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Phosphate-limited growth and uptake kinetics of the marine prasinophyte *Tetraselmis suecica* (Kylin) Butcher

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ABSTRACT

The marine prasinophyte *Tetraselmis suecica* was grown in a chemostat culture system over a series of phosphate-limited growth rates ranging from 0.164 to 0.755 d^{-1}. The relationship between phosphate concentration and growth rate was well described by the Monod equation with a half-saturation constant of 3.45 nM. The kinetics of short-term uptake of phosphate spikes were well described by the Michaelis–Menten equation, maximum uptake rates being 2–3 orders of magnitude higher than associated growth rates. The relationship between cellular carbon-to-phosphorus ratios and growth rates was consistent with the Droop formulation of nutrient-limited growth, and chlorophyll *a*/carbon ratios were linearly related to growth rate. The protein content of the cells inferred from calculated nitrogen-to-carbon ratios ranged from a minimum of ~7% at zero growth rate to a maximum of ~59% at a relative growth rate of 1.0. Control of the relative growth rate of *T. suecica* via selection of the phosphate concentration in the growth medium and dilution rate of the culture appears to be an effective and practical way to manipulate the biochemical composition of this species in order to maximize its nutritional value to invertebrate consumers in aquaculture systems.

1. Introduction

Algae of the genus *Tetraselmis* have been used as a source of nutrition for invertebrates in aquaculture for decades (Camacho et al., 1990; de la Pena and Villegas, 2005; D'Souza and Kelly, 2000; Fábregas et al., 1985, 1995, 1996; Laing and Helm, 1981; Ukeles and Bishop, 1976; Wikfors, 1986). Much of this previous work has focused on the nutritional value of the algae, as evidenced either from its biochemical composition (de la Pena and Villegas, 2005; Fábregas et al., 1985; Wikfors, 1986) or from the survival, growth, and reproductive rates of its invertebrate consumers (D'Souza and Kelly, 2000; Fábregas et al., 1996; Piña et al., 2006). In some cases the algae have been grown in batch culture, and the physiological condition of the culture has been inferred from cell division rates as opposed to being controlled. In other cases the cultures have been operated in a continuous (Camacho et al., 1990; D'Souza and Kelly, 2000) or semi-continuous (Fábregas et al., 1995; Laing and Helm, 1981; Piña et al., 2006) manner, perhaps motivated by the feeling that for economic reasons batch culture, "may not be the most appropriate approach" (Camacho et al., 1990). When the transition from log phase to stationary phase growth has been associated with exhaustion of either inorganic nitrogen or phosphate, it has been accompanied by a dramatic reduction in the protein content of the algae, from ~60% of ash-free dry weight (AFDW) in log phase to 25% in late stationary phase (e.g., de la Pena and Villegas, 2005). During the log-to-stationary phase transition the lipid content remains approximately constant at 20% of the AFDW, and changes in protein are mirrored by changes in carbohydrate, the sum of the two averaging ~80% of the AFDW (de la Pena and Villegas, 2005). Changes in the protein content of the algae are of great relevance to the nutritional value of the cells, since the survival, growth, and reproduction of invertebrate consumers are closely correlated with the protein content of *Tetraselmis* (e.g., Fábregas et al., 1996).

Of the five previous continuous or semi-continuous culture studies of *Tetraselmis*, D'Souza and Kelly (2000) used only a single steady state growth rate, 0.2 d^{-1}. Their "low N" cells appear to have been nitrate-limited as evidenced by their very low protein and high carbohydrate contents, 12 ± 6% and 63 ± 17%, respectively, of AFDW. Fábregas et al. (1995) and Piña et al. (2006) each used only a single semi-continuous dilution rate, equivalent to continuous growth rates of 0.693 d^{-1} and 0.357 d^{-1}, respectively. The protein content of their cells averaged 58 ± 11% and 45 ± 3% of the AFDW, respectively, presumably a reflection of the fact that their cultures were growing at 3.5 and 1.8 times the growth rate used in the D'Souza and Kelly (2000) study. The studies of Camacho et al. (1990) did not involve steady state growth conditions. Instead, cultures were brought to a pre-determined density and then diluted at a constant rate from early morning to late afternoon. In some cases the dilution rate was well above the growth rate of the culture, as evidenced by

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a steady decline in biomass during the day. Laing and Helm (1981) grew their cultures in nutrient-replete media. Growth rates were limited by temperature or inorganic carbon concentrations and varied between roughly 0.4 and 0.9 d^{-1}.

Steady state continuous cultures are, strictly speaking, in log phase growth regardless of the dilution rate. Given the dramatic changes in the protein content of *Tetraselmis* between log and stationary phase growth conditions (de la Pena and Villegas, 2005), it seems reasonable to ask whether controlled manipulation of the growth rate of a nutrient-limited culture grown in continuous mode could be used to identify and maintain the growth rate associated with the optimum biochemical composition of the cells from a nutritional standpoint. A comparison of the results obtained by D'Souza and Kelly (2000) at $\mu = 0.2\ d^{-1}$ with those obtained by Piña et al. (2006) and Fábregas et al. (1995) at $\mu = 0.357\ d^{-1}$ and $0.693\ d^{-1}$, respectively, suggests, for example, that the protein content of the cells may be more-or-less proportional to growth rate. The work reported here was a first attempt to explore the relationship between growth rate and biochemical composition of *Tetraselmis suecica* in a systematic manner.

2. Materials and methods

The *T. suecica* culture was obtained from the culture collection of the Center for Marine Microbial Ecology and Diversity at the University of Hawaii (culture code: TETRA 01) and was grown in a continuous culture system similar to that described by Laws and Bannister (1980). Temperature in the range 18–22 °C has been shown to have no effect on the productivity of *T. suecica* (Laing and Helm, 1981) and in this study was maintained at 22 ± 0.1 °C by circulating water from a thermoregulated water bath through the jacket of a double-walled glass reaction flask, which served as the growth chamber. Irradiance, provided continuously from a bank of daylight fluorescent lights, was 440 μmol quanta $m^{-2}\ s^{-1}$ as measured with a QSL 2100 quantum scalar light meter at the center of the empty growth chamber. The growth medium consisted of artificial seawater (Instant Ocean®) with the salinity adjusted to 35. The medium was enriched with nitrate and vitamins as specified for f/2 medium (Guillard and Ryther, 1962) and trace metals as specified by Sunda and Hardison (2007). Phosphate, the limiting nutrient, was added at a target concentration of 0.5 μM. The concentration of phosphate in the reservoir was determined as soluble reactive phosphorus (SRP) (Strickland and Parsons, 1972) on an AutoAnalyzer. The growth chamber and 40-liter medium reservoir were sterilized by autoclaving [Steris model SG-120], and the growth medium was sterile filtered (0.2 μm) into the reservoir. Fresh medium was introduced into the growth chamber at a controlled rate via a peristaltic pump. The culture was stirred by a Teflon-coated magnetic stir bar and via bubbling with sterile air (0.2 μm sterile filter), which also served to drive the overflow by pressurizing the headspace above the culture.

Cell counts were recorded with a Beckman Coulter model Z1 particle counter, and dilution rates were recorded daily. Sampling of the growth chamber for characteristics other than cell counts did not begin until four doubling times had elapsed at each dilution rate, by which time cell counts had stabilized with a coefficient of variation of ±2%. Samples for determination of particulate carbon and chlorophyll *a* (chl *a*) were collected on glass fiber (GF/F) filters. Concentrations of chl *a* were measured in methanol extracts on a Cary model 50 UV–Visible spectrophotometer using the protocol of Holm-Hansen and Riemann (1978). Particulate carbon was measured on an Exeter Analytical model CE 440 elemental analyzer. Due to the fact that the instrument's nitrogen reduction column reached the end of its normal lifetime just before the samples from this study were run, no information was obtained on concentrations of particulate nitrogen.

Concentrations of Pi in the growth chamber were estimated by a bioassay technique. Sixty milliliters of culture was withdrawn from the growth chamber and transferred to a 100-milliliter beaker placed in front of the same light bank used to illuminate the growth chamber. The contents of the beaker were stirred with a small Teflon-coated magnetic stir bar. At time zero the beaker was spiked with 100 μl of deionized water containing 0.6 pmol of carrier-free ^{33}P labeled phosphate (1.16×10^4 Bq), thereby increasing the concentration of Pi in the beaker by $0.6/(60\times10^{-3}) = 10$ pM. Ten-milliliter aliquots were withdrawn at selected time intervals and immediately filtered through 0.8 μm membrane filters. The total activity of ^{33}P in the beaker was determined by counting duplicate 100-microliter aliquots of the medium. Samples for determination of ^{33}P activity were counted on a Packard Tri-Carb model 3100 TR liquid scintillation counter using Ultima Gold LLT (Perkin-Elmer) as a scintillation fluor.

Assuming the ten-picomolar ^{33}P spike to be negligible compared to the ambient concentration of phosphate in the growth chamber (P_{gc}), the product of P_{gc} and the fraction of the ^{33}P spike taken up per unit time equals the uptake rate of Pi in the growth chamber, which we equated to the product of the dilution rate and the difference between the concentration of phosphate in the nutrient reservoir (P_r) and P_{gc}. This implies that

$$f \cdot P_{gc} = \mu\left(P_r - P_{gc}\right) \Rightarrow P_{gc} = \frac{\mu P_r}{f+\mu} \quad (1)$$

where μ is the dilution rate and f is the fraction of ^{33}P taken up per unit time. The duration of the bioassay was constrained by the requirement that the rate of ^{33}P uptake be constant throughout the incubation, i.e., that drawdown of P_{gc} not noticeably affect its rate of uptake. In practice this limited the duration of the incubations to no more than 100 s at the lowest P_{gc}.

Experiments to determine the ability of the culture to take up a spike of Pi were carried out at two growth rates (0.164 and $0.755\ d^{-1}$) in a similar manner, but the ^{33}P tracer was accompanied by small additions of ^{31}P stock solution (36 mM) sufficient to raise the phosphate concentration by amounts ranging from less than 1 μM to 11–12 μM. Pi uptake rates were calculated using standard tracer kinetic equations (Strickland and Parsons, 1972) assuming negligible isotope discrimination.

3. Results

The relationship between ambient Pi concentrations and growth rate (μ) was well described by a hyperbolic function of the form

$$\mu = \frac{\mu_m \cdot Pi}{K_m + Pi} \quad (2)$$

where μ_m is the asymptotic growth rate in the limit as Pi→∞, and K_m is the value of Pi when $\mu = \mu_m/2$ (Monod, 1949) (Fig. 1). Remarkably, K_m was only 3.5 nM, which is well below the limit of detection for SRP (20–40 nM) by standard colorimetric methods (Karl and Tien, 1992; Strickland and Parsons, 1972). The maximum growth rate observed during batch phase growth after initial inoculation of the growth chamber was 0.8–0.9 d^{-1}, which is consistent with the observations of Laing and Helm (1981) in the temperature range 18–22 °C.

The kinetics of uptake of Pi spikes were remarkably similar at low ($0.164\ d^{-1}$) and high ($0.755\ d^{-1}$) growth rates (Figs. 2–3). In both cases the relationship between uptake rate (V) and phosphate concentration was described by the Michaelis–Menten hyperbola (Michaelis and Menten, 1913):

$$V = \frac{V_m \cdot Pi}{K_s + Pi} \quad (3)$$

where V_m is the asymptotic uptake rate in the limit as Pi→∞, and K_s is the value of Pi when $V = V_m/2$. The two values of V_m were virtually