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Vat incubator with immersion core illumination – a new, inexpensive setup for mass phytoplankton culture

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Abstract

One of the shortcomings in studies of bivalve grazing has been the difficulty of culturing and making available sufficient quantities of algae. This was overcome using a 250 l capacity vat incubator with immersion core illumination (VIICI) in connection with experiments involving the diatom *Nitzschia pungens* f. *multiseries*, which produces domoic acid, the cause of amnesic shellfish poisoning. *Nitzschia* cultures grown in this incubator yielded maximum cell concentrations of $158–166 \times 10^6$ cells l^{-1}, a peak intracellular domoic acid level of 2.0 pg $cell^{-1}$ and a maximum division rate of 0.3 d^{-1}. The VIICI design is ideally suited for laboratory mass culture of phytoplankton, and has potential for wide application in phycotoxin, toxicological and environmental research, as well as for aquaculture.

Introduction

Various aspects of amnesic shellfish poisoning have been under study since its first recorded occurrence in Prince Edward Island, Canada in 1987 (Addison & Stewart, 1989) particularly the growth of the diatom *Nitzschia pungens* f. *multiseries* which, under certain conditions of growth, produces the causative toxin domoic acid (Subba Rao *et al.*, 1988). Experiments that involved feeding mussels (*Mytilus edulis*) with *Nitzschia* however, lagged because of the logistics of supplying large volumes of toxin-laden diatom culture.

Earlier mass culture techniques were unsuitable because of certain pitfalls such as limited culture volumes, restricted illumination, excessive space requirement (Claus, 1981) or highly specialized equipment (Spectorova *et al.*, 1982). To provide a solution for our studies on the uptake and loss of domoic acid (DA) in mussels and scallops (*Placopecten magellanicus*) we designed a unique, low-cost, vat incubator with immersion core illumination (VIICI) and raised as much as 150 l of toxic *Nitzschia*.

Materials and methods

Phytoplankton cultures

The DA-producing diatom, *Nitzschia pungens* f. *multiseries* clone NPBIO (Subba Rao *et al.*, 1988) was maintained in 250 ml Pyrex Erlenmeyer flasks in FE medium (soil extract, 20 ml l^{-1} + Wood Hole medium F) at 10 °C and 490–500 μmol photon m^{-2} s^{-1} photosynthetically ac-

tive radiation (QSL-100 LiCor meter, Biospherical Instruments Inc., San Diego CA). The stock was subcultured every 7–10 d using an inoculation ratio of 1:10 of culture to medium.

Culture apparatus

The vat incubator with immersion core illumination (VIICI) for culturing 150 l of phytoplankton consisted of a 200 l translucent polypropylene vat fitted with a central light cylinder (130 cm height, 15 cm diameter, and 0.63 cm wall thickness) of cast acrylic (Fig. 1). Four fluorescent tubes (80 cm, General Electric cool white) generating a total of 140–415 μmol photon m^{-2} s^{-1} were positioned in the cylinder (Fig. 1b, Table 1). Two additional double-tube (108 cm) fixtures were suspended 40 cm above the growth tank. The estimated total cost for the materials and fabrication of one VIICI was $600 US.

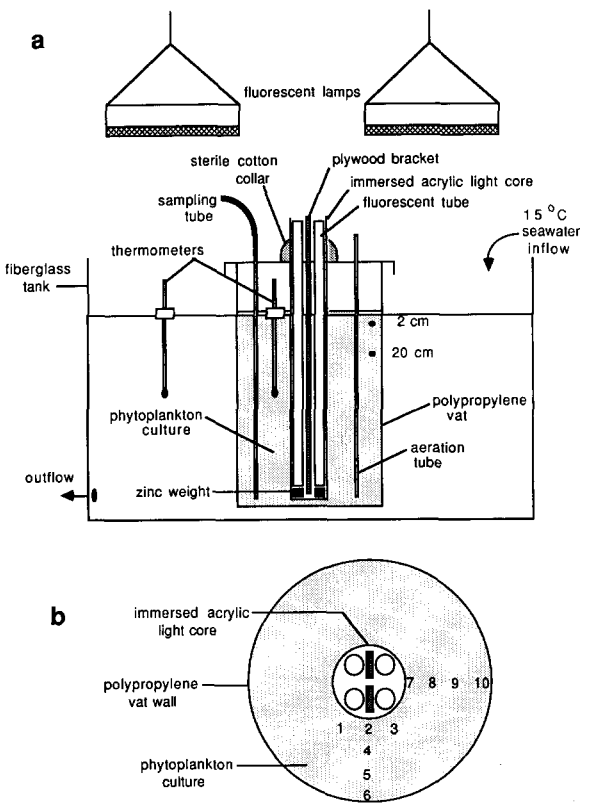

Fig. 1. (a) Schematic diagram of VIICI (Vat Incubator with Immersion Core Illumination) in the 15 °C seawater bath. The points at 2 and 20 cm inside VIICI indicate the depths at which light was measured. (b) Top view. The numbers between the light core and polypropylene wall indicate positions where light was measured.

The VIICI was sterilized for 2 d with UV radiation (75.4 cm, 07V tube, Aquafine Corp., Valencia CA). Each of two 20 l carboys with 10 l of FE medium was inoculated with 2 l of exponentially growing (10 days growth) *Nitzschia*. The resulting 24 l of culture were used to seed 140–150 l of fresh, sterile FE medium in the VIICI. Temperature was maintained between 10 and 15 °C by using a fiberglass bath through which Bedford Basin seawater was circulated (Fig. 1a). To ensure good suspension of the algae in such a large volume, the culture was manually stirred daily with a flame-sterilized, acrylic paddle. An air pump (Hagen Optima, 7.0 W, 5.0 l min^{-1}) connected to a timer helped to keep the culture in suspension through 15 min on/off cycles. Samples were taken occasionally to observe growth, cell activity and purity of culture.

Enumeration

Samples from cultures (20 ml) were preserved with a 1% 1:1 formaldehyde-glutaraldehyde so-

Table 1. Light intensities (μmol photon m^{-2} s^{-1}) at depths of 2 and 20 cm in the vat incubator with immersion core illumination (VIICI), before inoculation with *Nitzschia pungens* f. *multiseries* and 14 days later. The numbered position at which the measurement was taken refers to Fig. 1b.

Measuring position	t_o		t_{14}	
	2 cm	20 cm	2 cm	20 cm
1	332	166	216	166
2	349	158	232	149
3	332	166	216	158
4	316	149	199	125
5	282	149	158	93
6	249	141	141	85
7	415	249	349	257
8	365	183	266	166
9	299	166	191	118
10	266	141	183	108

lution. One ml was settled for 24 h in a 25 ml chamber and counted by the Utermohl method using a Nikon Diaphot-TMD, inverted microscope (Phase Contrast-2 ELWD turret). Live samples from VIICI were enumerated using a Neubauer haemocytometer (Humphrey & Subba Rao, 1967).

Determination of domoic acid

Samples of culture (20–40 ml) were filtered through 3 μm Nuclepore filters and stored at −25 °C in plastic petri dishes. For analysis a filter was transferred to a scintillation vial, the petri dish rinsed twice with 1 ml of seawater, and the rinse water was added to the vial. The vial was capped and sonicated in a water bath for 15 min (Sonic 300 Dismembrator, Artek, 20 ± 0.4 kHz) to rupture the *Nitzschia* cells and release the DA. The contents of the vial were then filtered through a 0.4 μm Nuclepore filter.

Domoic acid content of the filtrate was determined by the 9-fluorenyl-methoxycarbonyl (FMOC) method (Pocklington *et al.*, 1990). The domoic acid was first reacted with the compound 9-fluorenyl-methylchloroformate to form a fluorescent derivative that could then be separated by high performance liquid chromatography (HPLC), and measured using a fluorescence detector.

Results

Cultures of *N. pungens* remained in the exponential growth phase for up to 10 days (Fig. 2a). The maximum culture density was 166×10^6 cells l^{-1}. A growth curve (Gompertz 1825) of the form

$$y = ae^{[-e^{(b-ct)}]}$$

was fitted to data from the exponential and early stationary phases of growth (days 2 to 23) of culture 131L, but not to the death phase (Zwietering *et al.*, 1990). In the equation for growth, $y = \ln(N_t/N_0)$, N_0 is the initial culture density and N_t is the

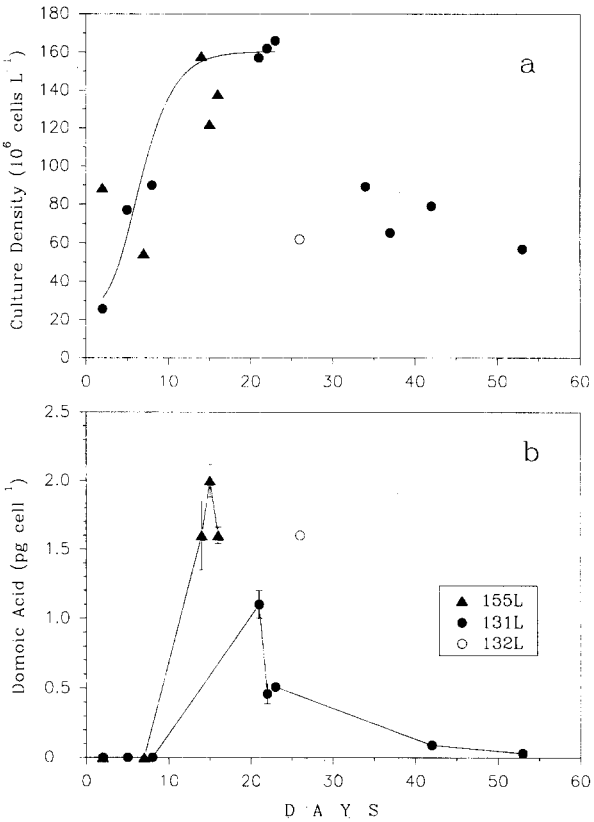

Fig. 2. (a) Concentration of *Nitzschia pungens* f. *multiseries* and (b) intracellular domoic acid levels in three VIICI cultures. Labels for cultures are indicative of the total volume. Growth curve in (a) fitted to data for 131L. Error bars in (b) are 1 S.D.

density at time t. The calculated values of a, b and c were 1.8, 1.7 and 0.40 respectively. The maximum culture density estimated by the fitted curve is given by $e^{(a + \ln N_0)}$ and was 155×10^6 cells l^{-1}. The maximum growth rate of the culture, μ_m, calculated as ac/e, was 0.3 divisions per day. Cell concentration and cellular domoic acid (Fig. 2b) peaked by the fifteenth day of growth and declined after about day 25.

Domoic acid was not produced by cultures in the exponential phase of growth. Production began only in the early stationary phase of growth, about the eleventh day. Intracellular DA usually ranged from 0.03–1.6 pg cell^{-1} (Fig. 2b). The maximum cellular DA level was 2.0 pg cell^{-1}.

Discussion

The growth rate of *N. pungens* f. *multiseries* in VIICI was 30–60% of the rates observed in smaller batch cultures grown at 10 °C and 530 μmol photon m^{-2} s^{-1} (Subba Rao *et al.* 1990), probably as a result of the light intensity in VIICI being 30–60% less. Nevertheless, cultures grew well in VIICI. Ultimately they reached densities four times greater than the smaller batch cultures, probably due to availability of nutrients and CO_2 supply via aeration, and they produced comparable amounts of domoic acid per cell.

The onset of DA production on entering early stationary phase is consistent with earlier observations (Subba Rao *et al.*, 1990). The range of DA levels, and the maximum cellular DA level reported here are in accord with recent work on small batch cultures (Subba Rao *et al.*, 1988, 1990).

Dependability of the VIICI

The VIICI apparatus is robust, dependable, and easily cleaned and sterilized. After 6 months of use, the only sign of wear in one unit was crazing of the cast acrylic light well which can be prevented by using a glass light well or perhaps by the use of chemicals for sterilization rather than ultraviolet light. The design would be improved by the construction of 3 or 4 small external fins at the base of the light well to facilitate agitation of the culture by turning the light well; this would obviate the use of an acrylic paddle, and reduce the chance of airborne contamination. Another possible improvement would be pH control by the introduction of CO_2 through the aeration tubes. The VIICI design is not necessarily limited to a volume of 155 l. It is conceivable that larger volumes could be grown by employing a larger polyethylene tank fitted with 160 cm fluorescent lamps.

Utility of the VIICI in mass culture

Mass culture of phytoplankton, the principal diet of marine bivalves, is especially important in aquaculture and in grazing experiments. For hatchery purposes, two important criteria must be met. The food supply must be continuous and the food organisms must be appropriate to the larvae. There are three general methods which hatcheries employ to meet these criteria (Claus, 1981; De Pauw, 1981; Laws *et al.*, 1988): (1) cultivation of phytoplankton in open outdoor ponds, (2) cultivation under controlled conditions, indoor or outdoor, and (3) bloom induction in natural seawater by the addition of fertilizer or sewage effluent. The main problem with the first two methods is the lack of control over the species or their abundance. The major concerns for the third method are water quality and contamination. Commercial methods for algal production are too large (3000 l to several hectares) and costly for laboratory purposes and cannot guarantee culture purity.

Mass cultured algae are often a mixture of the hardy weed species such as *Chaetoceros calcitrans*, *C. simplex*, *Isochrysis galbana*, *Pavlova lutheri*, *Tetraselmis suecica*, *T. pseudonana*, *Phaeodactylum tricornutum*, *Thalassiosira weisflogii* and *Dunaliella tertiolecta*, in contrast to those algae that are more difficult to culture. These species alone do not constitute the natural food for many bivalves; at best they are food items only under hatchery or laboratory conditions. Laboratory experiments generally involve monospecific cultures and although monospecific blooms of the weed species have not been reported in the wild, raising them in scaled-up VIICI units should not be difficult. Other techniques for mass culture of phytoplankton are expensive or unsuitable for *Nitzschia*. Dialysis techniques, essentially for small volumes, are expensive (Ney *et al.*, 1981) and could present greater problems in the maintenance of culture purity.

Utilizing toxin-producing cultures grown in the VIICI, mussels fed on *Nitzschia* yielded a DA level of 13 $\mu g\ g^{-1}$ wet wt of soft tissue, a level approaching the legal limit of 20 $\mu g\ g^{-1}$ set by National Health and Welfare, Canada (Gilgan *et al.*, 1990). The maximum level attained by scallops was 4 $\mu g\ g^{-1}$. The VIICI has also been employed in an experiment in which seawater from

a toxic bloom of *N. pungens* in Cardigan Bay, Prince Edward Island, was retained for one week and used in a grazing experiment with *Mytilus*. It is therefore possible that VIICI can have wide application in grazing experiments and studies of bioaccumulation of phycotoxins, pesticides and pollutants. The VIICI apparatus is a dependable culture chamber ideally suited to the laboratory.

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