SPIROLIDE PRODUCTION AND PHOTOPERIOD-DEPENDENT GROWTH OF THE MARINE DINOFLAGELLATE *ALEXANDRIUM OSTENFELDII*

Uwe John[1], Michael A. Quilliam[2], Linda Medlin[1] and Allan D. Cembella[2]

[1]Alfred-Wegener-Institut, Postfach 120161, D-27570 Bremerhaven, Germany;
[2]Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford St., Halifax, Nova Scotia, Canada B3H 3Z1

ABSTRACT

The effects of physiological status on spirolide production were studied in nutrient-replete batch cultures of a toxic strain of the dinoflagellate *Alexandrium ostenfeldii*. Although complete cell synchronisation was not achieved by dark adaptation, the concentration of motile vegetative cells apparently increased in the light and decreased in the dark. The concentration of extracted chlorophyll *a* followed the same trend as the cell concentration, with no apparent shift in the amount of chlorophyll *a* per cell in relation to the light/dark (L/D) phase. Analysis of spirolides by liquid chromatography coupled with mass spectrometry (LC-MS) showed that the toxin profile did not vary significantly over the L/D cycle, and consisted primarily of a des-methyl-C derivative (>90% molar), with minor constituents C, C3, D, D3 and des-methyl-D. The total spirolide concentration per unit culture volume was directly related to the concentration of cells and chlorophyll *a*, but there was a dramatic increase in cell quota of spirolides at the beginning of the dark phase and a corresponding decrease in the light. The biosynthesis of these polyketide-derived metabolites is apparently governed by light-dependent events during the cell division cycle.

INTRODUCTION

The marine dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech *et* Tangen has been recently identified as the source of toxic spirolides [1]. These potent macrocyclic imines were first isolated and characterised from shellfish viscera [2,3], and later identified in the plankton from Nova Scotia, Canada [4,5]. New rapid and highly sensitive methods to quantify spirolides in only few plankton cells by liquid chromatography-mass spectrometry (LC-MS) [6] have been applied to the analysis of a spirolide-producing *A. ostenfeldii* clone [1].

The biosynthesis of other toxic metabolites by *Alexandrium* spp. is known to be regulated by a complex interplay of environmental and intrinsic genetic factors (reviewed by [7]). Typically, changes in environmental variables, such as light, salinity, turbulence, temperature, and macronutrients, influence the cell quota of toxin (Q_t), either by direct effect or via a feedback interaction with cell growth rate. As the cell divides, Q_t is partitioned between the daughter cells [7,8]. The synthesis of PSP toxins in *Alexandrium* occurs during vegetative growth in the G1 phase of the cell cycle [8], thus any prolongation of G1 phase (decrease in growth rate [μ]) may result in higher Q_t even if the rate of toxin synthesis is constant. Physiological studies on dinoflagellate production of tetrahydropurine neurotoxins (e.g., saxitoxin derivatives) [7] and polyether toxins [9] have generally indicated that the toxin composition is characteristic of the strain, and that the toxin profile is rather refractory to change [10,11], except under extreme environmental stress.

In photoautotrophic dinoflagellates, the photoperiod influences many diurnal physiological processes, including cell division, nutrient assimilation, vertical migration and bioluminescence rhythms. The direct dependence of cellular processes on light/dark (L/D) cycles can be exploited to phase or synchronise the cell division cycle. Dark-induced synchronisation followed by entrainment on a defined L/D cycle has been previously used to study the cascade of events involved in toxin production in the dinoflagellates *Alexandrium fundyense* [8] and *Prorocentrum lima* [12].

There are few studies on the effects of photoperiod and cell division cycle events on the production and accumulation of polyketide-derived metabolites. We attempted to use dark-induced synchronisation of *A. ostenfeldii* cultures to determine the effects of photoperiod on the cell quota of spirolides through successive cycles of cell division. Such studies are a prerequisite to establish the links between toxin biosynthesis and discrete stages of the cell division cycle. Furthermore, these data can be used to determine the optimum photoperiod for maximum growth and to quantify the effects of light induction on other cellular processes, such as chlorophyll synthesis.

MATERIALS AND METHODS

Experiments were conducted on a clonal isolate of *Alexandrium ostenfeldii* (AOSH1) from Ship Harbour, Nova Scotia in unialgal batch cultures using aseptic techniques. Stock cultures (1.0 L) in exponential growth phase were inoculated into 12 L of L1 growth medium in triplicate 15 L Belco glass carboys. Cultures were grown with gentle aeration to maintain homogeneity at 15±1 °C under a 14:10 light/dark (L/D) photocycle at an ambient photon flux density of 260 µmol m^{-2} s^{-1}. After 106 h of dark adaptation, culture samples were collected by sterile syringe at 2 h intervals throughout three L/D cycles for measurements of chlorophyll *a* (extracted and *in vivo*), cell number, cell size and spirolide concentration. During the dark period, samples were collected under red light (<0.1 µmol m^{-2} s^{-1}) to avoid photo-induction.

Growth of cultures prior to dark adaptation was monitored by optical microscopic counts (125X). During the experiment, cell concentrations were determined using a Coulter Counter (Multisizer II). *In vivo* chlorophyll *a* fluorescence of whole cultures (10 ml) was measured by fluorometry (Turner Designs Model 10). Particulate

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chlorophyll samples were filtered (Whatman GF/C) and extracted in darkness with 90% acetone (72 h) at –20 °C for quantitation by fluorometry.

Spirolides were analysed from three culture fractions: cells, filtrate (cell free medium), and whole culture. Duplicate samples of whole culture were filtered through 0.5 ml spin-cartridges (Millipore Ultrafree-MC, 0.45 μm) by centrifugation at 500 x *g*. The filtered cells were extracted by spin-filtration with 1 ml of 100% methanol [5]. Extracellular spirolides in the cell free medium were determined by direct injection of the filtrate. Spirolides were analysed by liquid-chromatography with ion-spray mass spectrometry (LC-MS) (PE-SCIEX API-III) [6] using purified standards.

RESULTS

After inoculation of stock cultures into fresh growth medium, *A. ostenfeldii* cells remained in lag phase for one week. Dark adaptation for 106 h was initiated after Day 8, when the mean cell concentration had reached 800 ± 150 (n=3) cells ml^{-1}. During dark adaptation, the mean cell concentration declined substantially to 468 ± 109 cells ml^{-1}. After transfer to the 14:10 L/D cycle, the cell concentration oscillated with the photoperiod, decreasing in the dark and increasing in the light phase (Fig. 1). This variation in cell concentration between the light and the dark phase was maintained throughout the experiment, for three L/D cycles. Non-motile cells, resembling pellicular cysts, accumulated on the bottom of the culture vessel, particularly during the dark phase. Based upon cell counts of motile vegetative cells alone, the net growth rate, calculated from T=0 to the end of the experiment, was low (μ = 0.18 div. d^{-1}).

The concentration of particulate chlorophyll *a* (ng ml^{-1}) in the cultures exhibited the same trend as the cell concentration (Fig. 2). As for the cell concentration, the amplitude of the oscillation in chlorophyll *a* between the light and dark phases increased with each successive L/D cycle through the experiment. There was no apparent shift in chlorophyll *a* per cell related to the L/D phases.

Analyses by LC-MS showed that the sum of spirolides extracted from the cellular fraction, plus that found in the cell-free culture medium, was similar to that extracted from the whole culture. Leakage or excretion of spirolides from healthy vegetative cells accounted for <3% of the total spirolide content of the *A. ostenfeldii* cultures. Total spirolide concentration per unit culture volume (whole culture) fluctuated in response to the L/D cycle, similar to the pattern exhibited by the cell number and chlorophyll *a* concentrations (Fig. 3). Spirolide levels in the culture peaked at the end of the light phase and plummeted by as much as 20% early upon entry into darkness. However, in contrast to the pattern of cellular chlorophyll *a*, there was a dramatic increase in cell quota of spirolides at the beginning of the dark phase, peaking by the middle of the dark phase, and a corresponding decrease in the light (Fig. 3). The variation in the cell quota of spirolides over the last two L/D cycles was >50%, when the increase was calculated from the middle of the light period to the maximum in the dark. Variation in total concentration of chlorophyll *a* and spirolides over

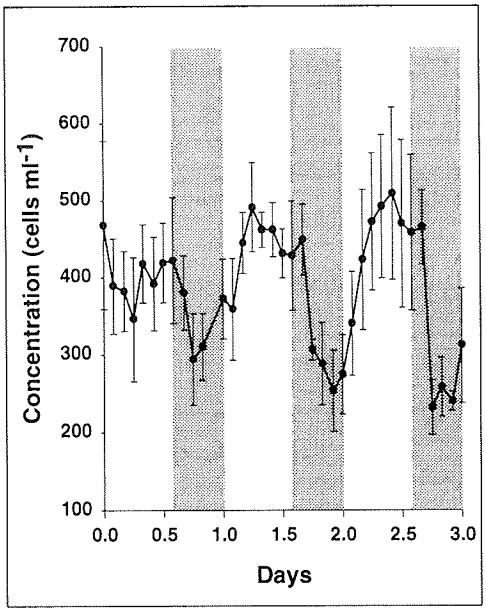

Fig. 1. Variation in cell concentration of *A. ostenfeldii* AOSH1 over several photocycles. Dark bars denote the darkness periods.

the L/D cycle was not due to cell size differences; mean cell diameter in the light was 27.9 (± 0.4 s.d.)μm, compared to 26.5 (± 0.6 s.d.)μm in darkness.

The spirolide profile of this isolate was very stable, and no substantial variations were noted in response to the photoperiod. Des-methyl-C comprised >90% of the total toxin on a molar basis, whereas derivatives C, C3 and des-methyl-D were minor components.

DISCUSSION

We report here the first evidence that extrinsic environmental factors, specifically photoperiod, can influence the rate of production and cell quota of macrocyclic imines in marine dinoflagellates. Toxin production in dinoflagellates is also known to be subject to genetic regulation [7,8,9], but physiological mechanisms and regulatory control of the biosynthetic pathways of toxin production are poorly understood.

Cell Growth and Photoperiodic Events

Compared with other *Alexandrium* isolates, this *A. ostenfeldii* strain is fastidious and less robust in mass culture. Even under recently optimised growth, reducing the light from 250 to 70-100 μmol m^{-2} s^{-1}, cells appear healthy, but growth rates remain <0.2 div. d^{-1} (A. Cembella, unpublished data). Long term acclimation to higher than optimal light intensity followed by prolonged dark exposure to achieve cell synchronisation may account for the apparent decline in cell numbers in darkness and the subsequent low net growth rate.

To reduce the deleterious effects of turbulence on cell growth, diffuse aeration was supplied at a level only sufficient to maintain roughly homogeneous distribution of motile cells and to minimise sedimentation. Prior to the experiment, samples were collected simultaneously

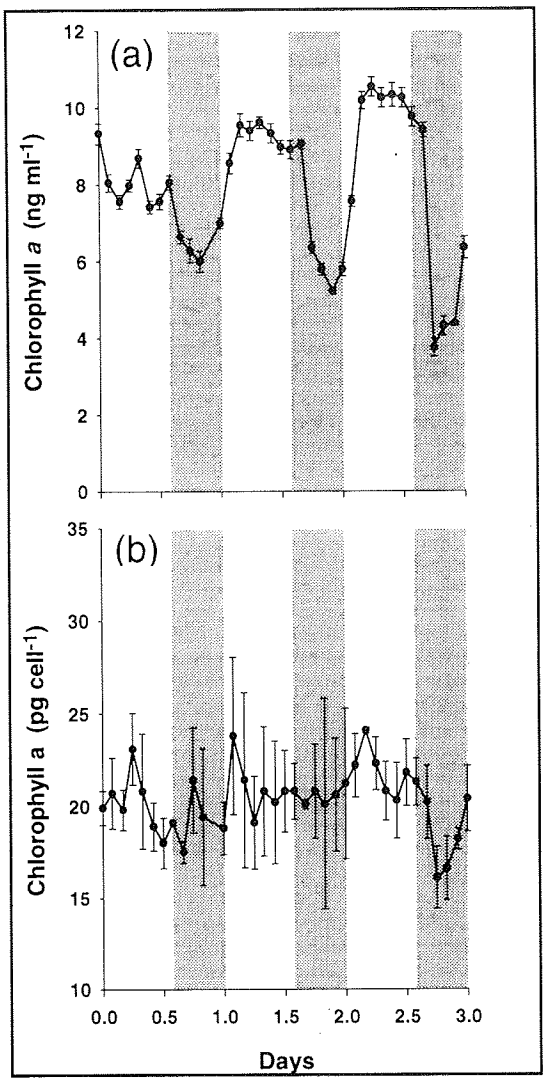

Fig. 2. Variation in chlorophyll *a* per unit culture volume (a) and per cell (b) over several photocycles. Note different scaling of the Y-axes.

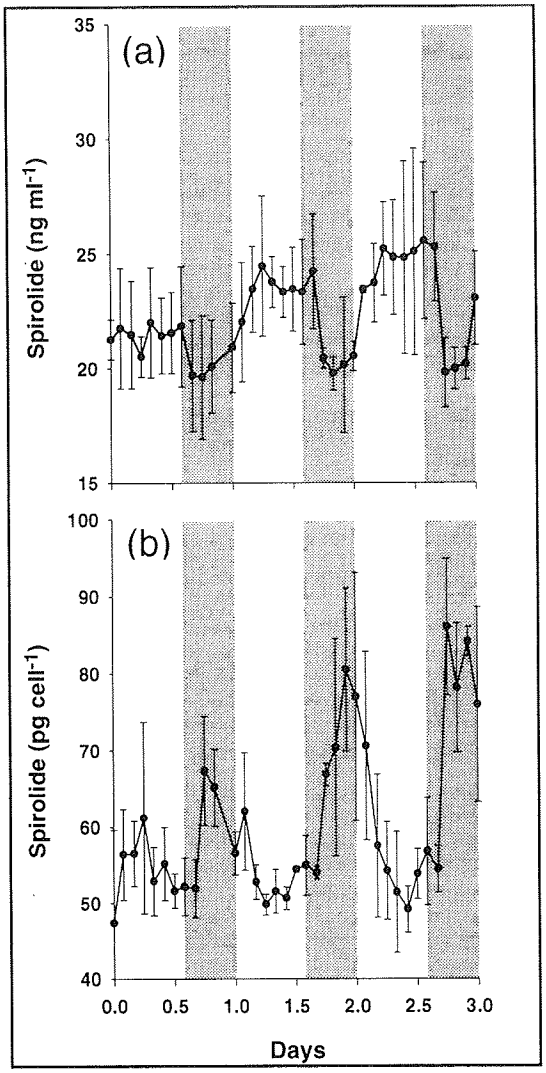

Fig. 3. Variation in total spirolide concentration per unit culture volume (a) and per cell (b) over several photocycles. Note different scaling of the Y-axes.

from several locations in the carboy to confirm homogeneity. The inlet port for cell sampling was situated several centimetres off the bottom of the carboy to ensure that motile (and presumably cycling) cells were primarily selected. The phasing of the cell concentration with the photoperiod, following a pattern of increasing cell concentration in the light and decreasing in the dark phase could be correlated with the vertical migration of motile cells. Higher deposition of dead cells, cell debris and pellicular cysts on the bottom during the dark period, and regeneration of vegetative cells from pellicular cysts in the light, might also account for the apparent growth kinetics. Nevertheless, visual observations confirmed that turbulence was sufficient to prevent layer formation of motile cells even in the dark. This phenomenon of vertical migration and encystment (pellicular cyst formation) during the dark phase has been described for *Alexandrium taylori* [14]. In this species, pellicular cysts give rise to motile cells at the beginning of the light phase, indicating that excystment and encystment may be controlled by light and regulated via the cell cycle.

For *A. ostenfeldii*, the rate of chlorophyll *a* production was approximately in balance with the cell division rate, as evidenced by the coupled oscillation in the total amount of chlorophyll *a* and cell numbers in the culture over the L/D cycles. If the cells were synchronised, chlorophyll *a* concentration should increase in the same stepwise manner as the cell concentration (see [13]). For *Prorocentrum lima*, Pan *et al.* [13] showed a L/D period-dependent increase and decrease of chlorophyll *a* cell quota, at least in the period before the cultures became asynchronous. For *A. ostenfeldii*, there was no shift in the chlorophyll *a* per cell in relation to the L/D phases. This might be due to the low cell division rate and/or to poor synchronisation.

Numbers of motile cells collected were insufficient for statistically valid identification of the cell cycle phases using nuclear DNA staining and flow cytometry. Since we were unable to attain a high level of cell synchronisation with *A. ostenfeldii* via dark acclimation, we did not observe the typical pattern of stepwise

increases in cell concentrations, as for *A. fundyense* [8] and *Prorocentrum* spp. [13].

Production of Spirolides

The dramatic increase in total spirolide per cell at the beginning of the dark period and the decrease during the light periods showed that spirolide biosynthesis is affected by light-dependent metabolic events. The >50% increase in the cell quota of spirolides after the L/D shift through several photocycles indicates a coupling of spirolide production to the photoperiod and cell cycle. By comparison, in *Prorocentrum lima*, the cell quota of the polyketide-derived DSP toxins increased in the light, but also extended through several phases of the cell cycle [12]. In contrast, although PSP toxin production by *A. fundyense* occurred in the light, synthesis was restricted to the G1 phase [8].

The transition of a fraction of the motile vegetative cells to pellicular cysts and the formation of dead cells may account for the variation in total spirolide concentration per unit culture volume. Pellicular cysts are a temporary quiescent stage produced through ecdysis of vegetative cells [14]. Since pellicular cysts are arrested in G_o-phase, maintaining only basal metabolism, these recurrent cells should have approximately the same cell quota of spirolides as vegetative cells before ecdysis. The maximal cell quota observed primarily from motile vegetative cells at the end of the dark phase is explicable as net spirolide production if this period also represents the late mitotic phases G2+M, just prior to cytokinesis.

The consistently low concentration of spirolide found in the medium (<3% of total spirolide of the whole culture) tends to indicate that leakage and excretion of spirolides from healthy vegetative cells, pellicular cysts and cell debris is not an important cycling mechanism. There is some preliminary evidence (M. Quilliam, unpublished data) that spirolides may be somewhat unstable in water at pH >5, although the decomposition rates in buffered seawater are unknown. Thus although it is conceivable that decomposition could account for low ambient spirolide levels in the medium, this is counter-indicated by the relative consistency in the spirolide profile (major derivative des-methyl-C) found in both the cellular fraction and the medium.

It is still unclear if spirolide biosynthesis is directly light-dependent, or if biosynthesis, intracellular transport and excretion are indirectly mediated via the effects of light on enzymes and other functional metabolites. In any case, the apparent lack of any photoperiod-dependent shift in spirolide composition indicates that the cascade of events leading to biosynthesis of the various spirolide analogues is on a time-scale shorter than that of the sampling intervals. By comparison, in *Prorocentrum lima,* the production of DTX4 derivatives was initiated in G1 phase and continued into S phase, whereas other derivatives, such as OA and DTX1, were produced later in S and G2 phases [12].

This study has provided significant insights into the light-dependence of spirolide production, but little information is available on the biosynthesis of polyketide-derived metabolites by dinoflagellates. Further effort will be directed towards the use of cell synchronisation techniques coupled with studies of gene expression of putative biosynthetic genes for spirolides.

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