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Cellular and extracellular production of carbohydrates and amino acids by the marine diatom *Skeletonema costatum*: diel variations and effects of N depletion

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ABSTRACT: A detailed study was made of cellular and extracellular production of carbohydrates and amino acids by the marine diatom *Skeletonema costatum* (Grev.) Cleve during different growth phases. Batch cultures were run with a 14:10 h light:dark cycle in N-limited media at 2 different nutrient strengths. The exponential growth rate was 2.0 div. d^{-1}, and balanced growth took place except for significant diel variation in chemical composition. Inorganic C and N were primarily assimilated during the photophase, and the elemental cell quotas increased accordingly. The level of storage polysaccharide, β-1,3-glucan, oscillated between 17% (end of scotophase) and 42% (end of photophase) of cellular organic C, and the corresponding protein:glucan ratio alternated between 2.3 and 0.7. Cell wall polysaccharides constituted 6 to 10% of cellular organic C. Concurrently, the cellular free amino acid pool oscillated between 8% (end of scotophase) and 22% (end of photophase) of cellular organic N. Glutamine emerged as the principal amino acid during photosynthesis, increasing from 0.2 to 12 fmol $cell^{-1}$, and the corresponding glutamine:glutamate ratio increased from 0.05 to 2. Upon NO_3^- exhaustion, the glucan level increased rapidly for 3 to 4 d, and then stabilized at 75 to 80% of cellular organic C with little diel variation. In contrast, the cellular N quota decreased by 80%, and the cell wall polysaccharide quota decreased by 35%. Consequently, the protein:glucan ratio decreased to <0.1. The cellular free amino acid pool decreased by 90% within 24 h of N depletion, and continued to decrease slowly throughout the stationary phase. Glutamine decreased most rapidly, and constituted <1% of the free amino acids in the stationary phase. Extracellular production accounted for 4% of total photosynthetic production during both exponential and stationary growth phase, but the absolute excretion rate (per cell) was markedly higher in the exponential phase. A transient high release occurred in the transition phase in 1 case, which was probably caused by cell leakage. Extracellular production by 'healthy' cells contained 33% polysaccharides, 15% monosaccharides and 5% free amino acids (as C). The composition of the extracellular amino acids differed from the intracellular ones, and changed considerably from exponential to stationary growth phase. This study illustrates the rapid response of carbohydrate and amino acid dynamics to ambient N and light conditions at the cellular level.

KEY WORDS: Carbohydrates · Free amino acids · Production rate · Excretion · Diel variation · N starvation · Marine diatom

INTRODUCTION

The diatoms (Bacillariophyceae) comprise the most abundant marine phytoplankton species, and are estimated to contribute 20 to 25% of the world net primary production (Werner 1977). The biochemical composition of diatoms is rather similar to that of other microalgae (Parsons et al. 1961, Haug et al. 1973). Rapidly growing cells contain 30 to 60% protein, 10 to 50% carbohydrate and 5 to 20% lipid (organic dry weight). However, chemical composition varies markedly as a function of growth conditions. Nutrient deficiency gen-

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erally leads to elevated levels of carbohydrate and lipid in relation to protein. Moreover, protein and carbohydrate pools oscillate in response to diel light conditions.

Polysaccharides account for the bulk of the algal carbohydrates, and the main cellular groups are storage and cell wall polysaccharides. The principal storage polysaccharide in diatoms is β-1,3-glucan (chrysolaminaran), a (1→3)-linked β-D-glucopyranan (DP 20-80) with occasional branching through C-2 and C-6 (Beattie et al. 1961, Ford & Percival 1965a, Paulsen & Myklestad 1978, McConville et al. 1986). The cellular glucan content accumulates markedly under nutrient deficiency (Myklestad & Haug 1972, Myklestad 1974). The glucan is consumed by the alga when kept in the dark, and apparently serves as a respiratory substrate (Handa 1969, Vårum & Myklestad 1984).

In the diatom cell wall, structural polysaccharides are important constituents of the organic casing, which coats the siliceous components (Schmid et al. 1981, Volcani 1981). This organic casing contains sulphated glucuronomannans and other heteropolysaccharides with variable composition (Ford & Percival 1965b, Allan et al. 1972, Hecky et al. 1973, Haug & Myklestad 1976).

Assimilation of N into amino acids and proteins requires energy as well as C skeletons provided by carbohydrates, illustrating the unique interactions between algal N assimilation, photosynthesis and respiratory processes (Turpin 1991). Incorporation of NH_4^+ into amino acids is primarily carried out by the sequential action of glutamine synthetase and glutamate synthase in the chloroplast (Syrett 1981, Falkowski & Raven 1997). However, NO_3^- is the predominant form of assimilable N in most aquatic environments, and must be reduced by nitrate reductase (cytosol) and nitrite reductase (chloroplast). The enzymatic activity of nitrate reductase often is a rate-determining step in N assimilation, and is highly regulated at a transcriptional level (Guerrero et al. 1981). The activity undergoes a diel cycle with a maximum at midday and minimum at night, which appears keyed to the cellular pool of organic C (Falkowski & Raven 1997, Vergara et al. 1998).

Conversely, photosynthetic and respiratory processes are dependent on N supply. Nitrogen limitation can be considered as a form of translational control, where the supply of amino acids limits the translation of mRNA and, hence, reduces protein synthesis (Falkowski et al. 1989). Reduction in the cellular levels of free amino acids is further reflected in lower levels of cellular protein, and usually by a concurrent increase in carbohydrate or lipid contents.

Extracellular production should also be considered as a significant part of the algal primary production (Myklestad 2000). The relative extracellular release as percentage (PER) is defined as extracellular production × 100 / total photosynthetic production. In general, phytoplankton generates 2 to 10 PER during rapid growth, increasing to 10 to 60 PER in the stationary phase. However, the absolute rate of release (per unit of biomass) may be higher during exponential growth (Myklestad et al. 1989). The physiological mechanisms for excretion are still poorly understood, but simple diffusion is probably important for transporting small molecules across the cell membrane, while biopolymers most likely are excreted by complex mechanisms (Myklestad 2000).

A completely different cause of release is leaky ('unhealthy') cells exposed to various physical or biological stress factors. Phytoplankton cells contain 15 to 50% of cell C as soluble compounds composed of small molecules, soluble proteins and reserve carbohydrate (Myklestad & Swift 1998). Thus, cell lysis will lead to considerably higher levels of extracellular material.

While protein is the major cellular component in rapidly growing cells, carbohydrate is the dominating substance among the extracellular products, especially polysaccharides (Myklestad 1995). Extracellular polysaccharides are generally complex heteroglycans (Smestad et al. 1974, 1975, Percival et al. 1980), and often form viscous or gelatinous mucilages. The next largest extracellular fraction may be proteins and amino acids, and other important substances are organic acids, sugar alcohols, lipids and fatty acids, vitamins and toxins. Investigations of marine diatoms have shown dramatic differences in excretion between different species. The genus *Chaetoceros* appears to have a generally high extracellular production (20 to 50 PER), particularly polysaccharides, while *Thalassiosira* spp. and *Skeletonema costatum* produce far less (1 to 7 PER) (Myklestad 1974, 1977).

This contribution presents a detailed study of carbohydrate and amino acid production in the marine diatom *Skeletonema costatum* during exponential growth and N starvation. As far as we know, this is the first study to investigate the combined cellular and extracellular production of these components. Dynamics of storage glucan and free amino acids in response to ambient NO_3^- and light conditions are emphasized. Furthermore, we investigate the rate and composition of extracellular production compared to cellular growth.

MATERIALS AND METHODS

Organism and culture conditions. The marine planktonic diatom *Skeletonema costatum* (Grev.) Cleve, clone Skel-5 isolated from the Trondheimsfjord (Myklestad 1974), was maintained in axenic stock cultures in the f/2 medium of Guillard & Ryther (1962) at 13°C.

The medium was based on filtered seawater of salinity ~32 psu from the Trondheimsfjord at 90 m depth, which was diluted to ¾ with Milli-Q water (Millipore), i.e. final salinity was ~24 psu. Cells were grown in modified N-limited f media containing 150 (Case 1) or 50 µM NO_3^- (Case 2). Other components were added at f/2 (Case 1) or f/10 (Case 2) concentrations, except phosphate, which was set at 1/10 of NO_3^- (i.e. 15 or 5 µM). Because the cells were growing in a closed system without aeration, 3 to 5 mM $NaHCO_3$ was added as supplement to the natural bicarbonate content of ¾ seawater (~1.7 mM).

The cultures were grown in photobioreactors (Granum & Myklestad 2002a) run as batch reactors at 13°C with a 14:10 h light:dark cycle. Illumination was provided by racks of alternating Phillips TLD 18W/33 and TLD 18W/96 tubes with an irradiance of 200 µmol m^{-2} s^{-1} as measured by a Biospherical Instruments QSL-100 light meter. pH in the culture was continually monitored with a pH electrode coupled to a Consort R301 controller and a TrendView Multitrend recorder. pH was kept within a range of 7.8 to 8.1 by relay-activated titration with 0.1 M HCl (in the light) and 0.02 M NaOH (in the dark).

Precultures were grown in Erlenmeyer flasks, and exponentially growing cells were incubated in a photobioreactor. When the cell density reached 20 to 30 µl^{-1}, the cells were further incubated in 2 parallel photobioreactors, and one of them was radiolabeled with 40 µCi l^{-1} $NaH^{14}CO_3$. Samples were taken every 24 h (4 h into the photophase), and some additional samples were taken at the end of the light and dark periods. Samples for measuring ^{14}C fixation were taken from the radiolabeled culture, while all other samples were taken from the unlabeled replicate. During the last 2 d in Case 1, extra bicarbonate (unlabeled) was added to the cultures to avoid inorganic carbon depletion (2 mM each day). Consequently, the specific ^{14}C activity was diluted to 20% on Day 5 and 7% on Day 6, and ^{14}C fixation was accordingly lower.

Sampling and analyses. Algal cells were counted with a Bürker haemocytometer, and the cell density was determined as the mean of 6 counts with SE 5 to 10%. The growth medium and culture samples from the exponential and stationary phase were tested for bacteria by filter tests with DAPI fluorescence (Porter & Feig 1980), and showed no significant contamination. The bacterial test was confirmed by direct microscopy.

The cells were harvested by filtration through 25 mm Whatman GF/C glass fiber filters under gentle vacuum (50 mm Hg), and stored at –20°C until analyzed. Cellular samples were used for analyses of chl *a*, organic C and N, carbohydrates (β-1,3-glucan and cell wall polysaccharides) and free amino acids. The filtrates were used for analyses of dissolved NO_3^-, dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), free amino acids and carbohydrates (mono- and polysaccharides).

Cellular chl *a* was extracted by cold acetone and measured with a Turner Design 10 AU fluorometer according to Holm-Hansen et al. (1965), and determined as the mean of 3 samples with SE 3 to 5%. Cellular organic C and N were measured with a Carlo Erba NA 1500 elemental analyzer (Kirsten 1979), and determined as the mean of 3 samples with SE 3 to 8%. Cellular protein, including free amino acids, was estimated as 90% of organic N × 6.25, and used for calculating protein/glucan ratios. Cellular β-1,3-glucan was extracted by dilute H_2SO_4 at 60°C, and the cell wall polysaccharides were subsequently hydrolyzed by cold 80% H_2SO_4 (Granum & Myklestad 2002b). Each carbohydrate fraction was determined by the phenol-sulphuric acid method (Dubois et al. 1956) as the mean of 3 samples with SE 1 to 5%.

Cellular free amino acids were extracted by Milli-Q water at 70°C (Flynn 1988). Both cellular extracts and dissolved (extracellular) free amino acids were determined by reverse phase HPLC with precolumn fluorescence derivatization with *o*-phthaldialdehyde (Lindroth & Mopper 1979) as modified by Flynn (1988), using a Waters Nova-Pak C_{18} column. Gly and Arg were not chromatographically resolved, and their combined concentration was based on a 1:1 ratio. Amino acid concentrations were determined as the means of 2 samples. The precision of this analysis is ±1 to 9% ($p < 0.05$) according to Flynn (1988).

Dissolved NO_3^- was determined by reduction and diazotizing reaction (Strickland & Parsons 1972) as the mean of 2 samples. Strickland & Parsons reported a precision of ±5% ($p < 0.05$) for this method. DIC and DOC were measured by high-temperature catalytic oxidation (Sugimura & Suzuki 1988) using a Dohrmann DC-190 analyzer with SE 3%, and both determined as means of 2 samples. Before DOC analysis, the samples were acidified to pH 2 and sparged with pure oxygen to remove inorganic carbon. New DOC production was calculated by subtracting the DOC of the growth medium (1.1 to 1.4 mg l^{-1}). Dissolved mono- and polysaccharides were determined by the TPTZ (2,4,6-tripyridyl-*s*-triazine) method (Myklestad et al. 1997) as the mean of 3 samples with SE 6 to 10%.

^{14}C fixation and biochemical fractionation. Cells from the radiolabeled culture were harvested on 25 mm Whatman GF/C filters as described above, and washed immediately with filtered seawater (3 × 1 ml). Cellular samples (filters) were purged of inorganic ^{14}C with HCl vapor, and extracellular samples (filtrates) by adding H_3PO_4 (pH < 2). Fixed ^{14}C radioactivity (dpm) was measured with a Wallac 1410 scintillation counter using Wallac OptiPhase HiSafe 3 scintillation liquid

(10 ml), and determined as the mean of 3 samples with SE 5 to 10%. The inorganic carbon assimilation rate ($\partial C/\partial t$) was calculated from the rate of ^{14}C fixation ($\partial A_{org}/\partial t$), the total activity of $NaH^{14}CO_3$ added to the culture (A_{tot}), the total DIC concentration and the carbon isotope discrimination factor ($f = 1.06$) according to Geider & Osborne (1992):

$$\frac{\partial C}{\partial t} = f \frac{\partial A_{org}}{\partial t} \frac{DIC}{A_{tot}} \quad (1)$$

Biochemical fractionation of cellular fixed ^{14}C was performed according to Granum & Myklestad (2001). A 'low MW' fraction was extracted by Milli-Q water at 70°C, and separated by cation exchange chromatography into 'glucan' and 'amino acid' fractions (Granum & Myklestad 1999). A 'lipid' fraction was extracted by cold chloroform, and a 'polysaccharide' fraction was extracted by 5% trichloroacetic acid at 95°C. The remaining insoluble fraction was regarded as 'protein'. Fixed ^{14}C in all fractions was measured as described above.

RESULTS

Cellular production

Batch cultures of *Skeletonema costatum* were grown with a 14:10 h light:dark cycle in N-limited media with 150 (Case 1) or 50 µM NO_3^- (Case 2), and sampled every 24 h (4 h into the photophase). The cells grew exponentially until NO_3^- was exhausted, and then went through a transition phase and into the stationary phase (Figs. 1A & 2A). The exponential growth constant was 1.4 d^{-1}, corresponding to 2.0 div. d^{-1}. Upon NO_3^- depletion, cellular organic N slightly decreased, but the cell numbers and cellular organic C still increased 5-fold. Accordingly, the cellular N quota decreased by 80%, and chl *a* $cell^{-1}$ decreased by as much as 90%. Cellular production rates were calculated as 30 pg C $cell^{-1}$ d^{-1} and 4.5 pg N $cell^{-1}$ d^{-1} in the exponential phase, and 3.9 pg C $cell^{-1}$ d^{-1} in the stationary phase, Days 5 to 7 (Table 1).

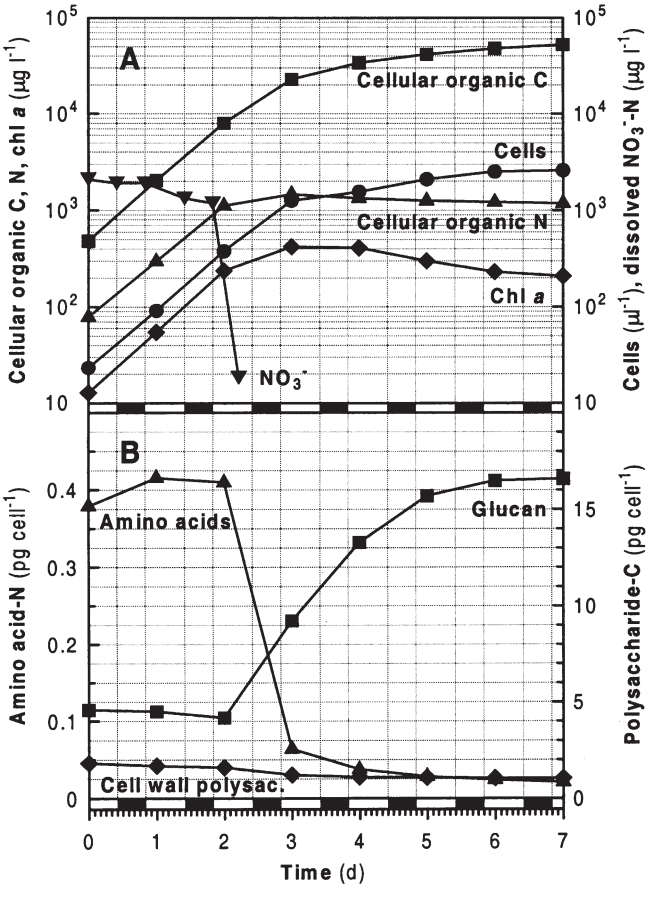

Fig. 1. *Skeletonema costatum*. Growth and chemical composition in 150 µM NO_3^- medium (Case 1). (A) Growth in cell numbers and cellular organic carbon, nitrogen and chl *a*. (B) Cell quotas of free amino acids, β-1,3-glucan and cell wall polysaccharides

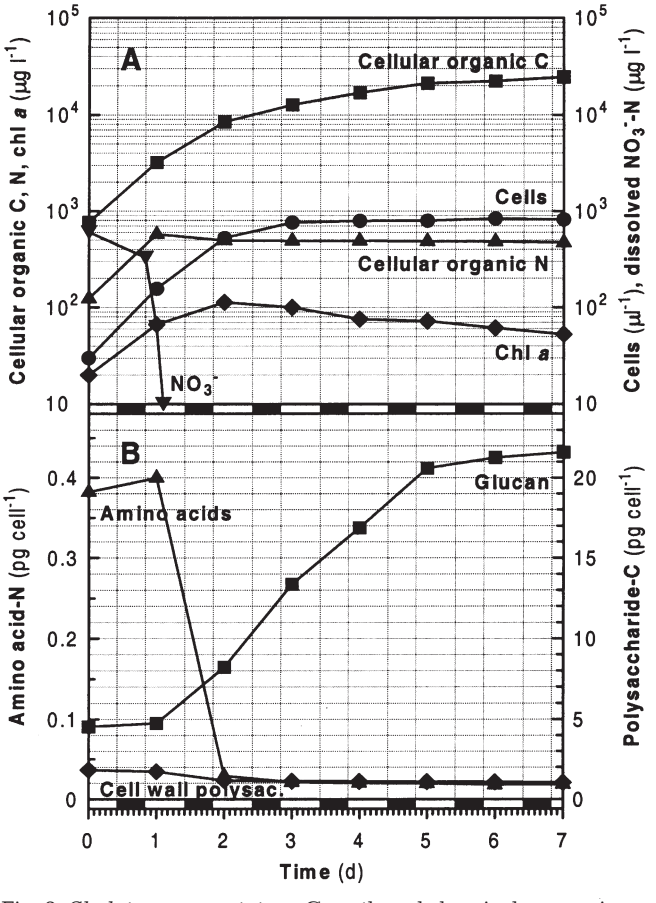

Fig. 2. *Skeletonema costatum*. Growth and chemical composition in 50 µM NO_3^- medium (Case 2). (A) Growth in cell numbers and cellular organic carbon, nitrogen and chl *a*. (B) Cell quotas of free amino acids, β-1,3-glucan and cell wall polysaccharides

Table 1. *Skeletonema costatum*. Cellular and extracellular production rates during exponential (Days 0 to 2) and stationary (Days 5 to 7) growth phase

	Growth phase	
	Exponential	Stationary
Cellular production (pg C cell^{-1} d^{-1})	30	3.9
Extracellular production (pg C cell^{-1} d^{-1})	1.3	0.16
Percentage extracellular release (PER)	4	4

The level of cellular β-1,3-glucan was only 4.5 pg C cell^{-1} during exponential growth (Figs. 1B & 2B). Upon NO_3^- exhaustion, the glucan content increased rapidly for 3 to 4 d, and then stabilized at a level of 17 to 21 pg C cell^{-1}. Consequently, the cellular protein: glucan ratio decreased from 1.7 to 0.08. In contrast, the cell wall polysaccharides decreased from 1.7 to 1.1 pg C cell^{-1} from exponential to stationary phase. The cellular free amino acid pool was 0.4 pg N cell^{-1} (22 fmol cell^{-1}) during exponential growth, but decreased by 90% within 24 h after N depletion, and was eventually reduced to 0.02 pg N cell^{-1} (1 fmol cell^{-1}). The variation in cellular chemical composition in Case 1 is summarized in Table 2.

Additional sampling at the end of the light and dark periods revealed significant diel changes in the chemical composition of the alga, although cell division was asynchronous. During exponential growth, the glucan level oscillated between 2.7 (end of scotophase) and 13 pg C cell^{-1} (end of photophase), constituting 17 to 42% of cellular organic C (Fig. 3A). The corresponding protein/glucan ratio alternated between 2.3 and 0.7. Concomitantly, the cell wall polysaccharides oscillated between 1.6 (end of scotophase) and 1.9 pg C cell^{-1} (end of photophase), constituting 6 to 10% of cellular organic C. Whereas total cellular organic C increased most rapidly during the first 4 h of the photophase, the glucan level increased most rapidly during the last 10 h. The cellular free amino acid pool oscillated between 0.2 (end of scotophase) and 0.8 pg N cell^{-1} (end of photophase), constituting 8 to 22% of cellular organic N (Fig. 3B). The levels of both amino acids and total cellular organic N increased most rapidly during the first 4 h of the photophase. In the stationary phase, cellular glucan and organic C showed much lower diel variations (Fig. 4A), and cell quotas of nitrogenous components gradually decreased without significant diel variation (Fig. 4B).

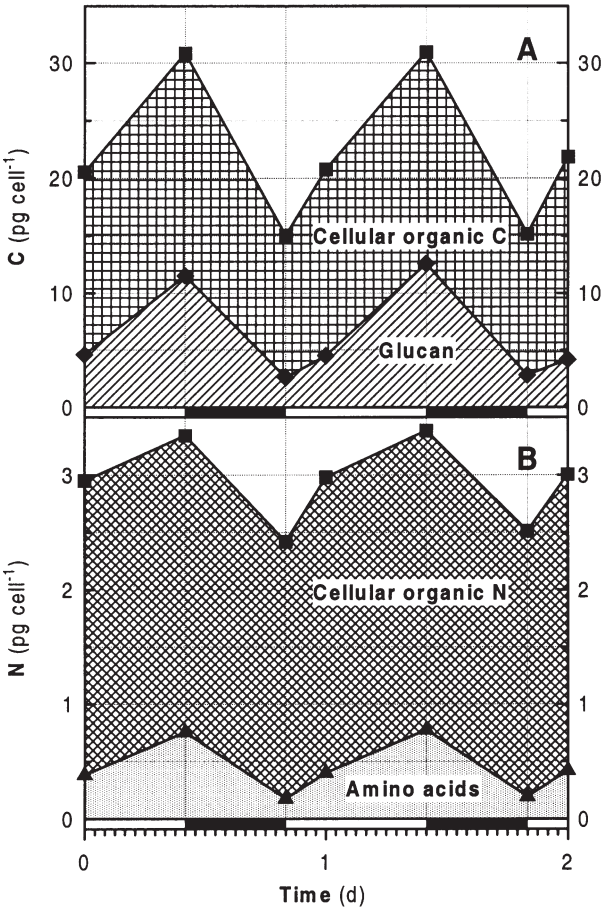

Fig. 3. *Skeletonema costatum*. Diel variation in chemical composition during exponential growth (Case 1, Days 0 to 2). (A) Cell quotas of β-1,3-glucan and cellular organic carbon. (B) Cell quotas of free amino acids and cellular organic nitrogen

Table 2. *Skeletonema costatum*. Cellular chemical composition during exponential (Days 0 to 2) and stationary (Day 7) growth phase in Case 1 (sampled 4 h into the photophase)

	Growth phase	
	Exponential	Stationary
Organic carbon (pg C cell^{-1})	20	21
Organic nitrogen (pg N cell^{-1})	3.0	0.5
Organic C:N ratio (molar)	7.8	50
β-1,3-glucan (pg C cell^{-1})	4.5	17
Protein:glucan ratio (weight)	1.7	0.08
Cell wall polysaccharides (pg C cell^{-1})	1.7	1.1
Free amino acids (pg N cell^{-1})	0.4	0.02

The composition of the cellular free amino acids also showed striking diel variations during exponential growth (Fig. 5, Table 3). Glutamine (Gln) oscillated between 0.2 (end of scotophase) and 12 fmol cell^{-1} (end of photophase), contributing 2 to 34% of the total free amino acids. Glutamate (Glu) showed much lower oscillations (4 to 6 fmol cell^{-1}), and accordingly, the Gln:Glu ratio alter-

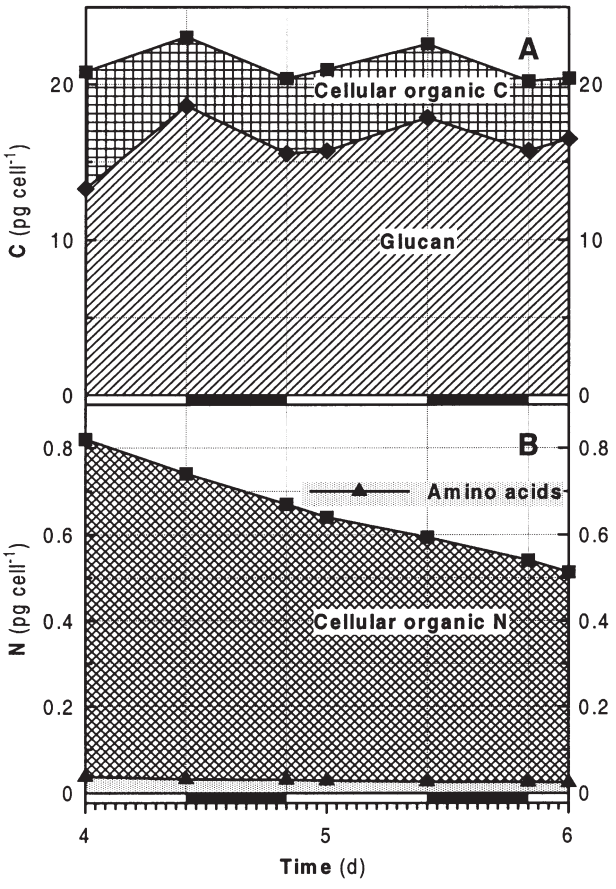

Fig. 4. *Skeletonema costatum*. Diel variation in chemical composition during nitrogen starvation (Case 1, Days 4 to 6). (A) Cell quotas of β-1,3-glucan and cellular organic carbon. (B) Cell quotas of free amino acids and cellular organic nitrogen

Table 3. *Skeletonema costatum*. Cellular free amino acid composition (molar %) during exponential growth (Days 0 to 2) at dawn (end of scotophase) and dusk (end of photophase) and stationary growth phase (Days 5 to 7)

	Growth phase		
	Exponential		Stationary
	Dawn	Dusk	
Aspartate	22	9	4
Glutamate	36	17	10
Asparagine	1	2	–
Serine	2	2	1
Glutamine	2	34	1
Glycine + Arginine	5	5	1
Threonine	2	3	1
Alanine	11	8	4
Tyrosine	1	3	4
Methionine	–	–	1
Valine	8	7	18
Phenylalanine	–	2	4
Isoleucine	3	3	22
Leucine	3	1	25
Lysine	5	4	4

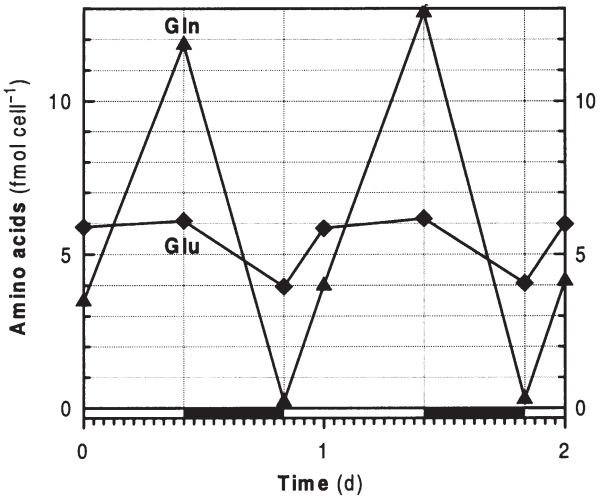

Fig. 5. *Skeletonema costatum*. Diel variations in cellular free glutamate (Glu) and glutamine (Gln) during exponential growth (Case 1, Days 0 to 2)

nated between 0.05 and 2. Even though the other amino acids also increased somewhat in the photophase (by factors of 1.3 to 10), they contributed much less than Gln, hence they mostly decreased in percentage. Upon N depletion, Gln decreased most rapidly, and constituted <1% of the amino acids in the stationary phase. Accordingly, the Gln:Glu ratio decreased to 0.05. No diel variation in the composition of cellular amino acids was observed during N starvation. Compared to the exponential phase, the proportions of the acidic amino acids (Glu and Asp) were much lower, while amino acids with large aliphatic side chains (Leu, Ile and Val) were much higher.

Extracellular production

During growth of the diatom, extracellular production of DOC, carbohydrates and free amino acids was measured every 24 h (4 h into the photophase) in both cases (Fig. 6). During exponential growth, the excretion rate was 1.3 pg C cell^{-1} d^{-1} in both cases, corresponding to 4 PER (Table 1). However, upon NO_3^- depletion in the transition phase the 2 cultures responded quite differently. In Case 1 a transient high release, 3 pg C cell^{-1} (28 PER), occurred between Days 3 and 4, while in Case 2 the extracellular release stabilized at a low rate. The high release coincided with microscopic observations of cell death affecting ~15% of the population. After this high-release event, however, extracellular production leveled off at a low rate. In both cases, excretion stabilized in the stationary phase (Days 5 to 7) at 0.16 pg C cell^{-1} d^{-1}, corresponding to 4 PER. The sudden elevated release in

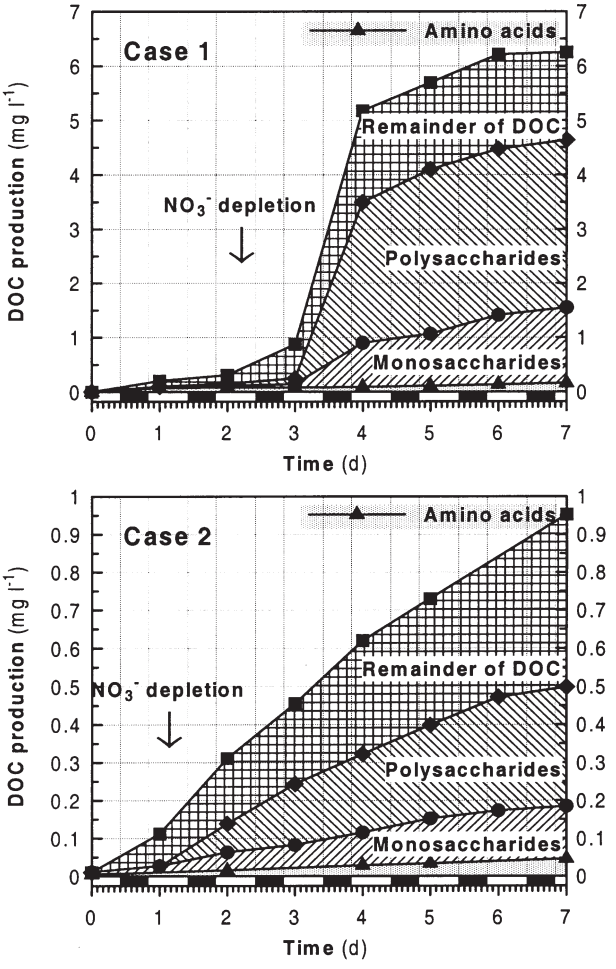

Fig. 6. *Skeletonema costatum*. Extracellular production of carbohydrates and free amino acids as fractions of total DOC during growth in 150 (Case 1) and 50 µM NO_3^- (Case 2) media

Table 4. *Skeletonema costatum.* Extracellular free amino acid composition (molar %) during exponential (Days 0 to 2) and stationary (Days 5 to 7) growth phase

	Growth phase	
	Exponential	Stationary
Aspartate	19	10
Glutamate	32	18
Asparagine	–	1
Serine	4	1
Glutamine	–	4
Glycine + Arginine	13	6
Threonine	–	2
Alanine	10	5
Tyrosine	–	3
Methionine	–	5
Valine	5	7
Phenylalanine	2	7
Isoleucine	9	10
Leucine	6	15
Lysine	–	5

Case 1 consisted mainly of carbohydrates, especially polysaccharides. After this release, DOC consisted of 50% polysaccharides, 20% monosaccharides and 2% free amino acids. The DOC produced by 'healthy' cells in Case 2 consisted of only 33% polysaccharides, 15% monosaccharides and 5% amino acids.

The composition of the extracellular free amino acids changed significantly from exponential to stationary growth phase (Table 4). The proportions of acidic and small amino acids (Asp, Glu, Ala, Gly and Ser) decreased, while large hydrophobic amino acids (Ile, Leu, Phe and Val) increased in accordance with intracellular changes. But in contrast with the intracellular pool, extracellular Gln was below detection level during exponential growth, and then slightly increased during the stationary growth phase.

Radiocarbon fixation

Parallel cultures were radiolabeled with ^{14}C-bicarbonate, and sampled every 24 h (4 h into the photophase) in both cases (Fig. 7). Growth rates and cellular/extracellular production rates calculated from ^{14}C fixation were similar to the rates based on chemical analysis (Table 1), except the last 2 d in Case 1. The low ^{14}C incorporation after Day 5 in Case 1 was probably caused by isotope dilution with unlabeled bicarbonate (see 'Materials and methods'). Biochemical fractionation of cellular organic ^{14}C was performed at 3 different points (Days 2, 3 and 7) in Case 1, and shows the same trends as the chemical analyses (Fig. 8). The ^{14}C partitioning into glucan was 5 to 10% lower in the stationary phase, but the isotope dilution probably contributes to this difference. The results also show that the cellular lipid content increased significantly from exponential to stationary growth phase (from 5 to 9% of organic ^{14}C).

DISCUSSION

Cellular production

The results show that *Skeletonema costatum* grows exponentially at 2.0 div. d^{-1} under the present light and temperature conditions, which is near the optimum growth rate (Sakshaug & Andresen 1986). Apart from diel changes in chemical composition, the alga underwent balanced growth in the exponential phase.

Diel dynamics were particularly characterized by oscillations in cellular β-1,3-glucan, increasing >4-fold cell^{-1} during the photophase (Fig. 3A). The glucan level increased most rapidly in the late part of the photophase, which is consistent with a study of the

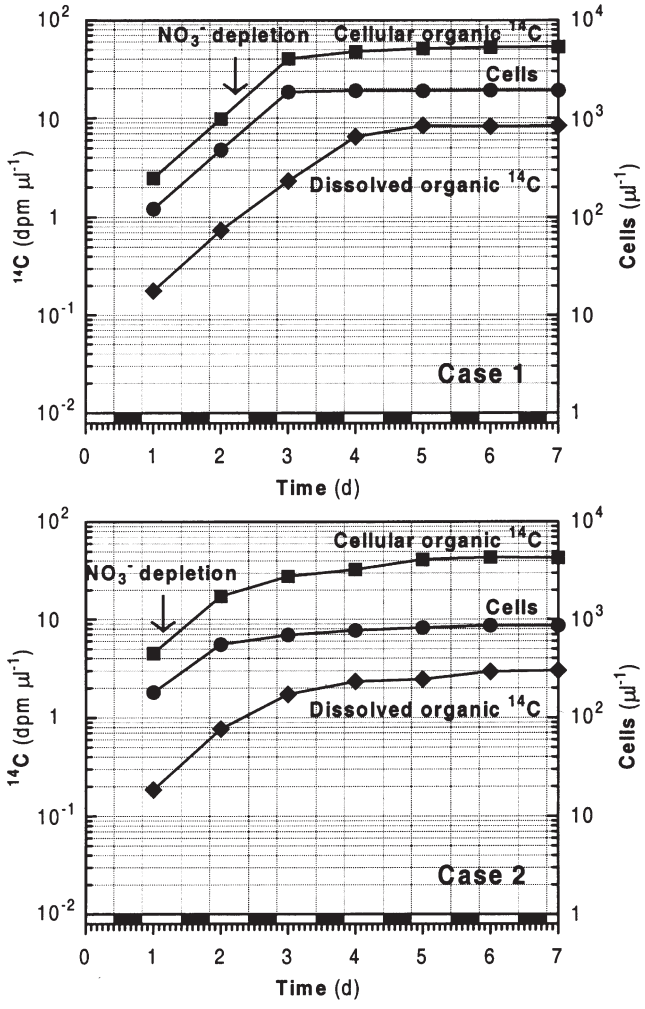

Fig. 7. *Skeletonema costatum*. ^{14}C fixation in cellular and extracellular (dissolved) fractions during growth in 150 (Case 1) and 50 µM NO_3^- (Case 2) media

same alga by Vårum et al. (1986). Also, Hitchcock (1980) observed similar diel variations in cellular carbohydrate in *Skeletonema costatum*. In field investigations, Morris et al. (1981) reported that ^{14}C incorporated into the polysaccharide fraction increased markedly during the day, while at night it was either constant or decreased. Hama & Handa (1992) found that the glucan fraction increased about 8-fold during the afternoon, and decreased by 80% during the night in natural phytoplankton dominated by *S. costatum* and a *Heterosigma* sp. (Raphidophyceae).

The rate of NO_3^- uptake was very low in the scotophase compared to the photophase, which was reflected in the diel variations in cellular free amino acids and organic N (Fig. 3B). In contrast with the glucan, the cellular pools of organic N and free amino acids accumulated more rapidly in the early photophase, indicating gradual saturation. Even though the cellular organic N quota decreased again in the scotophase, the total cellular organic N slightly increased. The results indicate that some inorganic N in intracellular pools was assimilated into amino acids and proteins during darkness. As NO_3^- uptake exceeds growth in the photophase, intracellular pools of NO_3^- and intermediate N assimilation products accumulate (Collos et al. 1992, Lomas & Glibert 2000). Raimbault & Mingazzini (1987) found strong diel patterns in NO_3^- storage in N-sufficient *Skeletonema costatum*, with maximum accumulation in the morning, and reduced uptake at night. This is also consistent with the diel cycle of nitrate reductase activity found in diatoms (Vergara et al. 1998). Moreover, similar diel variations in cellular protein and organic N have been reported in other studies with *S. costatum* (Hitchcock 1980, Vårum et al. 1986).

The composition of the cellular free amino acids also showed striking diel variation during exponential growth. Glutamine emerged as the principal amino acid during the photophase, effectively increasing the Gln:Glu ratio (Fig. 5). This ratio probably reflects the main route of NH_4^+ incorporation, the glutamine synthetase-glutamate synthase pathway (Vanlerberghe et al. 1990, Falkowski & Raven 1997). Thus, the high rate of N assimilation leads to an increasing Gln:Glu ratio in the photophase, and vice versa in the scotophase.

The results clearly demonstrate uncoupling of photosynthetic CO_2 fixation, NO_3^- uptake and protein synthesis during exponential growth of the diatom. Thus the cells were able to perform biosynthetic processes in the dark using stored glucan as C and energy

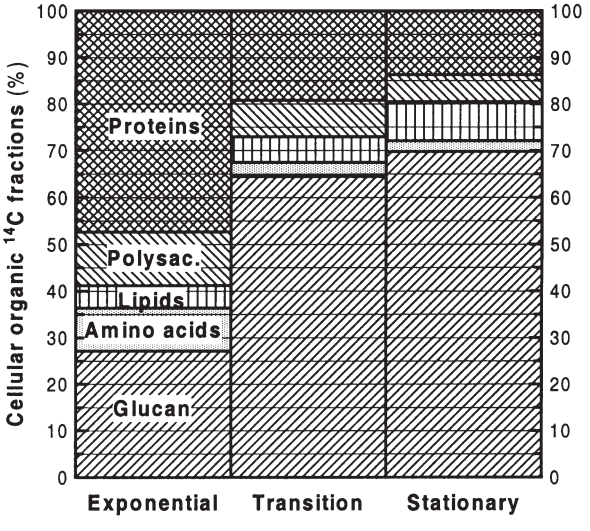

Fig. 8. *Skeletonema costatum*. Biochemical fractionation of cellular organic ^{14}C during exponential (Day 2), transition (Day 3), and stationary (Day 7) growth phase (Case 1)

source. Moreover, the cells accumulated excessive N pools in the photophase to buffer the effect of low NO_3^- uptake and reduction in darkness. Protein synthesis in the dark using glucan reserves has been indicated by several investigations (Cuhel et al. 1984, Vårum & Myklestad 1984, Granum & Myklestad 1999). This is probably an important strategy for algae to optimize growth under diel light conditions. In a recent investigation we have specifically demonstrated that β-1,3-glucan provides precursors for amino acids and proteins in *Skeletonema costatum* (Granum & Myklestad 2001).

Encountering NO_3^- exhaustion in the medium, cell numbers still increased 5-fold while the cellular N quota decreased accordingly (Figs. 1A & 2A). Moreover, the cellular free amino acid pool decreased by 90% within 24 h, and the cellular chl *a* quota decreased by 90% within 5 d. The results indicate that the alga adapted to N starvation by reducing cellular N contents to a minimum and entering a low activity stage. Dortch (1982) found similar effects of N starvation on nitrogenous components in *Skeletonema costatum* and other diatoms, including depletion of internal NO_3^- and NH_4^+. The concurrent reduction in growth rate and cellular N quota is in accordance with the Droop model, where specific growth rate is hyperbolically related to the cell quota of the limiting nutrient (Droop 1973).

At the point of NO_3^- exhaustion, Gln decreased most rapidly, and the cellular Gln:Glu ratio eventually decreased to 0.05. The proportions of small and acidic amino acids decreased compared to large aliphatic amino acids in the stationary phase, resulting in a higher average C:N ratio for the cellular free amino acids. Admiraal et al. (1986) also reported a disproportional reduction of the N-rich amino acids, and Flynn & Al-Amoudi (1988) showed that the Gln:Glu ratio decreases under N deprivation in diatoms. However, our results show that the effect of N depletion can easily be confounded with the effect of dark periods in N-sufficient cells, inducing equally low Gln:Glu ratios.

Upon N depletion, cellular β-1,3-glucan increased rapidly for 3 to 4 d before it stabilized at 75 to 80% of cellular organic C (Figs. 1B & 2B). The results indicate a high photosynthetic activity for several days under N starvation. During this period, the biosynthesis of N-free carbohydrates was apparently not hampered. In the final stage, the diel variation in glucan was very low (Fig. 4A). The results suggest that the cellular pool of glucan reached maximum levels within 3 to 4 d, after which a small surplus of glucan synthesis in the photophase balanced maintenance respiration in the scotophase. Previous studies have also shown that the glucan content of *Skeletonema costatum* may accumulate up to 80% of cellular C under strong nutrient deficiency (Myklestad 1974, Vårum & Myklestad 1984). Moreover, extensive accumulation of glucan under nutrient-deficient conditions has been demonstrated in natural phytoplankton populations dominated by diatoms (Haug et al. 1973, Hama & Honjo 1987). The combined glucan accumulation and sharp reduction in protein synthesis makes the protein:glucan ratio a sensitive indicator of the nutritional status in phytoplankton. A protein:glucan ratio of <1 normally indicates nutrient deficiency (Haug et al. 1973, Myklestad 1974). In this study, however, we obtained a slightly lower value than 1 at the end of the photophase in nutrient-sufficient *S. costatum*.

The radiocarbon results corroborate the chemical analyses in our investigation, and the biochemical fractionation provides some additional information. The ^{14}C partitioning shows that cellular lipids accumulated significantly (from 5% to 9% of organic ^{14}C) during N starvation in addition to the large glucan accumulation. However, both glucan and lipid fractions were probably underestimated after Day 5 due to significant dilution of specific ^{14}C activity (see 'Materials and methods'). Whereas new production of nitrogenous components (amino acids and proteins) was extremely low, significant metabolism of carbon reserves (glucan and lipids) still occurred in the late stationary phase. Due to mobilization of ~12% of cellular glucan during each dark period (Fig. 4A), the relative fraction of glucan could be underestimated by as much as 5%.

Nutrient-dependent changes in the chemical composition of oceanic diatoms influence cellular buoyancy, and have important ecological and biogeochemical implications (Richardson & Cullen 1995, Villareal et al. 1996). Carbohydrate ballasting seems to be one of the mechanisms for vertical migration of diatoms. Nutrient-deplete cells in the euphotic zone can accumulate glucan, causing increased cellular density and sinking below the nutricline. Upon nutrient replenishment and mobilization of glucan, cellular density is reversed and the cells rise toward the surface again. Such vertical migration of phytoplankton with uncoupled carbon and nutrient assimilation enhances transport of deep nutrients to the euphotic zone, thereby increasing oceanic primary production. Physiological changes induced by nutrient depletion may also be connected to the formation of resting cells and resting spores. Kuwata et al. (1993) found that resting spores of the marine diatom *Chaetoceros pseudocurvisetus* accumulated large amounts of glucan (70% of cellular organic C) and lipids (17%) with concurrent reduction in the cellular N quota. β-1,3-glucan is not, as far as we know, synthesized by marine bacteria, and is therefore particularly interesting as an ecological index of phytoplankton growth.

Extracellular production

Different diatoms show widely different levels of excretion, but *Skeletonema costatum* has generally been found in the low range of species investigated (Myklestad 1974, 1977, Mague et al. 1980). This was confirmed in the present study, with only 4 PER in both exponential and stationary growth phases (Table 1). However, the absolute rate of release (per cell) was markedly higher during exponential growth, which is consistent with a similar study of the marine diatom *Chaetoceros affinis* (Myklestad et al. 1989).

A transient high release occurred 1 to 2 d after NO_3^- exhaustion in Case 1, which coincided with 15% cell death. This event was confirmed by release of extracellular organic ^{14}C in the radiolabeled culture. Moreover, the high fraction of released polysaccharides indicates cell lysis with leakage of β-1,3-glucan. The reason for this occurrence is uncertain, but there have been similar reports of an abrupt increase in excretion at the transition between the exponential and stationary phases (Williams 1990). This release may arise from a temporary imbalance of internal pools during the deceleration phase of growth, induced for instance by exhaustion of the external N supply. For instance, Ignatiades & Fogg (1973) reported that excretion by *Skeletonema costatum* increased from 2 to 4 PER in exponentially growing cells to 14 to 65 PER under nutrient deficiency. Berges & Falkowski (1998) showed that a specific protease was induced in the diatom *Thalassiosira weissflogii* under N stress, and suggested that the process was a form of autocatalyzed cell death.

Apart from the transient high-release event in Case 1, the excretion rates during the exponential and stationary growth phase were similar in both cases. A large fraction of the DOC produced by 'healthy' cells was accounted for by monosaccharides (15%) and polysaccharides (33%), while only 5% was identified as free amino acids. The constituents of the residual DOC remain uncertain, but proteins probably constitute a significant fraction. The composition of the extracellular free amino acids changed from exponential to stationary phase much in accordance with the intracellular ones. In contrast with the intracellular pool, extracellular Gln was below detection level during exponential growth. A possible reason for this could be the compartmentation of the primary N assimilatory enzymes, glutamine synthetase and glutamate synthase, within the chloroplasts, where only Glu is exported to the cytosol for further transaminations (Falkowski & Raven 1997).

Although excretion represents a physiological loss term, algae may gain in ecological terms from long-term symbiotic advantages within a planktonic community (Williams 1990). Fogg (1983) proposed that excretion was an overflow mechanism, or C sink, when photosynthesis takes place more rapidly than is required to supply the needs of growth. However, our results indicate no such mechanism, but rather that cellular β-1,3-glucan is the C sink in *Skeletonema costatum*. Release of glucan due to cell lysis and inefficient grazing (sloppy feeding) is probably important in the microbial ecology of the sea due to the great abundance of diatoms (Myklestad et al. unpubl.).

Concluding remarks

In this study, typical cell quotas of organic C and N, carbohydrates and amino acids were established for the marine diatom *Skeletonema costatum* grown under a light:dark cycle in N-limited batch cultures. Such growth conditions may be analogous to the spring bloom in coastal waters (culmination by N depletion). Balanced growth took place in the exponential phase apart from significant diel changes in cellular chemical composition, particularly the levels of β-1,3-glucan and free amino acids. Exhaustion of NO_3^- resulted in rapid accumulation of cellular glucan and concurrent reduction in cellular nitrogenous components, especially free amino acids. Our observations show that when parameters such as C:N, protein:glucan and Gln:Glu are used as indicators of nutrient deficiency, the diel changes in nutrient-sufficient cells must be taken into account.

A low excretion rate for *Skeletonema costatum* was confirmed in this investigation, constituting 4 PER. Polysaccharides (33% of C), monosaccharides (15%) and free amino acids (5%) contributed substantial fractions of extracellular production by 'healthy' cells.

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