

Production of cell mass and eicosapentaenoic acid (EPA) in ultrahigh cell density cultures of *Nannochloropsis* sp. (Eustigmatophyceae)

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This work attempts to identify growth conditions for maximal productivity of cell mass and of eicosapentaenoic acid (EPA) in ultrahigh cell density cultures of *Nannochloropsis* sp. Using flat plate reactors with a narrow (1–2 cm) light path and rigorous stirring exposed to high photon flux densities (1000–3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), record population densities ($1.2\text{--}1.4 \times 10^{10}$ cells ml^{-1}) were obtained. Consequently, the EPA content of the culture (mg l^{-1}) was higher by some two orders of magnitude than reported hitherto for cultures of much lower cell concentrations. In continuous cultures, highest culture EPA yield coincided with maximal output rate of cell mass. The very high population densities and output rates of cell mass and of culture EPA were possible provided culture medium was replaced at least every 48 h. Inhibitory activity, for which a bioassay was developed, was thereby removed. When nutrients were added frequently in order to prevent nutrient limitation without removing the inhibitory activity in the cultures, cell proliferation ceased after reaching some 30% of the attainable maximal cell number, and the culture gradually deteriorated. Inhibitor-induced culture deterioration was fully reversible when the growth medium was replaced with fresh medium.

Key words: EPA, flat plate reactors, inhibitory activity, *Nannochloropsis oculata*, photoacclimation, productivity, ultrahigh cell density

Introduction

The complexity involved in designing reactors for mass production of photoautotrophs rests with light, the major input for culture growth. Unlike all other inputs required for growth, light attenuates exponentially upon penetrating the culture, imposing stringent limits on optimal culture density. Relatively low cell concentrations, falling in the approximate range of 0.5–4 g dry cell mass per litre, are therefore typical for photoautotrophic mass cultures. This is in stark contrast with cultures of heterotrophic micro-organisms, on which a vast and successful industry is based and in which cultured cell concentrations are some 50 g l^{-1} , greatly contributing to the relatively low production cost of heterotrophic cell mass and products. The major challenge involved in industrial mass production of light-requiring micro-organisms relates, therefore, to strong irradiance (e.g. sunlight): how best to utilize it to obtain maximal yields of cell mass or products.

Work in this laboratory over the past few years has focused on development of a new approach to mass-produced microalgae, placing emphasis on achieving a high photobioreactor efficiency (Richmond, 1996). Essentially, we seek to emulate prevailing production systems of heterotrophic micro-organisms by establishing

ultrahigh cell density cultures with which to obtain sustainable output rates of high cell concentrations.

Aiming to use photobioreactor volume most effectively, experiments were focused on plate glass reactors of a narrow (i.e. 1–2 cm) light path, stirred vigorously and exposed to strong light (e.g. 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Ultrahigh cell densities of *Spirulina* (i.e. up to c. 50 g dry mass l^{-1}) as well as record areal output rates of cell mass were readily obtained under these conditions. Cell growth was strongly light-limited even when exposed to very high radiation doses because of the extreme mutual shading accompanying very high cell concentrations. Essentially complete absorption of all irradiance reaching the reactor surfaces takes place and efficient use of strong light and of photobioreactor volume was shown (Hu *et al.*, 1998).

In this study we use a similar approach to test some physiological aspects characteristic of ultrahigh cell density cultures of *Nannochloropsis* sp., a unicellular marine eustigmatophyte widely used in mariculture as a very good source of omega-3 polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA). The physiology of biosynthesis of lipids and polyunsaturated fatty acids, with particular reference to EPA, has been thoroughly investigated in this alga (Sukenik *et al.*, 1989, 1993; Roessler, 1990; Hodgson *et al.*, 1991; Sukenik, 1991; Renaud *et al.*, 1991) and reviewed recently (Sukenik,

1999). Also, the effect of light intensity on lipid content and composition was studied by Sukenik *et al.* (1993) as well as by Renaud *et al.* (1991), who found high photon densities significantly reduced the EPA content of *Nannochloropsis oculata*. The effect, however, was obtained with optically thin cultures and a relatively low dose of light per cell. Major attention in this work was thus focused on identifying conditions conducive to productivity of cell mass and of EPA in ultrahigh cell density cultures exposed to high photon flux densities.

Materials and methods

Organism

The marine eustigmatophyte *Nannochloropsis* sp. was obtained through the courtesy of Mr O. Zmora, Israel Oceanographic and Limnological Research, National Center of Mariculture, Eilat.

Photobioreactors

Batch cultures. Two identical, 14 mm light path, flat plate glass reactors (Hu *et al.*, 1996a), 185 mm wide and 700 mm high, were used for batch cultures. The total irradiated surface of the reactors (i.e. including both the front and back surfaces) was 0.15 m² when air was bubbling, net culture volume (without bubbling gas) being 1 litre. Stirring was provided by bubbling 2% CO₂-enriched air (1–2 l min⁻¹ per litre of culture) as described by Hu *et al.* (1996a).

'Old' culture medium was obtained as follows. After centrifugation of the algal suspension to precipitate the cell mass (3000 g for 5 min), the cells were resuspended in the same supernatant, having been centrifuged once again at 15 000 g for 20 min to remove all particulate matter and debris. Nutrients were added to the 'old' culture medium every 3 days to prevent nutrient deficiencies. 'New' culture medium was established by centrifuging the culture (3000 g for 5 min) every 2 days, and resuspending the cells in fresh growth medium.

Continuous cultures. A flat plate glass photobioreactor, 550 mm high and 500 mm wide with a light path of 11 mm, was placed in a water tank maintained at a temperature of 27 ± 1 °C. Cultures were stirred with compressed air containing 2% CO₂ at a flow rate of 0.5 l min⁻¹ l⁻¹ culture. Chemostat operations started when culture growth reached mid-logarithmic stage. Dilution rates (*D*) of 0.0043, 0.0086, 0.012, 0.017, 0.021, 0.026 and 0.030 (h⁻¹) were set by adjusting the speed of a peristaltic pump (LKB). Biomass concentration was measured several times a day. Chlorophyll, protein and fatty acid profiles were determined at the steady state of each dilution rate.

Growth conditions

Temperature was set by circulating tap water around the reactors, maintaining culture temperature at 27 ± 1 °C.

Illumination for batch cultures consisted of cool white fluorescent lamps set along one side of the reactor, providing 400 μmol photons m⁻² s⁻¹, and 1500 W halogen lamps set in front of the opposite side of the reactor, supplying from 600 to 2600 μmol photons m⁻² s⁻¹, according to the distance of the lamps from the reactor. Light was measured with a Li-Cor quantum sensor (model Li-185A). In the continuous cultures, one reactor side was illuminated with a bank of fluorescent lamps providing a photon flux of 300 μmol photons m⁻² s⁻¹, the opposite side being illuminated with halogen lamps at a photon flux of either 700 μmol photons m⁻² s⁻¹ or 1700 μmol photons m⁻² s⁻¹, obtained by adjusting the distance between lamps and reactor front.

pH was monitored by a Microprocessor pH meter (WTW) and was maintained between 7.2 and 8.0 by adjusting the CO₂ flow in the air stream used for mixing the culture. Dissolved oxygen (DO) was monitored using Dissolved Oxygen Meter YSI Model 58. Unbuffered artificial sea water (ASW) medium was prepared as described by Zou & Richmond (1999).

Measurements and chemical analysis

Proteins were assayed as described by Lowry *et al.* (1951). Cell number and cell mass density (dry cell mass and ash-free dry mass) were determined as described by Zou & Richmond (1999).

EPA was analysed according to Cohen *et al.* (1987). Concentration of chlorophyll *a* was measured with a spectrophotometer at 666 nm and calculated using a specific absorption coefficient of 13.9 (Hu & Richmond, 1994). Optical density (OD) of the culture suspension was measured by a Klett-Summerson photoelectric colorimeter using a green filter, with transmission of 520–580 nm.

Bio-assay of inhibitory activity

Culture samples to be assayed were centrifuged at 20 000 g for 20 min to remove the cells and particulate matter. An aliquot of 1.5 ml from the supernatant was used for each bioassay, to which 0.5 ml of freshly prepared cell suspension (6–8 mg chlorophyll l⁻¹), which served as inoculum, was added. In addition, 0.5 ml of concentrated (× 5) nutrient solution and 1 ml growth medium were added. In the control, the 1.5 ml assayed supernatant was replaced with 1.5 ml ASW. After 3 days of culture in 16 ml tubes placed on a shaker (7.5 strokes min⁻¹) under 150 μmol photons m⁻² s⁻¹ from cool-white fluorescent lamps, chlorophyll concentration, OD and cell number were measured to determine the extent of inhibition of culture growth.

Results and discussion

Establishing *Nannochloropsis* sp. cultures of ultrahigh cell density

Highest cell density cultures of *Nannochloropsis* sp. were

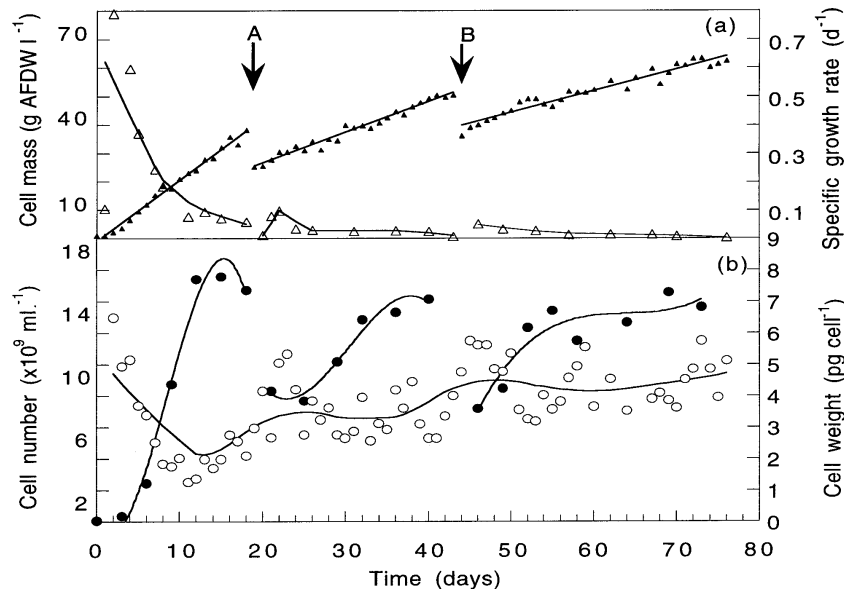


Fig. 1. Growth kinetics of ultrahigh cell density *Nannochloropsis* cultures in relation to photosynthetic photon flux density (PPFD) and population density. (a), Specific growth rate (open triangles) in relation to cell mass (filled triangles). (b) Cell mass (open circles) in relation to cell number (filled circles). Arrows indicate: switching from 1000 to 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (A); switching from 2000 to 3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (B). AFDW, ash-free dry cell mass.

Table 1. Eicosapentaenoic acid (EPA) and cell density in recent reports

Algal species	Light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Cell concentration ($\times 10^7 \text{ cells ml}^{-1}$)	Biomass concentration (g l^{-1})	Cell EPA (pg cell^{-1})	EPA culture		References
					EPA (%TFA)	content (mg l^{-1})	
<i>Nannochloropsis</i> sp.	33	2.0		0.46	38	9.3	Sukenic (1991)
	150	2.0		0.35	18	7.0	
	290	2.0		0.38	14	7.6	
<i>N. oculata</i>		2.0–2.6	0.06–0.13	0.20–0.21	22.4–26.4	4.3–5.1	Renaud & Parry (1994)
<i>N. oculata</i>	65	0.2–0.4	0.02	1.43	27.8–33.8	2.9	Hodgson <i>et al.</i> (1991)
<i>N. oculata</i>	100	1.3–2.4		0.10–0.18	16.4–39.8	2.1–3.6	Dunstan <i>et al.</i> (1993)
<i>Nannochloropsis</i> sp.	1000–3000	1000–1900	40.6–67.3	0.08–0.18	14.9–26.2	1042–2335	This work

TFA, Total fatty acids.

obtained in flat plate reactors with a narrow (i.e. 1–2 cm) light path and vigorous stirring, exposed to high photon flux densities (Fig. 1a).

Exposure to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ resulted in a maximal density (ash-free dry cell mass) of *c.* 40 g l^{-1} whereas cell concentrations of 50 and over 60 g l^{-1} were obtained by providing photosynthetic photon flux densities (PPFD) of 2000 and 3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively. Such extremely high concentrations of cell mass (not reported hitherto) were conditional, however, on frequent replacement of the growth medium with fresh medium, to remove inhibitory substances. The initial relatively high growth rate of *c.* 0.6 d^{-1} (Fig. 1a) declined rapidly, and within 10 days the growth rate was *c.* 10% of its initial value. When the PPFD was increased from 1000 to 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 1a, arrow A) following a *c.* 30% dilution of the cell mass, the growth rate increased somewhat, soon declining to *c.* 3% of the maximal (μ_{max}). Likewise, increasing the PPFD to 3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 1a, arrow B) initially increased the growth rate, which thereafter declined progressively, finally reaching zero on day 70. We estimate that 60 g of ash-free dry cell mass per litre represented the highest concentration range of cell mass

attainable in *Nannochloropsis* cultures (Fig. 1a). Unlike cell mass, which reached a maximum after some 70 days, the maximal cell number of *c.* 12 to 14 billion cells ml^{-1} (or close to two orders of magnitude higher than reported previously, see Table 1), was reached within some 12 days from the start (Fig. 1b). Since the culture was somewhat diluted at the end of the period of exposure to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (see Materials and methods), the initial cell number at the onset of exposure to 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was *c.* 1×10^{10} cells ml^{-1} . Cell number reached the maximal 1.4×10^{10} cells ml^{-1} value within 20 days. Increasing the photon flux to 3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (following another culture dilution) resulted in a gradual increase in cell number which reached the same maximum more slowly, after some 30 days (Fig. 1b).

Cell mass, in contrast, exhibited a different pattern. Initially, when cell number was relatively low (up to 5 days from the start), cell mass was 5–6 pg per cell. As cell concentration increased over time, cell mass rapidly decreased to 3–4 pg per cell. According to Sukenic *et al.* (1989) high light causes an increase in fatty acid content of cells; thus the high cell mass obtained under high light conditions could have resulted from an increase of triacylglycerols in accumulation bodies (Fisher *et al.*, 1998).

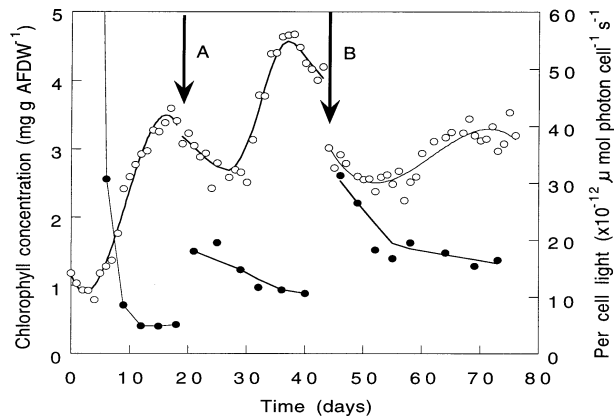


Fig. 2. Acclimation to high photosynthetic photon flux density (PPFD) and increasing cell concentrations as reflected by cell chlorophyll. Arrows as in Fig. 1. Filled circles, photon irradiance expressed on a per cell basis; open circles, chlorophyll concentration AFDW, ash-free dry cell mass.

Acclimation to changing light regimes

For a given light path and rate of stirring, the light regime to which the average cell is exposed is a function of both the PPFd and the population density (Hu *et al.*, 1998). Since each rise in PPFd was associated with an initial reduction in cell mass caused by diluting the culture (Fig. 1a, b), cells during the culture period were in effect exposed to rather extreme variations in the light regime (Fig. 2, arrows A and B), which required continual light acclimation (Fisher *et al.*, 1996). This in turn was reflected in sharp variations in cell chlorophyll which were related to the light available for the average cell (light per cell). A decrease or increase in this parameter produced a corresponding increase or decrease in cell chlorophyll. At some point, this trend was reversed and from day 36 on, chlorophyll per cell began to decline as light per cell decreased further – a phenomenon first observed for very high cell concentrations of *Spirulina* (Hu *et al.*, 1997). The explanation proposed is that when the areal concentration of photosynthetic units exceeds a high point associated with extreme self-shading including severe light limitation, the dependency of light absorption on the PSU is greatly diminished, due in effect to excess chlorophyll in the culture. Thus when self-shading becomes extremely high (e.g. 1000–2000 mg chlorophyll l^{-1}), chlorophyll synthesis ceases, resulting in declining chlorophyll per cell. Some improvement in the light regime due to a reduction in self-shading is thereby conceivably achieved.

Effect of the light source and the light regime, as modified by the dilution rate, on EPA values and productivity

Increasing the dilution rate (D) in continuous cultures exposed to two different levels of irradiance provided an opportunity to establish several light regimes affected by varying both the population density (and hence the PPFd per cell), as well as the intensity of the light source. In cultures exposed to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, increasing the dilution rate resulted in a gradual decrease in

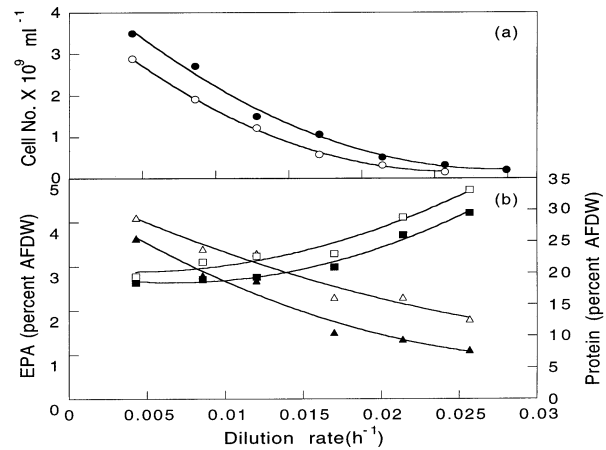


Fig. 3. Relative effects of photosynthetic photon flux density (PPFD) and of cell density (a) regulated by the dilution rate, on cell EPA and protein (b). Open circles, cell density at 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; filled circles, cell density at 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; open triangles, EPA (as % ash-free dry cell mass) at 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; filled triangles, EPA (as % ash-free dry cell mass) at 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; open squares, protein at 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; filled squares, protein at 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Reactor light path = 11 mm.

EPA content (Fig. 3). When the light source was doubled (to 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) very much the same pattern of response was obtained, showing a consistent effect of increased light per cell on the reduction of cell EPA, reported by Sukenik *et al.* (1987, 1989) and Renaud *et al.* (1991).

The impact of increasing dilution rates (associated with decreasing population densities) on the light regime and on EPA as a percentage of dry cell mass was far greater than that of doubling the intensity of the light source from 1000 to 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$: EPA decreased by 54% and by 63% as the population density decreased from 3 to 2×10^9 cells ml^{-1} in the 1000 and 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ treatments, respectively. Doubling the intensity of the light source, in contrast, effected an average EPA decrease of only 15–30%. The light regime (Richmond, 1999), therefore, serves as a more meaningful parameter than the mere intensity of the light source in assessing the effect of light on cell EPA.

Protein content of the cell mass gradually increased with increasing dilution rate (D), accompanied by decreasing cell concentration and mutual shading (Fig. 3). It reflected a population of younger cells as seen from the increase in the specific growth rate μ (at steady state, $D = \mu$). This raised a query: was the decrease in cell EPA a direct result of a higher PPFd on cell metabolism, or did it essentially stem from the effect that increased light per cell exerted on accelerated cell division, resulting, in turn, in a younger cell population? Since doubling the light intensity at high cell concentrations and very low growth rate (e.g. D of 0.005 per hour and cell number of 3.5×10^9 ml^{-1}) had only a small (less than 10%) effect on cell EPA, the latter possibility seems better supported.

As expected for a light-limited photoautotrophic culture, maximal output rate was obtained at about one half

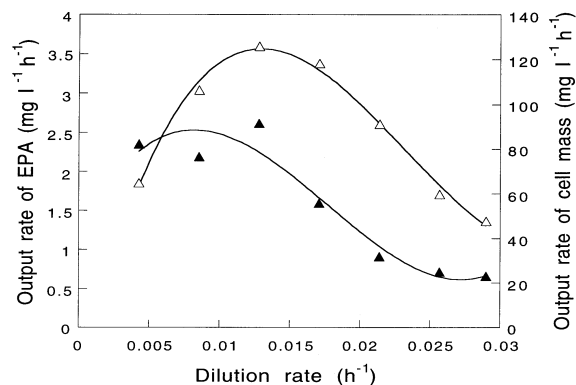


Fig. 4. Steady-state output rates of EPA and biomass exposed to 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in continuous cultures. Reactor light path = 11 mm. Filled triangles, output of EPA; open triangles, output of biomass.

the maximal growth rate (estimated as somewhat higher than 0.03 $D \text{ h}^{-1}$, Fig. 4). Maximal output rate of cell mass at 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was obtained in the range 0.011–0.017 D (Fig. 4), yielding *c.* 120 mg dry cell mass $\text{l}^{-1} \text{ h}^{-1}$. Maximal output of EPA was obtained in the somewhat lower range of 0.004–0.013 $D \text{ h}^{-1}$. Because the major use of *Nannochloropsis* is in the aquaculture industry, in which it serves as a valuable food chain component (Lubzens *et al.*, 1995), the fact that maximal output of culture EPA approximately coincided with the maximal output of cell mass is therefore of practical significance. For gross EPA production, optimal growth conditions yielding highest cell mass seem preferable to conditions which increase cell content of EPA but reduce growth, *i.e.* low light (Sukenic *et al.*, 1989; Sukenic, 1991). A similar conclusion was reached for γ -linoleic acid production in *Spirulina* cultures exposed to low nitrogen (Hu *et al.*, 1997).

The combined effects of PPF and cell density on culture EPA (but not on productivity of EPA in continuous cultures as shown in Fig. 4) may be summarized as follows (Table 2). Increasing the light source from 1000 to 2000 and thereafter to 3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ caused a progressive decrease in EPA as a percentage of the total fatty acids (*c.* 25%) and a much larger (58%) decrease in EPA content (EPA as % of AFDW). Although the 3-fold increase in PPF resulted in a significant increase in culture density, culture EPA content decreased nevertheless by *c.* 31% (from 2302 to 1574 mg l^{-1}), due to the

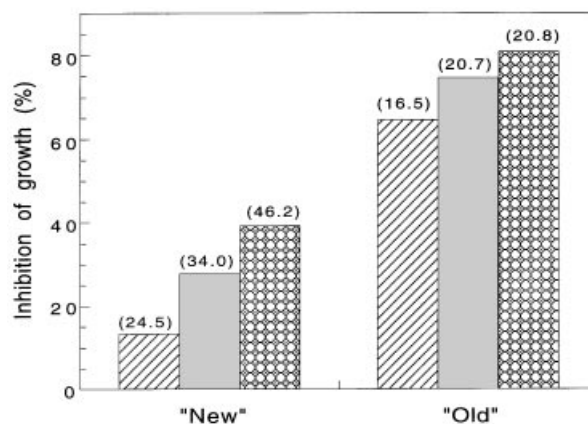


Fig. 5. Bioassay of inhibitory activity in 'new' and 'old' growth media on the tenth day (hatched), fourteenth day (shaded) and nineteenth day (patterned) of culture. Numbers in parentheses above the bars (g l^{-1}) represent culture cell density from which aliquots were taken for bioassay.

significant increase in light per cell caused by the higher intensity of the light source. It is worth noting that the maximal culture content of EPA in the ultrahigh cell density cultures reported here was higher by some two orders of magnitude compared with reports in the literature (Table 1).

Autoinhibitory activity

A salient feature of ultrahigh-density cultures concerns growth inhibitors, which have been reported in other algal species (Javanmardian & Palsson, 1991; Imada *et al.*, 1991; 1992; Richmond & Zou, 1999). Ultrahigh cell density cultures in *Nannochloropsis* can be sustained only if inhibitory activity is not built up, being routinely removed from the cultures once cell density reaches the level at which growth-autoinhibitory activity becomes evident. When the inhibitory activity was not removed, and instead of frequent replacement of the entire growth medium with fresh medium only the nutrient formula was added into the culture every 3 days to avoid nutrient limitation, growth began to be curtailed after some 10 days, when culture density reached *c.* 20 g l^{-1} . (In other experiments, net culture growth ceased at a much lower cell concentration, *e.g.* 6 g l^{-1} .) Following a few days in which no net growth took place, cell concentration rapidly

Table 2. Combined effects of photosynthetic photon flux densities and cell concentration on culture eicosapentaenoic acid (EPA) content

Light intensity ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$)	Culture age (days)	EPA (% TFA)	EPA (% AFDW)	TFA (% AFDW)	Culture concentration (g DW l^{-1})	Light per cell mass ($\mu\text{mol g}^{-1} \text{s}^{-1}$)	EPA content (mg l^{-1})
1000	18	26.2	5.7	21.7	40.6	1.85	2302
2000	24	19.0	3.2	16.8	53.6	2.80	1700
3000	29	16.8	2.3	13.9	67.3	3.34	1574

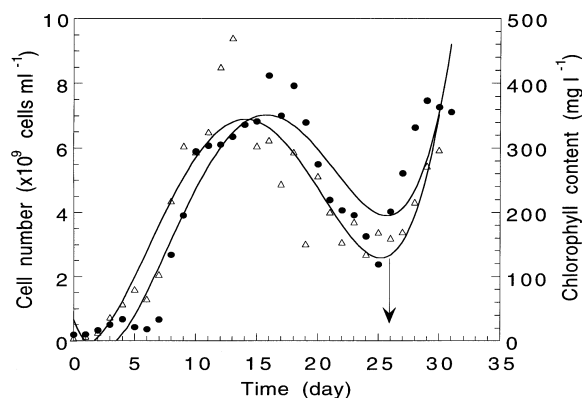


Fig. 6. Recovery of cell growth and cell chlorophyll content upon replacing the 'old' growth medium with fresh medium. Arrow, culture medium replaced with fresh medium. Open triangles, cell count; filled circles, chlorophyll content.

declined under these circumstances. In contrast, when the entire culture medium was replaced frequently, culture growth continued unabated. A bioassay developed to assess the extent of inhibitory activity (Fig. 5) revealed that some inhibitory activity existed even in ultrahigh density 'new' cultures in which the growth medium was replaced every 2 days. The magnitude of the inhibitory activity increased with culture density, becoming most significant (causing *c.* 80% inhibition of growth compared with the control) in the oldest cultures with the highest cell density.

In 'old', i.e. growth-inhibited, cultures the path of culture deterioration was reflected in declining population density and was associated with a sharp decline in EPA. As expected, this became more severe with increasing PPF. EPA as a percentage of AFDW in 'old' cultures exposed to 3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was some 80% lower than in 'new' cultures exposed to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Was the potent growth inhibitory activity observed and assayed in 'old' cultures reversible? Recovery experiments, in which the culture medium of 'old' cultures was finally fully replaced with fresh medium, show clearly that even in the extreme case shown, in which the 'old' culture has long been deteriorating as indicated by the sharp decline in cell count (*c.* 75% lower than the maximal cell count observed some 10 days earlier), recovery was complete (Fig. 6), as indicated by the quick rise in cell count and chlorophyll content (Fig. 6). The same trend was observed in cell EPA, which rose to normal after a few days during the course of recovery. The effect of the growth inhibitory activity was thus fully reversible: once removed from the culture, cell division and metabolism resumed normal patterns immediately.

The advantages of having outputs of high cell densities in highly efficient reactors, thereby reducing costs of processing as well as capital costs, are self-evident. The appearance of autoinhibitory activity at cell densities which are far from the maximal attainable, however, requires their frequent removal, which would place a heavy burden on the cost of production.

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