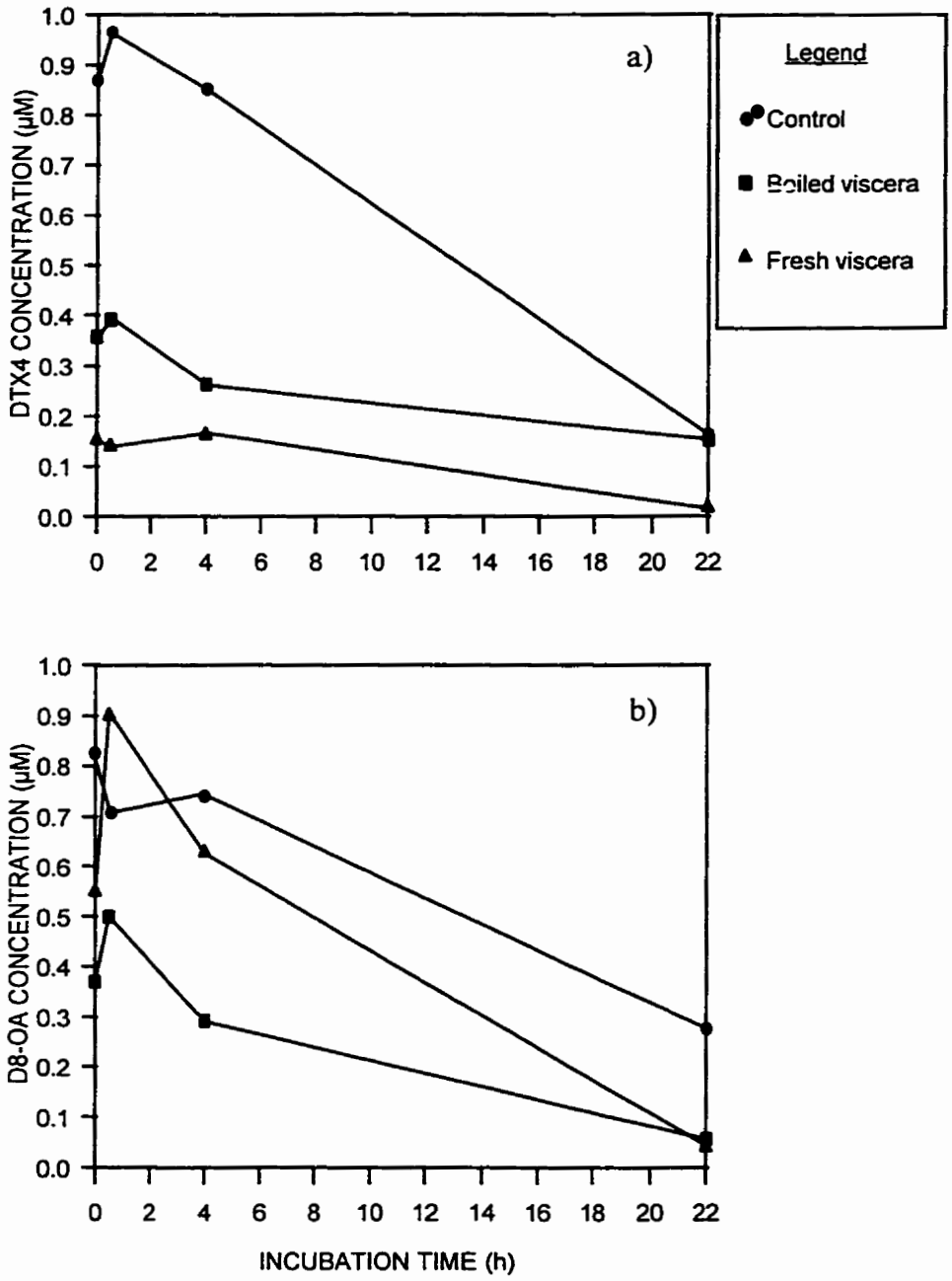


Fig. 4.3. Concentrations of DTX4 (a) and D8OA (b) homogenates incubated with intact *Prorocentrum lima* cells, as analyzed by LC-UV.

initial increase was much more pronounced in the enzymatically active "fresh" viscera homogenate.

4.4. Discussion

In the presence of ruptured *Prorocentrum lima* cell material, hydrolysis of DTX4 to D8OA was complete within 30 minutes of incubation, followed by a more gradual conversion of D8OA to OA during the remaining 22 hours. These results are consistent with the findings of Quilliam and Ross (1996) which suggest that the rapid conversion of DTX4 and the much slower conversion of D8OA may be a result of two distinct esterase pathways. The addition of bay scallop viscera homogenate to incubations already containing ruptured *P. lima* may have slightly increased the conversion rate of DTX4 to D8OA, however the overall contribution of scallop visceral enzymes to these metabolic reactions can be considered insignificant.

Although conversion of DTX4 to D8OA was evident in visceral and gonadal homogenates, similar results occurred in control incubations, and in boiled tissue samples in which enzymatic activity had been arrested. In these samples, significant degradation of DTX4 did not occur until after 4 hours of incubation, suggesting that transformation was occurring due to spontaneous hydrolysis of DTX4 rather than a result of enzymatic activity. Transformation of D8OA to OA only took place in incubations containing *P. lima* esterases, demonstrating the greater stability of D8OA relative to DTX4.

Incubation of intact *P. lima* cells with scallop tissue homogenates revealed that during the initial hours of incubation, transformation of DTX4 to D8OA was most evident in the fresh viscera sample, suggesting that *P. lima* cells were lysed by visceral enzymes causing *P. lima* esterases to hydrolyze cellular DTX4. During this period, however, significant DTX4 degradation also occurred in the presence of boiled viscera. Since enzymatic activity was destroyed by boiling, *P. lima* cells must have been ruptured by exposure to non-enzymatic chemical activity derived from viscera tissue. Although the Tris HCl buffer was intended to maintain a stable pH environment (pH 7.5) in the incubations, it is possible that digestive acids released from viscera tissue may have caused *P. lima* cells to lyse. Acidic conditions in mussels guts were also suggested by Bricelj et al. (1990) as

environments conducive to hydrolysis of PSP toxins produced by the dinoflagellate *Alexandrium fundyense*. Finally, hydrolysis of both DTX4 and D8OA in all incubations after 22 hours was clearly a result of *P. lima* cell lysis due to poor conditions for cell maintenance.

The results of the *in vitro* incubation study can be applied to findings obtained from the feeding experiments to construct a hypothesized pathway of DSP toxin metabolism in bay scallops (Fig. 4.4). Following ingestion of DSP-toxigenic *P. lima* cells by scallops, the cells would be ruptured due to physical and chemical digestive processes that occur in the scallop gut, including the grinding action of the chitinous crystalline style in the stomach (Beninger and Le Pennec, 1991) and the catabolic activity of a wide spectrum of enzymes secreted by the style and digestive glands (Seiderer et al., 1982). As demonstrated in the present study, lysis of *P. lima* cells results in the release of esterases that rapidly hydrolyze DTX4 to D8OA and more slowly degrade D8OA to OA. The differential rate of breakdown of the two toxins would explain the absence of DTX4 and relatively high proportions of D8OA in scallop tissues during the feeding experiments. Although, bay scallops do not appear to have esterases capable of efficiently hydrolyzing D8OA to OA, it is possible that other species of bivalve molluscs may be capable of such enzymatic activity. In this case, it would be expected that OA would be the predominate toxin accumulated in tissues, resulting in increased net toxicity due to the phosphatase inhibition activity of OA.

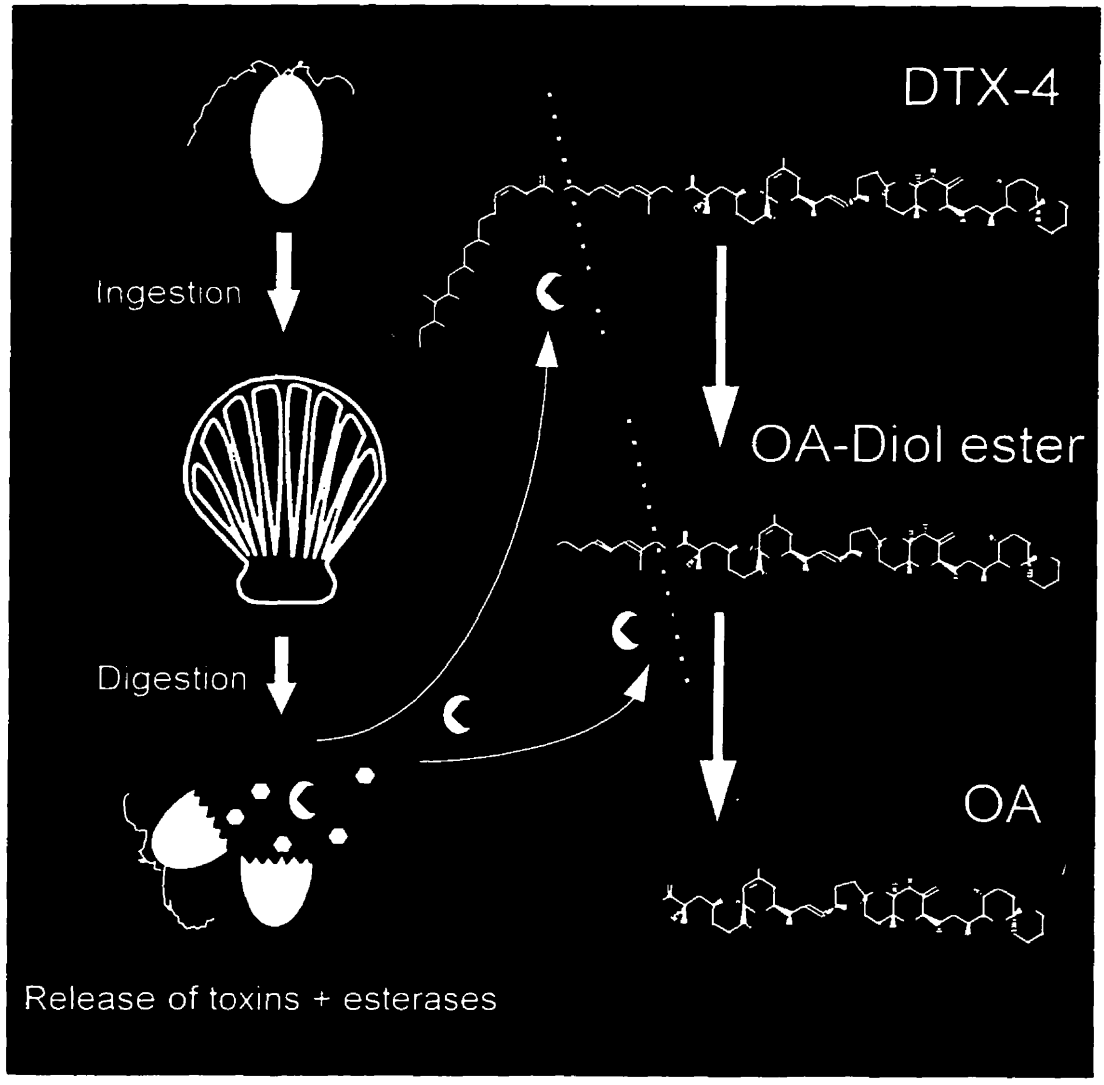


Fig. 4.4. Diagramatic representation showing biotransformation of DTX4 to OA-diol ester (D8OA) and OA via digestion and rupture of *P. lima* cells in bay scallop stomach/digestive tract.

Chapter 5. General summary and conclusions

5.1 Feeding physiology and survival

Exposure to DSP toxigenic *Prorocentrum lima* cells had very little effect on the feeding physiology of juvenile and adult *Argopecten irradians*, nor did any mortalities occur. Clearance rates of bay scallops feeding on *P. lima* were comparable to those reported in other studies of *A. irradians* ingesting non-toxic diatom cells at similar biovolume concentrations (Pierson, 1983; Bricelj and Kuenstner, 1989; Bricelj and Shumway, 1991; Palmer, 1980). Previous studies involving bivalve molluscs feeding on PSP toxigenic dinoflagellates have described a wide range of physiological effects exhibited by bivalves, including feeding inhibition and mortalities (Bardouil et al., 1993; Lesser and Shumway, 1993), while others reported no apparent effects or feeding inhibition (Bricelj et al., 1990; Bricelj et al., 1991). Similarily, Whyte et al. (1995) observed no adverse reactions when *Mytilus edulis* were exposed to cells of *Pseudo-nitzschia multiseries* containing the neurotoxin domoic acid. Aside from the present study, the only examination of the effect of DSP toxigenic algae exposure on bivalve feeding activity reported that *P. lima* had no influence on physiology, behaviour or survival in *Mytilus edulis* (Pillet and Houvenaghel, 1995). Under natural conditions, evidence of shellfish mortalities during exposure to dense blooms of DSP toxigenic *Dinophysis* spp. is seldom, if ever described, however there are many reports of shellfish mass mortalities upon exposure to blooms of paralytic shellfish toxin producing dinoflagellates (Shumway, 1990). Furthermore, blooms of non-DSP toxic *Prorocentrum mininum* have caused large-scale mortalities of juvenile bay scallops (Wikfors and Smolowitz, 1993) and oysters (Luckenbach et al., 1993). Yet juvenile bay scallops exhibited 100% survival when exposed to toxic cells of *P. lima* in the present study. It is clear that, although DSP toxicity in shellfish is a serious problem for human consumption, adverse effects on scallop survival and feeding activity are minimal.

Okadaic acid and its derivatives are powerful cytotoxins that cause phosphorylation in a broad range of animals and plants (Van Egmond, 1993; Dickey et al., 1993; Terao et al., 1993), however as noted by Windhust et al. (1996), there is very little known regarding

the effect of these compounds on marine animals. The evidence depicted in the present study, suggesting that bay scallops are not affected by exposure to DSP toxigenic cells, implies that these animals are able to protect themselves from the effects of the toxins. Shumway et al. (1987) postulated that when bivalves are exposed to toxigenic algal cells, they will often cope with the toxin by either closing their valves, thereby arresting feeding activity completely, or by employing feeding mechanisms to reduce the amount of toxin accumulated. Pre-ingestive methods to reduce ingestion of toxic cells would include reduced filtration rates or rejection of cells as pseudofeces. Throughout the long-term feeding studies, bay scallops exhibited neither of these strategies when exposed to *P. lima*. At concentrations greater than 400 cells mL^{-1}, however, pseudofeces production was observed and clearance rates were significantly reduced relative to the non-toxic control diet. Pre-ingestive rejection of *P. lima* cells only at high cell concentrations may have been employed by the scallops as a secondary adaptation if post-ingestive feeding mechanisms were overwhelmed by too many cells in the gut (Bayne et al., 1984). Similar results were reported by Pillet and Houvenaghel (1995) who noted that *M. edulis* feeding rates were lower for DSP toxic *P. lima* cells than non-toxic *P. micans* cells at cell concentrations of 1000 cells L^{-1}, however there were no differences at concentrations of 100 cells L^{-1}

Post-ingestive feeding mechanisms used by bivalves to select for and against ingested particles were demonstrated by Shumway et al. (1985) using flow-cytometric methods to examine cell composition in fecal ribbons. Although *Ostrea edulis* preferentially ingested *Prorocentrum minimum* cells relative to other algal species, these authors argued that the high incidence of *P. mininum* fragments and intact cells in the fecal ribbons indicated that these cells were selectively rejected from the animal's gut. Post-ingestive strategies by bay scallops are proposed here to reduce DSP toxin absorption when exposed to *P. lima* as a food source. Evidence for post-ingestive rejection of *P. lima* cells is provided by the observed decrease in absorption efficiency (AE) exhibited by adult bay scallops following a change of diet from *T. weissflogii* to *P. lima*, and by the frequency of live *P. lima* cells in fecal ribbons. While absorption efficiencies of bay scallops feeding on *T. weissflogii* cells were remarkably similar to those reported by Bricelj and Kuenstner (1989) and by Pierson (1983) for *A. irradians* feeding on the same diatom species, lower absorption efficiencies

for *P. lima* cells resembled those of scallops exposed to cells of poorer nutritive quality than T. weissflogii (Pierson, 1983). Cranford and Grant (1990) noted that AE values for sea scallops were correlated with the organic matter content of the ingested food. Since *P. lima* and *T. weissflogii* were determined to be equal in relative organic matter content (80%), the decrease in AE during *P. lima* exposure was likely a result of *P. lima* cells being less digestible than the diatoms. An alternative hypothesis is that the decreased absorption of *P. lima* organic material by the scallops may have been an attempt to limit DSP toxin absorption. Although AE values for bay scallops exposed to *P. lima* cells were comparable to those of other bivalves (eg. *Mercenaria mercenaria*, *Mytilus edulis*) exposed to cells of PSP toxin-producing *Alexandrium* spp. (Bricelj et al., 1990, 1991), PSP toxins were accumulated into tissues at much higher levels than those determined for DSP toxins in the present study.

The present study is the first to demonstrate the survival of DSP toxigenic algal cells in bivalve fecal ribbons. Similar studies (Scarratt et al., 1993; Bricelj et al., 1993) showed how PSP toxin-producing *Alexandrium* cells could survive and reproduce following passage through the gut of *Mytilus edulis*. Scarratt et al. (1993) reported that after 3.9 h of depuration, almost all of the *Alexandrium* cells were gone from the fecal ribbons and recommended that 12 h of purging should be sufficient to rid mussels of toxic cells prior to transferring stock to new waters. Passage of *P. lima* cells in bay scallop guts occurred over a much longer timescale, as demonstrated by the presence of live cells in fecal ribbons after as much as 3 days of depuration. Since scallops were continually ingesting *T. weissflogii* cells during the depuration period, it is appears that the residence time for *P. lima* cells greatly exceeded the normal gut passage time for particles in the bivalve guts (Scarratt et al., 1993). Bomber et al. (1988) reported that live *P. lima* cells have been found attached to the viscera of tropical fish and suggested that the ability of these cells to survive in fish viscera for extended periods represents an important system of dispersal for this species. Similarily, the slow passage of *P. lima* cells in scallop guts represents a risk of dispersing cells to new aquaculture sites via stock transfer. Furthermore, the ability of *P. lima* to adapt and survive in a wide variety of benthic environments (McLachlan et al.,

1994; Bomber et al., 1985) implies that cells released into a new site via fecal deposition from transferred stock would have a high probability of long-term survival and growth.

5.2 Toxin uptake, compartmentalization and detoxification

Although DSP toxin concentrations in shellfish tissue commonly surpass regulatory levels (0.2 μg g whole tissue^{-1}) in Japan and Europe where blooms of toxic *Dinophysis* spp. are common, levels rarely approach those reported for PSP toxin accumulation in bivalve tissue (> 100 μg STXeq g^{-1}) under natural conditions (Shumway and Cembella, 1993) and during laboratory feeding studies (Bricelj et al., 1990). Peak DSP toxin concentrations reported for bivalves exposed to natural blooms of *Dinophysis* spp. are consistently in the range of 0.5 to 2 μg g^{-1} whole tissue (Underdal et al., 1985; Lembeye et al., 1993; Zhao et al., 1993) and 1 to 10 μg g^{-1} digestive gland tissue (Sechet et al, 1990; Hageltorn, 1989; Haamer, 1995; Della Loggia et al., 1993; Quilliam et al, 1993; Carmody et al., 1995; Aune and Yndestad, 1993). The highest DSP toxin levels reported in the literature, 17 μg g^{-1} digestive gland (Rodriguez et al., 1989) and 40 μg g^{-1} digestive gland (Gago et al., 1991), occurred in mussels exposed to *Dinophysis* spp. blooms in the Spanish Rias. In the present study, bay scallop viscera tissue attained peak toxin levels at 3 to 6 μg g^{-1}, which agrees very well with values reported in the literature. Thus, it appears that the low DSP toxin accumulation efficiency exhibited by bay scallops emulates feeding processes that occur in scallops and mussels during exposure to DSP toxigenic algae under natural conditions. These observations are supported by a field study by Haamer et al. (1990) in which DSP toxin levels were much lower than expected for mussels feeding on *Dinophysis* cells in a Swedish fjord. The authors suggested that an OA blocking mechanism may operate within the mussels and hypothesized that such processes could include valve closure, reduced clearance rates, decreased absorption or an increase in depuration. The results of the present study argue against a reduction in feeding processes as a toxin blocking mechanism, and support the hypothesis that bivalves are able to maintain relatively low DSP toxin levels primarily via efficient toxin elimination.

In juvenile and adult bay scallops, the majority of the DSP toxin load was confined to the viscera. Although toxin concentrations were relatively high in adult gonadal tissue, rapid detoxification rates for gonads implied that toxicity in the gonads was represented by

a labile component confined to the intestinal tract, rather than bound to the tissue. This evidence agrees with observations for *Mytilus edulis* in which DSP toxin concentrations in digestive tissue were seven (Vernoux et al., 1994) to ten (Pillet et al., 1995) times greater than that of the other tissues. Data for paralytic shellfish toxins also indicate that toxin loads are greatest in visceral tissue during periods of toxin uptake (Bricelj et al., 1990), yet following the loss of labile toxins during depuration, most of the bound PSP toxins are often localized in non-digestive tissue including mantle and gill tissue (Bricelj and Cembella, 1995). In bay scallops, DSP toxins were only bound to visceral tissue as demonstrated during depuration by the slow loss of toxins from this compartment as compared to all other tissues. This evidence suggests that during periods when DSP toxigenic dinoflagellates are present in the water column, all of the scallop tissues represent a potential toxin risk due to the presence of labile toxins from recently ingested toxic cells. However during depuration periods, toxicity would only be expected to be associated with visceral tissue, and possibly gonads, due to the inclusion of the intestine with this tissue. For areas prone to DSP toxicity, it may be advisable for shellfish growers to culture bivalve species which do not include the viscera tissue as a market product. Scallops, for example, can be sold as adductor muscle alone, which does not sequester DSP toxins above regulatory levels. However mussels are consumed as a whole animal product, and could be kept off market for extended periods if DSP toxins persist in visceral tissue.

Detoxification of the viscera was identified by an immediate and substantial loss of DSP toxins during the initial 16 h of depuration followed by a much slower loss over the subsequent weeks. Similar detoxification patterns have been observed for the loss of PSP toxins from *Mytilus edulis* (Silvert and Cembella, 1995) and *Mercenaria mercenaria* (Bricelj et al., 1990, 1991), prompting Silvert and Cembella (1995) to hypothesize that such a pattern can be modelled as a two-stage process consisting of a rapid loss of labile toxins followed by a slow release of the bound fraction.

Bricelj et al. (1991) used general exponential equations to describe the second-stage loss of PSP toxins from tissues of *Mercenaria mercenaria*. The same approach was employed in the present study to describe the release of DSP toxins from bay scallop

tissues. Unfortunately, since other studies have only attempted to report depuration of DSP toxins from shellfish in descriptive terms, it is difficult to compare data in the existing literature. To facilitate such comparisons, exponential decay equations were fitted to data extracted from DSP studies in which toxin concentrations in depurating shellfish were reported (Fig. 5.1). Thus, DSP toxin loss rates can be compared between studies as percentage loss d^{-1} or toxin half-life in tissues (Table 5.1).

As expected, detoxification rates of bay scallop non-visceral tissues were much faster than those calculated for visceral tissues from other studies. Bay scallop viscera detoxified at a rate of 8.4% d^{-1}, which compared extremely well with data reported by Japanese researchers for loss of DSP toxins from the blue mussel *Mytilus edulis* (Yasumoto et al., 1978) and from the Japanese scallop *Patinopecten yessoensis* (Tazawa et al., 1989). Kikuchi et al. (1992) reported similar detoxification rates for *P. yessoensis* depurated on a diet of the diatom *Chaetoceros septentrionalis*, yet when an alternative diatom *Thalassiosira* was used, loss rates were halved. Marcaillou-Le Baut et al. (1993b) reported loss rates similar to the present study for *M. edulis* depurated in natural ponds, yet animals depurated in laboratory tanks on a diet of the flagellate *Tetraselmus* were very slow to detoxify. Natural basins also proved very effective sites for depuration of Adriatic mussels (*Mytilus galloprovinciallis*) which were toxin-free within 22 days (Poletti et al., 1996). Rates of DSP toxin loss from mussels at aquaculture sites in Mahone Bay, Nova Scotia were found to be slightly faster in one study (Quilliam et al., 1993), yet slower in another case (Gilgan et al., 1995), than for bay scallops in the present study. This similarity is significant since the isolate of *Prorocentrum lima* used as the DSP toxin source in this study originated from Mahone Bay and is considered a suspect as the causative organism of DSP in that region.

Overall, the DSP detoxification rate determined for bay scallop visceral tissue in the present study was comparable to rates calculated for elimination of DSP toxins from other bivalve species in the laboratory and *in situ* (Quilliam et al., 1993; Gilgan et al., 1995; Yasumoto et al, 1978; Tazawa et al., 1989). However, such comparisons must be made with caution due to the variety of toxin analysis methods used for each study. As discussed previously in this thesis, mouse assays are not necessarily specific to DSP toxins

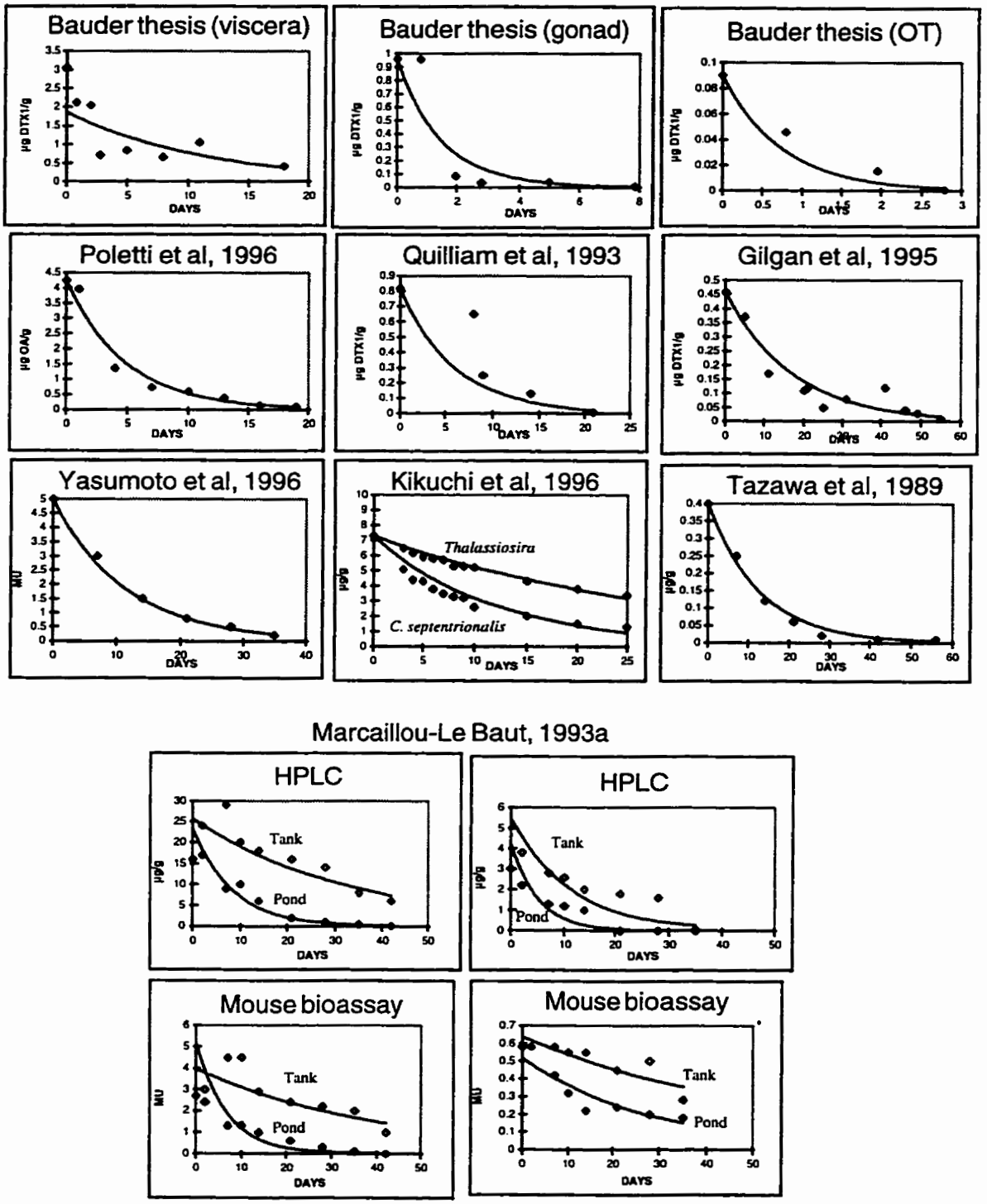


Fig. 5.1 Release of DSP toxins from bivalve mollusc species. Exponential decay curves fitted to data from studies listed in Table 5.1.

Table 5.1 Detoxification rates of DSP toxins from digestive tissues of bivalve mollusc species. Detoxification data from cited studies were fitted to the general exponential loss equation: $T_t = T_0 e^{-\lambda t}$, where T_t = % of toxin concentration at start of detoxification, T_0 = initial toxin concentration, λ = exponential decay coefficient and t = time (d). Toxin concentrations from mouse bioassay data was converted according to Rodriguez et al. (1989), where 1 μg DSP toxin g^{-1} = 4 mouse units (MU) g^{-1}.

DATA SOURCE	SPECIES	DETOXIFICATION SITE	DETECTION METHOD	TOXINS	T_0 (μg g^{-1})	$-\lambda$	r^2	HALF LIFE (d)	% LOSS d^{-1}
Bauder (present study)	*A. irradians*	Tank; fed *T. weissflogii*	LCMS	D8OA+OA +DTX1					
viscera					3.055	0.088	.62	7.9	8.4
gonad					0.964	0.683	.80	1.0	49.5
other tissue					0.090	1.350	.88	0.5	74.1
Poletti et al. (1996)	*Mytilus galloprovincialis*	Natural basin, Italian Adriatic	LCMS	OA	4.25	0.210	.97	3.3	18.9
Quilliam et al. (1993)	*M. edulis*	*in situ*; Mahone Bay, NS	LCMS	DTX1	0.82	0.167	.81	4.1	15.4
Gilgan et al. (1995)	*M. edulis*	*in situ*; Mahone Bay, NS	HPLC-FD	DTX1	0.46	0.059	.81	11.7	5.7
Yasumoto et al. (1978)	*M. edulis*	Tank	Mouse assay		20	0.088	.99	7.9	8.4
Kikuchi et al. (1992)	*P. yessoensis*	Tank; fed *Thalassiosira*	Micobial inhibition	OA	7.3	0.033	.98	21.0	3.2
Kikuchi et al. (1992)	*P. yessoensis*	Tank; fed *C. septentrionalis*	Micobial inhibition	OA	7.3	0.081	.89	8.6	7.8
Tazawa et al. (1989)	*P. yessoensis*	*In situ*; Japan	ELISA	OA	0.4	0.079	.90	8.8	7.6
Marcaillou-Le Baut et al. (1993a)	*M. edulis*	Tank	HPLC-FD	OA	16	0.030	.77	23.1	3.0
		Aquaculture pond	HPLC-FD	OA	16	0.118	.97	5.9	11.1
		Tank	HPLC-FD	OA	3	0.089	.63	7.8	8.5
		Aquaculture pond	HPLC-FD	OA	3	0.196	.86	3.5	17.8
		Tank	Mouse assay	OA	10.8	0.025	.64	27.7	2.5
		Aquaculture pond	Mouse assay	OA	10.8	0.147	.78	4.7	13.7
		Tank	Mouse assay	OA	2.4	0.017	.70	40.8	1.7
		Aquaculture pond	Mouse assay	OA	2.4	0.035	.85	19.8	3.4

and could result in inflated toxin values due to the presence of fatty acid derivatives in the extracts used for the assays. This may explain the extremely slow detoxification rates calculated for mouse assay data reported by Marcaillou-Le Baut et al. (1993a). Furthermore, it is difficult to compare detoxification rates between various bivalve species due to species-specific differences in feeding physiology, as shown for PSP toxin depuration studies (Shumway, 1990).

Several studies have related very slow DSP detoxification rates for mussels under natural conditions. In Norway (Underdal et al., 1985; Dahl and Yndestad, 1985; Sechet et al., 1990) and Sweden (Haamer et al., 1990) low levels of DSP toxicity have been found to remain in mussel tissue throughout the winter following toxin uptake from exposure to blooms of *Dinophysis* spp. the previous autumn. Similar patterns of winter DSP toxin persistence have been reported for mussels depurated on the Italian (Della Loggia et al., 1993; Boni et al., 1993) and French Mediterranean coasts (Belin, 1993). Dahl and Yndestad (1985) suggested that mussels were slow to detoxify due to low metabolic rates resulting from cold water and poor food conditions during the winter. According to Bayne (1985), mussels respond to the environmental stress of poor food quality by reducing clearance rates, lengthening gut residence times and increasing absorption efficiency. Since low cell concentrations of *Dinophysis* cells often occur sporadically at the above sites during the winter (Della Loggia et al., 1993; Underdal et al., 1985), winter DSP toxin persistence in mussels may be a combined result of unusually efficient absorption of DSP toxins from ingested *Dinophysis* cells and very slow gut clearance and rates. This hypothesis was supported by Haamer's (1995) observation that mussels in Sweden detoxified most rapidly at the onset of the spring bloom when high concentrations of non-toxic diatoms were once again available for mussel consumption.

5.3 Toxin composition and biotransformation

The recent discovery of ester derivatives of okadaic acid (Hu et al., 1992, 1993, 1995) poses questions critical to the understanding of DSP toxin chemistry, especially regulatory strategies for detection of these toxins in shellfish for public consumption. Although okadaic acid-diol esters (D8OA) do not appear to be phosphatase inhibitors, both its biochemical precursor (DTX4) and end-product (OA) exhibit such cytotoxic activity (Hu

et al., 1995). New evidence indicating that OA can be a powerful tumour promoter needs to be investigated further to determine whether OA-esters are also tumourgenic. In any case, given the bioconversion pathways linking OA ester derivatives to OA (Quilliam and Ross, 1996), regulatory authorities should consider such compounds as potential toxins. Prior to the present study, no information was available regarding the accumulation and transformation of OA-esters in shellfish tissue, either *in vivo* or *in vitro*.

Quilliam and Ross (1996) hypothesized that DTX4 may be hydrolyzed in shellfish via the activity of digestive enzymes. *In vitro* experiments performed in the present study demonstrated that DTX4 is indeed rapidly hydrolyzed in bay scallop viscera, however the transformation is solely a result of endogenous esterases derived from ruptured *Prorocentrum lima* cells. Conversion of DTX4 to D8OA occurs almost immediately upon digestion of *P. lima* cells in the gut of scallops. The absence of DTX4 in scallop tissues following ingestion of *P. lima* cells indicates that this compound is unlikely to be present in shellfish tissue. It is more likely that any DTX4 will be hydrolyzed to form D8OA or OA. During feeding studies, D8OA comprised a major portion of the total DSP toxin load in all tissue compartments of bay scallops. This evidence, combined with the relatively slow rates of conversion of D8OA to OA, should be a concern for regulatory authorities which rely on detection methods specific to OA and DTX1. Although the HPLC-FD ADAM method yields better quantitative data than mouse bioassays (Quilliam, 1995) it is unable to detect D8OA or other OA-esters which do not have a free carboxylic acid endgroup. Thus, there is a high probability that potential DSP-toxicity in the form of OA-esters is being overlooked by shellfish monitoring programs. Numerous reports of erratic OA concentrations in shellfish tissues (Pillet et al., 1995; Haamer et al., 1990; Marcaillou-Le Baut et al., 1993a) may simply be a result of biotransformation processes occurring in tissue samples. In the present study, D8OA and OA concentrations in scallop tissues fluctuated inversely throughout the feeding studies, however when total toxin concentrations were taken into account, the results were not nearly as erratic. Previous studies of DSP toxicity in shellfish did not analyze for OA-esters and may have misinterpreted fluctuating toxin concentrations due to bioconversion processes as erratic toxin uptake. Although in the present study, analysis of OA-derivatives by LC-MS proved

to be a very effective tool for determining the composition of DSP toxins in scallop tissues, routine DSP monitoring programs must rely on simpler, less costly methods. Since HPLC-FD analysis cannot detect potential toxins in the form of OA-esters, regulatory authorities should consider using LC-UV detection to analyze for diol esters and DTX4. Perhaps an even simpler and more conservative solution would be to hydrolyze all OA-esters in shellfish extracts to OA, thereby converting potential toxins to OA for HPLC-FD analysis.

Significant biotransformation pathways were not apparent for DTX1 in bay scallop tissues. However, slight decreases in DTX1 concentrations in scallop tissues relative to P. lima cells suggested that DTX1 may be either selectively eliminated by scallops or less efficiently retained in the tissues. An alternative explanation is that DTX1 had undergone biochemical conversion to a compound not detected in the LC-MS analysis. Yasumoto et al. (1989) suggested that DTX3 may be formed *in vivo* in scallop (*Patinopecten yessoensis*) digestive glands via acylation of DTX1. This hypothesis was based on the fact that DTX3 was detected in scallop tissue yet was never found in *Dinophysis* cells implicated in causing DSP toxicity. Similar evidence of the presence of DTX3 in mussel viscera from Ireland was reported by Marr et al. (1989). Since DTX3 was not analyzed for by LC-MS in the present study, the possibility remains that a portion of DTX1 ingested by bay scallops was biotransformed to DTX3 via acylation. Further analysis of bay scallop viscera extracts using LC-MS configured for detection of DTX3 is required to investigate such a possibility.

5.4 Conclusions

This study has demonstrated that the epibenthic dinoflagellate *Prorocentrum lima* can act as a vector for DSP toxins in shellfish tissue. In the bay scallop, *Argopecten irradians*, toxigenic *P. lima* cells were ingested without any inhibitory effect on clearance rates, except at concentrations greater than 400 cells mL^{-1}, at which point scallops reduced feeding rates by intermittent valve closure and pseudofeces production. Detrimental effects on feeding physiology were not apparent in either adult or juvenile bay scallops during exposure to *P. lima*, nor were any mortalities observed, suggesting that DSP-toxigenic algal blooms pose little threat to bay scallop populations.

Although DSP toxin concentrations in bay scallop tissues surpassed accepted closure levels in some countries (0.2 $\mu g\ g^{-1}$ whole tissue; Andersen, 1996) within 18 h of exposure to P. lima cells, after 2 weeks of exposure overall DSP toxin retention was less than 1% of that ingested by the scallops. This suggests that scallops are able to prevent significant DSP toxin accumulation via efficient detoxification mechanisms. Furthermore, it does not appear that DSP toxins are easily bound to scallop tissues, with the exception of the viscera which retained DSP toxins during three weeks of depuration. Gonadal tissues can become highly toxic during exposure to toxigenic microalgae due to newly ingested cells and labile toxin in sections of the intestine that loop through the gonads. Depuration rendered gonads free of toxins within three days, indicating that DSP toxins were not sequestered in this tissue.

In general, the results of the present study, as well as data reported in the literature, suggest that DSP toxins can be efficiently depurated from shellfish when the animals are exposed to a non-toxic food supply. However, in the case of *P. lima*, there is a real risk of contaminating clean waters with this dinoflagellate due to its ability to survive in fecal pellets released from depurating shellfish.

Conversions of DSP toxins in bay scallop tissues occurred as a result of the activity of esterases released from lysed *P. lima* cells subjected to physical and biochemical digestive processes in the scallop gut. The major DSP toxin component in ingested *P. lima* cells, DTX4, is hydrolyzed immediately in scallops to form D8OA. Transformation of D8OA to OA occurs over a span of several hours, resulting in these compounds existing in roughly equal proportions in scallop tissues. Unlike the fluctuations observed for OA-derivatives in scallop tissues, DTX1 levels were relatively stable. However, slight decreases in the relative proportion of DTX1 in scallop tissues relative to ingested cells implies that either DTX1 is selectively eliminated over OA-derivatives in scallops, or that DTX1 is converted to an undetected compound, possibly DTX3.

Prorocentrum lima is indeed capable of causing DSP toxin uptake in bay scallops under laboratory conditions. Yet, the question still remains: is it possible for *P. lima* to cause DSP toxicity in bivalve molluscs in the natural environment? Although occasional blooms of *P. lima* have been observed in the water column (Van Egmond, 1993), this

epibenthic dinoflagellate is primarily found growing on either the benthos or on surfaces of macroalgae (Faust, 1995; Bomber et al., 1985). Macroalgal cover, often termed "slub" by east coast Canadian shellfish growers, is commonly found attached to mussel socks and scallop lantern nets. In Spain, DSP toxigenic cells of *P. lima* have been found growing on macroalgae associated with mussel rafts (ICES, 1992; Lee et al., 1989). Although DSP toxicity in mussels is common in Spain, most evidence suggests that the toxicity is caused by dense blooms of *Dinophysis* spp. (Reguera, 1993). In Mahone Bay, Nova Scotia, where chronic low levels of DSP have been detected in cultured mussels since 1991, DSP toxins have never been detected in concentrated net haul material taken during blooms of *Dinophysis* spp. (M. Quilliam, Institute for Marine Biosciences, pers. comm.). Yet, examination of macroalgal growth on mussels socks has revealed the presence of *P. lima* growing on the seaweed in very close proximity to the mussels (J. Lawrence, Dalhousie University, Dept. Oceanography, pers. comm). The results of the present study suggest that *P. lima* cells would likely be ingested by shellfish if the cells were resuspended from the macroalgae, or perhaps from benthic sediments underneath the socks.

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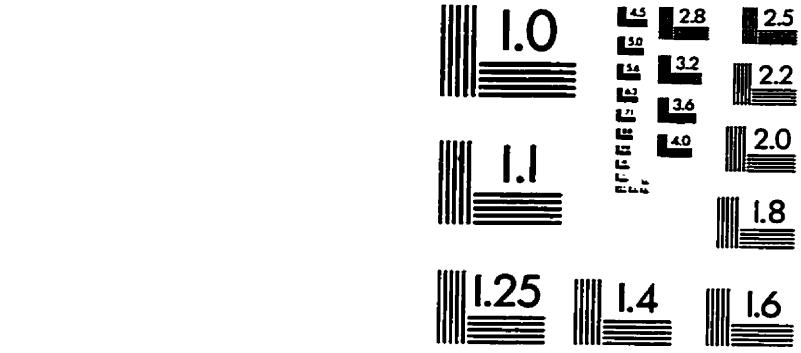
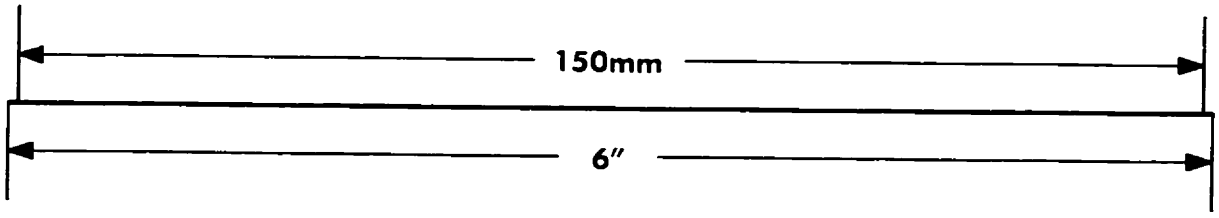
TEST TARGET (QA-3)





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